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Silvia Mazzaferro

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par

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AMELIORATION DE LA BIODISPONIBILITE ORALE DU DOCETAXEL AU MOYEN DE SYSTEMES NANOPARTICULAIRES

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AMELIORATION DE LA BIODISPONIBILITE ORALE DU DOCETAXEL AU MOYEN DE SYSTEMES NANOPARTICULAIRE

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INTRODUCTION GENERALE

1. Introduction

La chimiothérapie représente un des trois pilastres du traitement du cancer à côté de la chirurgie et de la radiothérapie. Alors qu'une administration par voie orale (per os) permettrait de réduire les coûts du traitement et d'améliorer la qualité de vie des patients, la majorité des chimiothérapies est encore administrée par voie intraveineuse. En effet, l'administration par voie orale permettrait de faire bénéficier aux patients d'un traitement à domicile en réduisant la durée d'hospitalisation [1-5]. Plusieurs études ont montré que 89 % des patients préféraient un traitement par voie orale [6] et que les patients se sentaient plus libres et moins malades car ce traitement s'accorde mieux avec la vie quotidienne [3]. Les patients pouvaient faire face plus facilement à la maladie en améliorant ainsi leur qualité de vie. Cette voie d'administration permet également de palier aux risques d'infection au niveau du cathéter, de thrombose et d'extravasion possibles par voie intraveineuse [7]. L'administration des traitements de chimiothérapie par voie orale présente donc un intérêt majeur au point de vue confort du patient.

Le docétaxel (Dtx) est l'un des plus puissants agents anti-cancéreux utilisé pour le traitement de patients atteints de cancers de la prostate, du sein, ou du poumon [8]. C'est un alcaloïde végétal appartenant à la famille des taxanes. Il a été synthétisé en France en 1989 par hémi-synthèse à partir des aiguilles de l'if européen *Taxus baccata.* Il favorise le maintien des microtubules au niveau des celles tumorales en inhibant leur dépolymérisation par liaison stable à la tubuline et entraîne ainsi un blocage de la mitose en phase G2 du cycle cellulaire. C'est un analogue du paclitaxel, de structure et d'activité voisines, mais il diffère surtout par son efficacité anti-tumorale puisqu'il est dix fois plus efficace que le paclitaxel sur les lignées de cellules tumorales [9]. Il est actuellement administré exclusivement par voie intraveineuse. Dès les premiers essais cliniques, il a été constaté que l'administration de la formulation commerciale, Taxotere®, s'accompagnait de réactions d'hypersensibilité plus ou moins

sévères attribuées à l'un des excipients utilisés (le polysorbate 80), ce qui nécessite en clinique la co-administration préventive de corticostéroïdes ou d'antihistaminiques pour palier ces effets secondaires [10]. Dans ce contexte, disposer de formulations administrables par voie orale, moins toxiques et mieux tolérées, représenterait une avancée majeure au plan clinique. Toutefois, plusieurs études ont montré que la biodisponibilité par voie orale du Dtx est très faible en raison de : *(i)* sa faible solubilité aqueuse, *(ii)* son faible passage au niveau intestinal, *(iii)* son efflux par les pompes d'efflux (P-gp) et son métabolisme par le cytochrome P450 présents au niveau intestinal.

Dans l'objectif d'une amélioration de la biodisponibilité orale du Dtx, plusieurs stratégies de formulation peuvent être envisagées :

(i) Améliorer la solubilité aqueuse

(ii) Agir au niveau de l'absorption, par le ralentissement du transit intestinal en employant des formes mucoadhésives capables d'augmenter la durée et donc l'efficacité de l'absorption.

(iii) Agir au niveau de l'épithélium, par l'inhibition des pompes d'efflux (P-gp) et du métabolisme entérocytaire.

Cependant, une importance majeure est portée à la toxicité locale liée à l'administration orale du Dtx. En effet, le mécanisme d'action aspécifique des agents anti-cancéreux conduit à une toxicité sur les cellules en prolifération rapide telles que les cellules du tractus digestif, de la moelle osseuse et des follicules pileux. Ainsi, lorsqu'une administration orale est envisagée, il faut se demander si les effets secondaires gastro-intestinaux augmentent à cause du contact direct de l'agent anti-cancéreux avec les enterocytes, ce qui peut alors conduire à une toxicité locale.

Dans ce contexte, le présent mémoire est organisé de la manière suivante. Dans un premier temps, nous nous sommes intéressés aux problématiques liées à l'administration orale des agents anti-cancéreux. Une revue bibliographique a été écrite avec l'objectif de discuter le potentiel et les limitations thérapeutiques d'une chimiothérapie par voie orale. Une première section vise à identifier les questions générales relatives à l'administration orale de médicaments anti-cancéreux, y compris l'adhérence des patients aux traitements par voie orale, les questions économiques, les considérations sur la gestion de leurs index thérapeutiques ainsi que leur toxicité au niveau des tissues intestinaux seront prises en compte. Dans ce premier chapitre, nous avons également décrit les différentes stratégies proposées pour améliorer la biodisponibilité orale des agents anti-cancéreux notamment la stratégie des prodrogues, la conception des nouvelles formes pharmaceutiques ainsi que la modulation du métabolisme entérocytaire.

La deuxième partie du mémoire, essentiellement expérimentale, est consacrée à décrire le nouveau système nanoparticulaires que nous avons conçu pour l'administration orale du Dtx. Tout d'abord, nous nous sommes intéressés à augmenter la solubilité apparente du Dtx en milieux aqueux à l'aide de cyclodextrines (CDs) afin d'obtenir une dispersion moléculaire, plus favorable à son absorption au niveau intestinal. Par la suite, la possibilité d'encapsuler le Dtx dans un système nanoparticulaire mucoadhésif a été explorée ainsi que l'absorption orale du Dtx libéré à partir des nanoparticules (NPs). Ce dernier a été étudié au moyen du modèle expérimental des chambres de Ussing afin d'évaluer les avantages en termes de biodisponibilité apportées par la formulation. Enfin, dans une dernière partie, la toxicité au niveau intestinal de ces NPs chargées en Dtx a été évaluée sur les intestins de souris xénogreffées après administration *in vivo* des formulations.

Une discussion générale conclue le mémoire en permettant de mettre en perspectives les résultats obtenus, puis de dégager les avantages et les inconvénients de la stratégie

consistant à utiliser des NPs mucoadhésives pour l'administration oral des agents anti-cancéreux.

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TRAVAUX ANTERIEURS

CHAPITRE I : Oral delivery of anti-cancer drugs

Oral delivery of anti-cancer drugs

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Abstract

Historically, most of anti-cancer drugs have been formulated for being delivered by the intravenous (i.v.) route. If this route is the most direct one leading to immediate and complete bioavailability, it is the most hazardous administration route and often results several hypersensitivity reactions, nephrotoxicity and neurotoxicity, and in all cases it needs hospitalization, nursing, and a palliative treatment. This review aims to discuss the potential and the limitation of oral chemotherapy. A first section aims to identify general issues related to the oral delivery of anti-cancer drugs, including patient adherence to oral treatments, economic issues and formulation considerations. Specific features of anti-cancer drugs to be considered for the development of reliable and safe formulations include the management of their therapeutic index and of the variability following oral absorption and most important their potential toxicity for intestinal tissues, and their relevance is discussed. A specific section describes different approaches (chemical modifications, formulation approaches and metabolism modulation), which have been proposed to improve the delivery of poorly absorbed anti-cancer drugs. Their relative merits are explored and discussed. Finally, the progresses of clinical development of oral chemotherapy are presented.

Keywords: Anti-cancer drugs, oral chemotherapy, patient's adherence, drug delivery systems, prodrugs, drug metabolism.

1. Introduction

Anti-cancer drug therapy is one of the three pillars of cancer treatment along with surgical treatment and radiation therapy. Generally the anti-cancer drugs are divided into three categories: cytotoxic, biological and hormonal agents. Cytotoxic agents are the traditional therapies that damage cancer cells by interfering with DNA or its precursor, inhibiting the cellular division. However, this kind of agents has the great drawback of killing healthy cells along with cancer cells [1]. Major types of cytotoxic agents include alkylating agents [2, 3], antimetabolites [4], and plant alkaloid [5-7]. Biological agents or targeted agents, includes monoclonal antibodies [8-12] and cancer vaccines [13-16]. This therapy (also called immunotherapy, biological response modifier therapy, or biotherapy) uses the body's immune system to fight cancer. Hormonal therapy interferes with hormone dependent pathways that promote the development or growth of cancer cells and plays an important role in treating breast and prostate cancers. It includes tamoxifen [17, 18] and aromatase inhibitors [19-21].

Historically, most anti-cancer drugs are administered intravenously (i.v.). The intravenous route is the most direct one and it by-passes the variable absorption patterns of the gastrointestinal tract. It leads to immediate and complete bioavailability and therefore, to accurate dosing. However, this route is also the most hazardous administration route, because potentially high concentration of the drug is delivered to normal tissues [22, 23]. I.v. chemotherapy regimens are designed to deliver the maximal tolerated dose of cytotoxic agent to kill cancer cells in a short period of therapy, followed by a period of several weeks without administration [24]. Cisplatin, for example, since 1979 has become an important component in chemotherapy for his broad spectrum of antitumor activity. Unfortunately, a great restriction of this drug is its several side effects due to the unspecific uptake of the drug into

all rapidly dividing cells. For this reason, the tolerated doses are very low. The major side effects include nephrotoxicity, neurotoxicity, ototoxicity and myelosuppression. For this reason, the dose delivered to patients could be sub-lethal to tumor which means they are then able to develop resistance to further drug treatment [25]. In addition, the pharmaceutical additives required to dissolve the drug are often responsible for the toxicity of the product. Taxol® and Taxotere® approved for breast, prostate and lung cancer are two clear examples. Taxol® is the commercial formulation of Paclitaxel. The high lipophilicity of this compound needs a particular vehicle composed of 1:1 blend of Cremophor EL (polyethoxylated castor oil) and ethanol which is diluted with 5-20-fold in normal saline or dextrose solution before administration. However, many problems have been reported related to this vehicle. The most important is related to his large amount of Cremophor EL necessary to deliver the required dose of paclitaxel, which causes several hypersensitivity reactions, nephrotoxicity and neurotoxicity. Consequently, premedication with corticosteroids and antihistamine is used to increase safety and reduce the intensity of this kind of reactions [26]. Furthermore, it was reported that this additive could modify the kinetic of the drug [27, 28]. In the same way, in Taxotere®, docetaxel is formulated with the non-ionic surfactant polysorbate 80 (Tween® 80) which has been implicated in the occurrence of severe anaphylactic hypersensitivity reactions [29]. Furthermore, i.v. chemotherapy needs hospitalization, nursing, and a palliative treatment. Although the use of ambulatory pumps and indwelling catheters enable home-based i.v. chemotherapy, this kind of administration remains inconvenient for patients. It is painful, can lead to haemorrhage and in the long term it is often associated with infection, bleeding and venous thrombosis [30]. Generally, the patient shows sight of depression and anxiety, he does not feel free and independent, and his daily life is influenced by the medication schedule.

During the past few decades, quality of life (QoL) has emerged as an important outcome in oncology [31]. Actually, the success of the therapy is due more and more to the adherence and the persistence of the patient. In this context, the oral chemotherapy becomes a very interesting alternative to the i.v. therapy. In several studies, patient preference for oral or i.v. treatment has been studied directly in a randomized crossover trial, comparing an oral drug regimen versus i.v. treatment. The majority of them at the end of the study chose to continue with the oral treatment. They found oral chemotherapy advantageous and it made them feel less sick. It helped them to face their illness better. The most important feeling elicited is the feeling of freedom, they can spend more time at home and the medication interfered less with the dailies activities. Finally, from an economical point of view the oral therapy is convenient because it limits the cost of hospitalization and the infusion equipment supplies [22, 30, 32-34].

Currently, 10% of cancer chemotherapy is provided to patients as an oral formulation, but the national Comprehensive Cancer Network predicts that by the year 2013 this percentage will jump to 25% [35]. More than 20 already available and some emerging oral cytotoxic agents are in development (Table I-II) [36]. Most of the oral anti-cancer drugs, are not based on new molecules, but are new formulations of the drugs already used pour the i.v. therapy.

Table I. Some examples of approved oral chemotherapy drugs.

Drug	Trade name	Form	Indication	Company	Authorisation
Busulfan	Myleran®	Coated tablets	Chronic myeloid leukaemia	GSK	2010°
Capecitabine	Xeloda®	Coated tablets	Metastatic breast and colorectal and Stage III (Dukes' C) colon	Roche	2001*
Chlorambucil	Chloraminophene®	Capsules	Chronic lymphatic (lymphocytic) leukemia, malignant lymphomas and Waldenstöm's disease	Techni Pharma	1970°
Cyclosphosphamide	Endoxan®	Coated tablets	Different cancer deseases (breast, ovarian cancer and leukemia)	Baxter	2002°
Etoposide	Vepesid®	Capsules	Lung cancer, leukaemia and cancer of the lymph glands	Alkopharma SARL	2005*
Erlotinib	Tarceva®	Tablets	Non small-lung carcinoma and prostate cancer	Roche Registration	2005°
Estramustine phosphate	Estracyt®	Capsules	Prostate cancer	Keocyt	2007°
Gefitinib	Iressa®	Tablets	Non small-lung carcinoma	AstraZeneca	2009*
Iapatinib	Tyverb®	Tablets	Breast Neoplasm	Glaxo Group	2008*
Ibandronic acid	Ibandronic acid Sandroz®	Tablets	Breast Neoplasm	Sandroz Pharmaceutics	2001*
Idarubicin	Zavedos®	Capsules	Acute myelogenous leukaemia	Ptizer	2004°

Drug	Trade name	Form	Indication	Company	Authorisation
Imatinib	Glivec	Capsules or tablets	Leukaemia, gastro-intestinal stromal tumor	Novartis Europharm	2001*
Lomustine	Belustine®	Capsules	Brain tumors	Prostrakan	2008°
Melphalan	Alkeran®	Coated tablets	Multiple myeloma,	Aspen Europ GMBH	2010°
Mercaptopurine	Purinethol®	Tablets	Acute lymphoblastic leukemia	Aspen Europ GMBH	2010°
Methotrexate	Methotrexate bellon®	Tablets	Different kinds of cancer	Sanofi-Aventis	2008°
Procarbazine	Natulan®	Capsules	Hodgkin's lymphoma and other malignant lymphomas	Sigma Tau	2004°
Tegafur-uracile	UFT®	Capsules	Metastatic colorectal tumors	Merk Sante S.A.S	2005°
Tegafur-gimeracil-oteracil	Teysuno	Capsules	Stomach cancer neoplasm	Thaino Pharma Europe	2011*
Temozolomide	Temodal®	Capsules	Brain tumors	Schering Plough Europe	1999°
Topotecan	Hycamtic®	Capsules	Small lung cancer, Uterin cervical and ovarian neoplasm	Smithkline Beecham PIC	2006*
Toremifene	Fareston®	Tablets	Breast Neoplasm	Orion Corporation	2004*
Thioguanine	Lanvis®	Tablets	Acute leukemia and chronic granulocytic leukemia	Aspen Europ GMBH	2010°

° *afssaps*

* European public assessment reports

Table II. Some examples of oral cytotoxic drugs in development

Agent	New strategies	Comments	References
Paclitaxel (PTX)	Ptx/Cyclosporine A	P-gp inhibitor; similar bioavailability with daily dosing schedule	[37]
	Ptx/GF120918	P-gp inhibitor; bioavailability ~ 40%	[38]
	Ptx-HPCD-nanoparticles	bioavailability ~ 80%	[39-40]
Docetaxel (DTX)	Dtx/ritonavir	CYP450 inhibitor; increment of systemic exposure by 50-fold.	[41]
	Dtx/Cyclosporine A	P-gp inhibitor; increment of bioavailibility	[42]
	Dtx/PLA-TPGS/MMT NPs	bioavailability ~ 78%	[43]
	ModraDoc001	Oral solid dispersion formulation	[44]
Topotecan	Standard Hycamtin® for i.v.	Less toxicity	[45]
Irinotecan	Lipid nanocapsules (LNCs) loaded SN38	Permeability improvement across Caco-2 cells	[46]
Satraplatin	JM216 (prodrug)	Milder toxicity; lack of cross resistance with cisplatin; same efficiency of other platinum drugs	[47]
5-Fluorouracil	Capacitabine (prodrug)	Promising in colorectal and metastatic breast cancer	[48]
	UFT (prodrug)	Good response rate in colorectal and gastric cancer	
	S-1 (prodrug)		

This review wants to discuss all these different aspects related to the oral chemotherapy in the aim to understand its potential benefits and limitations. In the first part of this review, the general specification for the oral delivery of anti-cancer drugs will be exposed. Although the availability problems of the long-used cytotoxic drugs and the patient adherence represent two major aspects in view of new oral formulations, it is also important to consider their toxicity. In this context, many questions worth to be asked: will the oral

administration increase this side effect? What about the local toxicity vis-a-vis the enterocytes?

In the second part of this review, different strategies to improve the bioavailability of anti-cancer agents will be reviewed. The progress of clinical development of oral chemotherapy is described and also the relative merits and challenges of different approaches for the improvement of oral delivery of anti-cancer drugs are explored.

This review is intended to be as exhaustive as possible since it was conceived as a work tool for readers wanting to go further.

2. General issues for the oral delivery of anti-cancer drugs.

As mentioned above, nowadays we can find different kinds of anti-cancer drugs formulated for the oral administration (Table I), but if we pay attention to the type of formulation used, we can notice that the major oral anti-cancer drugs are formulated as capsules or tablets. These pharmaceutical forms are very simple, but required a therapeutic agent with fewer problems of solubility and bioavailability. ~~Generally, this kind of active agents belongs to the Class I/II of the Biopharmaceutical Classification System and they are easier to be formulated.~~

Nevertheless, it should be remarked that most of anti-cancer drugs belong to the Class IV of the Biopharmaceutical Classification System, which comprises substances with both low solubility in aqueous fluids and low apparent permeability. Moreover these substances are substrates of biological transporters and/or metabolized in the intestinal barrier. For these reasons the choice of the parental administration seems to be more obvious.

Success in cancer treatment has traditionally been measured in terms of cure rate, increased survival, and tumor response. The researchers did not wonder if an oral

chemotherapy might be better than a parental treatment. The choice how to formulate the anti-cancer agents normally fell into the more convenient and effective formulation. For intravenous injections the bioavailability is maximal because the chemotherapeutic agent is administered directly to the bloodstream. For oral administration, the compound must be released from its carrier and pass from the gastrointestinal tract to the bloodstream without being inactivated. The level of bioavailability depends on the sensitivity of the agent to the conditions of the gastrointestinal tract and its ability to pass from the tract into the bloodstream.

Recently, particular attention has been attached to the patient's quality of life during the therapy. It became of particular importance to allow a more comfortable life for the patients during the final stage. Moreover, the fact that major developments have been made in cancer research, in particular in diagnostic field, has as consequence, an improvement of the patient's number with a cancer at a treatable stage. In this context, the oral chemotherapy seems to be the better candidate allowing a normal life to the patient, spent less in the hospital and more at home.

When foreseeing oral delivery and before to launch into the development of new molecules or new formulations, different areas of great concern should be take in consideration. First of all it is necessary to consider the toxicity aspects. Chemotherapy is well known to be one of the most difficult therapies with the most strong side effects. In particular the contact of a cytotoxic agent *vis-à-vis* with the intestinal wall, leads to suppose great and dangerous damages at the intestinal cells.

Secondly, the patient adherence and the economic issue play an important role in the development of a new medicament. Adherence, as defined by the International Society for Pharmacoeconomics and Outcome Research (ISPOR), is *"the degree or extent of conformity*

to the recommendations about day-to-day treatment by the provider with respect to the timing, dosage and frequency". Adherence is a synonymous of compliance, but often preferred because it is generally believed to have a less pejorative and less judgmental connotation [49]. In the matter of adherence problem, providing patients with a good educational background on when and how to take their medications [35], as well as finding new techniques for measuring adherence and persistence is of paramount importance. It is important to highlight with the oral treatment that, not only the patient role changes, but the role of the oncologist and the pharmacist changes too.

Finally, as mentioned before, a crucial problem is the *bioavailability*: it is a result of limited aqueous solubility, degradation in gastrointestinal fluids and/or affinity for intestinal and liver cytochrome P450 (CYP3A4) and P-glycoprotein (P-gp) [22]. For this reason, more work and better systems are required to overcome the number of obstacles to oral delivery.

2.1. Patient's adherence to treatment

The era of oral chemotherapy began in Avril 1998 with the Capacitabine FDA approval [50]. From this moment, it became more and more a very attractive alternative to i.v. therapy because of its convenience and easy administration [36].

Despite these major developments, the future of oral anti-cancer treatment remains unclear. An important issue, which needs to be discussed, is the patient's adherence. Different studies compared the patient preference for oral versus i.v. treatment, and in the most part of them the result showed a preference for the oral treatment if this does not sacrifice efficacy [51, 52]. The main reason is the improvement of patient's quality of life: the medication can be taken at home, interferes less with the daily activity [30] and in particular the oral chemotherapy makes the patients feel less ill and help them to face their illness better. The feeling that the patients interlined was the feeling of freedom [33].

Although the patients seem enthusiastic for the oral chemotherapy, this kind of therapy raises a great problem: the patient's adherence, especially in chronic conditions such as cancer [53]. Adherence is often referred to as compliance and the editors of "Compliance in Health Care" defined it the extent to which a patient's behavior (in terms of taking medications, following diets or executing lifestyle changes) coincides with medical care or health advice [54]. A patient is adherent when no doses are missed, no extra dose are taken, and no dose are taken in the wrong quantity or in the wrong time [49]. If, in the case of i.v. chemotherapy, the patient is in the hospital, and the treatment is attended directly by a health care provider, with the oral chemotherapy the adherence and the measure of adherence become more complicated [35]. In the parental therapy, the physician knows exactly how much medicament was given over which period of time and on which day. This level of control is not possible with the oral chemotherapy. Many of the responsibilities of managing the regimen and monitoring for doses and toxicity shift from oncologist to the patient [55]. The patient becomes the real actor of the therapy; he must promptly initiate the therapy at the correct time of the day, at the correct dosage and alert the clinician of adverse symptoms in a timely way. Adherence can become a challenging task for many patients. For this reason the decision to take oral chemotherapy must be based on a collaborative discussion between the patient and the physician, with appropriate support from oncology staff [56].

The majority of oncologists considered advantageous the oral chemotherapy, but not all the patients are good candidates for oral treatment. Usually, the oncologists use their "own autonomy index" to select patients, depending on different criteria such as age, previous experiences, side effects. They took into consideration the patient's wishes to have this kind of treatment and also the presence of a social network for special patients (the old and children). The relationship between the oncologist and the patient, the organization of consultations and how to consider risk change. New tasks for oncologists are to communicate

information to their patients, to verify that they have understood and to make their patients want to adhere to their treatment [57].

In general, adherence to chronic medication therapy in adult ambulatory care is generally fair to poor. Approximately 50 % of patients will discontinue taking the medication within 6 months [50]. The main reasons of non-compliance include misinterpretation of physician instructions, denial, forgetfulness or confusion, dosing frequency and side effects [57-60]. In particular in children and adolescents compliance is a very complex issue. Cameron Tebby in his review [53] discussed different factors involved in the noncompliance of pediatric patients. Among the influent factors found there are human error, complexity of regimen, duration of therapy, side effects, interaction between the provider and the patient and several factors about the family, such as demographic, psychological and cognitive, social or situational [53, 61]. In the same way, non-adherence is a real problem in the elderly population. In this kind of patients another factor that can often lead to non-adherence is the "polypharmacy". The term means "many drugs" and indicates the use of more medication [55].

Suboptimal adherence to oral therapy can have sever consequences: can impede the efficacy of the oral regimens, the toxicity of the drug may increase with the consequence of an increment of costs due to the necessity of more physician visits, higher hospitalization rates and longer stays [49, 62]. For this reason great attention was paid to find effectives methods to measure patient's adherence. There are different methods of monitoring adherence and they are divided in two categories: direct and indirect methods.

The direct methods include the directly observation of the therapy, easy with the parental administration in clinic, more difficult with the oral therapy takes at home, and the measurement of the pharmacokinetics parameters. In this case, drug or metabolite levels in

serum or urine may provide objective measures of adherence, but such level may vary widely because of individual pharmacokinetics. Moreover, non-adherent patients can manipulate results by becoming adherent immediately before the physician visit.

Between **the indirect methods** we can find *(i)* patient self-report: is the more traditional methods, but frequently inaccurate because of poor patients memory or reluctance to report non-adherence; *(ii)* pill counts and microelectronic monitoring system (MEMS): these devises are able to record each time they are opened. The data are collected and processed by a computer to generate a graphic representation of the number of doses taken daily, the number of missed or extra doses, and the dosing intervals. The main problems with this kind of methods are that there was no proof that the tablet was taken, the act of opening a pill counter does not necessary mean that the patient really ingested the pill, and they are too expensive for an applicability to large scale use [60, 63, 64]. Regardless of the technique used to assess adherence, physician must realize that the lack of adherence typically reflects the complexity of the regimen rather than willful or manipulative behavior from the patients [50].

In this context, one strategy for promoting appropriate treatment compliance is to educate the patient, to stimulate an individual patient's motivation to follow instructions and his perception of the risks and benefits. Albrecht and Hoogstraten in their work [65], underlined that compliance is often linked with satisfaction of therapy, in terms of information-communication, understanding acceptance and perceived technical competence. Simons et al. in a recent work [66] investigated the effect of an intensified multidisciplinary pharmaceutical care programme on the adherence of patients treated with capacitabine. The study was carried out by dividing patients into two groups (control and intervention group). Patients in the intervention group showed an increased, but not significantly different mean overall adherence of 97.9% compared with the control group (90.5%). Nevertheless, mean daily adherence was significantly higher in the intervention group (96.8%) than the control

group (87.2%). Moreover variability of both adherence parameters was reduced when pharmaceutical care was provided and at the end of the study, the probability of still being treated with capacitabine was 83% in the intervention group against 48% in the control group.

The patients and the family, in case of children or adolescents, become the real actors of the therapy and their education represent a mandatory factor to ensure the success of the therapy [60]. In this context, each protagonist, physicians, nurses, pharmacists, oncologists have an important role to play.

There are different ways to educate the patient, from the print material, individual or group sessions, to video or audiotapes and Computer-assisted instruction (CAD). Susan Moore in her review [60] discussed the advantages and disadvantages of each method. She underlines that the most part of patients gets benefit by imagining themselves as partners in a therapeutic process, understanding how the medications works, how it should be taken and how manage the side effects. In this context, the role of oncology nurses becomes really important. In fact, more and more, the nurses are in charge of the educational programs. It is essential that the nurses know the patient and they let be known, establishing with the patients a real relationship based on trust. They have to stimulate a genuine dialogue with patients, listening and accommodating the individual needs and circumstances, emphasizing the patient's personal choices, setting goals and focusing on the patient's perspectives. In this way they may be able to provide patients with the tools they need to adhere to treatment [67, 68].

In the recent years, in the UK, the National Health Service (NHS) has embraced home delivery and home nursing, and has developed strong, mutually beneficial relationships with the independent providers of home healthcare services. The UK Oncology Nursing Society (UKONS) indentified the skills necessaries to enhance patient safety. A number of private

sector healthcare companies have established services which offer support, monitoring and follow-up for patients receiving oral chemotherapy within the UK [69].

2.2. Economic issue

Pharmaco-economic analyses were carried out in clinical trial to evaluate the cost effectiveness of new oral drugs and to make comparison with the cost of infusion administration. Lokich et al. in 1996 [70] studied the comparison of costs for infusion versus bolus administration for different anti-cancer drugs and in different tumors and chemotherapy regimens. The model developed for the study, identified six cost centers: physician visit, clinic visit, laboratory, drug costs, disposable costs and durable medical equipment (DME) costs. The results showed that the major differences in costs were related the drug dosage and the toxicity profile and finally the costs for both therapies are similar. In a second work [71] the authors analysed the charges and the reimbursement for both (oral and i.v.) chemotherapy regimens. Even in this case data did not show a substantial difference between the two therapies, but there were two limitations to this study analysis: *(i)* the study was a retrospective analysis and conclusions would be more substantive if it were performed prospectively; *(ii)* the number of cycles analysed for each treatment was too small and excluded toxicity, radiology cost and hospitalization. They suggested to be careful to analyse the real cost of chemotherapy and the future studies should be prospectively, with a sufficient numbers of cycles and including hospitalisation and diagnostic studies.

To date, pharmaco-economic analysis have been carried out for different anti-cancer drugs, capacitabine [72], capacitabine/cisplatin [56], ibandronate [73, 74]UFT [75].

In the United Kingdom (UK), Cassidy et al. [72] conducted a study comparing oral capacetibine versus intravenous 5-FU/LV. For the total cost of the therapy they took in consideration the direct medical costs to the NHS (National Health System) including:

- cost of chemotherapy drugs;
- cost of visits for the study drug administration;
- cost of hospital use;
- cost of physician consultations for adverse events and for treating them;
- cost of ambulance trips.

When the *societal costs* were added, the total costs were approximately 3500 £ for the oral capacitabine versus 8500 £ of 5-FU/LV. For this reason, from an economical point of view, they termed capacitabine as a "dominant" treatment strategy. The costs saving was over 5000 £ per patients. The same study, has been carried out in the United States (US) considering similar parameters, and the results showed that the life-time costs saving with capacitabine versus 5-FU/LV was 1935 $ per patient [76].

Others studies confirmed these results and supported the benefit of the development of oral chemotherapy. In Spain, an economical assessment compared the costs of the association of oral cisplatin/capacitabine and i.v. cisplatin/fluororacil in patients with gastric cancer. The annual drug costs per person of the cisplatin/capacitabine regimen were estimated to 1333 € higher than cisplatin/fluororacil. However, if considering the drug administration and adverse events costs, the estimated annual costs reached 2688 € per person in the cisplatin/capacitabine versus 4014 € of cisplatin/fluororacil treatment [56].

In addition, in some states as the UK, there is a tax, the Value Added Tax (VAT, 17.5%) for the drugs that are purchased by hospitals. On the contrary, when the drugs are prescribed and dispensed to an individual within the community setting, are exempt of VAT. This provides significant benefits to both patients and commissioners of homecare services in terms of greater convenience, reduces drug costs and pharmacy waiting times [69].

2.3. Specific features of anti-cancer drugs to be considered for oral delivery

As we could see, oral administration of anti-cancer drugs is faced to many problems. Moreover, further aspect related to therapeutic index, dose adjustment considerations, pharmacokinetics inter and intra-individual variability and most important its potential toxicity have to be considered. Individuals have a highly variable capacity to metabolize and eliminate drugs, which originates from a combination of physiological variables, intrinsic (genetic) characteristics and environmental factors that determine each patient's phenotype. In particular, many anti-cancer drugs are characterized by unique and peculiar pharmacokinetic (PK) and pharmacodynamic (PD) profiles, and also by a narrow therapeutic window. Thus, a small variation in the administered dose can lead to severe and life-threatening toxicity in some individuals, and poor antitumor effects in others. During the last 40 years, body surface area (BSA) has certainly made a considerable contribution to dose adaptation, and it can still be correctly employed, albeit for a very limited number of anti-cancer agents. Other measures seem to be more appropriate for some agents, including PK monitoring for methotrexate and enzyme phenotyping strategies for agents like docetaxel. For the long list of anti-cancer agents where BSA-based dosing does not seem to be accurate, it is suggested that flat-fixed dosing strategies should be implemented and the routine use of normalizing the dose to BSA should be abandoned [77].

Considerations about dosing strategies and inter-intra-individual variability are of particular importance, because strictly related with the toxicity aspects. In same case, the side effects of chemotherapy can be so severe, that can escalate beyond what is tolerable or safe for the patient and therapy may be suspended until the side effects can be controlled. This period is called "therapy holiday". But, it is not highly recommended because it can increase costs as a result of wasted medications, lost treatment benefits on the cancer itself, resulting disease progression and service utilisation that occurs when patient health status deteriorated

[78]. Moreover, the several side effects are often a limiting factor for the use of same anti-cancer drug [79]. The most part of cytotoxic agents used in therapy are well known to be emetic. After i.v. administration the side effects include always nausea, vomiting and diarrhoea. The cause of these side effects, is probably linked with the a-specific action of cytotoxic agents against rapidly proliferating cells of GI tract [80]. The question now is: " will this gastrointestinal toxicities increase with the oral administration?" The answer to this question is not easy and universal. Each anti-cancer agent has its own toxicity's profile depending of its mechanism of action, the therapeutic regimen, duration of treatment and dose schedule. Intraperitoneally administration of 5-FU induces apoptosis or cell cycle arrest in intestinal cells. In the same way the administration of methotrexate (MTX) induces focal vacuolization and ultra-structural damage to the immature intestinal crypt cells. This damage lead to a morphological change associated with a reduction of the intestinal mucosal mass, mucosal protein and DNA content, body weight loss and an important increase in intestinal transit time and permeability. This entero-toxicity can be reduced with the oral co-administration of oral glutamine [79, 81]. Numerous clinical trials have been associated administration of glutamine with radiation and chemotherapy with promising results [79]. Other common side effects related with chemotherapy are stomatitis and difficulty swallowing. Even in this case, has been shown that the administration of low oral doses of glutamine after chemotherapy led to a reduction of the duration and the severity of mouth pain [80]. Oral mucositis represent a major non-hematologic complication in cytotoxic chemotherapy: pain, odynospahgie, dysgeusia, and subsequent dehydration and malnutrition reduce the quality of life of patients (for review see [82]).

Comparing different clinical study of oral versus i.v. administration of different kind of anti-cancer drugs such as vinorelbine, topotecan, etoposide, we can summarize that concerning the hematological toxicities, neutropenia and/or leucopenia were the principal side

effects in both treatment. Between the non-hematological side effects, gastrointestinal toxicities, i.e. nausea, diarrhea and vomiting, were the predominant adverse effects and they were only slightly higher after oral than i.v. administration [45, 52, 83-85]. However, in same case, it was reported a lower incidence of these side effects after oral administration of capetabicine and UFT versus i.v. administration of 5-FU and leucovorin for the treatment of colorectal cancer [52, 86].

Particularly attention concerning toxicity considerations is required when the oral chemotherapy is in association with the administration of ATP-binding cassette (ABC) transporters (or efflux pumps) or cytochrome P450 (CYP450) blockers. This inhibition presents two side of the coin. If, it leads to an important enhancement of the plasma concentration of the cytotoxic agent, with a resulting improvement of the bioavailability of drug, on the other hand, at the same time, it can lead to an enhancement of toxicity. The efflux pumps are expressed in different kind of normal cells, but in particular, three tissues need closer examination: hematological stem cells, gut end endothelial cells in the blood-brain barrier. The administration of a P-glycoprotein (P-gp) inhibitor can resulting in an exacerbation of neutropenia, incidence of intestinal mucositis and an increased amount of anti-cancer drug in the central nervous system. The same considerations hold good for the inhibition of CYP450 [87]. Van Waterschoot et al. [88] studied the toxicity of the oral administration of docetaxel in the mice lacking all CYP3A and P-gp genes (*Cyp3a/Mdr1a*$^{-/-}$), in comparison with wilde-type mice. In this fully deficient condition, mice deteriorated quickly and all died in 4 day. The most important cause was the degeneration and necrosis of the intestinal mucosa throughout the entire intestinal tract. For these reasons the association must be carefully studied; ideally the blockage must be specific and the duration need to be long enough to allow for efficacy, but short enough to limit toxicity [87]. Different clinical studies showed promising results, and the toxicity observed following oral anti-cancer drug

administration in combination with different kind of blockers, were mild or slightly more frequently than the i.v. treatment [42, 89-91].

The use of the drug delivery systems may reduce the toxicity of the anti-cancer agents. In fact, in same case the formulation can protected the GI tract against ulceration and mucositis. This was proved for the oral administration of indomethacin, a non-steroidal anti-inflammatory drugs, which use is limited by their ulcer-necrotic effects on the GI mucosa. Encapsulation of the drug into nanocapsules allowed maintaining the therapeutic activity eliminating the side effects [92-94]. Furthermore the use of oral formulations may reduce adverse effects through lowered peak plasma concentrations. Local and systemic adverse effects due to high concentrations of drug can be minimize by the use of controlled release delivery systems [95]. In term of formulation strategy, may be interesting to formulate the inhibitors of the efflux pumps and the cytochrome to control their release and their site of action. In the specific case of oral administration can be useful mucoadhesive carriers to limit the inhibition effect in the GI tract. Moreover, in the following 4.2 sections we listed different kind of polymeric excipients frequently used for the preparation of the drug delivery systems that showed an inhibitory action against both MDR mechanisms.

3. Oral delivery of anti-cancer drugs is often faced to identified formulation issues

3.1 Inadequate aqueous solubility

Most of anti-cancer drugs are a very sparingly water-soluble drugs making difficult to formulate them efficiently. Improving the apparent solubility of such substances may solve only one aspect of the problem but is obviously requested as a starting point for the design of efficient pharmaceutical formulations. In this context, different strategies were essayed to improve anti-cancer drug solubility by using pharmaceutical excipients, drug delivery systems or chemical reactions to form prodrugs. Concerning pharmaceutical excipients used for the solubilisation of anti-cancer drugs, cyclodextrins (CDs), water miscible co-solvents (ethanol, methanol, methylene chloride or acetonitrile, ~14 mM in isopropanol [96] and water-insoluble organic solvents (oils, triglycerides, vitamin E…) or their associations are generally used. Furthermore, micelles, liposomes, micro and nanocapsules, dendrimers, emulsions, microemulsions and nano-emulsions were largely used to increase water solubility of anti-cancer drugs [97-99].

One strategy to improve anti-cancer drug aqueous solubility is to use CDs. which are macromolecules composed of cyclic oligosaccharides of D-(+) glucopyranose units, all in chair conformation, linked by α-(1,4) glucosidic bonds. This molecular structure confers to cyclodextrins the shape of a truncated cone, with the outer side formed by the secondary 2- and 3-hydroxyl groups and the narrow side by the primary 6-hydroxyl groups. Thanks to this conformation, cyclodextrins have lypophilic inner cavities and hydrophilic outer surfaces. Complexation of poorly water-soluble drugs with natural or chemically-modified CDs represents an interesting strategy for increasing their apparent water solubility (For review see [100-104] and offers further possibilities for their pharmaceutical formulation ranging from

conventional to colloidal dispersions [39, 105-107] for review see [105, 108-110]. Interactions generally occur between cyclodextrins and lypophilic molecules or lypophilic groups beard by the molecules, resulting in the formation of complexes [105-107, 111]. CD/drug complexes can be much more water-soluble than the lypophilic molecule [112]. For years, CDs were used to increase the solubility of the poor water-soluble drug in order to increase their bioavailability. However, the hydrophilic external surface can represent a drawback, because it can results in a lack of affinity for biological barriers [113]. In this context, researchers focused their interest in the CD derivatives and in particular in the advantages of the CDs encapsulated into colloidal carriers. Duchêne et al. [102] showed the increase of loading capacity of poly(isobutyl cyanoacrylate) nanospheres by employing hydroxypropyl CD and the possibility of the spontaneous formation of either nanocapsules or nanospheres by nanoprecipitation of amphiphilic CD diesters. In the recent years, the use of CDs has been successfully applied for the increase of encapsulation of different lypophilic drugs [114] such as benzophenone, tamoxifen, paclitaxel [105, 115] and saquinavir [116].

3.2. Rapid intestinal transit

The enhancement of water-solubility is not enough to improve the intestinal absorption of the drug. The drug has to reach intact the absorption site and for this reasons the development of the drug delivery systems (DDS) can be useful. In the development of oral nano-carriers it is important to consider their interaction with the biological barriers after administration. The first barrier encountered by the nano-systems is the gastrointestinal lumen. They should be stable at the harsh conditions of the stomach (pH ~ 2) then to reach the small intestine where takes place 90% of the all absorption [117, 118]. In this context, the use of different kinds of polymers like polysaccarides (chitosan or dextran), acrylic polymers (Eudragit), phospholipids or cellulosic derivatives (Cellulose acetate phthalate -CAT or Cellulose acetate trimellitate-CAP and Hydroxypropyl Methylcellulose Phthalate-HPMCP),

confer to nanoparticles the gastro-resistant quality [119, 120]. If they pass intact the stomach they can reach the small intestine where the nanocarries have to diffuse across the unstirred water layer in spite of rapid transit [121].

For an oral drug administration the consequent short transit time in the gastrointestinal tract can be inadequate to release a significant fraction of encapsulated drugs, precluding in many cases the realization of a high local drug concentration over extended periods of time, hence leading to low bioavailability and poor efficacy. To overcome the short transit time, many researchers have sought to enhance the mucoadhesion in order to improve their retention at mucosal surfaces. The phenomenon of mucoadhesion is a combined result of different mechanisms. First, the mucoadhesive polymer gets wet and swells (*wetting theory*); then, non-covalent bonds are created within the mucus-polymer interface (*electrostatic and adsorption theory*). Furthermore, the polymer and the protein chains would interpenetrate (*diffusion theory*) and entangle together to form further non-covalent and covalent bonds within the mucus-polymer interface (*electrostatic and adsorption theory*) [122-124]. Furthermore, the use of mucoadhesive DDS can (i) improve the effectiveness of the drugs by maintaining the plasma drug concentration at the therapeutic levels for prolonged periods of time, (ii) inhibiting the dilution of the drugs in the body fluids and (iii) allowing targeting and localization of the drug at a specific adsorption site [125, 126].

3.3. Drugs metabolism and efflux pumps

Another problem consists on the ability of cancer cells to become resistant at the same time to different drugs, known as multidrug resistance (MDR), is a significant impediment to successful chemotherapy. Two are the principal mechanisms of resistance to anti-cancer drugs: the first one is due to genetic and an epigenetic alteration of the tumor cells themselves

that decrease the drug sensitivity, and the second one is the difficulty to deliver the anti-cancer drug to tumor cells [127].

In a particular way, the expression and the activity of ABC transporters and the metabolic CYP450 enzyme expressed in the gastrointestinal tract impair the bioavailability of the anti-cancer drug [128] [129, 130]. Physiologically, ABC transporters play an important role of body defence; they are recognized for their ability to modulate the normal absorption, distribution, metabolism, excretion, and toxicity of xenobiotic [131-133].

An important strategy to achieve the oral chemotherapy is the concurrent administration of inhibitors of the ABC transporters and CYP450 with the aim of increasing the drug bioavailability [88, 134]. However, the inhibition of the metabolism mechanism may influence the distribution and bioavailability of other xenobiotic, altering the normal equilibrium of the body.

4. How to improve the oral bioavailability of anti-cancer drugs

The approaches to overcome the obstacles, previously mentioned, are different; they include *(i)* chemical approach (prodrugs), *(ii)* the formulation approach (drug delivery systems) and *(iii)* the co-administration of P-gp and CYP3A4 inhibitors to increase the bioavailability of these compounds.

4.1. The prodrugs strategy

What is a prodrug?

Albert A. coined the term "prodrug" in 1958. He defined prodrugs as "*chemicals with little or no pharmacological activity, undergoing biotransformation to a therapeutically active metabolite*" [135, 136]. In the context of cancer therapeutics, prodrug therapy is a strategy that aims to favourably alter the therapeutic index. Prodrugs are inactive but are

converted *in vivo* to active cytotoxic compounds. Conversion to the active form can occur by a number of mechanisms, usually by specific enzymes. This strategy may be combined with specific drug design to make the drug more tumor selective [137]. Antibody-directed enzyme prodrug therapy (ADEPT) involves prodrug-activating enzymes targeted to tumors using monoclonal antibodies against tumor-associated antigens prior to the administration. The status of ADEPT studies has been largely reviewed [138, 139] and will not be discussed here.

There are several classifications of prodrugs, some of them are based on research-related criteria and others based on chemical arguments. The first one divides the prodrug in two classes:

(i) Intentional prodrugs: are compounds obtained by chemical derivation or modification of a known active agent in order to improve its pharmaceutical and the pharmacokinetic properties. The major part of prodrugs belongs to this type.

(ii) Fortuitous prodrugs: are prodrugs not designed as such. The biotransformation is fortuitous, and their discovery appends only after isolation and testing of the drug's metabolites.

From a chemical point of view, it is possible to distinguish four major classes:

(i) Carrier-linked prodrugs: the active agent is linked to a carrier/promoiety and the activation occurs by chemical reaction;

(ii) Bioprecursors: a compound is metabolised by molecular modification in a new compound, which represent the active drug.

(iii) Macromolecular prodrugs: the promoiety is a macromolecule such as a polyethylenglycol;

(iv) Drug-antibody conjugates: the carrier is an antibody raised against tumor cells [140].

When a medical chemist designs a new prodrug, he is determinated to improve the bioavailability and to decrease the side effects of the lead compound. The targeted objectives for developing oral prodrugs are (i) to solve the formulation problems namely the solubility and the stability [141], (ii) to improve the drug absorption, (iii) to prolong the duration of the action and finally (iv) to decrease the systemic toxicity of the native molecule [136].

In this context, in the research area on prodrugs, three are the aspects that must be considered to achieve this objective: pharmaceutical, pharmacokinetic, and pharmacodynamic aspects (Fig. 1).

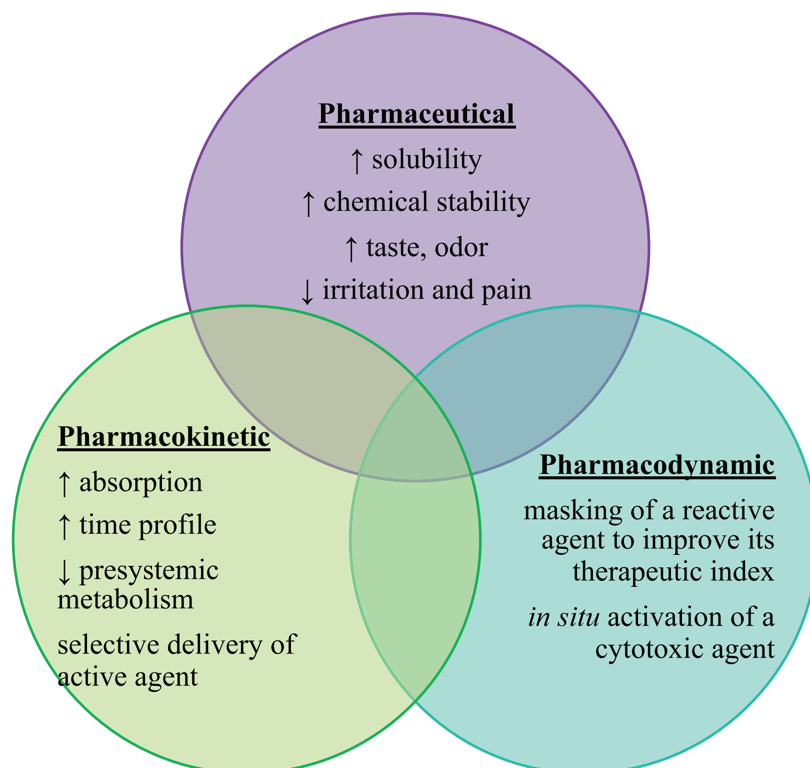

Figure 1. Objectives in prodrug research

5-FU prodrugs

Since its discovery in 1957, 5-fluorouracil (5-FU) was used in cancer therapy [142]. 5-FU remains one of the most commonly prescribed chemotherapeutic agents. It is active both as a single agent in colorectal cancer [48, 75, 143, 144] and as part of combination regimens for breast, head/neck, and upper gastrointestinal malignancies. 5-FU has been administered in several schedules, including daily and weekly intravenous (i.v.) bolus as well as by continuous i.v. infusion. Continuous i.v. infusion appears to yield improved response rates and overall survival, with fewer adverse effects compared with i.v. bolus dosing. However, 5-FU has numerous drawbacks. First of all, it is relatively toxic, causing myelosuppression, oral mucositis, nausea, diarrhea and vomiting; in the case of continuous regimen, the hand-foot syndrome (dermal pain in hands and foot) [48] and, even if less of 10% of the cases, cardiotoxicity [145]. Gastrointestinal disorders are mainly due to the phosphorylation of 5-FU in the digestive tract. Toxicity also derives from the lack of selectivity of the drug toward tumors. Its efficiency is limited by the short $t_{1/2}$ of 5-FU in plasma, by its low i.v. bioavailability due to the activity of DPD (enzyme dihydropyrimidine dehydrogenase), and by the resistance to 5-FU of some tumors with strong expression of thymidine phosphorylase (TP) or low reserves of reduced folates. Its mode of administration is also problematic. I.v. administration is not without risk of complications (venous thrombosis or infection around the catheter). Different routes of administration and a variety of schedules have been used to increase the efficacy of 5-FU with a modest response rates [146]. The use of oral 5-FU was abandoned decades ago because of its irregular absorption. Plasma levels of 5-FU are quite unpredictable after oral administration with marked intra- and inter-individual differences due to the variable activity of DPD, especially in the gastrointestinal mucosa [147, 148].

For these reasons, one of the challenges of cancer research is the development of prodrugs of 5-FU that diminish or circumvent some of these disadvantages: reduction in toxicity by avoiding certain routes of degradation (prodrugs that are not a substrate for the enzymes of degradation) or by targeting the tumor site (prodrugs that release the drug selectively in tumor cells); enhancement of activity by reducing catabolism (use of DPD inhibitors) or by increasing anabolism, and improvement in quality of life of the patient by developing oral prodrugs.

The prodrugs of 5-FU are characterized by a pyrimidine ring with a fluorine atom in position 5. They differ from 5-FU in a variety of chemical alterations. Their main benefit is related to the oral route of administration. They are designed to be well absorbed intact from the gastrointestinal tract and subsequently enzymatically converted into 5-FU in the liver or within the tumor itself, in order to expose the tumor to 5-FU for a longer time but at lower concentrations than those observed after an i.v. bolus, hence minimizing toxicity. Orally administered fluoropyrimidines thus provide protracted 5-FU delivery, which offers advantages that include schedule flexibility and reductions in professional health care resource requirements, administration costs, and toxicity-related hospitalization.

We can distinguish three generations of prodrugs:

- The first generation includes 5-fluoro-2'-deoxyuridine (5-FdUrd; Floxuridine®). The main limitation of 5′d5-FUrd derives from its gastrointestinal toxicity, attributed to liberation of 5-FU in the small intestine under the action of thymidine phosphorylase (TP) [149] a tumor-associated angiogenesis factor [150].

- The second generation includes the drug already used in clinic such as Ftorafur (FTO, 1-(2-tetrahydrofuryl)-5-fluorouracil, Tegafur or Futraful) and 5′-deoxy-5-fluorouridine (5′d5-FUrd, doxifluridine or Furtulon®), that shown a broad spectrum of antitumor activity when administered intravenously or orally [151]. It has been used in the treatment of adenocarcinomas and appears to have an efficacy similar in compared with i.v. 5-FU, but its associated neurological toxicities have limited enthusiasm for this drug [152].

- The third generation compounds include the enzymatically activated prodrug capecitabine, and the DPD-inhibitory compounds (UFT [FTO + uracil] eniluracil, S-1 [FTO plus 5-chloro-2,4-dihydroxypyridine plus potassium oxonate], and BOF-A2). We will focus our attention on compounds of this third generation. Details on the third generation compounds clinical trials can be easily found in the literature [48, 75, 143, 152-154].

Capecitabine (XelodaTM, Roche Pharmaceuticals; Nutley, NJ) is an oral fluoropyrimidine that is now commercially available. It is an inactive oral prodrug of 5-FU and is rapidly and almost completely absorbed from the intestine [155, 156]. It is subsequently converted into 5-FU in a three-stage mechanism involving several enzymes. In the first step, this prodrug is metabolized into 5′-deoxy-5-fluorocytidine (5′-dFCR) by hepatic carboxylesterase. 5′-dFCR is then deaminated into 5′d5-FUrd by cytidine deaminase, mainly localized in liver and tumor tissues [157]. Finally, 5′d5-FUrd is transformed into 5-FU under the action of thymidine phosphorylase (TP) [158], an enzyme with higher activity in tumor than in normal tissues. Higher levels of 5-FU are thus produced within tumors with minimal exposure of healthy tissue to 5-FU [157]. Nowadays, FDA has approved capacitabine as a

first line therapy in patients with metastatic colorectal cancer, as a single agent in metastatic breast cancer patients who are resistant to both anthraxcycline and paclitaxel-based regimens or when further anthracycine treatment is contraindicated. This prodrug has shown promising results alone or in combination with other chemotherapeutic agents in prostate, breast, pancreaticobiliary, gastric, renal cell and head and neck cancers [159]. The most commonly reported toxic effects of capecitabine are diarrhea, nausea, vomiting, stomatitis and hand–foot syndrome. Capecitabine has a well-established safety profile and can be given safely to patients with advanced age, hepatic and renal dysfunctions [160].

Ftorafur-uracil (UFT) combination: (OrzelTM, Bristol Meyers Squibb; Princeton, NJ) is a combination of tegafur, a prodrug of 5-FU, and uracil (modulator of the catabolism of 5-FU) in a molar ratio of 1:4 [161]. Tegafur (ftorafur, 1-[2-tetrahydrofuryl]) was originally developed by Japanese researchers in the 1970s in an attempt to improve the therapeutic index of the fluoropyrimidines [162]. Tegafur is a slow-release prodrug formulation of 5-FU is converted to 5-FU by hepatic cytochrome P450 pathway [163]. It has been shown that UFT was able to improve the post-operative survival after surgery for non-small cell lung cancer (NSCLC). The 5-years survival rate in patients treated with UFT was 76.5 %, significantly higher than in the control group (58.6 %) [164]. However, the clinical use of tegafur was limited due to significant central nervous system toxicity (depression, headache, lethargy, and dizziness) due to accumulation of the metabolite γ-hydroxybutyrate [165]. Oral tegafur is readily absorbed through the GI tract resulting in a plasma half-life of 5-12 hours. By combining tegafur with uracil in a 1:4 molar ratio, the compound known as UFT was created. Uracil enhanced the half-life of converted 5-FU by competing for its degradation by DPD, which is the rate-limiting enzyme in the catabolism of 5-FU. This leads to higher intracellular concentrations of 5-FU with increased antitumor activity in preclinical models [166] and in

tumor tissues when given to humans [167]. Many reviews in the literature summarize clinical UFT trials [48, 75, 143, 154, 168].

The association of UFT with leucovorin. In order to enhance the therapeutic efficacy of Tegafur, the association UFT+ leucovorin (LV, folinic acid) has been developed. Leucovorin is a metabolism modulator and can act as a pharmacological source of reduced folates that are important cofactors of the cytotoxic mechanism of 5-FU. UFT/LV regimen consist in daily oral dosing of 300 mg/m^2 /day plus oral LV (75-90 mg/ m^2 /day) for 28 day every 5 week, In order to mimic the pharmacology of continuous infusion of 5-FU [161]. Although some clinical studies suggested that high doses of LV are necessary for an optimal biomodulation, others phase I-II clinical trials showed that lower LV doses offered similar efficacy [169, 170]. Several studies in the literature compared the oral UFT/LV with the i.v. 5-FU in combination with leucovorin. All studies made similar conclusion: the outcome of patients treated with UFT/LV did not differ significantly from that of patients treated with i.v. FU/LV, similar values have been obtained in terms of median survival, tumor response, duration of response, and time of response in both treatments. However, UFT/LV significantly improved safety compared with 5-FU/LV. Diarrhea, nausea and vomiting, as well as, stomatitis, mucositis and myelosuprression were less frequent. The patients experienced fewer episodes of febrile neutropenia and documented infection. Moreover, 5-FU/LV regimen needed more frequent concomitant medications, including use of antibiotics, growth factors and antiemetic [52]. The efficacy and pharmacokinetic parameters, as well as the toxicity of UFT/LV were compared in different patient populations (Japanese and American) and the oral regimen was considered to have similar activity against metastatic colorectal cancer [171].

S-1 (Taiho Pharmaceutical Ldt.; Tokyo, Japan) is a combination of tegafur, 5-chloro-2,4-dihydroxypyridine (CDHP) and potassium oxonate (OXO) in a molar ratio of 1:0.4:1. S-1 was designed to enhance anti-cancer activity and reduce gastrointestinal toxicity trough the combination of two modulators: CDHP inhibits activity of DPD and OXO prevents intestinal phophorylation of 5-FU by pyrimidine-phosphoribosyl-transferase [172]. The development of S-1 in colorectal cancer is primarily being pursued at this time by the European Organisation for Research and Treatment of Cancer (EORTC) and the Early Clinical Studies Group (ECSG). In their first reported experience [173], 36 patients were treated with S-1 at a dose of 35 mg/m^2 twice daily after meals (the first four patients at a dose of 40 mg/m^2 twice daily had gastrointestinal toxicity). The most common side effects at this dose were diarrhea, nausea, fatigue, and anorexia. Four patients had a partial response and further development work is underway. Phase I-II study were carried out to evaluate the efficacy of S-1 in patients with different kinds of solid tumors, such as non-small-cell lung cancer (NSCLC), advanced gastric cancer and pancreatic cancer showing promising results to use S-1 as a single agent or in combination with other drug [174-179]. Many other reviews in the literature summarized clinical S-1 trials [180-183].

Other 5-FU prodrugs

In addition to the 5-FU prodrugs, mentioned above, it is possible to find in the literature other prodrugs such as Ro 09-1390 (N^4-Trimethoxybenzoyl-5'-fluorocytidine), a prodrug of 5'-Deoxy-5fluorouridine (Doxifluridine) (5'-DFUR), which is converted to 5-FU by pyrimidine nucleoside phosphorylases in the tumors as well as in the intestinal tract.

Doxifluridine is an active cytotoxic agent against the stomach, colorectal and breast cancer, but at high doses it caused intestinal toxicity, and diarrhea was the dose-limiting

factor in clinical trials. A study in which the doxifluridine was associated at low-dose of leucovarin, showed a diminution of its toxicity and an augmentation of efficacy [184].

Ro 09-1390 presented the advantage to be converted into 5'-DFUR after it entery in the bloodstream, causing little intestinal toxicity. Moreover, Ro 09-1390 was less toxic than 5'-DFUR in terms of lethality and weight loss, maintaining similar antitumor activity [185]. Other prodrugs of 5-FU are designed for different routes of administration. Some examples are the lipophilic alkylcarbonyl-5-FU prodrugs or compounds such as 1-(2'-oxopropyl)-5-FU (OFU 001) developed to be administrated topically [154].

Platinium-based prodrugs

Among the platinum-based drugs, a new prodrug is the great interest: **satraplatin** [*bis*-(acetato)-ammine dichloro-(cyclohexyl-amine) platinum VI), JM216]. This compound showed anti-cancer activity against several platinum sensitive and resistant cell lines including lung, ovary, cervix and prostate. It is readily absorbed by the gastrointestinal mucosa and once in the blood stream is reduced to yield to more than six different products. [25]. After administration, its major active metabilite (JM 118, *cis*-ammine dichloro (cyclohexylamine) platinum (II)) binds the DNA and induces the cell cycle arrest and apoptosis. Compared with the other platinum drugs, satraplatin binds the DNA in a particular way in order to form stable asymmetrical ligands, which contribute to its unique properties. Since 2004, when the FDA approved oxaliplatin for colorectal cancer, the field of platinum-based drugs development seemed to have hit a wall of waning interest. With development of this new compound, the platinum-based drugs promise to have resurgence to the forefront of cancer therapy. *In vitro* and *in vivo* studies have shown antineoplastic activity of satraplatin and its active metabolites against prostate, ovarian and colon cancer. Clinical trials were

carried out to study the potential of satraplatin; phase I clinical trial for pharmacokinetics and toxicity showed that the dose-limiting toxicities were thrombocytopenia and neutropenia, which were reversible and noncumulative. The most common grade 3 to 4 toxicities were gastrointestinal, including nausea, vomiting and diarrhea, each occurring in ~10 % of patients. Phase II and III clinical trials have been conducted to assess the efficacy of satraplatin, alone or in combination with other cytotoxic therapies, for the treatment of various cancers. It showed promising activity in prostate, small cell lung cancer and in association with radiotherapy in non-small cell lung cancer [47].

A further platinum prodrug of particular interest is **picoplatin** (*cis*-amminedichloro, 2-methylpyridine, platinum (II); JM473). It seemed to be active against a wide range of cisplatin-, oxaplatin-resistant cells *in vitro*, and *in vivo*, showed antitumor activity by both intravenous and oral routes. Clinical trials are ongoing for picoplatin in combination with docetaxel against prostate cancer and with 5-FU with LV against colorectal cancer. As, satraplatin, the dose-limiting toxicities were reversible thrombocytopenia and neutropenia, but no marked of nephrotoxicity or neurotoxicity were observed [25, 186]

Placlitaxel prodrugs

An interesting perspective about paclitaxel prodrugs has been written by Skwarczynski et al. 2006 [187]. The authors present various advantages and achievements for paclitaxel prodrug strategies including carrier linked prodrugs, bioprecursors, site-specific chemical delivery systems, macromolecular prodrugs, and paclitaxel antibody conjugates. Concerning the oral prodrugs, a promising water-soluble prodrug of paclitaxel is the PEGylated paclitaxel prodrug. The bioavailability and pharmacokinetic parameters of PEGylated paclitaxel prodrug after oral administration were studied and compared with the paclitaxel. The mean absolute bioavailability (AB%) of paclitaxel by the prodrug was 6.3%,

4-fold higher than the oral paclitaxel (1.6%). Probably the significantly enhancement of paclitaxel bioavailability by using the prodrug, is due to the avoidance of the P-gp efflux mechanism and the reduction of the CYP450 metabolism in the intestinal cells [188]. Moreover, the co-administration of inhibitors of P-gp efflux pump and CYP450 (naringin or quercetin) led to a further increase of the bioavailability of this prodrug [189, 190]. Recently, Lee et al. developed prodrugs of paclitaxel and his synthetic analogue, docetaxel, in form of conjugate with low molecular weight chitosan (LMWC), in which LMWC acted as a carrier [191, 192]. In both case, the two systems exhibited promising properties for the oral chemotherapy: the conjugate systems showed several important features including *(i)* increasing of water solubility of the drugs; *(ii)* mucoadhesive property in the gastrointestinal tract; *(iii)* ability to by-pass the P-glycoprotein efflux pumps and the CYP450 mediated metabolism, enhancing the bioavailability and the antitumor activity of both cytotoxic agents.

Polymer-drug conjugates

An important group of prodrugs initially proposed by Helmut Ringsdorf in 1975 [193] is the **polymer prodrugs** or **polymer-drug conjugates**. They consist of hydrophilic polymer linked via a spacer or a linker to a hydrophobic small molecule [154]. The major polymers of particular interest for this kind of conjugates include N-(-2-hydroxypropyl)methacrylamide (HPMA) copolymer, poly(glutamic acid) (pGlu), PEG, dextran, and cyclodextrin-based polymer. Different review in the literature developed widely the polymer prodrugs [154, 194-199]. All these works are of particular interest, but in the major part of them, the polymer drug conjugates are developed for the parenteral administration. Few examples of polymer prodrugs orally administrated concern paclitaxel and docetaxel prodrugs previously mentioned. The why this kind of prodrugs is widely studied for the parental administration and not for the oral administration, may result from the principle behind these prodrugs. Generally, macromolecules are inclined to accumulate preferentially in tumors than in the

normal tissues, because of enhanced permeability of tumor tissue vasculature and their deficient lymphatic system. Therefore, the polymer-drug conjugate can enter in the tumor cells where specific enzymes cleave the prodrug linker in order to release the cytotoxic agent. The effect of tumor accumulation of a polymer conjugate is named enhanced permeation and retention effect (EPR) [154, 197, 199]. For all these reasons, it is obvious that a parental administration is more appropriate than an oral administration, by which the polymer-drug conjugate cannot reach the tumor tissue due to the intestinal barrier. The purposes of both administration routes are different: with the oral therapy the aim is to enhance the intestinal absorption of the drug and not to target the drug on the tumor tissue. This is the case of the docetaxel, placlitaxel prodrugs or other polymer conjugates with molecules presented a low oral bioavailability [200].

Others prodrugs

Others interesting prodrugs came from the topoisomerase I inhibitors family. The parent compound is the camptothecin, an alkaloid extracted from the oriental tree *Camptotheca acuminata*. Camptothecin acts through reversible inhibition of DNA topoisomerase I, a nuclear enzyme responsible of replication, transcription and subsequent strand religation of DNA. The use of this compound was limited due to severe and unpredictable toxicities including myelosuppression, diarrhea and hemorrhagic cystitis. For this reason, different analogues, topotecan, irinotecan (CPT-11), 9-amono-20(s)-camptothecin (9-AC), 9-nitrocamptothecin (9-NC) or Rubitecan and Lurtotecan, were synthesized to reduce toxicity and enhance antitumor efficacy [201, 202]. Among these compounds, two are prodrugs: irinotecan (CPT-11) and rubitecan (9-nitrocamptothecin (9-NC)).

Irinotecan (CPT-11) is a prodrug of SN-38, which is 100-1000 fold more cytotoxic than the parent drug. This molecule has shown a broad spectrum of antitumor activity in

preclinical and clinical studies in various cancers, including colorectal, lung, cervical and ovarian cancer. The conversion of CPT-11 to SN-38 is due to various enzymes with major contribution of the carboxylrsterases (CEs). Because these enzymes are widely expressed in the human liver and the gastrointestinal tract, it is possible to administer irinotecan by oral route [203]. Phase I studies investigated the oral irinotecan formulated as semisolid matrix capsules, alone or in combination with capecitabine in patients with advanced solid tumor. A dose of 70 mg/m^2/d of irinotecan for 5 consecutive days every 3 weeks, has showed to be safe and recommended for further studies [204]. In the same way, the combination irinotecan/capecitabine seemed to be safe, feasible and demonstrated antitumor activity with a schedule regimen of irinotecan 50 mg/m^2/d given daily for 5 consecutive days, and capecitabine 1,000 mg/m^2/d twice daily for 14 consecutive days, repeated every 3 weeks. In both studies, the most important toxicities were delayed diarrhea, which was manageable by the use of loperamide, nausea, anorexia and colitis. Hematologic toxicity was mild [204, 205]. Recently, two consecutive prospective clinical trials of the Soft Tissue Sarcoma Committee of the Children's Oncology Group (STS-COG), have demonstrated that 42% of children affected by rhabdomyosarcoma, treated with two cycles of irinotecan (20mg/m^2/d for 5 days, for 2 weeks), had a favourable response. In the second study, irinotecan was combined with vincristine, which increased the window response rate and reduced the rate of progression. The main adverse effects were diarrhea and abdominal pain [206]. Others phase I-II clinical trials were carried out to evaluate the efficacy of oral administrated irinotecan alone or in combination with others drugs against different solid tumors [207-210].

Rubitecan (9-nitrocamptothecin, 9-nitro-20(S)-captothecin, 9-NC, RFS 2000, Orathecin^{TM}) is a prodrug of 9-amono-20(s)-camptothecin (9-AC) [201]. Preclinical studies showed that rubitecan had an antitumor activity against a broad spectrum of tumor. These results were obtained *in vitro* and *in vivo* by using human tumor xenograft model.

Unfortunately this level of activity is not translated into similar activity in clinical trials [211]. Phase II studies were performed in patients with advanced colorectal cancer (CRC), non-small cell lung cancer (NSCLC) and metastatic head and neck cancers. Rubitecan was administred orally at the range doses of 1.5-2 $mg/m^2/d$ for 5 days per week. In all cases, Rubitecan was well tolerated, but clinically inactive. The majority of the adverse events, myelosuppression, gastrointestinal toxicity and fatigue, were mild to moderate intensity [212-215]. However, it has shown a sufficient activity against pancreatic and ovarian cancer alone or in combination with other drugs or radiation therapy [216-218].

Finally, another interesting prodrug is **tributyrin**. It is a prodrug of butyrate, a small polar compound able to produce terminal differentiation and apoptosis in a variety of cell line *in vitro*. Differentiation therapy has the aim to produce a more differentiated state in which the cells were not able to proliferate, and may even function as mature cells. However, the mechanism of action of butyrate is unknown. Some authors proposed different mechanisms including, reduction in anaerobic glycolysis, modulation of gene expression, induction of apoptosis and altered expression of cell surface molecules [219]. Tributyrin was designed to overcome many of the problems of the parental drug. Interestingly, this molecule can be administrated orally. Clinical and pharmacological studies were carried out in patients with solid tumors. The doses required to achieve plasma concentration levels > 100 μM, were highs: 150-200 mg/kg three times daily. Despite this high dose, tributyrin was well tolerated with a high rate of compliance. Toxicity was minimal and no dose-limiting toxicity was observed. However, the best result was a stabilization of the disease without evidence of progression [220].

4.2. Modulation of drug metabolism with co-administration strategy

The ATP-binding Cassette drug transporters

ABC transporters are proteins expressed in normal tissues. They play an important role in the absorption, the distribution and the elimination of many substances, including the excretion of toxins from the liver, kidney, gastrointestinal tract and the limit permeation of toxins to vital structures, such as brain, placenta and testisv [221-224]. The ABC transporters are a super-family divided in different sub-families: from ABCA to ABCG. They contain two sets of transmembrane domains, typically containing six membrane-spanning α-helices and all bind ATP through two nucleotide binding folds (ATP-binding domains) [225]. The hydrolysis of the transporter's ATP, allowed to keep the energy to drive the transports of various substrates against a concentration gradient to reduce the intracellular concentration of the substrate [87]. In the gastrointestinal tract we can find three major subfamilies of ABC transporters, such as P-glycoprotein (MDR1, gene ABCB1), the cancer resistance protein (MRPs, gene ABCCs), in particular MRP2 [226] and the breast cancer resistance protein (BCRP, gene ABCG2) (Fig. 2) (See Box 1).

Box 1: **ATP-binding Cassette drug transporters (ABC transporters)**

P-glycoprotein

P-glycoprotein (P-gp, mdr1, ABCB1) is a 1280 amino acid long [227] of 170 kDa. It consists on two symmetric domains, each one contains six transmembrane segments and intracellular ATP binding site [9, 10, 23]. There are various models to explain the mechanism of xenobiotic extrution, but the exact site of substrate interaction with the protein it is not well known. Three are the models of efflux mechanism proposed: pore model, flippase model and hydrophobic vaccum cleaner (HVC) (Fig. 3) [227].

Rosenberg et al.[11, 228], in their recent works, compared a three dimensional structure for P-gp in the presence of a non-hydrolyzable ATP analogue (AMP-PNP, adenylyl-imidodiphosphate) with the three-dimensional structure of P-gp in the absence of nucleotide. In this case, two transmembrane domains (TMDs) form a barrel-like structure with a central pore, open at the extracellular face of the membrane and closed at the cytoplasmic face. The TMDs of P-gp, upon binding nucleotide, show a conformational change in three discrete domains that open one side of the pore throughout all the depth of the lipid bilayer. In this way the TMDs allow the direct access of hydrophobic drug from the bilayer to the central pore prior the extrusion. Both ATP-binding and hydrolysis were essential for functioning of P-gp, where one molecule of the drug is effluxed at the expense of two molecules of ATP [227]. The most common substrates of P-gp come from different families of anti-cancer drug, the anthracycline family, the taxanes, the vinca alkaloid, and other anti-cancer drug like VP-16, mitoxantrone and Actinomycin D [223].

Cancer resistance proteins (MRPs)

If P-gp was discovered in 1976, the first MRP, MRP1, was discovered only 18 years ago, in 1992. Therefore, it is not well know much about its clinical significance [14, 229]. MRPs are divided in seven sub-families (MRP1-MRP7); two additional members, MRP8 and MRP9, have been reported more recently [230]. The polypeptide is 171 kDa and contains two nucleotide-binding domains, each preceded by a multi transmembrane helices region [231, 232]. As show in Figure 2, some members of this family (MRP1, MRP2 MRP3, MRP6 and MRP7) present a similar structure that P-glycoprotein, but it has an additional membrane spannig domain (MSD_0) composed of five transmembrane helices resides, an intracellular loop (L_0) and an extracellular N-terminus [233]. Kou-Cheng Peng at al. [234] showed that the MRP proteins are present in the cells delimiting the bases of the crypts of normal mouse and human small intestine, mainly in the basolateral membranes, except for the MRP2 that is localized in apical membranes of the proximal tubule of the kidney and of the duodenum and jejunum [235]. The localization in the apical part of small intestine confers an important role to MRP2 for the oral bioavailability of anti-cancer drugs [226].

The substrate specificity of the MRP is different for each sub-family. MRP1 is able to transport organic anions and diverse glutathione, glucuronate and sulfate conjugates and, while MRP2, confers resistance to vinca alkaloids (vinblastine and vincristine), and anthracycline (doxorubicine and daunorubicine) [229]. In addition, recent *in vitro* studies identified that paclitaxel and docetaxel are efficiently transported by MRP2 [236]. *In vivo* functions of MRP3 have not been established; the substrates are similar to the two first members of the family, glucoranate and glutathione conjugates are good substrates, but also monoanionic bile [237]. Concerning MDR, the third pump can confer resistance to methotrexate etoposide and teniposide [128]. The fourth pump of the group, MRP4, presents a unique characteristic consisting on the ability to transport a range of endogenus molecules that have a key role in cellular communication and signaling, including cyclic nucleotide, ADP, eicosanoids, urate and conjugated steroid hormones. As a drug transporter, MRP4 stands out for its broad substrate spectrum covering antivirals, antibiotic, cardilvascular and cytotoxic (methotrexate, 6-thiguanine, 6-mercaptopurine, topotecan) [238]. In the same way that MRP4, MRP5 is able to transport the cyclic nucleotides cAMP and cGMP [239]. Recent works extended the substrate Spectrum of MRP5 to folic acid and several antifolates, the classic methotrexate and two novel generations (raltitrexed and OSI-7904) [240]. Concerning the sixth member of the family, Belinsk MG. et al [241] reported that the protein confered low levels of resistance to several common anti-cancer drugs, including etoposide, doxorubicin and daunorubicine, as well as actinomycin. They also found the ability of MRP6 to confer a low level of resistance to cisplatin. Finally, the last three members of the family, MRP7 MRP8 MRP9, are at the early stages of investigation. A distinctive feature of MRP7 is its ability to confer resistance to docetaxel. In addition, three or fourfold levels of resistance were observed for paclitaxel, vincristine and vinblastine. The drug resistance capabilities of MRP8 are similar to those of MRP4 and MRP5 and concerning MRP9, the drug resistance ability and its physiological functions are not well known [139, 242].

Breast cancer resistance protein (BCRP)

The group of Doyle et al., in 1998 allowed to identify the BCRP transporter in the mitoxantrone-resistant breast cancer cell line (MCF-7/MX) [243]. It is the second member of subfamily G designed as ABCG2. Two features distinguish the BCRP from the other efflux pumps. The first one is that, unlike P-gp and MRP, which are organizes in 2 repeated halves, BCRP proteins are half transporters composed of a single NBD followed by one MSD (Fig. 2). There is increasing evidence to suggest that BCRP may operate as either homodimers or heterodimers. The second feature is the configuration of the protein in which the NBD precedes the MSD, whereas P-gp or MRP has an opposite domain arrangement, the MSD that follows the NBD [244].

BCRP is localized primarily in the plasma membrane in accordance with its capacity to efficiently extrude drug substrates from the cell. It is expressed in several tissues with highest levels in the placenta and lower levels in the liver, kidney, small intestine, brain, and ducts and lobules of the breast. The substrate specificity of BCRP shows overlap with that of P-gp, suggesting that two transporters have a similar role in the pharmacokinetics of substrates drugs. Thereby BCRP might affect the oral absorption, tissue distribution, and/or hepatobiliary and intestinal elimination of xenobiotic substrates. Furthermore, its high expression in the placenta may be relevant in protecting the fetus from drug exposure [75, 144, 245, 246]. The substrates of BCRP are chemotherapy drugs belonging to antracyclines family (daunorubicine, doxorubicine), antracenes as mitoxantrone, camptothecin derivates (topotecan; SN-38, irinotecan) and nucleoside analogs (AZT, prazosin, flavoperidol), but also organic molecules such as fluorophores, organic anions and a variety of chemical toxicants [48, 75, 244].

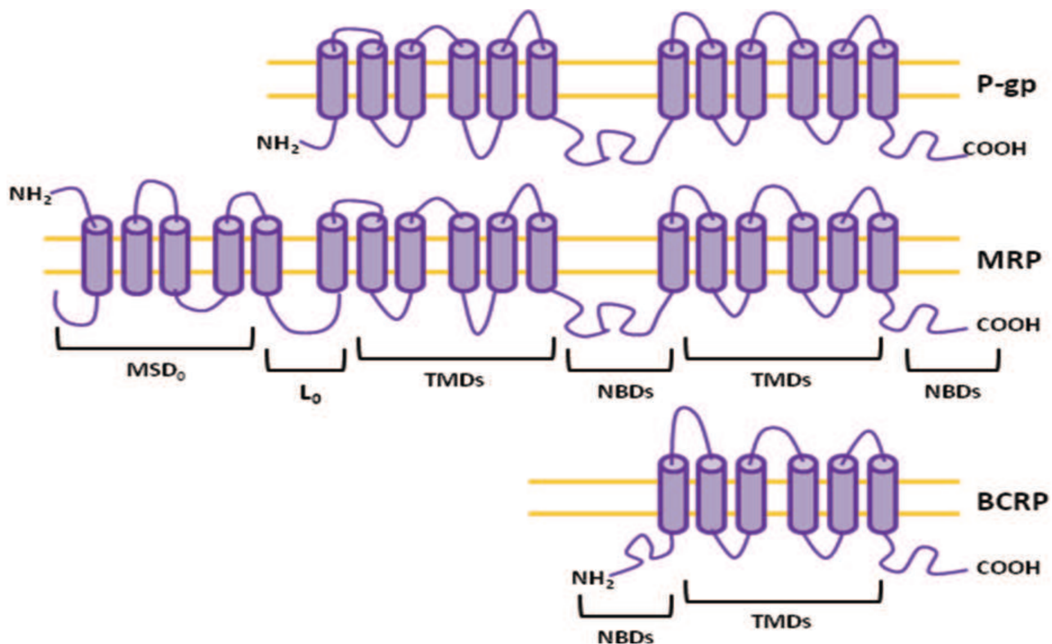

Figure 2. Structures of three categories of ABC transporter. TMDs: transmembrane domains; NBDs: ATP-binding sites; MSD_0: membrane spannig domain; L_0: intracellular loop. Adapted from [247]

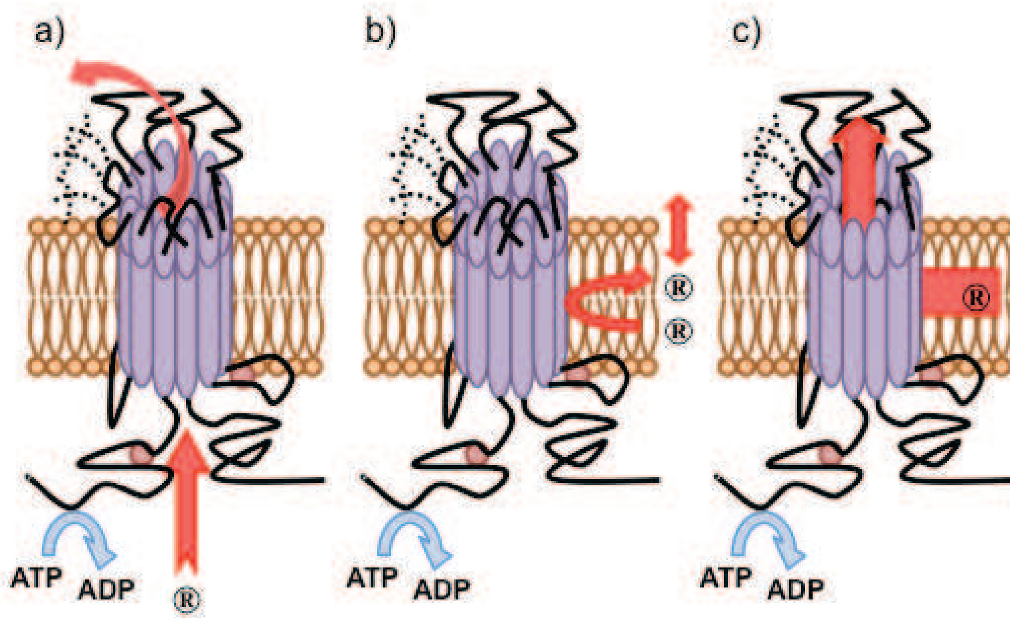

Figure 3. Proposed mechanism for the Pg-p transporters: **a)** ***Pore model***: drugs associated with P-gp in the cytosolic compartment and are transported out through a protein channel. **b)** ***Flippase model:*** P-gp acts as a drug exporter by flipping drugs from the inner leaflet of the plasma membrane to the outer leaflet against a concentration gradient. **c)** ***Vacuum Cleaner Model*****:** intra-membranous molecules that do not belong to the membrane are recognized by P-gp, enter P-gp from the membranous site and leave the cell. Adapted from [227]

Sparreboom et al. [248] showed the contribution of the P-gp to the bioavailability of paclitaxel, compared the bioavailability of the drug in ABCB1 knockout and wild-type mice. Their results showed that the disruption of the *mdr1a* P-gp gene in mice resulted in a drastic alteration in the pharmacokinetics of paclitaxel. The bioavailability, after oral administration, increased from 11.2% in the wild-type to 35.2% in the ABCB1 knockout mice. The ability to transfer paclitaxel from the circulation into the gut is greatly reduced, establishing a major role for the P-gp in this process. The fact that the bioavailability of the drug did not increase to 100%, is due to the first-pass hepatic extraction and/or metabolism in the liver and intestinal mucosa. This study represented a proof of concept that the inhibition of the efflux pump can really improve the bioavailability of oral drugs, in particular anti-cancer agents. To achieve this object the co-administration of compounds able to block the pump named the MDR inhibitors seems to be an interesting possibility.

Nowadays, several molecules are identified as MDR blockers or modulators and they are divided in four generations here enumerated in Table III.

Tableau III. Principals p-gp inhibitors

First Generation	**Second Generation**	**Third Generation**	**Polymeric Inhibitors**
Verapamil Cyclosporine A Ketoconazole Lidocaine Quinidine Erythromycin Tamoxifen Reserpine	PSC 833 R-verapamil KR30031 VX-710 MS-209	GF120918 LY335979 OC144093 XR9576	PEG Pluronic P85 Tween® 80 Chitosan-4-thiobutylamidine Glutathion Cysteine TPGS 1000

The first generation includes drugs currently used in clinical for different kinds of diseases and which showed an additional P-gp inhibitory property. The typical examples are verapamil and cyclosporin A. A clinical study showed that the co-administration of

cyclosporine A increased the oral bioavailability of paclitaxel from 4% to 47% [89]. In the same way, the administration of 15 mg/kg of cyclosporine A 30 minutes before the oral administration of docetaxel (75 mg/m^2), was able to increase the absolute bioavailability from 8% (docetaxel alone) to 90% (docetaxel/cyclosporine) [42]. It is clear that this kind of compound, having its own pharmacological property, cannot be used as auxiliary agents in clinical applications, but they are often used as a reference or a control inhibitors in studies focusing on the identification of efflux pump substrates or to evaluate the activity of a novel inhibitors and also to prove the effective improvement of the oral drug bioavailability [249-254].

The second generation includes derivates of the first generation compounds selected or designed to reduce the non-MDR pharmacological actions. An example is the non-immunosuppressive cyclosporine analogue PSC 833 or [3'-keto-Bmt1]-[Val2]-cyclosporine or Valspodar. In comparison with the parent agent, valspodar seemed to be more potent and less nephtoxic, hepatotoxic and neurotoxic [255]. Van Asperen et al. [256] in 1997, showed that the oral bioavailability of paclitaxel increased more than 10-folds in mice treated with the PSC 833. Further studies confirmed its ability to modulate the MDR. The most part of phase I-II clinical trials were carried out to investigate the safety of the combination of cytotoxic agents (paclitaxel, doxorubicin and cisplatin), administered intravenously, with oral valspodar. Some of them noted that valspodar altered the pharmacokinetic of the drugs, in particular paclitaxel and doxorubicine, with a following increase of the side effects as myelosuppression, providing a rationale for dose reducing of paclitaxel when administered in combination with the inhibitor [255, 257, 258]. Furthermore, in a recent phase III study patients with advanced ovarian or primary peritoneal cancer were treated with the association paclitaxel/carboplatin alone or in combination with valspodar. Results showed that the

addiction of valspodar did not improve the time to disease progression (TTP) or the overall survival time (OS) [259]. Another example is verapamil analog KR30031 able to increase the paclitaxel oral bioavailability about 7.5-fold as compared with the paclitaxel alone [253]. Unfortunately, these compounds, *in vivo* studies did not show a significant inhibition of MDR at tolerated doses [260].

The third generation includes the drugs with a high affinity and specificity to MDR transporters. The inhibition activity, reached at relatively low concentrations, is the only pharmacological action of these molecules. *In vivo* studies showed that the plasma AUC of paclitaxel achieved in wild-type mice when given with GF120918 became comparable with that in knockout mice [38]. The same authors, in a following *in vitro* and *in vivo* studies confirmed that GF120918 is one of the most effective inhibitors in comparison with other inhibitors from different generations (cyclosporine A, PSC833, LY335979 and R101933) [261]. Another inhibitor, OC144-093 (ONT-093), less potent of GF12918 was studied to evaluate the enhancement of the oral bioavailability of docetaxel. The combination of oral docetaxel and ONT-093 showed a good safety, and the bioavailability increased from 8 % to 26 %. However the magnitude of the enhancement is considered insignificant for further development of this combination [251]. Other inhibitors such as LY335979 ((2R)-anti-5-[3-[4-(10,11 difluoromethanodibenzo-suber-5-yl) piperazin-1-yl]-2-hydroxypropoxy]quinoline trihydrochloride) and R101933, were studied *in vitro* and *in vivo*, but in combination with i.v. cytotoxic drug [249, 262-265].

Finally, the last group of inhibitors includes a **novel generation** of polymeric pharmaceutical excipients and low molecular weight compounds such as Tween® 80, Pluronic 85, PEG, [134, 266] thiolated-chitosan, and Glutathion [267]. Föger et al. [268] showed that delivery systems based on Pluronic P85, Myrj 52 (polyoxyethylene-40-stearat) and especially

thiolated-chitosan significantly increased the oral bioavailability of P-gp substrate Rhodamine-123 (Rho-123) [269]. Recently, Martin Werle [266] in his review article discussed and resumed different synthetic polymers able to inhibit efflux pumps as well as their application in cancer therapy and drug delivery. Furthermore, the most part of these excipients are commonly used in pharmaco-technology to develop, microspheres, nanosized drug carriers, liposomes, mixed micellar systems and hydrogels. These systems in addition to be able to control the delivery of the drugs, may be conceived to evade MDR [270]. A further promising compound is also the D-α-tocopheryl polyethylene glycol 1000 succinate (TPGS), a non-ionic water soluble derivate of Vitamine E. *In vitro* studies showed that TPGS and TPGS analogues containing different PEG chain length, were able to block polarized transport of Rhodamine 123 and paclitaxel in epithelial Caco-2 cell transport assay [271, 272]. Varma et al. [252] compared the co-administration of paclitaxel with verapamil and TPGS 1000. The results showed that the AUC of paclitaxel was 1.5-fold higher when co-administrated with TPGS, than when administered with verapamil.

The mechanisms of action of these compounds could be different and sometimes could be unknown. However, it is possible to categorize several inhibitors according to their mode of action on the targeted transporter proteins. The most part of inhibitors are analogue of the transported substrates and can inhibit both competitively and non-competitively drug extrusion through MDR1 or MRP. These agents are able to interact with the transporters or their drug-binding sites with higher affinity than any cytotoxic drug, and might be either efficiently transported or not by the pumps. A second group of MDR modulators includes inhibitors of ATP binding, or ATP utilization in the drug pumps. Of course, very low specificity against MDR can be expected from such compounds because ions pumps or protein kinases will be affected [273]. At these two categories belong all the inhibitors of the

first three generations as well as the low molecular compounds. On the contrary the mechanism of polymeric inhibitors often seems to be mediated by altering of cell membrane lipids with a fluidization of the membrane. This mechanism is most likely responsible for the inhibitory activity of PEG and polymers, like polysaccharides and thiomers that cannot access the cells. About thiomers, a mechanism based on the formation of disulfide bonds between the own free thiol groups and cysteins of P-gp inside the transporter channel has also been suggested. Moreover, some of these polymeric compounds, like Pluronic 85 or TPGS 1000, can inhibit the efflux pumps also interfering with ATP related mechanism such as ATP depletion [266].

The metabolic cytochrome P450

Cytochrome P450 is the main oxidative enzyme system in charge of the metabolism of the most part of organic substances. It consists on approximately 12 families and 17 subfamilies, but is only a small number of these enzyme families that is responsible for the majority of drug oxidation [23].

The CYP450 3A is the main enzyme localized in both the intestine and the liver, and responsible of the first-pass metabolism of a several number of xenobiotics including many drugs [274]. A lot of anti-cancer drugs e.g., paclitaxel, docetaxel, the vinca alkaloids, antrhacyclines, several camptothecin derived, topoisomerase I inhibitors, etoposide and some of novel anti-cancer agents known by code names (ET743, ABT839), are metabolized by the isoform CYP450 3A4 [275] [276]. The drug crosses the intestinal lumen and goes into the enterocytes by passive diffusion and/or facilitated or active transport by uptake transporters. Within the enterocytes it can be metabolized by CYP450 3A before to reach the mesenteric and subsequently portal veins. The residual fraction of the drug that arrives to reach the portal blood can be taken-up into hepatocytes, where it can be metabolized. Consequently, a small

amount of the drug can reach the systemic circulation. Furthermore, the concurrent presence of the efflux pumps increased the efficiency of the first-pass metabolism acting as a coordinated absorption barrier that restricts oral drug entry [274]. In a recent work Watersrchoot at al. [88] defined the synergistic collaboration of two systems. The study showed that the combinated absence of both CYP450 3A and P-gp has a disproportionate effect on the systemic exposure of docetaxel, which could have important clinical implications. The authors showed that its area under the curve ($AUC_{0-\infty}$) after oral administration was significantly higher in *Mdr1a/1b*$^{-/-}$ (2.8-fold) and *CYP450 3a*$^{-/-}$ (11.5-fold) mice than in wild-type mice. Furthermore these results indicated that CYP450 3A by itself plays a more important role in the pharmacokinetcs of docetaxel than *Mdr1a/1b*. However, in the *Cyp450 3a/Mdr1a/1b*$^{-/-}$ combination knockout strain, they found a 70-fold increase in AUC after oral docetaxel administration.

Even in this case, the strategy consists of the co-administration of the inhibitors of CYP450 3A. Actually, these compounds, called inhibitors, are just drugs that are metabolised by the CYP450 3A presenting a major affinity for the enzyme. One of the strong inhibitor is ritonavir, an HIV protease inhibitor. Different studies showed that the concomitant administration of ritonavir in an anti-cancer therapy with docetaxel strongly enhanced the systemic exposure of oral docetaxel in mice [41] as well as in patients with solid tumors [277]. The problem of ritonavir is the same that the first generation P-gp inhibitors, it has a different principle pharmacological action as anti-HIV. The main risk of the development of this combination is the increase the side effects, even if Oostendorp et al. [277], in their work, previously mentioned, found that the combination was well tolerated.

Several studies were carried out by using ketoconazole, an imidazole-piperizine compound, orally active as antimytotic agent. So far, the essays that have been made with

these inhibitors consisted in an oral co-administration in an i.v. therapy of docetaxel. Engels et al. showed that the co-administration of standard dose of ketoconazole resulted in a relative increase in exposure of docetaxel of 290%, associated with a considerable increase of toxicity. Thereby the clearance decreased of 49% [278]. Moreover, five-fold increase in ketoconazole dose does not result in a uniform reduction of docetaxel clearance and does not reduce the inter-individual variability in docetaxel AUC or clearance. They drew the conclusion that may be the choice for ketoconazole as CYP450 3A inhibitor may not be ideal because of the large inter- and intra-individual variability in absorption [279]. However, ketoconazole is usually used, as reference product to test new inhibitors or to evaluate which system among the efflux pumps and the metabolic CYP450 enzyme is responsible for the low bioavailability of cytotoxic agents [253, 254].

In recent years Curcumin, a natural phenolic pigment component in *Curcuma longa*, has attractive attention because of its potential application as a relatively safe therapeutic adjuvant for cancer treatment [280-283]. In particular, it has been reported that curcumin may act as modulator of P-gp and CYP450 expression [284, 285]. Zhang et al. [286] showed that the administration of 60mg/kg/day of curcumin for 4 consecutive days, not only changed the P-gp and CYP3A protein level in the rodent intestine, but also modified the levels of these proteins in the rodent liver and kidney. These results were then confirmed by several authors. It has been showed that the inhibitory action of curcumin may appear at low concentration (IC_{50} = 16.3 μM) [287]. Recently, *in vivo* studies showed that the administration of cucumin for 4 days prior to oral administration of docetaxel, resulted in a marked increase of the bioavailability of docetaxel by about 8-fold, from 5.5% in the control group (rats received docetaxel alone) to 43.7% in the treatment group. On the contrary, rats administrated with curcumin 30 min before docetaxel (co-administrated group) did not show significant difference in AUC and C_{max} values compared with the control group [254]. This can be

explained by the poor bioavailability of curcumin (about 60%) because of its poor absorption, rapid metabolism and rapid systemic elimination. Nevertheless, when rats were treated with curcumin for 4 consecutive days, the accumulative absorption of curcumin into the general circulation was adequate to modify the expression of P-gp and CYP 3A in the rats [254, 286].

Speaking about natural compounds, it is well known that the co-administration of the grapefruit juice may alter the bioavailability of some oral drugs, increasing their plasma concentration [288, 289]. This action is due to the active components of the grapefruit juice, the furocoumarins, able to inhibit the CYP 3A4 enzymes. Five of the most important furocoumarins are isolated and tested. The inhibitory potency was in the order of paradisin A > dihydroxybergamottin > bergamottin > bergaptol > geranylcoumarin at 0.1 μM to 0.1 mM concentrations [290, 291]

4.3 Drug delivery systems (DDS) for the oral administration of anti-cancer drugs

An interesting approach for the enhancement of the oral absorption of the drugs is the development of new oral DDS, in particular oral nanocarriers. In the last few years, the explosive growth of nanotechnology has burst into challenging innovations in pharmacology, allowing real progresses to achieve temporal and spatial site-specific delivery.

In general nanocarriers may protect the drug from degradation, enhance drug absorption by facilitating diffusion through epithelium, modify pharmacokinetic and drug tissue distribution profile, and/or improve intracellular penetration and distribution [121, 292]. Nanoscale drug delivery vehicles have shown the ability to encapsulate a variety of anti-cancer drugs. The solubility and the stability of the drugs can be improved, providing an opportunity to revaluate potential drugs previously ignored because of poor pharmacokinetics [293]. Interestingly, the surface of DDS can be engineered to improve their mucoadhesion

resulting in longer contact time in the GI tract. Furthermore, the small size of DDS allowed to penetrate mucus and to improve the contact between the DDS and the absorption site.

However, a plethora of research works can be found on the i.v. administration of nanocarriers and few *in vivo* evaluated DDS was cited in the literature. It is worth to note that the research works on the i.v. administration of DDS containing anti-cancer drugs are useful from a formulation and technological point of views. Important information can be extracted from these works concerning the protocol for anti-cancer drug encapsulation, solubility, stability studies, loading and release of the drug from nanocarriers.

This part of the review will be focused on nanoparticle technology used for the oral delivery of anti-cancer drugs. These technologies included polymeric nanocarriers (nanospheres, nanocapsules, polymeric micelles and dendrimers), and phospholipids-based nanocarriers (liposomes, nanoemulsions and microemulsions).

4.3.1 Polymeric nanocarries

Polymeric nanocarriers are nano-structured systems that are prepared from polymers (For reviews see [121, 294]. Depending upon the method of preparation it is possible to obtain different kinds of nanocarriers (Fig. 4), such as nanoparticles (NPs) (nanospheres and nanocapsules), polymeric micelles and dendrimers.

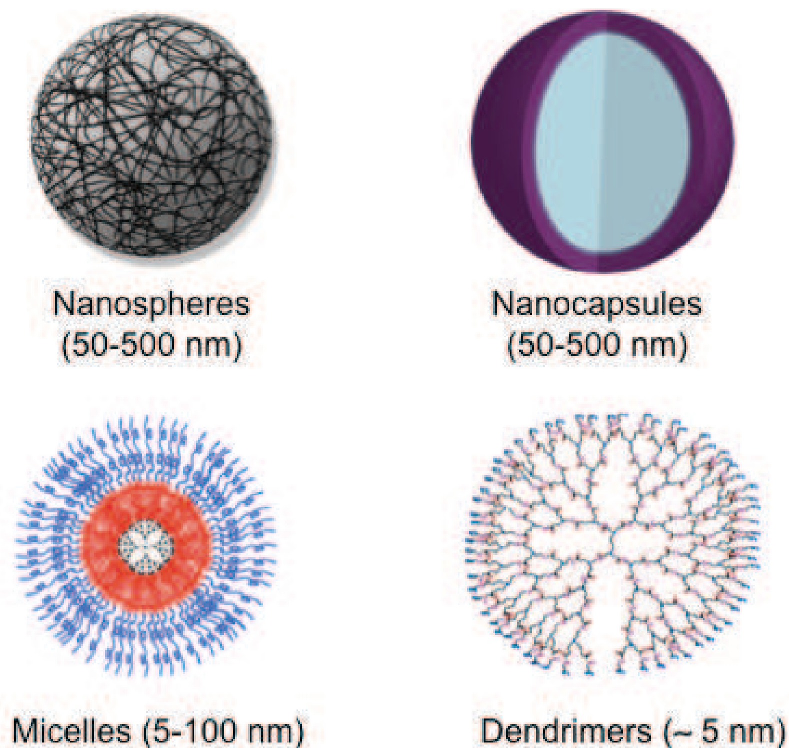

Figure 4. Structures of different nanocarries for oral drug delivery. **Nanoparticles**: the drug is dissolved, entrapped, encapsulated or attached to a nanoparticles and depending on the method of preparation, nanoparticles, nanospheres or nanocapsules can be obtained. **Micells**: nano-sized particles composed of polymer chains and are usually spontaneously formed by self-assembly in aqueous medium forming typically a so-called core-shell structure. **Dendrimers**: spheroid or globular nanostructures that are precisely engineered to carry molecules encapsulated in their interior void spaces or attached to the surface.

Polymeric nanoparticles

Nanoparticles of biodegradable polymers may provide an alternative solution for oral delivery of anti-cancer drugs across the gastrointestinal barrier due to their extremely small size and their appropriate surface coating to escape from the recognition by P450/P-gp [295-302]. Furthermore, anti-cancer drug encapsulation allowed resolving limitations due to their low water solubility. Finally, mucoadhesive nanoparticles could be obtained in the aim to increase their cellular uptake in the gastrointestinal tract. So far, the most used polymeric nanoparticles for the encapsulation of anti-cancer drugs were composed of poly(alkyl

cyanoacrylates) (PACA) [303]. PACAs are hydrophobic polymers and their use emerged in the 1980s and have gained increasing interest in therapeutics, especially for cancer treatments, which generally involve highly toxic molecules in contact with healthy tissue [303]. Doxorubicin [304] and paclitaxel [305] were successfully encapsulated into PACA nanospheres. Soma et al. [306] suggested the co-encapsulation of doxorubicin and cyclosporine A within the same nanospheres.

Although most of research works were conducted on the i.v administration of the nanoparticles loaded with anti-cancer drug, the pre-formulation studies allowed resolving the problems related to drug solubility, encapsulation, stability and release. Information extracted from these research works could be useful for the oral delivery of these formulations. The bioadhesive properties of its corresponding monomer, alkylcyanoacrylate, are well known since 1966 when it has been used as surgical glue. Interestingly, PACA is bioerodible polymer, mainly degraded *in vivo* by esterases of pancreatic juice in the intestinal tract [307]. The products of degradation (alkylalcohol and poly(cyanoacrylic acid) are soluble in water and easily eliminated [308]. Different studies showed the effective ability of the PACA nanoparticles to load different kinds of active agents such as, peptides [309], in particular insulin [310-312], vaccines [313], cytotoxic agents [305] and others kinds of hydrophobic compound like curcumin [314]. Today PACA nanoparticles are considered to be the most promising polymer colloidal drug delivery systems and they are already in clinical development for cancer therapy [303].

However, the hydrophobic and the anionic nature of PACA nanoparticles do not make them attractive candidates for bioadhesive systems. In this context, the surface modification of this kind of nanoparticles with polysaccharides has been suggested to improve the mucoadhesion properties. Many research works were oriented towards the design of colloidal

systems based on **core-shell nanoparticles** composed by PACA cores coated with different polysaccharides [315-317]. One of the most promising polysaccharides with a favourable profile, includes biodegradability, biocompatibility and absence of toxicity, used in biomedical and pharmaceutical fields, is chitosan [318]. Chitosan is a cationic polysaccharide obtained from the partial deacetylation of chitin, a natural abundant mucopolysaccharide. Compared to traditional excipients, chitosan showed a superior characteristics and especially the high flexibility in its use. It has been already used for conventional formulations, in direct compression tablets, as a tablet disintegrant, for the production of controlled release solid dosage forms or for the improvement of drug dissolution. However, its special bioadhesive nature and its permeation enhancing property, made chitosan an exciting excipient, especially for nasal or oral delivery of polar drugs [319-321].

The mucoadhesive property of chitosan was dramatically improved by grafting thiol groups on chitosan. Because of the presence of the thiol groups, they can form disulfide bonds with the cystein-rich subdomains of mucus glycoproteins [322]. Different thiolated chitosan derivatives have been synthesized: chitosan- thioglycolic acid conjugates, chitosan-cysteine conjugates, chitosan-4-thio butyl-amidine (chitosan-TBA) conjugates [323] and chitosan-thioethylamidine (TEA) derivatives [324]. Different research groups took advantage of the mucoadhesion enhancement of the thiomers and they developed the PACA core-shell nanoparticles coated with chitosan and chitosan thiolated. Because of the presence of the chitosan chains, positive charged and the thiol groups in the surface this kind of nanoparticles may adhere to the mucus layer following the mechanisms previously mentioned in the section 3.2 [125, 325].

An interesting work on the use of mucoadhesive polymers for the oral administration of anti-cancer drugs concerns Paclitaxel. Agüeros et al. [39] incorporated a solid inclusion

complex between 2-hydroxypropyl-β-cyclodextrin (HP-β-CD) and paclitaxel, in poly(methyl vinyl ether-co-maleic anhydride) [Gantrez® AN] nanoparticles. In presence of HP-β-CD the aqueous solubility of paclitaxel increased up to 7-fold compared with the solubility in pure water and more important the formation of Ptx/HP-β-CD inclusion complex increased the paclitaxel loading in the nanoparticles. Comparing the formulations with and without cyclodextrin, the incorporation of the drug as a complex increased about 500-times the drug loading compared to the control where the drug was not associated with the cyclodextrin. Moreover, they studied the effect of these nanoparticles on the permeability of intestinal epithelium using the Ussing chamber technique. Results showed an important increase of the paclitaxel dose absorbed (~ 10%) when it was formulated as Ptx/HP-β-CD nanoparticles, compared with the near-zero absorbed dose of the commercial formulation (Taxol®). *In vivo* pharmacokinetic studies were then carried out to confirm the potentiality of this kind of nanoparticles by comparing three different formulations. These formulations were obtained by using three different cyclodextrins: β-cyclodextrin (β-CD), 2-hydroxypropyl-β-cyclodextrin (HP-β-CD) and 6-monodeoxy-6-monoamino-β-cyclodextrin (NH-β-CD). All the formulations led to a dramatically increase of the paclitaxel plasma concentrations. The AUC ranged from 14μg h/mL for Ptx/NH-β-CD NP to close to 65μg h/mL for Ptx/βCD and Ptx/HP-β-CD nanoparticles. In particular, the relative oral bioavailability of Ptx/βCD NP and Ptx/HP-β-CD NP was calculated to be higher than 80%.

Examples of others potential biodegradable polymers for the improvement of oral drug delivery include poly(lactide-*co*-glycolide) (PLGA) [326], poly-anhydrides and poly(methyl methacrylate) poly-lactic acid (PLA) [299], poly-glycolic acid (PGA), poly(ε-caprolactone) (PCL), diblock copolymers Poly(sebacic acid)-*co*-poly(ethylene glycol) (PSA-PEG) [327], palmitic acid, and poly(3-hydroxybutyrate) (PHB) [297, 328, 329]. The presence of PEG at

the surface improved the stability of these nanoparticles because PLA, PLGA and palmitic acid contain ester bounds that can be cleaved in an acidic medium [330].

Many research works demonstrated the importance of surface nanoparticle properties for their cellular uptake. For example, the cellular uptake of PLGA nanoparticles loaded with the fluorescent marker Coumarin-6 and coated with vitamin E succinated polyethylene glycol 1000 (Vitamin E TPGS) showed 1.4 folds higher than that of PVA-coated PLGA nanoparticles and 4-6 folds higher than that of nude polystyrene nanoparticles [331]. Images of confocal laser scanning microscopy, cryo-SEM and transmission electron microscopy clearly evidenced the internalization of nanoparticles by the Caco-2 cells, showing that surface modification of PLGA nanoparticles with vitamin E TPGS notably improved the cellular uptake [331].

Vitamin E TPGS can be absorbed intact readily in the gastrointestinal tract, and as mentioned above, this molecule acts as P-gp inhibitor, enhancing the bioavailability of the anti-cancer drug. Furthermore, the use of Vitamine E TPGS as emulsifier in the elaboration of PLGA nanospheres improved the encapsulation efficiency of the paclitaxel, which can approaches 100% [300]. In the following studies, a new type of nanoparticles for the oral administration of docetaxel, the PLA–TPGS/MMT nanoparticles was developed [43, 332]. Furthermore, the efficiency of three different kinds of nanoparticles composed of PLGA, PLA-TPGS and PLGA-MMT was compared. *In vivo* experiments allowed demonstrating that PLA-TPGS and PLA-TPGS formulations exhibited a half-life 26.4 and 20.6 times longer respectively, than the half-life of docetaxel *i.v.* administrated. The oral bioavailability could be enhanced from 3.59% for Taxotere® to 78.0% for the PLA-TPGS/MMT nanoparticles and even 91.3% for the PLA-TPGS nanoparticles.

Chitosan-PLA nanoparticles blended with montmorillonite (MMT) were developed to control the release of paclitaxel. *In vitro* studies showed a more pronounced release in a basic medium than the acidic medium, which suggests an intestinal absorption. MMT provides the mucoadhesive capability of the nanoparticles in addition to its detoxifying property [328].

Iqbal et al. [333] elaborated a new formulation containing poly(acrylic acid) cysteine (PAA-cysteine) at different molecular weights (100 and 250 kDa), and glutathione (GSH) for the oral administration of paclitaxel. The aim of the study was to identify an appropriate system to dissolve the drug and, simultaneously, to evaluate the potential capability of the PAA-cysteine conjugates to inhibit the P-gp efflux pumps and CYP450 metabolism. They proved through *in vitro* studies that the two formulations containing PAA-cysteine (100 and 250 kDa)-GSH improved the transport of paclitaxel trough the cell Caco-2 monolayer (6.7- and 7,4-fold respectively) in comparison with the paclitaxel alone. *In vivo* studies confirmed these results with an enhancement of the plasma concentration and the bioavailability. The area under the plasma concentration curve increased 4.7-fold for PAA-cysteine 100kDa and 5.7-fold for PAA-cysteine 250 kDa in comparison to the oral formulation of paclitaxel alone.

Hydrogel nanospheres composed of poly(methyl methacrylic acid) (PMMA) grafted to PEG were used for the oral delivery of bleomycin [334]. Hydrogel nanoparticles obtained by UV-initiated free radical polymerization is pH-sensitive. The pKa (4.9) of MMA group makes the pH shift from the fasted stomach to upper intestine (1.5-6.5) appropriate to change the ionization of the carboxyl groups. This system allowed to release bleomycin at the pH of the intestine due to polymer swelling [334].

Micellar-based DDS

Micelles are small sized systems (~10 to 30 nm) formed when amphiphilic molecules are placed in water. They consist of an inner core of hydrophobic segments able to improve

apparent water solubility of lipophilic substances and an outer hydrophilic corona serving as a stabilizing interface between the hydrophobic core and the external aqueous environment. The hydrophilic shell allows to maintain the micelles in a dispersed state and to decrease undesirable drug interactions with cells and proteins through steric-stabilization effects. Depending on the delivery purpose, the size, the charge, and the surface properties of micelles can be modulated.

Vitamine E-TPGS was able to form micelles too, above the critical micellar concentration (CMC) and it has been showed that this ability with its inhibitory P-gp activity improved the pharmacokinetic profile of paclitaxel [252].

Like micelles, amphiphilic polymers were able to self-associate in aqueous medium to form "**polymeric micelles**" (~10 to ~100 nm), consisting of a hydrophobic core stabilized by a corona of hydrophilic polymeric chains exposed to the aqueous environment. Polymeric micelles can be used as efficient carriers for poorly water-soluble drugs. This topology is similar to that of surfactant micelles, hence polymeric micelles can be expected to solubilize hydrophobic drugs within their core. However, there are significant differences between the two types of assemblies from the physicochemical viewpoint. The polymer concentration at which the association first takes place, sometimes known as the critical association concentration (CAC), is lower by several orders of magnitudes than typical surfactant CMC values. Consequently, polymeric micelles are more stable to dilution in biological fluids (for review see [335-337]). Furthermore, multifunctional polymeric micelles showed cancer-targeting capability when used by intravenous injections (for review see [337, 338]).

Polymeric micelles have been extensively studied as delivery medium for injectable drug formulations of poorly water-soluble anti-cancer drugs such as taxans [339-341], doxorubicin [342-344], etoposide [344] and Cisplatin [345]. However, very few works

reported the oral delivery of anti-cancer drugs by using polymeric micelles. Particularly, pluronic micelles composed of poly(propylene) block surrounded by two blocks of poly(ethylene) [346, 347] demonstrated a particular flexibility for anti-cancer drug solubilization, delivery [348] and targeting when administrated intravenously [349]. Pluronic P85 at concentrations lower than the critical micelle concentration inhibits Pgp efflux systems [350, 351]. Furthermore, at high concentrations pluronics are able to form hydrogel at the body temperature. Chemically formed copolymers of poly(acrylic acid) (PAA) and pluronics possess many physical and pharmacological features that make dosage forms adapted for the treatment of cancers after their oral administration (for review see [352]). Pluronic segments include the hydrophobic Pluronics such as L61 ($EO_3PO_{30}EO_3$) and L92 ($EO_8PO_{52}EO_8$) or the relatively hydrophilic F127 ($EO_{99}PO_{67}EO_{99}$) [353]. The Pluronic-PAA **microgels** loaded with both paclitaxel and doxorubicin collapsed at the low pH of the stomach and expand thus releasing the loaded drugs at the pH of the lower gastrointestinal tract. These mucoadhesive microgels [354] can be double loaded with paclitaxel and doxorubicin and enable longer retention time and prolonged release in the colon and allowed encapsulated chemotherapeutics to partially escape from recognition by P-gp [351, 355].

The increase in the bioavailability of a lipophilic drug upon oral administration is caused by drug solubilization in the gut by naturally occurring biliary lipid/fatty acid-containing mixed micelles produced by the organism as a result of the digestion of dietary fat. The micellar form of the drug is transferred across the intestinal mucosal membrane into the enterocyte where it enters the lipoprotein biosynthetic pathway and incorporated into chylomicron particles after its release into the intestinal lymphatics [356].

Because of their particular structure, polymeric micelles showed the ability to increase the apparent aqueous solubility of hydrophobic compounds incorporating the drug into its

hydrophobic core (for review see [97] , [337]. Shin et al. [344] in a recent work demonstrated that poly(ethylene glycol)-*block*-poly(D,L lactic-acid) (PEG-*b*-PLA) micelles can solubilize multiple poorly water-soluble drugs as paclitaxel, etoposide and docetaxel. They showed that the micelles with the combination of 2-3 drugs were able to dissolve the drugs at relevant concentration and that they were stable for 24 h.

Dendrimers

Other useful drug delivery systems proposed are **dendrimers**. The advantages of these macromolecules are their easy method of production and the effectiveness of being able to control the amount and the specificity of the attached functional groups. They can be applied for all different administration routes (oral, i.v., transdermal etc…) [357] and used to improve aqueous solubility of poorly water soluble drugs (Milhem et al., 2000, Devarakonda et al., 2004, 2005,). Camptothecins, paclitaxel and doxorubicin have been successfully encapsulated in the dendrimers [358-360]. In particular the dendrimers loaded with doxorubicin were tested for the oral route and *in vivo* studies showed a doxorubicin bioavailability 200-fold higher than the free drug [360]. They used the biocompatible polyamidoamine (PAMAM) dendrimers, which in previously study showed the ability to permeate across intestinal epithelial barriers [361].

SN-38 (7-ethyl-10-hydroxy-camptothecin) was complexed with 4.0G PAMAM dendrimers to improve oral bioavailability of SN-38, a biologically active metabolite of irinotecan hydrochloride [362]. Irinotecan hydrochloride, an anti-cancer agent, has oral bioavailability of about 8% only and displays gastrointestinal toxicity. SN-38 has potent antitumor activity i.e. approximately 1000-fold more active than irinotecan hydrochloride. Authors performed this study to investigate the potential of PAMAM in improving the delivery of SN-38. They synthesized the complex of SN-38 with PAMAM dendrimers and

evaluated its stability, permeability and cellular uptake by Caco-2 cells and found that the complex was stable at pH 7.4, and the drug was released at pH 5.0. A 10-fold increase in permeability and more than 100-fold increase in cellular uptake was observed with respect to free SN-38.

However, with increasing interest in the possibility of using dendrimers for oral delivery, toxicity has also been examined using a variety of gastrointestinal tract models (For review see [363, 364]. The influence of PAMAM dendrimers on the integrity, paracellular permeability, and viability of Caco-2 cell monolayers was also monitored by measuring the transepithelial electrical resistance (TEER), mannitol permeability, and leakage of lactate dehydrogenase (LDH) enzyme, respectively [163]. This study demonstrated that TEER values decreased and mannitol permeability increased as a function of dendrimer concentration, incubation time and generation number.

Interestingly, many works demonstrated that the Conjugation of PAMAM to the drug allowed to decrease the dendrimer toxicity. For example, the conjugation of SN38 to PAMAM dendrimers (G 3.5) has the potential to improve its oral absorption while minimizing gastrointestinal toxicity [365].

4.3.2. Phospholipids and lipid-based formulations

Phospholipids have a special amphiphilic character able to form various structures in aqueous media depending on their specific properties offering various possibilities of liquid, semi-solid and solid lipid-based formulations for oral anti-cancer delivery (for review see [120]. The principal lipidic drug delivery systems are shown in Figure 5 including micro-/nanoemulsions, liposomes, solid lipid nanoparticles (SLNs) and self-emulsifying drug delivery systems (SEDDS).

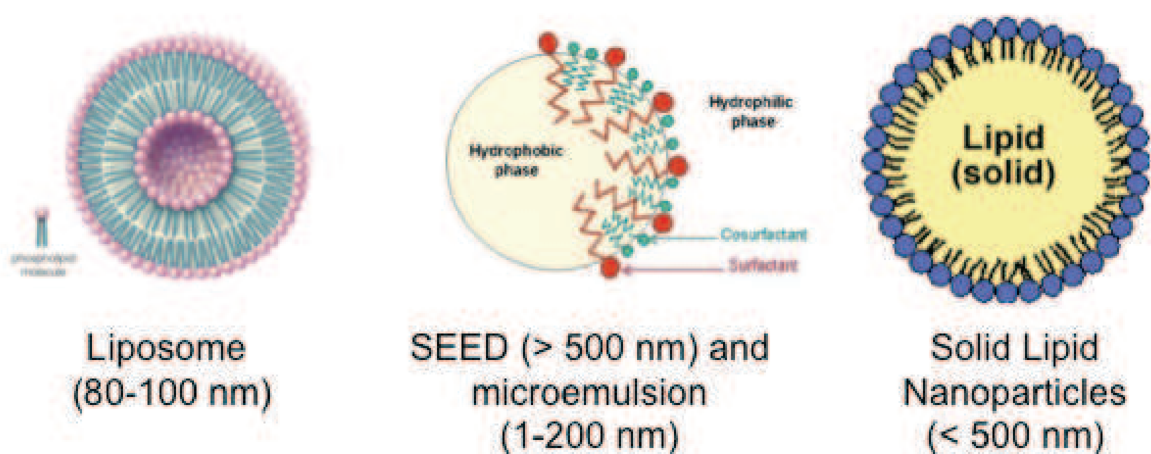

Figure 5: Phospholipids formulations. **Liposomes**: nano size artificial vesicles of spherical shape that can be produced from phospholipids and cholesterol which combined with water forming a bi-layered sphere. **SEDDS:** mixture of oil and surfactants that forms fine oil-in-water emulsion in aqueous media. **Microemulsion:** thermodynamicaly stable solution composed of water, oil and surfactant. The difference between the SEDDS and the microemulsion are the size of droplets, which are smaller in the microemulsion. **Solid lipid nanoparticles:** nanoparticles composed of physiologically tolerated lipid components, solid at room temperature.

Self-emulsifying drug delivery systems (SEDDS)

SEDDS are mixtures of oil and surfactants sometimes including co-solvent which form fine oil-in-water emulsion in aqueous media under conditions of gentle agitation [366] [367]. The delivery system is expected to self-emulsify rapidly in the aqueous content of the stomach in the form of *(i)* microemulsions (transparent dispersed systems with oil droplet of less than 30 nm), *(ii)* or fine opaque nano-emulsions of droplet size of 50-200 nm, *(iii)* or coarse emulsions with oil droplet size larger than 500 nm. Droplets are smaller than the ones *in vitro* because bile salts would be incorporated into the surfactant layers of the emulsion droplets. The great increase of the interfacial area for partitioning of the drug between oil and water promotes release of the drug molecules from the droplets and their absorption. SEDDS are able to dissolve a great quantity of hydrophobic drug including anti-cancer drugs and P-gp

inhibitors (for review see [98, 99]. The encapsulation of TPGS into SEDDS allowed to improve the intestinal absorption of Paclitaxel. Non-ionic SEDDS (o/w) formulation free of Cremophore® was designed as drug delivery systems for paclitaxel. The optimum formulation, containing oil, Tween® 80 and L-α-phosphatidylcholine, could solubilize at least 500 ppm of paclitaxel [368].

Furthermore, Self-microemulsifying drug delivery systems (SMEDDS) (that upon aqueous dilution form only microemulsion) were conceived for improved oral administration of paclitaxel with or without P-gp inhibitors. Interestingly, when P-gp inhibiting lipidic excipients were present in the formulation, the oral bioavailability of anti-cancer drugs was significantly improved [369]. For example, a SMEDDS composed of α-tocopherol, TPGS, propylene glycol, sodium deoxycholate and Cremophor RH40 was used to improve the oral bioavailability of paclitaxel [369]. Compared to Taxol®, the oral bioavailability of paclitaxel SMEDDS increased by 28.6% to 52.7% at various doses. Furthermore, with the co-administration of 40 mg/kg the Cyclosporine A, the values of $AUC_{0\text{-}24}$, C_{max}, $T_{>0.1}$ and $AUC_{>0.1}$ of paclitaxel in SMEDDS at 5.0 mg/kg dose increase dramatically by 49.8, 28.6, 74.0 and 168% compared to the corresponding values of Taxol® [369].

Microemulsion (ME)

Microemulsion formulations are very similar systems to the SEDDS. MEs are thermodynamically stable solutions composed of water, oil and surfactant. They can be administrated in the form of water-in-oil microemulsion or surfactant-oil mixture [370]. Like SEDDS they are used to increase the solubility of the poorly water-soluble compounds [371].

Oral administration studies showed that microemulsion enhanced intestinal absorption in different ways depending on the surfactant used [367].

The oral administration of a microemulsion formulation containing dimethylisosorbide, Tween® 80 and DL-α-tocopherol and incorporating paclitaxel in presence of P-gp inhibitor (KR-30032) to rats allowed to increase the paclitaxel oral bioavailability from 4.6% to 41%. This effect is dose dependent and was saturable above 20mg/kg of KR-30032 [253].

The solubility of docetaxel was increase up to 30 mg/mL using the o/w microemulsion formulation composed of Capryol 90 (oil), Cremphor EL (surfactant) and Transcutol (co-surfactant). Transport studies through Caco-2 cell monolayer, showed that the P*app* of docetaxel from the microemulsion was around 43 times higher than from the control group and around 27 times higher than from the Taxotere® (7.68×10^{-5} cm/s *vs* 0.18×10^{-5} and 0.29×10^{-5} cm/s, respectively). Moreover, *in vivo* studies, the oral bioavailability of the microemulsion formulation in rats (34.42 %) increased dramatically compared to that of the oral Taxotere® [372].

Liposomes

Liposomes are structured in concentric bilayers of phospholipids. The use of **liposomes** as oral drug delivery systems is difficult due to the poor stability of the vesicles under the physiological conditions of the gastrointestinal tract [120]. The use of liposomes in oral drug delivery has been widely studied and reviewed [373]. Three major destabilizing factors are pH, bile salt, and pancreatic enzymes in the gastrointestinal tract. Most liposome formulations cannot be used for oral delivery because H^+ cations in the gastric medium can diffuse in the inner phase of liposomes and destabilize them. The bile salts are able to penetrate into liposomal bile bilayer [374, 375]. Disruption of the vesicular lipid bilayer in the gastrointestinal tract led to the exposure of the encapsulated molecule and therefore to the loss of their protective functions [376, 377]. To stabilize the liposomes and allow their application

in oral delivery, polymerized liposomes have been developed [377, 378]. The increase of cholesterol content in the formulation or the addition of saturated phospholipids improves the rigidity of liposomes and decreases the possibility of enzymatic degradation [373, 379-381]. In the literature, we can find different studies about the preparation of oral liposomes for the administration of peptides or proteins [382, 383] and vaccines [384] [378]. A β-sitosterol-containing orally administered liposomes were used for preventing tumor metastasis [385]. This research work proved that increasing host defence may prevent tumor cells from metastasis.

Liposomal coating by using polymers (chitosan [386], polyethylene glycol [387]), carragheenans and collagen [388] and gangliosides GM1 and GM types [389] represents an interesting alternative to improve their stability in the GI tract. Moutardier et al. [388] prepared liposomes with carragheenan and collagen core for the oral delivery of different anti-cancer drugs (Vincristine, 5-FU and methotrexate) for the treatment of colorectal cancer. The aims of the study were to optimize the oral bioavailability of compounds and to reduce their cytotoxicity towards healthy cells in the gastrointestinal tract. In comparison with classical liposomes, liposomes with polymeric core have the advantage to be less sensitive to bile salts, to temperature, to mobility and to the exposure to degradative enzymes.

Solid lipid nanoparticles (SLNs)

Solid lipid nanoparticles are nanoparticles composed of lipids that are solid at room temperature and are degraded by the pancreatic lipase found in the intestinal fluid [390, 391]. In the latest years, SLNs offered new perspectives in the formulation of poorly water soluble drugs. It has been demonstrated that the SNLs produced a significant improvement in the bioavailability of All-*trans* retinoic acid (ATRA), an anti-cancer drug for the treatment of human malignant gliomas. LianDong Hu et al. [392] prepared ATRA-loaded SNLs with

mucoadhesive properties. They used Tween® 80 and Pluronic F68, which may have contributed to an increase in the permeability of the intestinal membrane and as previously mentioned, they may contributed to inhibit the P-gp efflux pumps [393]. Recently, the potential therapeutic effects of quercetin for cancer treatment has been discovered. Quercitin is a flavonoid compound with poor water solubility and consequently a low bioavailability. Li et al. [394] designed and characterized quercetin-loaded SNLs and the results *in vitro* and *in vivo* were very promising with the relative bioavailability of Qt-SNLs to quercitin suspension of 571.4%. Others cytotoxic drugs has been encapsulated in SNLs as Camptothecin [395] and Paclitaxel [396] [397]. All these works indicate that SLNs provided a promising new formulation to enhance the oral bioavailability of the anti-cancer drugs.

5. Perspectives and conclusions

Oral chemotherapy seems to be a real prospective in the future of oncology. Different fields of science are working hardly to improve oral delivery of anti-cancer drugs. Many problems must to be solved. From a pharmacological point of view, the bioavailability and the toxicity are the most important limitations for the oral administration of anti-cancer drugs. As we could see, the synthesis of prodrugs, the use of drug delivery systems and the strategy to inhibit the drug metabolism at the intestinal level, are the very interesting strategies. However, this represents one of the aspects of chemotherapy. If there will be new drugs available for the oral chemotherapy, it is worth to know that the protagonists of therapy should be prepared to this fundamental change. In fact, the role of oncologist, physician, nurses and in particular of patient, really change. Many of the responsibilities of managing the regimen, monitoring for doses and toxicity are shifted from the oncologist to patient and the adherence to the therapy may become a great problem. In the case of i.v. chemotherapy, the hospitalization allows the monitoring of the treatment directly by the health care provider. This is not possible with the oral chemotherapy. The patient and the family, for children or

adolescents must promptly initiate the therapy at the correct time of the day, at the correct dosage and alert the clinician of adverse symptoms in a timely way. For this reason, a new task for the oncologists and more and more for the oncology nurses will be to educate patients, stimulating an individual patient's motivation to follow instructions and his perception of the risks and benefits.

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TRAVAUX EXPERIMENTAUX

CHAPITRE II: Bivalent Sequential Binding of docetaxel to methyl-β-cyclodextrin

Bivalent Sequential Binding of docetaxel to methyl-ß-cyclodextrin

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Abstract

New docetaxel (Dtx) and cyclodextrin (CD) inclusion complexes having improved apparent water solubility (up to 9.98 $mg.mL^{-1}$) were obtained from phase solubility diagrams. γ-CD and SBE-β-CD offered only poor solubility enhancements while considerable increases in apparent solubility were obtained with Me-β-CD (20 % w/w) and HP-β-CD (40 % w/w) (9.98 $mg.mL^{-1}$ and 7.43 $mg.mL^{-1}$ respectively). The complexation mechanism between Dtx and Me-β-CD was investigated by circular dichroism spectrometry, two-dimensional ^{1}H-NMR (NOESY) in D_2O, isothermal titration calorimetry (ITC) and molecular docking calculations. Circular dichroism and NOESY confirmed the existence of non-covalent interactions between Dtx and Me-β-CD and suggested that the *tert*-butyl group (C_6-C_9) and two aromatic groups (C_{24}-C_{29} and C_{30}-C_{35}) of Dtx interacted with the Me-β-CD molecules. The combination of ITC results to molecular docking calculations led to the identification of an unconventional sequential binding mechanism between Me-β-CD and Dtx. In this sequential binding, a Me-β-CD molecule first interacted with both *tert*-butyl and C_{30}-C_{35} aromatic groups (K_1: 744 M^{-1}). Then a second Me-β-CD molecule interacted with the C_{24}-C_{29} aromatic group (K_2: 202 M^{-1}). The entropy of the first interaction was positive, whereas a negative value of entropy was found for the second interaction. The opposite behavior observed for these two sites was explained by differences in the hydrophobic contact surface and functional group flexibility.

Keywords: Anticancer drug; docetaxel; cyclodextrins; isothermal titration calorimetry; complexation thermodynamics; solubilisation studies; sequential binding; molecular docking.

1. Introduction

Docetaxel (Dtx) is a potent anti-cancer drug that displays a broad spectrum of antitumor activity [1] being used in clinical trials for the treatment of prostate and breast cancers as well as lung carcinoma. Dtx is a very sparingly water-soluble drug (0.0019 $mg.mL^{-1}$) making difficult to formulate it efficiently. Dtx is currently administered by the parenteral route and is commercially formulated as a solution containing polysorbate 80 which causes severe allergic reactions and peripheral neuropathology [2]. In clinical practice, the incidence of these severe hypersensitivity reactions requires the oral administration of dexamethasone and antihistamine before Dtx infusion. Thus, the search for alternative routes of administration is of practical importance when looking for suitable formulations either for parenteral or oral delivery. For parenteral delivery, the increase in the apparent solubility of Dtx should be looked for using acceptable excipients. When foreseeing oral delivery, it should be remarked that Dtx belongs to the Class IV of the Biopharmaceutical Classification System, which comprises substances with both low solubility in aqueous fluids and low apparent permeability. These substances are substrates of biological transporters and/or metabolized in the intestinal barrier. In this situation, improving the apparent solubility of such substances may solve only one aspect of the problem but is obviously requested as a starting point for the design of efficient pharmaceutical formulations. In this context, one strategy to improve Dtx solubility is to use cyclodextrins (CDs). Complexation of poorly water-soluble drugs with natural or chemically-modified CDs represents an interesting strategy for increasing their apparent water solubility (For reviews see [3-7]) and offers further possibilities for their pharmaceutical formulation ranging from conventional to colloidal dispersions [8-15] for review see [5].

Shaped as a hollow truncated cone, CDs are composed of cyclic oligosaccharides of D-(+) glucopyranose units, all in chair conformation, linked by α-(1,4) glucosidic bonds. This

molecular structure confers to CDs the shape of a truncated cone, with the outer side formed by the secondary 2- and 3-hydroxyl groups and the narrow side by the primary 6-hydroxyl groups. Thanks to this conformation, CDs have lipophilic inner cavities and hydrophilic outer surfaces. Interactions generally occur between CDs and lipophilic molecules or lipophilic groups borne by the molecules, resulting in the formation of inclusion complexes. CD/drug complexes can be much more water-soluble than the non-complexed drug. However, in spite of the numerous advantages of CDs for the improvement of apparent drug solubility, few research works detailed the solubilisation of Dtx by using CDs [16]. In the literature most data about the complexation of taxane derivates with CDs concern paclitaxel [17-24]. So far, the investigation of the CD/Dtx molecular interaction has not been reported in the literature yet and remains a fundamental question that needs to be solved.

In the work to be presented here, phase-solubility diagrams were first used to screen the potential of different CDs to enhance aqueous solubility of Dtx and to identify the best CD capable of improving Dtx aqueous solubility. Then, an in depth physico-chemical methods including Nuclear Magnetic Resonance (^{1}H-NMR) coupled with two-dimensional nuclear overhauser effect (NOESY), isothermal titration calorimetry (ITC) experiments and molecular modeling were used to investigate the mechanism of CD/Dtx complexation.

2. Materials and methods

2.1. Reagents

Anhydrous docetaxel (Dtx) (M_w= 807.85 $g.mol^{-1}$), was purchased from Chemos GmbH (Germany). Random Methyl-β-cyclodextrin (Me-β-CD Rameb®, M_w= 1320 $g.mol^{-1}$) was purchased from Cyclolab (Budapest, Hungary), Hydroxypropyl-β-cyclodextrin (HP-β-CD, M_w= 1309 $g.mol^{-1}$) from Acros Organics (Belgium), γ-cyclodextrin (γ-CD, M_w= 1297 $g.mol^{-1}$) Cavamax® W8 from ISP (Netherlands) and sulfobutylether-β-cyclodextrin (SBE-β-CD, M_w= 2241 $g.mol^{-1}$) Captisol® was a gift from CyDex Pharmaceuticals, Inc (USA). All chemicals were of analytical or reagent grade and were used without further purification. Solutions were prepared by weight using MilliQ® water (Millipore, France). Analytical grade methanol and ethanol were obtained from Carlo Erba (Italy). Deuterium oxide (D_2O) (99.96 % D) was obtained from Sigma-Aldrich (Germany) and chloroform-d (99.8 % D) from Carlo Erba Reactifs-SDS (France).

2.2. Phase solubility diagrams and apparent solubility determinations.

Phase-solubility studies were carried out according to the method described by Higuchi and Connors [25]. An excess of commercial Dtx was placed in a glass vial (20 mL) with 2 mL of solubilizing agent, which was an aqueous solution of Me-β-, HP-β- or SBE-β-CD at concentrations ranging from 5 % to 40 % w/w and from 5 % to 10 % w/w only for γ-CD, due to solubility limitations.

Vials containing an excess of Dtx powder dispersed in a solution of CD were kept in a shaker for 108 hours at 25 °C. This duration was estimated to be long enough for reaching the complexation equilibrium. Then, the samples were filtered through a 0.22 μm membrane filter (Millex, SLAP 0225, Millipore, France).

The solubility of Dtx in water was determined according to the same protocol without the presence of CDs.

2.3. Determination of Dtx concentration.

After appropriate dilution with HPLC mobile phase, the samples containing Dtx were analyzed by reversed phase HPLC using a symmetry C_{18} column (250 x 4.6 mm) and UV detection at 231 nm (Waters system , France). The mobile phase was an isocratic mixture of methanol/water (70:30 % v/v). HPLC analyses were performed at a flow rate of 1 $mL.min^{-1}$. Stock solutions of Me-β-CD/Dtx and HP-β-CD/Dtx complexes (400 $\mu g.mL^{-1}$ and 100 $\mu g.mL^{-1}$ respectively) were prepared according to the method described in the previous section. The concentration of Dtx should be below the solubility of the inclusion complex. Both stock solutions were diluted with the mobile phase. Valid concentrations for quantification were in the range 0.5-400 $\mu g.mL^{-1}$ for the Dtx with Me-β-CD, and 0.1-100 $\mu g.mL^{-1}$ for HP-β-CD. The equations of the calibration curves were, $y = 59.42(\pm 0.19)x + 62.05(\pm 28.40)$, $r^2 = 0.9999$ and $y = 59.83(\pm 0.32)x + 33.706(\pm 12.41)$, $r^2 = 0.9995$ for Me-β-CD and HP-β-CD respectively. However, it was not possible to prepare sufficiently concentrated stock solutions of Dtx complexes prepared with SBE-β-CD and γ-CD due to the poor Dtx solubilisation effect obtained when using these two CDs. A Student's t-distribution test was carried out to compare the slope and the intercept of the two previous equations. The p-values were higher than 5 % (28.7 % and 36.5 % respectively), supporting that the two linear least square lines could be considered statistically equal. For this reason the concentrations of Dtx for SBE-β-CD and γ-CD were estimated from the one obtained with Me-β-CD/Dtx complex.

2.4. Circular dichroism spectrometry.

Circular dichroism spectra of Me-β-CD/Dtx inclusion complexes were determined by using a Jasco J-810 Circular Dichroism Spectropolarimeter. Me-β-CD/Dtx inclusion complexes were prepared according to the protocol described in section 2.2 by using a 10 % w/w Me-β-CD solution. The circular dichroism spectra were recorded in the 200-400 nm wavelength domain at room temperature. Circular dichroism spectrum for Me-β-CD/Dtx inclusion complex was compared to those obtained for Me-β-CD and Dtx separately.

2.5. Nuclear Magnetic Resonance (NMR) experiments.

^{1}H-NMR spectra were obtained by operating at 300 MHz (Brucker 300 Avance) and 800 MHz (Bruker Avance 3, TCI Cryoprobe) for the free Dtx and at 600 MHz (Bruker Avance, TXI SB 5 mm) for the inclusion complex and the Me-β-CD alone. The two-dimensional nuclear Overhauser effect (NOESY) spectra of the complex were performed with a Bruker Avance, TXI Cryoprobe at 600 MHz.

The inclusion complex of Dtx and Me-β-CD (10 % w/w) was prepared as described in section 2.2 by replacing water by D_2O. Free Dtx solutions were prepared by placing an excess of commercial Dtx powder in a glass vial (20 mL) with either 2 mL of D_2O (for the ^{1}H-NMR at 800 MHz) or $CDCl_3$ (for the ^{1}H-NMR 300 MHz). Then, the samples were filtered through a 0.22 μm membrane filter (Millex, SLAP 0225, Millipore, France). Chemical shifts were reported in ppm (δ) downfield from tetramethylsilane (TMS) internal reference.

2.6. Isothermal titration calorimetry (ITC) experiments.

An isothermal calorimeter (ITC) (MicroCal Inc., USA) with a cell volume of 1.44 mL has been used for determining from a single titration curve simultaneously the enthalpy of the interaction between Dtx and Me-β-CD and when appropriate, equilibrium constant corresponding to the formation of a complex between those species. The ITC instrument was

periodically calibrated either electrically using an internal electric heater, or chemically by measuring the dilution enthalpy of methanol in water. This standard reaction was in excellent agreement (1-2 %) with MicroCal constructor data.

Typically, aliquots of 5 μL of Me-β-CD solution (10 mM) filled into a 283 μL containing syringe were used to titrate a 0.703 mM Dtx solution placed in the titration cell which was accurately thermostated at 25 °C. The corresponding heat flows were recorded as a function of time. Intervals between injections were 120 s and stirred at 394 rpm. Due to the poor aqueous solubility of Dtx, solutions were obtained by dissolving the Me-β-CD or Dtx into a water/ethanol mixture (1:1 v/v).

Enthalpograms consisting in series of heat flows were collected automatically. Prior to their analysis, a background enthalpogram consisting in a series of injections of a Me-β-CD solution in a water/ethanol 1:1 v/v into a water/ethanol 1:1 v/v mixture placed in the titration cell was subtracted from each experimental enthalpogram to account for possible dilution effects. The interaction process between the two species has been analysed by using different models proposed in the Windows-based Origin 7 software package supplied by MicroCal. Based on the concentrations of the titrant and the sample, the software used a nonlinear least-squares algorithm (minimization of Chi2) to fit the series of heat flows to an equilibrium binding equation, providing the best-fit values of the stoichiometry (N), binding constant (K) and change in enthalpy (ΔH). From these results, the free energy (ΔG) and the entropy (ΔS) were deducted according to the equations: $\Delta G = -RT\ln K = \Delta H - T\Delta S$.

2.7. Molecular modeling.

To gain insight into the interaction between Dtx and Me-β-CD, molecular modeling was used. The Me-β-CD structure used in this work was generated from the curated coordinates of ligand QKH (structure 2QKH [26]), downloaded from HIC-Up database [27], by manually adding methyl groups on all free hydroxyl groups in order to obtain a fully

permethylated structure (GaussView 5, Semichem Inc). All dihedral angles of the methoxy groups were then homogenised, the resulting conformations being compatible with an unhindered CD cavity. The curated 3D coordinates of Dtx (TXL), ligand present in structures 1TUB [28] and 1IA0 [29] were also downloaded from HIC-Up database [27](Kleywegt, 2007) and used without modification.

CD dimers were generated from two identical monomers aligned on the cavities' axes, with the outer sides pointing one to another. As the optimal distance between CD monomers is not known, eleven dimers were generated, with distances between cavities' centers ranging from 10 Å to 20 Å, which were then used as receptors in the docking procedure.

Molecular docking was carried out using Glide [30] within the Schrödinger Molecular Modeling Suite (http://www.schrodinger.com/). The receptor was prepared using the Protein Preparation Workflow, then the grid was calculated centered on cavity center for 1:1 complexes and on half-distance between cavities centers for 2:1 complexes. Standard precision (SP) protocol was used for the docking process, followed by post-docking minimization. All other parameters were used with the default values.

Molecular modeling images were generated using Chimera softwar [31] and the PDF3D representation of the 2:1 Me-β-CD/Dtx complex (see the Supplementary Information) was obtained using the CACTVS Chemoinformatics Toolkit (http://www.xemistry.com/).

3. Results and discussion

A preliminary screening of the potential of different pharmaceutically acceptable CDs (Me-β-, HP-β-, SBE-β- and γ-CD) for increasing the apparent aqueous solubility of Dtx has been investigated. As shown in Figure 1.a, different phase-solubility diagrams were obtained, depending on the CD used. The examination of the phase solubility diagrams gave an overview of the process of complexation. Higuchi and Connor [25] classified the phase solubility diagrams into "A" and "B" types. "A" type curves indicate the formation of soluble

complex, while “B” types are observed when insoluble complexes are formed. Type “A” diagrams are represented by a linear increase in solubility “A_L”, or by a linear increase with a positive or negative deviation from linearity “A_p” and “A_N” respectively. “B” type curves are subdivided into “B_S” (complex of limited solubility) and “B_I” (insoluble complex) (Fig. 1.b).

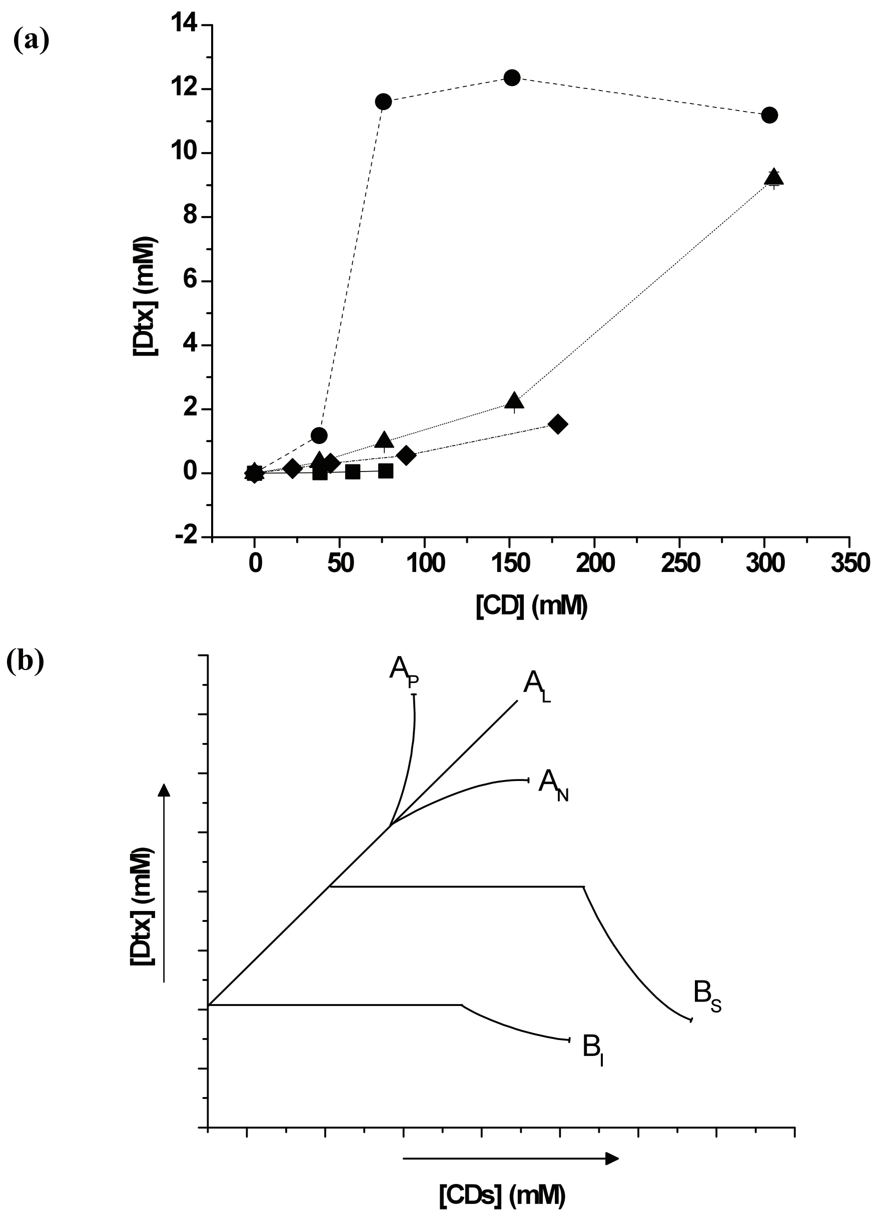


Figure 1. (a) Experimental phase solubility diagram of Dtx for several types of CDs (n=3). (•) Me-β-CD, (▲) HP-β-CD, (♦) SBE-β-CD, and (■) γ-CD. (b) Typology of phase solubility diagrams according to Higuchi and Connor [25], representing: "A": soluble inclusion complex. "B": formation of insoluble inclusion complex. "A_L": linear increase of drug solubility as a function of CD concentration. "A_P": positively deviated curve. "A_N": negatively deviated curve. "B_s": complex with limited solubility. "B_I": insoluble complex.

"A" type curves were experimentally obtained by using γ-, SBE-β- and HP-β-CD (Fig. 1.a). A linear increase in Dtx solubility as a function of CD concentration was obtained with the γ-CD (type "A_L"). However, the solubility enhancement of Dtx was very poor (from 0.0019 $mg.mL^{-1}$ to 0.06 $mg.mL^{-1}$) (Table I) and it was impossible to increase the concentration of γ-CD above 10 % w/w because of its own limited water solubility. The SBE-β-CD and the HP-β-CD led to "A_P" type curves and the highest Dtx concentration (1.23 $mg.mL^{-1}$ and 7.43 $mg.mL^{-1}$ respectively) necessitated an important amount of CD (40 % w/w) (Table I). Interestingly, higher concentrations of Dtx (9.38 and 9.98 $mg.mL^{-1}$) were obtained at lower Me-β-CD concentrations (10 and 20 % w/w) compared to SBE-β-CD and HP-β-CD (40 % w/w) (Table I). This considerable increase in Dtx solubility has never been reported in previous works and may be of interest in formulation practice.

Table I. Highest observed apparent solubilities of Dtx in different CD solutions**.** The apparent solubility enhancement factor (*F*) was obtained by dividing the solubility of Dtx in presence of CDs by the solubility of Dtx in water without CD*. T° = 298 K (25°C), n=3.

	[CD]		**Maximal apparent solubility of Dtx**		
CD	**(% w/w)**	**(mM)**	**($mg.mL^{-1}$)**	**(mM)**	***F***
None*	0	0	0.0019	0.0023	1
γ-CD	10	77	0.06 ± 0.01	0.07 ± 0.01	31
SBE-β-CD	40	178	1.23 ± 0.01	1.53± 0.01	665
HP-β-CD	40	305	7.43 ± 0.17	9.20 ± 0.21	4000
Me-β-CD	10	75	9.38 ± 0.06	11.61 ± 0.07	4937
	20	151	9.98 ± 0.16	12.36 ± 0.08	5374

The Me-β-CD diagram could not be assigned to any type and can be divided into four parts: *(i)* at low Me-β-CD concentration, the diagram should be assigned to A_L type suggesting the formation of 1:1 inclusion complex. *(ii)* However, at higher concentration of Me-β-CD, the curve positively deviated from linearity suggesting the formation of a 2:1 (Me-β-CD/Dtx) complex (type "A_P") [32]. *(iii)* The solution reached saturation at Me-β-CD concentration above 10 % w/w. *(iv)* Further increase of Me-β-CD above this concentration had a slight effect on the Dtx apparent aqueous solubility (type "A_N"). Finally, for Me-β-CD concentration higher than 20 % w/w a slight decrease of Dtx solubility was observed (type "A_N"). On this basis, Me-β-CD complexes were further characterized. Considering that the concentrations of Dtx with 10 % and 20 % w/w of Me-β-CD were very close (9.38 and 9.98 $mg.mL^{-1}$), the lowest concentration of the CD (10 %) was preferred for the characterization studies.

Circular dichroism spectroscopy was then used to confirm the existence of interactions between Dtx and Me-β-CD. Circular dichroism spectroscopy is based on the measurement of differences in the absorption of left-handed polarized light versus right-handed polarized light, which arises due to structural asymmetry [11, 33]. The relatively large increase in signal intensity suggested the existence of interactions between Dtx and Me-β-CD. Figure 2 presents the circular dichroism spectra of Dtx in the absence or presence of Me-β-CD. Me-β-CD did not show a circular dichroism simply because it does not absorb *uv* light in the considered wavelength range. On the contrary, Dtx molecules in solution produced a circular dichroism spectrum. A negative circular dichroism band at 280-300 nm was observed. This band was very weak due to the low concentration of Dtx in experimental samples, which was related to the low aqueous solubility of this drug. On the contrary, in the presence of Me-β-CD, the

intensity of this band increased, which was due to the enhancement of apparent Dtx aqueous solubility, considering that Me-β-CD did not show any circular dichroism band.

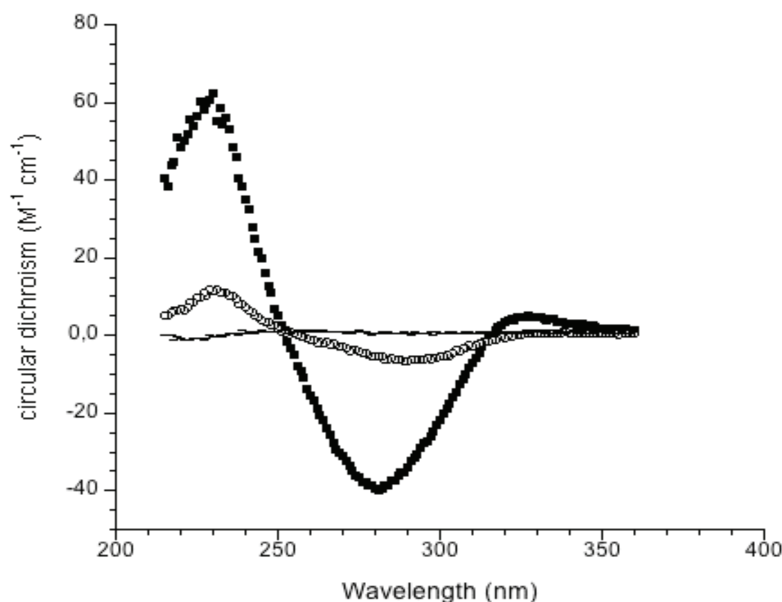


Figure 2. Circular dichroic spectra of free Dtx (0.0019 $mg.mL^{-1}$) (○), Me-β-CD (-) (10% w/w) and their inclusion complex (Me-β-CD/Dtx) (0.75 $mg.mL^{-1}$) (■).

To further investigate the nature of the interactions of Dtx with Me-β-CD, such as the possibility of an inclusion of a Dtx molecule or a specific chemical group within a Me-β-CD cavity, ^{1}H-NMR and the 2D-NOESY experiments were necessary. A first experiment was carried out by ^{1}H-NMR in order to compare the spectra of the Me-β-CD/Dtx complex with the one of the free drug and Me-β-CD. It was very difficult to dissolve a sufficient quantity of Dtx in D_2O necessary to perform the analysis. Considering that Dtx water solubility was 0.0019 $mg.mL^{-1}$, only a Spectrometer NMR at 800 MHz equipped with a special probe (cryoprobe) combined to a high number of the scan (512) were able to increase the NMR sensitivity. To ascertain the attribution of the peaks, it was then necessary to compare Dtx spectrum obtained in D_2O by using ^{1}H-NMR 800 MHz with the one obtained with Dtx dissolved in $CDCl_3$ by using ^{1}H-NMR 300 MHz. We checked that the chemical shifts characteristic of Dtx molecule (7.00-8.00 ppm) were similar for $CDCl_3$ (Fig. 3.a) and D_2O

(Fig. 3.b). Noteworthy that the chemical shifts obtained with $CDCl_3$ were similar to the ones described by previous research works [34].

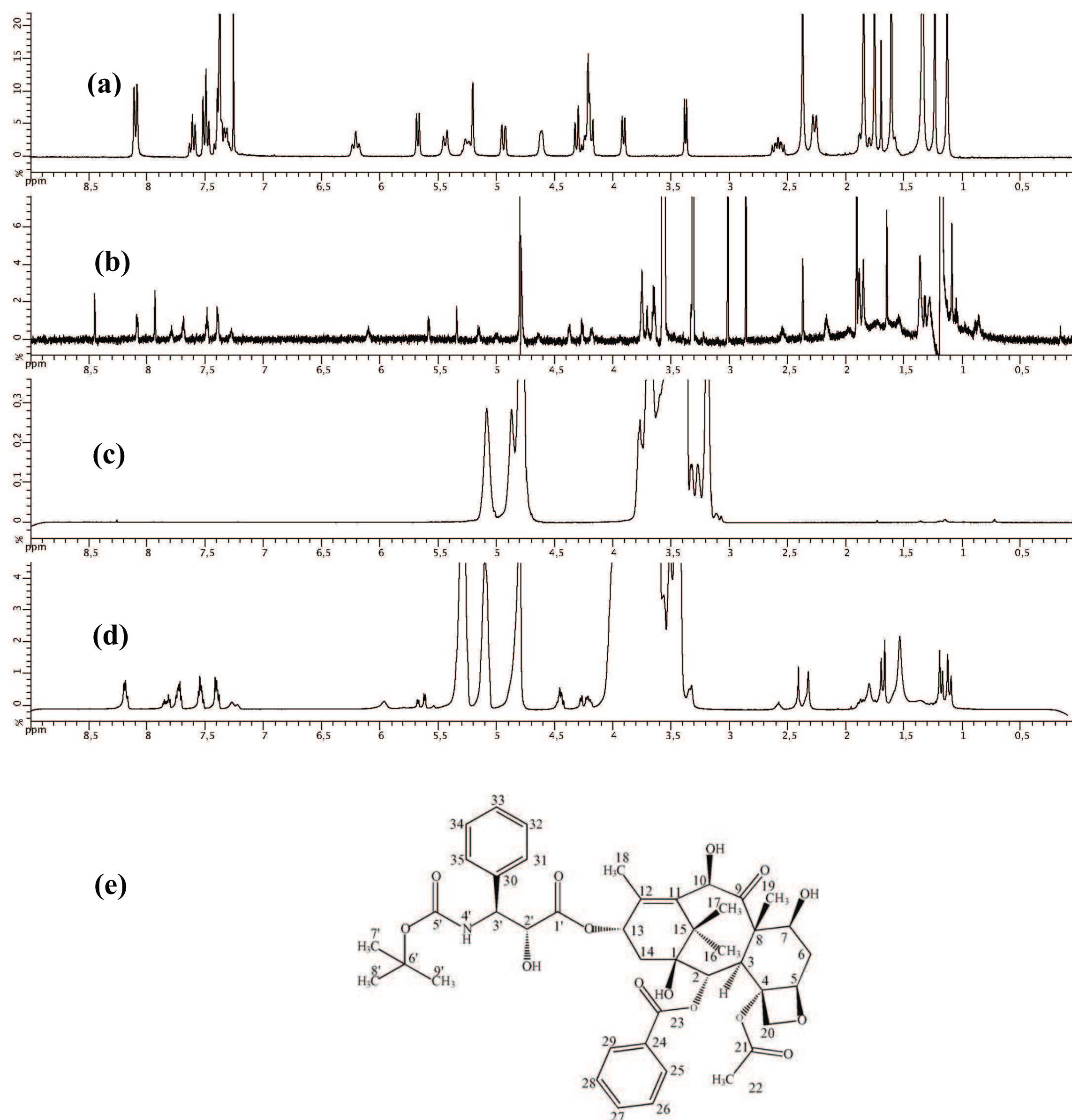


Figure 3: ^{1}H-NMR spectra: (a) free Dtx in $CDCl_3$ (300 MHz, NS=32, [Dtx]=10 mg.mL^{-1}); (b) free Dtx in D_2O (800 MHz, NS=512, [Dtx]=0.0019 mg.mL^{-1}); (c) free Me-β-CD in D_2O (600 MHz, NS=32, [Me-β-CD]=10% w/w); (d) Me-β-CD/Dtx complex (600 MHz, NS=32, [Dtx]=5.0 mg.mL^{-1}), (e) chemical structure of Dtx.

A spectrum of the Me-β-CD/Dtx complex was obtained by using ^{1}H-NMR 600 MHz instead of 800 MHz and by using a usual number of the scans (n=32) because higher concentrations

of Dtx could be obtained in D_2O in presence of Me-β-CD. As shown by phase solubility diagrams, the Dtx water solubility was significantly improved by using Me-β-CD at a concentration of 10 % w/w (Table I). The spectrum of the Me-β-CD/Dtx is presented in Figure 3.d. The peaks were localized in the range 3.2-5.3 ppm. The inclusion complex spectrum coincided with the sum of the spectra of free Dtx (Fig. 3.b) and Me-β-CD (Fig.3.c). Furthermore, in the complex spectrum, it is important to notice the pronounced chemical shift changes in Dtx and Me-β-CD protons typical of the inclusion complex formation.

The chemical groups of Dtx directly involved in the complexation process with Me-β-CD were then investigated using two-dimensional Nuclear Overhauser Effect measurements. Figure 4 shows the cross peak NOESY bands in the aromatic region (7.2-8.1 ppm) and methyl region (1.0-1.8 ppm). The largest cross peak coincided with the intermolecular interactions between the hydrogen of Dtx (C_{24}-C_{29}, C_{30}-C_{35} and C_6-C_9) and the internal hydrogens of the Me-β-CD cavity. These interactions could arise only if a Me-β-CD/Dtx complex is formed. These data suggested that the two aromatic rings and the *tert*-butyl group of Dtx were able to interact with Me-β-CD.

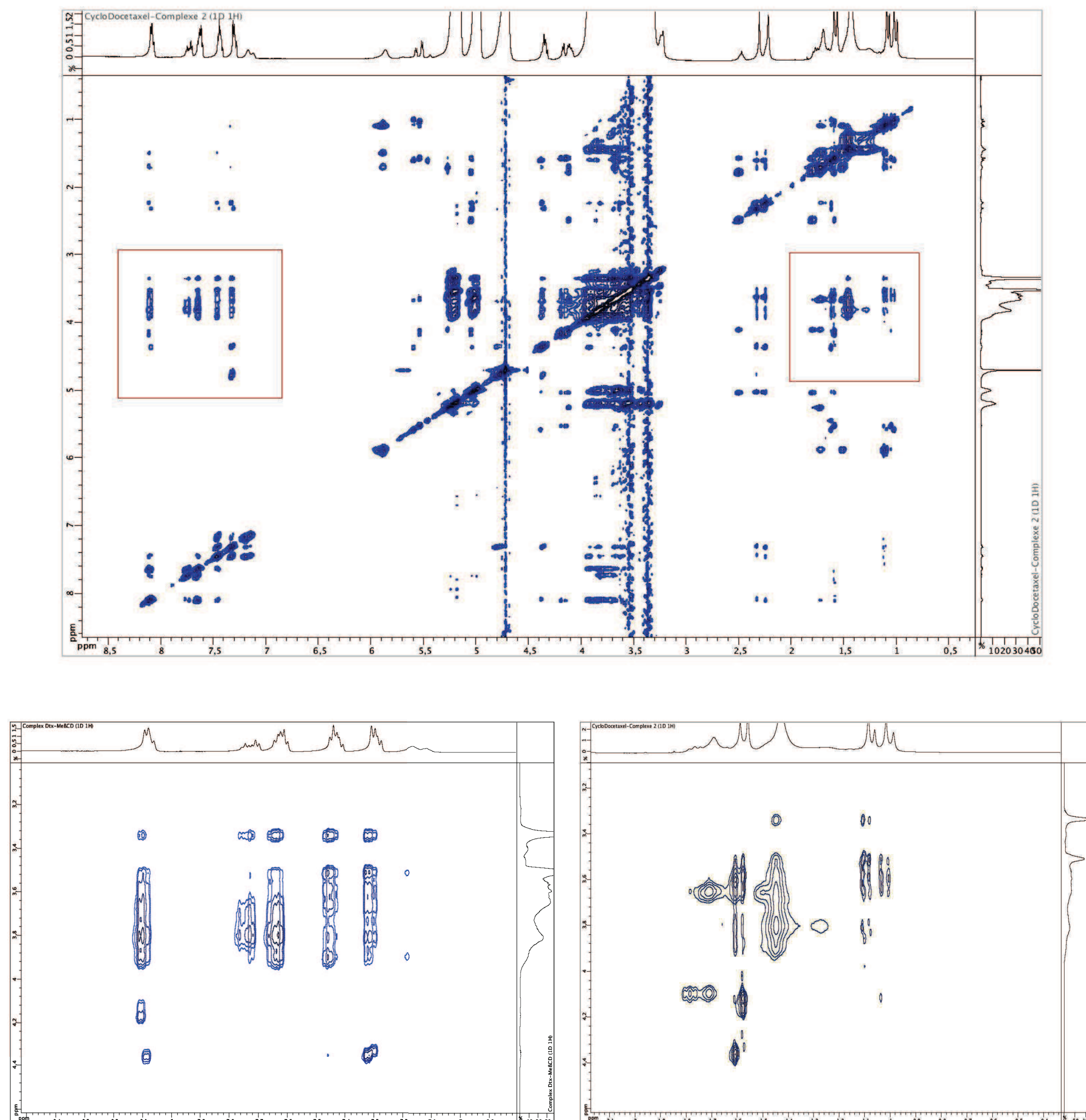


Figure 4. Two-dimensional spectrum of Me-β-CD/Dtx complex (600 MHz, D_2O). The largest cross peak coincided with the intermolecular interactions between the hydrogen of Dtx (C_{24}-C_{29}, C_{30}-C_{35} and C_6-C_9) and the internal hydrogens of the Me-β-CD cavity.

The ^{1}H-NMR results were confirmed by molecular modeling. In the first step, Dtx was docked into the cavity of one Me-β-CD molecule (1:1 stoichiometry), in order to identify all favorable interaction modes. Two clusters of conformers were obtained, each one corresponding to a distinct interaction mode, as shown in Figure 5. In the first complex (Fig. 5.a and 5.b), the *tert*-butyl and the C_{30}-C_{35} phenyl groups are positioned in the Me-β-CD cavity, towards the narrow and the outer side, respectively. In the second one, the C_{24}-C_{29} phenyl group is positioned exactly in the center of the Me-β-CD cavity (Figures 5.c and 5.d).

In both cases, most hydrogen atoms from *tert*-butyl and phenyl groups are in close proximity with the Me-β-CD cavity hydrogen atoms, so these modes of interaction identified by molecular modeling are in full compliance with the NMR data.

In the second step, we wanted to test if it would be possible to form a Me-β-CD/Dtx complex with a 2:1 stoichiometry, an interaction mode with all three hydrophobic groups shielded from solvent contact, while conserving the interacting sites observed in the 1:1 complexes. Thus, cavities of different sizes were generated from two identical Me-β-CD monomers with the outer sides pointing one to another, aligned on the cavities axes, with distances between cavities centers ranging from 10 Å to 20 Å. This orientation is supposed to provide maximum host-guest contact surface and minimum steric hindrance between Me-β-CD monomers. Dtx was then docked into these cavities and the optimum distance between cavities centers was found to be 13 Å. The resulting 2:1 inclusion complex, represented in Figure 6 (see also Supplementary Information for a PDF3D representation), shows the same Me-β-CD/Dtx interactions as in the 1:1 complexes. Therefore, the complex shown in Figure 6 can be considered as representative for Me-β-CD/Dtx interaction mode, although an equilibrium between the 2:1 and 1:1 forms cannot be excluded [35].

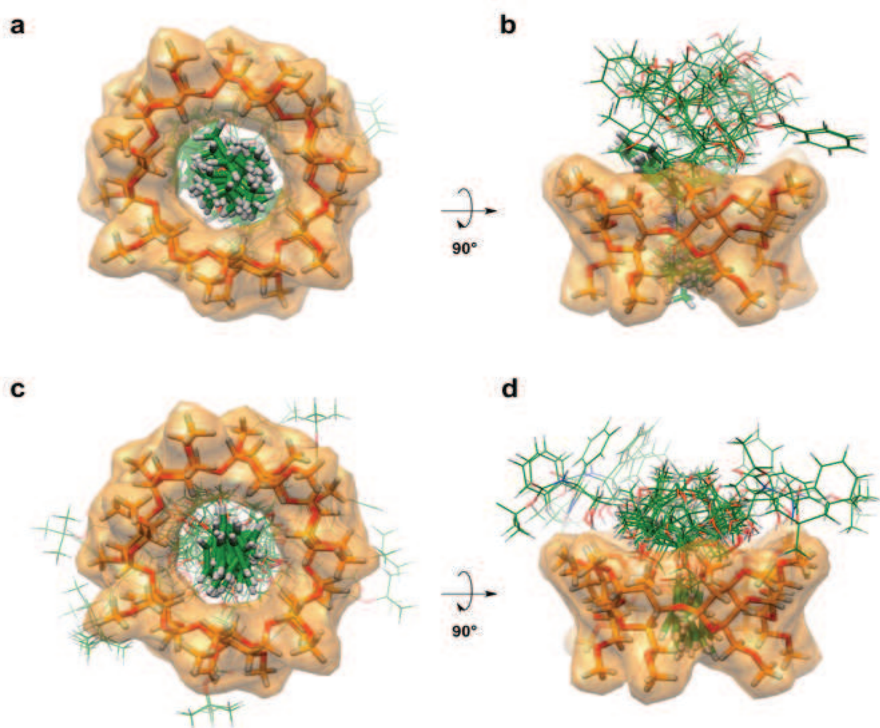


Figure 5. Representation of the two interaction modes (vertical and lateral views) between Me-β-CD (orange) and Dtx (green) obtained by molecular docking (1:1 stoichiometry). The hydrophobic groups interacting with the Me-β-CD cavity are shown in stick representation.

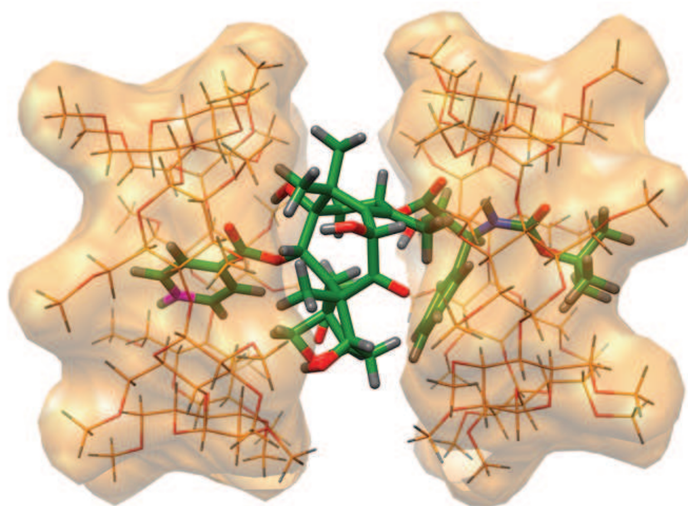


Figure 6. Representation of the complex between Me-β-CD (orange) and Dtx (green) obtained by molecular docking (2:1 stoichiometry). See Supplementary Information for an interactive PDF3D representation of this complex.

Let's determine now the association constants and the thermodynamic parameters of the interaction between Dtx and Me-β-CD. ITC technique can help to determine whether an association process occurs between two species and allows the evaluation of the association constant (K), the stoichiometry (N), the enthalpy (ΔH) and the entropy (ΔS) of the Me-β-CD/Dtx interaction from which the Gibbs free energy (ΔG) of the process can be derived [36-40]. The enthalpogram corresponding to the interaction between Dtx and Me-β-CD is presented in Figure 7.

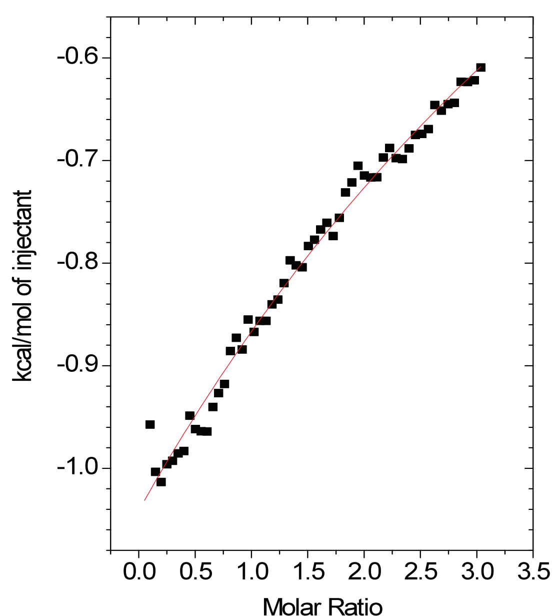


Figure 7: Typical ITC enthalpogram corresponding to the binding interaction developed during the titration of Dtx (0.703 mM) contained in the measurement cell by Me-β-CD (10 mM) contained in the titration syringe at 25°C giving a differential binding curve which was fit to sequential two-sites binding model yielding the following parameters: N=2, K_1=744 M^{-1}, ΔH_1= -12.1 kJ.mol^{-1}, K_2=202 M^{-1}, ΔH_2= -27.6 kJ.mol^{-1}.

Different interaction models available in the ITC apparatus software were tested to fit this enthalpogram, including a one-set of site model, a two-set of sites model and a sequential binding model. The enthalpogram was first fitted to the one-set of site model, which describes

the interaction with a defined number of identical binding sites. According to the ITC software, the best fit without constraint (i.e. without constraint on the value of the stoichiometry) led to a stoichiometry of the interaction equal to 4 (Table II), meaning that 4 molecules of Me-β-CD interacted with 1 molecule of Dtx. Obviously, such a stoichiometry would be very unlikely as shown by the molecular modeling which suggested that only two molecules of Me-β-CD were able to interact with one Dtx molecule.

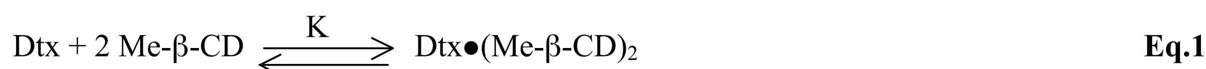
Table II. Different fitting models leading to the determination of the complex affinity constants (K), and thermodynamic parameters corresponding to inclusion complex formation of Dtx (0.703 mM) with Me-β-CD (10 mM) at 298 K (25°C).

Fitting model	*N*	*K* (M^{-1})	*ΔH* ($kJ.mol^{-1}$)	*TΔS* ($kJ.mol^{-1}$)	*ΔG* ($kJ.mol^{-1}$)
One-site model without constrain on N	4	73	-25.5	-14.9	-10.6
One-site model N=2 was imposed	2	48	-487.6	-487.0	-9.5
Two set of sites model	2	K_1: 130 K_2: 73	ΔH_1: 10.2 ΔH_2: -113.4	$T\Delta S_1$: 22.3 $T\Delta S_2$: -102.8	ΔG_1: -12.0 ΔG_2: -10.6
Sequential two-binding sites model	2	K_1: 744 K_2: 202	ΔH_1: -12.1 ΔH_2: -27.6	$T\Delta S_1$: 4.2 $T\Delta S_2$: -14.4	ΔG_1: -16.3 ΔG_2: -13.1

The enthalpogram was then fitted into the same one-set of site model but the stoichiometry of the interaction was imposed and fixed to two CDs for one Dtx molecule. According to this model, the association constant of both interacting groups was 48 M^{-1} and ΔH was equal to -487 $kJ.mol^{-1}$. The value of association constant is too low and the absolute

value of ΔH is too high when compared to the association constants and enthalpies observed for the interaction of β-CD derivatives with molecules bearing *tert*-butyl and aromatic groups. Generally, K is in the range of 130-400 M^{-1} and ΔH in the range of (-10)-(50) $kJ.mol^{-1}$ [34, 40-42] for review see [43].

The one-set of site model considers that all the binding sites are identical as no distinction between the interacting sites is made (Eq.1).


Dtx + 2 Me-β-CD $\xrightleftharpoons{K}$ Dtx●(Me-β-CD)$_2$ **Eq.1**

However, according to molecular modeling, a Me-β-CD could interact with Dtx according to two distinct modes. The *tert*-butyl and C_{30}-C_{35} phenyl groups interacted with one Me-β-CD and the C_{24}-C_{29} phenyl group interacted with a second Me-β-CD.

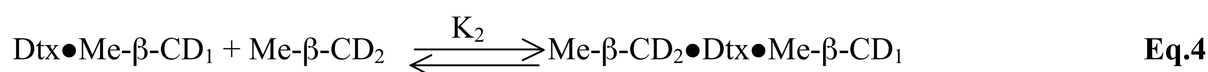
For investigating this possibility, the enthalpogram was then fitted to a two set of sites model. This model yields two distinct binding constants corresponding to the interaction of two Me-β-CDs molecules simultaneously with one Dtx molecule. In this model, association constants are expressed by the following equations:

$$K_1 = \frac{[Dtx \bullet Me - ß - CD_1]}{[Dtx][Me - ß - CD_1]} \qquad K_2 = \frac{[Dtx \bullet Me - ß - CD_2]}{[Dtx][Me - ß - CD_2]} \qquad \textbf{Eq.2}$$

However, this model considers that the two sites are identical and independent, which was unlikely according to molecular modeling and ^{1}H-NMR (NOESY) results. Finally, a sequential two-step binding model was found to be more suitable for describing the interactions. This model describes interactions for systems presenting non-identical sites. The two sets of sites model discussed earlier, assumes that the two interactions occur independently one from the other. In the sequential bonding model, the first Me-β-CD_1 molecule which binds to Dtx always binds to site 1 (both *tert*-butyl and C_{30}-C_{35} aromatic group), then the second Me-β-CD_2 molecule which binds to Dtx always binds to site 2 (C_{24}-

C_{29} aromatic group). Thus, two distinct binding constants are observed but in addition, these binding events are sequential. In this scheme, the Me-β-CD_1 molecule would first interact with the Dtx *tert*-butyl and aromatic group (C_{30}-C_{35}, K_1: 744 M^{-1}) (Eq.3) and then the second Me-β-CD_2 molecule would interact with the second aromatic group (C_{24}-C_{29}, K_2: 202 M^{-1}) (Eq.4). As expected for this type of model, the ΔG of the second interaction was less negative than the first interaction (ΔG_1: -16.38 $kJ.mol^{-1}$ and ΔG_2: -13.15 $kJ.mol^{-1}$) [44, 45].

$$\text{Dtx} + \text{Me-}\beta\text{-CD}_1 \overset{K_1}{\rightleftharpoons} \text{Dtx}\bullet\text{Me-}\beta\text{-CD}_1 \qquad \textbf{Eq.3}$$



$$\text{Dtx}\bullet\text{Me-}\beta\text{-CD}_1 + \text{Me-}\beta\text{-CD}_2 \overset{K_2}{\rightleftharpoons} \text{Me-}\beta\text{-CD}_2\bullet\text{Dtx}\bullet\text{Me-}\beta\text{-CD}_1 \qquad \textbf{Eq.4}$$

$$K_1 = \frac{[\text{Dtx} \bullet \text{Me} - \text{ß} - \text{CD}_1]}{[\text{Dtx}][\text{Me} - \text{ß} - \text{CD}_1]} \quad K_2 = \frac{[\text{Me} - \text{ß} - \text{CD}_1 \bullet \text{Dtx} \bullet \text{Me} - \text{ß} - \text{CD}_2]}{[\text{Dtx} \bullet \text{Me} - \text{ß} - \text{CD}_1][\text{Me} - \text{ß} - \text{CD}_2]} \qquad \textbf{Eq.5}$$

The possibility for sequential binding events in CD complexation have been reported for molecules with native CDs, [46, 47], CD dimers and CD polymers [48-50] but so far, it has never been reported for Dtx and Me-β-CD complexes. The only available association constant for Dtx interaction with β-CD derivates has been experimentally obtained from solubility diagrams [16]. A major source of systematic errors lies in the inadequate application of the 1:1 stoichiometry to calculate K. Loftsson and co-workers have questioned phase solubility diagram method for the determination of association constants [32]. They pointed out that the apparent association constant observed from phase solubility diagrams reflects drug solubilisation by many possible mechanisms other than simple inclusion inside the CD cavities. Indeed, several research groups have shown that CDs may form both inclusion and non-inclusion complexes and that many different types of complexes can coexist in aqueous solutions [51, 52]. In addition, both CDs and CD/drug complexes are known to form aggregates and it is thought that these aggregates are able to solubilize drugs and other hydrophobic molecules through micellar-type mechanisms [32, 53].

In the present study, the combination of NMR, molecular docking and ITC represented and efficient strategy for evaluating realistic association constants related to the complexation of Dtx with Me-β-CD. Indeed, the superiority of ITC over all other methodologies is the possibility to get deep understanding of the molecular interaction between Dtx and Me-β-CD through the determination of the association constant and thermodynamic parameters. This determination is independent from the solubilisation of the drug since both Dtx and Me-β-CD are in their soluble form. From a thermodynamic point of view, ITC allowed to gain insights into the nature of the non-covalent interactions occurring between Dtx molecule and Me-β-CD leading to the determination of complexation thermodynamic parameters. We demonstrated from molecular docking calculations that no hydrogen bonds were established between Dtx and Me-β-CD in the inclusion complex. The study of ΔH and ΔS obtained from ITC data led to the determination of the driving force of the interaction. The interaction is dominated by van der Waals' forces when the process is enthalpy-driven with minor favorable or unfavorable entropies ($|\Delta H|>|T\Delta S|$). However, hydrophobic interactions between two apolar molecules at room temperature have been known as entropy-driven processes, where the entropy of the interaction is large and positive while the enthalpy is small ($|\Delta H|<|T\Delta S|$). For the interaction of Me-β-CD with Dtx, the analysis of thermodynamic data led to the conclusion that the association process was exothermic ($\Delta H<0$), predominantly driven by enthalpy and moderately by entropy ($|\Delta H|>|T\Delta S|$) for both interacting sites. This thermodynamic signature strongly supports van der Waals-type interactions as the dominant driving force for Dtx complexation with Me-β-CD.

Concerning the entropy, a minor positive entropic contribution ($T\Delta S_1$= 4.27 kJ.mol^{-1}) was observed for the first interaction site (*tert*-butyl and C_{30}-C_{35} aromatic groups) and negative entropy for the second interaction site (C_{24}-C_{29} aromatic group) ($T\Delta S_2$= -14.47

kJ.mol^{-1}). These thermodynamic behaviors are in accordance with molecular modeling. Positive entropy changes arise from an important flexibility of the interacting species while negative entropy changes usually arise from a significant reduction of the translational and conformational freedoms of host and guest upon complexation [10]. The first interaction (Me-β-CD with *tert*-butyl and C_{30}-C_{35} aromatic groups) was characterized by a greater hydrophobic contact surface, but also by a flexibility induced by the carbamate linker connecting these two groups, whereas in the second interaction (Me-β-CD with C_{24}-C_{29} aromatic group), the interacting groups had a restricted mobility and smaller hydrophobic contact surface, with the phenyl group being kept more tightly in the center of the Me-β-CD cavity. These differences between the two modes of interaction are considered to be responsible for the positive and negative entropic contributions obtained from the analysis of ITC data.

The effect of guest flexibility on association constant and thermodynamic parameters has been reported in many cases and reviewed by Rekharsky and Inoue in their review [43]. They reported that unsaturated compounds have lower conformational degrees of freedom than the analogous saturated compounds. For example, the association constants for the complexation of trans-3-hexenoate and 6-heptenoate with α-CD were approximately half those corresponding to hexanoate and heptanoate [54]. Comparison of the relevant ΔH and ΔS values for these interactions indicates that this effect was entropic in origin [54].

Another example of drastic changes in the entropic terms caused by increasing guest flexibility can be seen in the comparison of the complexations of 1-phenylimidazole and 1-benzylimidazole, for which the affinity toward β-CD was 15 times greater for 1-benzylimidazole than for 1-phenylimidazole. This was attributed to an increased freedom resulting of the addition of an extra methylene group [55]. Similar conclusions were drawn

with α-CD complexes of these molecules [55]. The main source of the large increase in ΔG for the complexation of 1-benzylimidazole with both α and β-CD is a highly favorable ΔS, which is partly compensated by an unfavorable ΔH [55]. These examples clearly demonstrate the decisive role of the guest's flexibility in the stability of CD complexes. Thus, increasing flexibility or degrees of freedom in a guest molecule leads to more favorable complexation entropy, since more of the possible "conformers" can fit properly into the cavity.

4. Conclusion

In conclusion, apparent Dtx aqueous solubility has been successfully increased about 5374 times to 9.98 $mg.mL^{-1}$ by using Me-β-CD. This represents the highest Dtx aqueous apparent solubility increment ever reported by using conventional CDs. Interestingly, the combination of solubility experiments to circular dichroism, NMR, ITC and molecular docking calculations helped to identify a bivalent sequential binding mechanism, where a Me-β-CD molecule first interacted with both *tert*-butyl and C_{30}-C_{35} aromatic groups, then a second Me-β-CD molecule interacted with the C_{24}-C_{29} aromatic group. This type of binding is not frequently encountered for interactions between CD derivates and guest molecules and has never been reported before in the literature for Dtx. The results reported in this work constitute useful information for both fundamental and applied pharmacy.

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CHAPITRE III: Intestinal permeation enhancement of docetaxel encapsulated into Me-ß-CD/poly(isobutyl cyanoacrylate nanoparticles coated with thiolated chitosan

Intestinal permeation enhancement of docetaxel encapsulated into Me-ß-CD/poly(isobutyl cyanoacrylate) nanoparticles coated with thiolated chitosan

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Abstract

In this study the potential of mucoadhesive nanoparticles comprising methyl-ß-cyclodextrin (Me-ß-CD) combined with poly(isobutyl cyanoacrylate) (PIBCA) and coated with thiolated chitosan to enhance the oral bioavailability of docetaxel (Dtx) was investigated. These nanoparticles were prepared by anionic emulsion polymerization of isobutyl cyanoacrylate. In order to encapsulate the highest amount of Dtx into nanoparticles, the polymerization was carried out in a solution of Me-ß-CD/Dtx inclusion complex. The resulting particles were spherical with diameters in the range of 200-400 nm and positively charged. Dtx loading efficiency was 70-80 %. *In vitro* experiments in simulated intestinal media containing pancreatin showed that Dtx was gradually released to reach 60 % after 24 h and 100 % after 48 h. The capacity of these nanoparticles to enhance the flux of Dtx crossing the intestinal membrane was investigated using the Ussing chamber technique. The amount of Dtx absorbed from NPs was found to be higher than from a control solution of Dtx in a 2 % v/v ethanolic/Ringer solution. Interestingly, these particles could decrease efficiently the efflux of Dtx in the serosal to mucosal direction, which was shown by the fact the permeation fluxes from NPs in the mucosal to serosal direction were comparable to the one obtained for a Dtx solution in the serosal-to-mucosal direction. Furthermore, when mucoadhesive interactions between nanoparticles and the mucosa were avoided, the intestinal flux of Dtx significantly decreased, confirming that mucoadhesion of the nanoparticles was a mandatory condition to enhance the bioavailability of Dtx.

Keywords: docetaxel, methyl-ß-cyclodextrin, nanoparticles, poly(isobutyl cyanoacrylate), thiolated chitosan, mucoadhesion, Ussing chamber, intestinal permeability.

1. Introduction

Docetaxel (Dtx) is a potent anti-cancer drug that displays a broad spectrum of antitumor activity [1]. It is registered for the treatment of prostate and breast cancers as well as lung carcinoma, head and neck cancer [2]. It is a semi-synthetic analogue of paclitaxel and its mechanism of action is based on the ability to bind to the ß subunit of tubulin, which interferes with the polymerization of microtubules, thereby damaging dividing cells. Its current commercial formulation, Taxotere®, is administered by the parenteral route and is formulated as a solution containing polysorbate 80, which causes severe allergic reactions and peripheral neuropathology [3] that require the oral administration of dexamethasone and antihistamine before infusion. During the past few decades, quality of life has emerged as an important outcome in oncology [4]. I.v. chemotherapy needs hospitalization, nursing, and a palliative treatment. Although the use of ambulatory pumps and indwelling catheters enable home-based, this kind of administration remains inconvenient for patients. In this context, the oral chemotherapy is becoming a very interesting alternative. The medication can be take at home, interferes less with the daily activity [5] and in particular the oral chemotherapy makes the patients feel less ill and help them to face their illness better [6].

However, Dtx belongs to the Class IV of the Biopharmaceutical Classification System [2], which comprises substances with both low solubility in aqueous fluids and low apparent intestinal permeability. This represents a major drawback when foreseeing oral delivery. Moreover, Dtx has been shown to be substrate of biological transporters and/or metabolized in the intestinal barrier [7]. Thus, efficient oral dosing of Dtx by the mean of conventional formulations could be inefficient.

We have already shown that the apparent Dtx aqueous solubility was successfully increased about 5000 times, from 0.0019 $mg.mL^{-1}$ to 9.98 $mg.mL^{-1}$ by using Me-β-CD [8]. This result represents the highest Dtx aqueous apparent solubility increment ever reported in

the literature by using conventional CDs. However, Dtx complexation by CDs only addresses one aspect of the problem, since oral delivery of Dtx/CDs complexes is likely to result in local precipitation phenomena in the GI tract.

In recent years, several strategies have been proposed for the oral delivery of Dtx including the co-administration of compounds able to block the efflux pumps or the cytochrome P450 (CYP450) responsible for the rapid drug elimination [9-12] and pharmaceutical approaches using oral solid dispersions [2], microemulsions [13] or conjugation of Dtx to low molecular weight (M_w) chitosan [14].

The aim of this work was to investigate the potential of nanoparticles for improving the oral bioavailability of Dtx, which is almost nil after oral delivery. In this purpose, core-shell nanoparticles were selected and composed of a poly (isobutyl cyanoacrylate) (PIBCA) core coated with a hydrophilic and mucoadhesive thiolated chitosan layer. Indeed, the ability of poly(alkyl cyanoacrylate) NPs to load different kinds of active agents, such as peptides, vaccines and cytotoxic agents has been extensively demonstrated [15-19]. Moreover, interesting release profile kinetics can be obtained, due a combination of drug entrapment and biodegradability of these particles in intestinal medium. These kinetics may be compatible with intestinal residence durations when mucoadhesion is considered. Furthermore, the addition of a hydrophilic peripheral layer has been shown to considerably enhance the mucoadhesion of these particles to the intestinal surface, which has been shown to improve drug fluxes across the epithelium [20, 21].

Among the characteristics of PIBCA nanoparticles coated with thiolated chitosan, mucoadhesion properties are the most important one for an oral administration. The presence of the positively charged chitosan chains, and the thiol groups at the surface could allow nanoparticles to adhere to the mucus layer [22, 23]. The immobilization of the drug carrying particles at the mucosal surface would result in: *(i)* a prolonged residence time at the site of

drug absorption, *(ii)* an increase in the local drug concentration gradient in front of the absorptive membrane, due to the prolonged contact of the particles with the mucus and *(iii)* a direct contact with enterocytes which represent the first step before particle absorption [24]. Moreover, thiolated chitosan led to a significant increase of the oral bioavailability of efflux pumps substrate molecules, such as rhodamine-123 [25, 26].

In this work, nanoparticles were prepared by anionic emulsion polymerization and in order to achieve the maximum concentration of Dtx into nanoparticles, polymerization has been conducted directly in a solution of Me-ß-CD/Dtx inclusion complex. This strategy has been shown earlier to improve considerably the yields of encapsulation for poorly water-soluble drugs. For example, it was already demonstrated for a series of steroids (i.e. hydrocortisone, prednisone and testosterone) or antiviral drugs (saquinavir), that combined PIBCA and hydroxypropyl-ß-cyclodextrin (HP-ß-CD) loaded-nanoparticles could be 2-100 folds more than for PIBCA nanoparticles prepared in absence of drug/CDs complexes [27-31].

In summary, the aim of this study was to develop a mucoadhesive nanoparticles constituted of Me-ß-CD combined with PIBCA coated with thiolated chitosan for the encapsulation of Dtx. In a first step, the system was characterized from a physico-chemical point of view and the loading ability was investigated. Then, the ability of nanoparticles to allow a time-controlled release was evaluated and final their capacity to enhance the intestinal permeation of Dtx was assessed using the Ussing chamber technique.

2. Materials and methods

2.1. Reagents

Anhydrous Dtx, 98.5 % was purchased from Chemos GmbH (Germany). [^{3}H] Dtx was purchased from Moravec Biochemical, Inc. (California, USA). Random Methyl-β-cyclodextrin (Me-β-CD Rameb®, M_w=1320 $g.mol^{-1}$) was purchased from Cyclolab (Budapest, Hungary). Isobutylcyanoacrylate (IBCA) was kindly provided as a gift by Henkel Biomedical (Dublin, Ireland). Chitosan Mw 400,000 $g.mol^{-1}$ and L-cysteine HCl were purchased from Fluka (Saint-Quentin-Fallavier, France). 2-iminothiolane HCl (Traut's reagent) was synthesized in the Department of Organic Chemistry (Biocis UMR CNRS 8076, Faculté de Pharmacie, Université Paris Sud, Châtenay-Malabry, France). Pancreatin, calcium chloride, monobasic potassium phosphate, dipotassium phosphate, sodium chloride, sodium hydroxide, sodium nitrite, nitric acid, bumetanide, forskolin and verapamil hydrochloride were provided by Sigma Aldrich (France). Magnesium chloride and sodium hydrogen carbonate were purchased from Prolabo (France). Methanol for HPLC analyses was obtained from Carlo Erba (Italy). Solutions were prepared by weight using MilliQ® water (Millipore, France). All chemicals were of analytical grade and used as received.

2.2. Chitosan modification and characterization

2.2.1 Depolymerization of chitosan

Chitosan was selectively depolymerized following the method developed Huang *et al.* [32]. Briefly, 100 mL of a 2 % (w/v) commercial chitosan (400,000 $g.mol^{-1}$) solution in acetic acid solution (6 %, v/v) was depolymerized at room temperature under stirring with 10 mL of $NaNO_2$ solutions in MilliQ® water at concentration of 9 $g.L^{-1}$, in order to obtain the desired final M_w: 20,000 $g.mol^{-1}$. After 1 h of reaction, chitosan was precipitated by raising the pH to

9.0 with NaOH (4 M). The white-yellowish solid was filtrated, extensively washed with acetone and re-dissolved in a minimum volume of acetic acid 0.1 N (around 20–30 mL). Purification was carried out by subsequent dialyses against MilliQ® water (Spectra/Por® 3 membrane MWCO: 3,500). The dialysed product was freeze-dried (Christ Alpha 1-4 freeze-dryer. Bioblock Scientific, Illkirch, France) and the yellowish lyophilized was then stored at 4 °C until use. The product obtained was called Chito20 depending of its theoretical M_w.

2.2.2. Thiolation of chitosan

The inclusion of thiol groups in the hydrolyzed chitosan was carried out following the method developed by Bernkop-Schnürch *et al*. [33]. One gram of chitosan was solubilized in 100 mL of acetic acid solution (1 % w/v). The pH of the solution was adjusted to 6.5 with NaOH (1 N). Then, the Traut's reagent (2-iminothiolane) was added in a chitosan : 2-iminothiolane weight ratio of 5:2. After an incubation period of 24 h at room temperature under continuous stirring, the resulting thiolated polymer was dialysed (Spectra/Por® 3 membrane MWCO: 3,500) against different aqueous media: 8 h against 5 L of 5 mM HCl, 8 h against 5 L of 5 mM HCl containing 1 % NaCl two times, 8 h against 5 L of 5 mM HCl and finally, 8 h against 5 L of 5 mM HCl (40 h in total). Dialysed products were freeze-dried (Christ Alpha 1-4 freeze-dryer. Bioblock Scientific, Illkirch, France) and stored at −20 °C until use. The resulting polymers are chitosan–4-thiol-butylamidine, named Chito20-TBA according with the original theoretical M_w of the respective unmodified polymer.

2.2.3. Determination of the deacetylation degree

The technique of nuclear magnetic resonance (^1H NMR) was used by many authors to determine the degree of deacetylation of chitosan [34-36]. ^1H NMR spectra were recorded with a Bruker MSL-400 spectrometer (Bruker Instrument Inc., Wissembourg, France) at 25 °C. Samples were dissolved in D_2O, which contained a small amount of DCl. From the NMR

spectrum of chitosan, a peak could be assigned unambiguously, representing the methyl protons ($\delta(CH_3)$ = 2.1 ppm). The cyclic structure of glucose residues could be also observed: (δ = 3.5–4.0 ppm for H-3 to H-6 and δ = 3.2 for H-2). The degree of deacetylation (D_{deac}) was calculated from these data following Eq.1:

$$D_{deac} = 1 - \left[\frac{2I_{CH3}}{I_{H-2;H-3;H-4;H-5;H-6}}\right]100 \qquad \textbf{Eq.1}$$

where I_{CH3} is the intensity of the methyl proton and $I_{H-2;H-3;H-4;H-5;H-6}$ is the intensity of all protons from the cyclic glucose structure except the proton on the anomeric carbon, according to Hirai *et al*. [37].

2.2.4. Molecular weight determination

The M_w of hydrolyzed chitosan was determined from capillary viscosity measurements. Briefly, the reduced viscosity of solutions of hydrolyzed chitosan of various concentrations (0.1–2.5 $g.L^{-1}$) in acetic acid 0.1 M, NaCl 0.2 M was measured in a Ubbelohde tube (53710/1 Schott Gerate) at 25 °C (Bath CT1450 Schott Gerate and cooling system CK100 Schott Ger- ate) using a viscometer AVS400 (Schott Gerate). The intrinsic viscosity $[\eta]$ was then deduced from the reduced viscosity measured for each solution of chitosan by extrapolation at zero concentration. The M_w was determined by using the Mark-Houwink Sakurada equation (Eq.2):

$$\eta = KM_w^a \qquad \textbf{Eq.2}$$

with K= 1.81×10^{-6} and a = 0.93 [38, 39].

2.3. Nanoparticle Preparation

Nanoparticles were prepared by anionic emulsion polymerization according to the method of Bertholon *et* al [40]. Briefly, 0.069 g of mixtures of hydrolyzed and thiolated chitosan (Chito20/Chito20-TBA) at different percentages (75/25 % and 100/0 %) was

dissolved in 5 mL of nitric acid in MilliQ® water (0.2 M), in a glass tube at 40 °C, under vigorous stirring and argon bubbling. After 10 min, 0.25 mL of IBCA were added under vigorous magnetic stirring. Argon bubbling was kept for additional 10 min and stopped. The reaction was allowed to continue at 40 °C under vigorous stirring for 50 min. The purification of nanoparticles was achieved by dialysis using a Spectra/Por membrane with a M_w cut off of 100,000 g.mol^{-1} (Biovalley, Marne-la-Vallée, France) twice 90 min and once overnight against 1 L of acetic acid 16 μM.

2.4. Me-ß-CD combined PIBCA nanoparticles

In order to achieve the maximum concentration of Dtx into nanoparticles, a modification of the protocol, described above, was introduced. The polymerization was carried out directly in a solution of 5 mL of Me-β-CD/Dtx inclusion complex (6 mg.mL^{-1}) containing nitric acid (0.2 M). After 50 min of polymerization, the suspension was ice cooled for 5 min and the pH was adjusted to 4.5 with NaOH (1 M). For the preparation of radioactive nanoparticles 50 μCi of [^{3}H] Dtx was added in the polymerisation medium before the IBCA inclusion. Nanoparticles free from Dtx were obtained according to the same protocol without Dtx. A solution of Me-ß-CD (10 % w/w) was used instead of the inclusion complex.

2.5. Physico-chemical characterization of the nanoparticles

2.5.1. Particle size distribution and ζ potential determination

The hydrodynamic diameter of the nanoparticles and the size distribution were determined at 20 °C by quasi-elastic light scattering using Zetasizer Nanoseries (Malvern Instruments Ltd. UK). The scattered angle was fixed at 90° and 60 µL of each sample was diluted in 2 mL of acetic acid 0.16 μM (Millex, SLAP 0225, Millipore, France). Zeta potential of nanoparticles was measured using Zetasizer Nanoseries (Malvern Instruments Ltd. UK). Dilution of the suspensions (1:33 (v/v)) was performed in NaCl (1 mM).

2.5.2 Scanning electron microscopy (SEM) and transmission electron microscopy (TEM)

Scanning electron microscopy was performed using a LEO 1530 apparatus (LEO Electron Microscopy Inc., Thronwood, NY) operating at 3 kV with a filament current of about 0.5 mA. Nanoparticle suspensions were diluted 1/3 in MilliQ® water. Liquid samples were deposited on vitreous carbon conductive double-side tape (Euromedex, France) and dried at room temperature. They were coated with a platinum laker of about 2 nm thick using a Cressington sputter-coated 208HR with a rotatory-planetary-tilt stage, equipped with a MTM-20 thickness controller.

Transmission electron microscopy was performed using a Philips EM 208 apparatus operating at 80 kV. Nanoparticles were directly observed after staining with phosphotungstic acid 1% (pH 7.4).

2.6. Determinination of the yield of Dtx loading into nanoparticles

The yield of Dtx loading (Y), expressed as a percentage, was calculated as described previously [41] according to Eq.3:

$$Y = \frac{L_{Dtx}}{T_{Dtx}} 100 = \frac{T_{Dtx} - F_{Dtx}}{T_{Dtx}} 100 \qquad \textbf{Eq.3}$$

where L_{Dtx} is the loaded Dtx (concentration of Dtx associated with the nanoparticles), F_{Dtx} the free Dtx (concentration of Dtx found in the dispersing medium after separation of the nanoparticles), and T_{Dtx} the total Dtx (concentration of Dtx recovered from the total nanoparticle suspension). In order to be comparable, all concentrations were reported to the same volume for each sample. Free unloaded Dtx contained in the dispersing medium was isolated from Dtx-loaded nanoparticles by ultrafiltration over Microcon centrifugal units YM-30 (30,000 MWCO). The concentration of Dtx associated with the nanoparticles and the total Dtx concentration recovered in the nanoparticle suspension were obtained after 0.1 mL of

nanoparticles were dissolved in 0.6 mL of DMSO at 40 °C over night. Samples containing Dtx were analyzed by reversed phase HPLC as following described (section 2.8).

We also checked the stability of the Dtx into nanoparticles during 15 days (see supplementary information).

2.7. *In vitro* released study

Release experiments were conducted in "sink" conditions at 37 °C by using two different mediums: Ringer solution with or without 1 % of pancreatin. Briefly, 100 µL of a freshly prepared suspension of Dtx-loaded nanoparticles were diluted in 5 mL of the medium at 37 °C and maintained under constant magnetic stirring. After scheduled incubation times the nanoparticles were removed from the incubation medium by centrifugation using Microcon® centrifugal filters YM-30 (Cut off 30,000 $g.mol^{-1}$) (15 min, $11x10^3$ rpm). The ultrafiltrate containing the released Dtx was analyzed using the HPLC technique as described in followed section.

2.8. Determination of Dtx concentration

Samples containing Dtx were analysed by reversed phase HPLC using a symmetric C_{18} column (250 x 4.6 mm) and UV detection at 231 nm (Waters system, France). The mobile phase was an isocratic mixture of methanol/water (70:30 % v/v). HPLC analyses were performed at 35 °C at a flow rate of 1 $mL.min^{-1}$. The validated range of concentrations (1.0 - 50 $\mu g.mL^{-1}$) was prepared from a standard solution of Me-ß-CD/Dtx inclusion complex at 1 $mg.mL^{-1}$ and diluted with the mobile phase. The resulting equation of the linear least square line was $y = 59564x + 127780$. Preliminary studies were carried out to evaluate the possibility to use the same validates range for all experiments. First of all the repeatability of the dilution method was checked (n=6). Then, different validation curves necessaries for each experiment were analyzed: in the first one, dilution was made with DMSO and in the second

one with Ringer solution in order to analyze the samples of the yield of encapsulation and release experiments respectively. The obtained curves were graphically compared with the one in which the dilutions were made only with the mobile phase and the three least square lines resulted superimposed (see supplementary information).

2.9. *"Ex vivo"* study by Ussing Chamber

2.9.1. Study of the pancreatin influence in the Ussing chamber experiment

Ussing chambers were used to determine the permeability of fresh intestinal tissue to Dtx formulated as nanoparticles or as ethalonic solution. In order to known if it was possible to add 1 % of pancreatin in the Ringer solution, the permeability of fresh intestinal tissue to a paracellular and transcellular marker ([^{14}C] mannitol, [^{3}H] Testosterone respectively) was studied. The methodology used was previously described [42]. Briefly, jejunum from fresh small intestine of sacrificed male Wistar rats (200–250 g; Charles River, Paris) was excised, rinsed with chilled physiological saline solution (NaCl 0.9 %) and cut into segments of 2–3 cm length. After visual examination of the tissue, sections containing Peyer's Patches were discarded. Jejunum portions were mounted in Ussing chambers (the intestinal surface tested was 1 cm^2) bathed with Ringer's solution at pH 6.8 containing glutamine 0.2 M with or without 1 % of pancreatin. The system was maintained at 37 °C and continuously oxygenated with O_2/CO_2 (95/5 %). After 30 min of incubation, the liquid of the donor chamber was replaced by the same volume of preheated (37 °C) Ringer solution containing mannitol (1 mM) and 5 μCi of [^{14}C] mannitol or testosterone (0.1 mM) and 5 μCi of [^{3}H] Testosterone. At pre-set time intervals (0, 0.5, 1, 1.5, 2, 2.5 and 3 h), aliquots of 200 μL were recovered from the receptor chamber and replaced with the same volume of fresh medium pre-equilibrated at the experimental temperature conditions (37 °C). Samples (10 μL) were also taken from the donor chamber at the beginning and at the end of the experiment to monitor any changes in

donor drug concentrations during the experiment and to safeguard mass balance. Samples analysis was performed by measuring the radioactivity of [^{3}H] and [^{14}C] by liquid scintillation (Scintillation liquid: Ultima gold from Perkin-Elmer, Apparatus LS 6000 TA, Beckman). Four tissue portions from four different rats were used to evaluate each formulation.

For comparisons, the apparent permeability coefficient (P_{app}) was calculated using the following Eq.4:

$$P_{app} = \left(\frac{dQ}{dt}\right) x \left(\frac{1}{AC_0}\right) \qquad \textbf{Eq.4}$$

where $\frac{dQ}{dt}$ is the flux of marker from the mucosal-to-serosal side of the mucosa, C_0 is the initial concentration of marker in the donor compartment and A is the surface of the membrane. In order to standardize the calculations, the values of P_{app} were calculated between 30 and 90 min after addition of the compound [43].

2.9.2. Study of Dtx intestinal passage

To study the intestinal passage of Dtx the same protocol described above was used. Once mounted the jejunum portion in the Ussing chamber and after equilibration at the temperature of the experiment for 30 min, Dtx-loaded nanoparticles were added to the donor compartment. Control tests were carried out with Dtx as ethanolic solution. For all experiences the concentration of Dtx in the donor chamber was 300 μg.mL^{-1} with 5 μCi of [^{3}H] Dtx. In the receptor compartment, 2 % (w/v) of Me-β-CD or ethanol were also included as Dtx solubilize agents. At pre-set time intervals (0, 0.5, 1, 1.5, 2, 2.5 and 3 h), aliquots of 200 μL were recovered from the receptor chamber and replaced with the same volume of fresh medium pre- equilibrated at the experimental temperature conditions (37 °C). Samples (10 μL) were also taken from the donor chamber at the beginning and at the end of the experiment to monitor any changes in donor drug concentrations during the experiment and to

safeguard mass balance. Sample analysis was performed by measuring the radioactivity of $[^3H]$ by liquid scintillation (Scintillation liquid: Ultima gold from Perkin-Elmer, Apparatus LS 6000 TA, Beckman). Four tissue portions from four different rats were used to evaluate each formulation.

The cumulative amount of Dtx permeated in the mucosal-to-serosal (M-S, absorptive, or apical to basolateral) or serosal-to-mucosal (S-M, secretory, or basolateral to apical) direction was calculated and plotted against time. Also, the influence of different parameters such as, *(i)* the temperature (evaluation of Dtx passage at 4 °C and 37 °C), *(ii)* the inhibition of the mucoadhesion capacity of nanoparticles or *(iii)* the inhibition of P-gp with verapamil or empty nanoparticles on the permeability of intestinal tissue to Dtx, was also studied. In order to determine the influence of the mucoadhesive capacity of PIBCA chito20/chito20-TBA nanoparticles on the absorption of Dtx, permeation studies were repeated avoiding the physical contact between the nanoparticles and the intestinal mucosa. To this purpose, a semi-permeable cellulose membrane (cut-off: 100,000 $g.moL^{-1}$) was placed in the donor chamber at the surface of the luminal side of the intestinal mucosa. Furthermore, the influence of the verapamil presence on the permeability of Dtx was studied by adding a solution of verapamil (0.2 M) in the donor chamber 30 min before.

For comparisons, the following parameters were calculated: the apparent permeability coefficient (P_{app}) (Eq.2) and the absorption enhancement ratio (R). Absorption enhancement ratio was calculated using the following Eq.5:

$$R = \frac{P_{app}(\text{sample})}{P_{app}(\text{control})} \qquad \textbf{Eq.5}$$

where P_{app} (sample) is the apparent permeability of jejunum to Dtx when included in the formulation tested and P_{app} (control) is the apparent permeability to the drug when included in the reference ethalonic solution.

During the all the experiences, electrical conductibility was recorded to determine the tissue viability (see supplementary information).

2.9.3 Statistical analysis

Data were expressed as the mean ± S.D. of at least three experiments. Statistical significance analysis was processed using the non-parametric Mann–Whitney U-test. P values of <0.05 were considered as statistically significant difference. All calculations were performed using KaleidaGraph® software program.

3. Results and discussion.

The aim of the present work is to investigate the possibility to enhance the intestinal permeability of Dtx by using mucoadhesive nanoparticles. Dtx-loaded nanoparticles were first characterized and the yield of Dtx encapsulated into nanoparticles was investigated. Finally, the release profile of Dtx from nanoparticles and its intestinal permeability using the Ussing chamber technique were studied.

3.1. Nanoparticle characterization

The system developed here, is a nanoparticle carrier constituted of PIBCA and Me-ß-CD, coated with thiolated chitosan. The presence of Me-ß-CD is essential to improve the apparent water solubility of Dtx forming an inclusion complex [8]. The possibility to form nanoparticles constituted of PIBCA and cyclodextrin has been previously reported in the literature showing the ability to prepare nanoparticles loaded with lipophilic drugs [30, 44]. In the anionic emulsion polymerization process the reaction is generally initiated by the hydroxyl groups of water present in the polymerization medium and more particularly of the glucose residue of chitosan [40]. The addition of Me-ß-CD brings new hydroxyl groups that may enter in competition with the hydroxyl groups contained in the other molecules [30].

Due to their amphiphilic properties, this system is able to auto-associate and form nanoparticles in a single-step method [40, 45]. To obtain core–shell particles in the nano-size range by emulsion polymerization of alkyl cyanoacrylates, low M_w chitosan was necessary [46]. Therefore, the oxidative degradation method developed by Huang *et al.* [32] was used here to synthesize a chitosan at a M_w of 20,000 g.mol^{-1} (Chito20), with a degree of deacetylation of 60 %.

Although the system without cyclodextrins has been well studied by varying the chitosan M_w and the thiol content [46, 47], The addition of Me-ß-CD in the polymerisation medium, might lead to a complete change of the physico-chemical properties of this system in comparison with the nanoparticles without Me-ß-CD. The physico-chemical characteristics of the obtained nanoparticles with or without CDs, are summarized in Table I.

Table I. Physico-chemical characterization of nanoparticles prepared by anionic emulsion polymerization. The mean hydrodynamic diameter (D), and surface charge determination (ζ potential) in NaCl 1 mmol.L^{-1} of nanoparticles with or without CDs. (n=3)

Formulations	D (nm)	ζ potential (mV)
PIBCA/(Chito20/Chito20-TBA) (75/25) %	127 ± 1	+27
Me-ß-CD/PIBCA/ (Chito20/Chito20-TBA) (75/25) %	235 ± 6	+45
PIBCA/(Chito20/Chito20-TBA) (100/0) %	195 ± 9	+43
Me-ß-CD/PIBCA/ (Chito20/Chito20-TBA) (100/0) %	408 ± 1	+47

The results reported without the use of Me-ß-CD are in agreement with our earlier studies [45]. The presence of Me-ß-CD in the polymerization medium led to increase the mean hydrodynamic diameter from 127 to 235 nm and from 195 to 408 nm for the nanoparticles Chito20/Chito20-TBA (75/25) % and (100/0) % respectively. The increase of nanoparticle size when cyclodextrins were added in the polymerization medium was already reported in the literature. The nanoparticle hydrodynamic diameters varied from 230 to 370 nm depending on CD used (native CDs and HP-ß, HP-γ, HP-α and sulfobutylether (SBE)-ß-CD) [31, 44].

Chitosan free amino groups were responsible for the measured positive ζ potential values obtained for all formulations, which might ensure the electrostatic interactions with the anionic groups of the mucus layer. The positive charge indicated that the cationic chitosan

was located at the surface of the nanoparticles and completely masked the negative surface charge generally found on control PIBCA nanoparticles stabilized with Pluronic F68 [23, 45].

Concerning the nanoparticles in presence of Me-ß-CD, the values of ζ potential varied from +23 to +45 mV for the nanoparticles containing 25 % of thiolated chitosan. On the contrary, for the nanoparticles containing the 100 % of unmodified chitosan the ζ potential did not change. This means that the presence of cyclodextrin did not influence the charge of the nanoparticle. As we previously reported, this result is expected since thiol groups were grafted on chitosan amino groups [45].

SEM and TEM microphotographs are shown in Figure 1. In agreement with our previous results [45], SEM and TEM microphotographs of two different formulations showed spherical particles in the nano-size range.

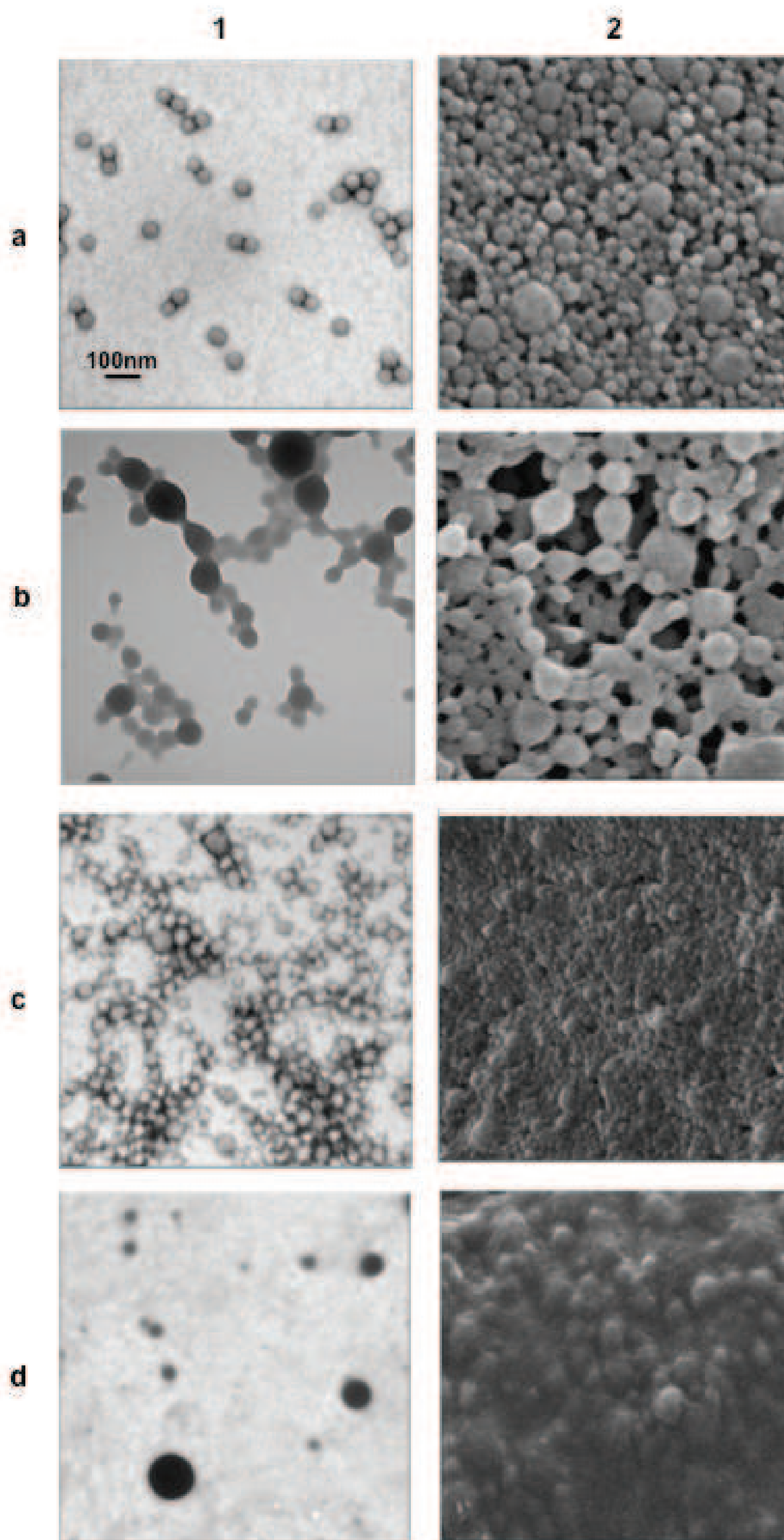

Figure 1. Transmission electron (1) and scanning electron microphotographs (2) of PIBCA-Chito20/Chito20-TBA (75/25) % and (100/0) % nanoparticles (a) and (b) respectively and Me-ß-CD/PIBCA Chito20/Chito20-TBA (75/25) % and (100/0) % nanoparticles (c) and (d) respectively.

3.2. Determination of yield of Dtx loaded onto nanoparticles.

In order to achieve the maximum rate of Dtx encapsulation into nanoparticles, the polymerization was carried out in a solution of Me-ß-CD/Dtx inclusion complex. The yield of the Dtx-loaded nanoparticles was determined using the new methodological approaches developed in a previous work [41]. The yields of encapsulation determined were 72 and 83 % for formulations with or without thiolated chitosan, as showed in Table II. As mentioned before, Duchene *et al*. in different works [30, 44] studied the possibility to prepare and characterize nanoparticles constituted of PIBCA and CDs. In particular, they studied the association of PIBCA nanoparticles with HP-ß-CD loading a series of different steroids as model drugs (danazol, hydrocortisone, progesterone, testosterone etc…). For all compounds an increase of loading capacity of nanoparticles was reported, in comparison with the system free from CDs.

Table II. Yield of Dtx encapsulation into nanoparticles

Formulation	**F_{Dtx} (mg.mL^{-1})**	**L_{Dtx} (mg.mL^{-1})**	**T_{Dtx} (mg.mL^{-1})**	**Loading Y (%)**
PIBC/Me-ß-CD (Chito20/Chito20-TBA) (75/25) %	1.3 ± 0.16	2.6 ± 0.45	3.6 ± 0.42	72 ± 8
PIBCA/Me-ß-CD/(Chito20/Chito20-TBA) (100/0) %	0.5 ± 0.16	2.8 ± 0.69	3.4 ± 0.59	83 ± 6

F_{Dtx}: concentration of Dtx found in the dispersing medium after separation of the nanoparticles. L_{Dtx}: concentration of Dtx associated with the nanoparticles. T_{Dtx}: concentration of Dtx recovered from the total nanoparticle suspension.

3.3. *In vitro* release study

Dtx release kinetics from nanoparticles were then determined in a Ringer solution, an isotonic saline solution containing sodium chloride, potassium chloride and calcium chloride, with or without 1 % (w/v) of pancreatin, in order to simulated intestinal fluids. Pancreatin is a mixture of several digestive enzymes produced *in vivo* by the exocrine cells of pancreas. It is mainly composed of amylase, lipase and protease. As showed in Figure 2.a, the release kinetics of Dtx were dramatically changed in the presence of pancreatin. In the Ringer solution, a plateau corresponding to 5 % of the loaded Dtx was rapidly attained and almost no release occurred in the next 24 h. On the contrary, in presence of 1 % (w/v) of digestive enzymes, Dtx was released in a biphasic way. In a first step, Dtx was slowly released during the first 6 h and then the release rate gradually increased and reached 60 % after 24 h.

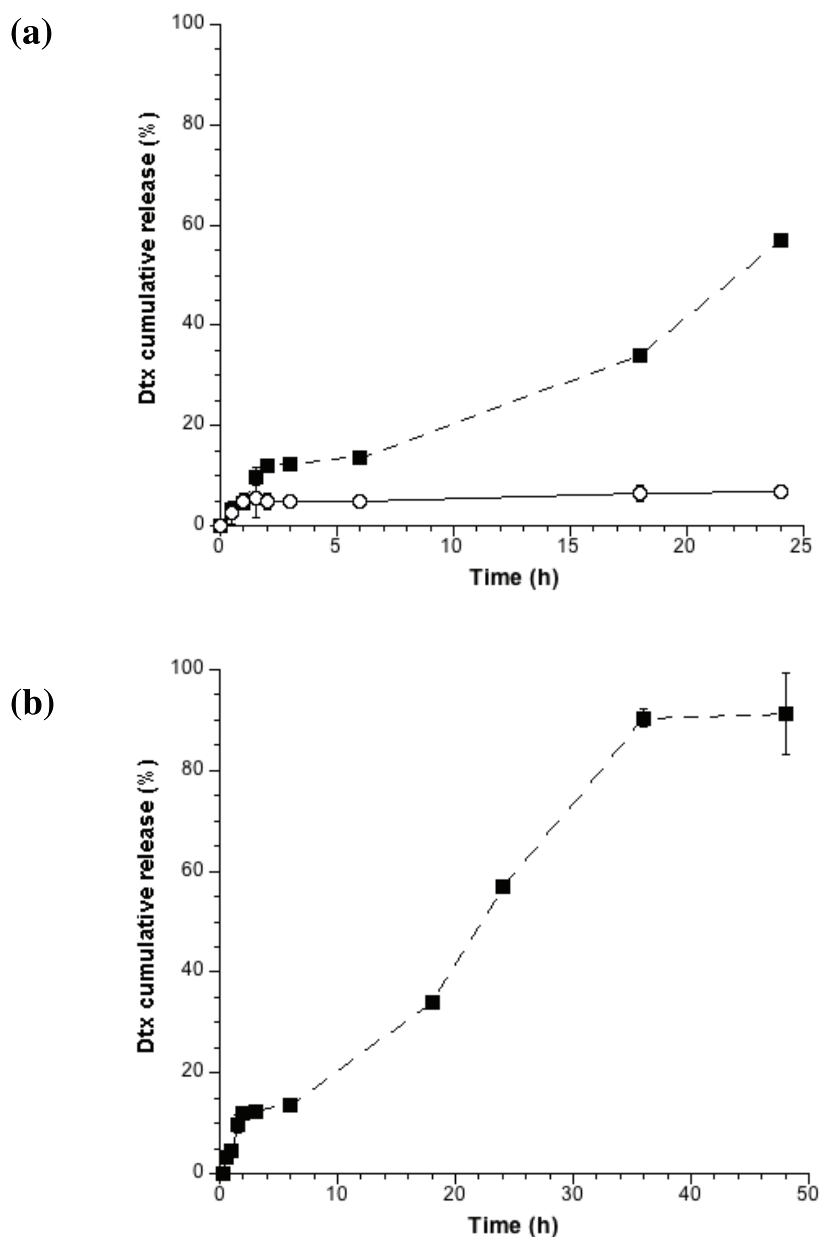

Figure 2. Release profile of Dtx from Me-ß-CD/PIBCA nanoparticles coated with Chito20/Chito20-TBA (75/25) %. (a) In two different media: (■) Ringer solution containing 1 % (w/v) of pancreatin and (**O**) Ringer Solution after 24 h. (b) In Ringer solution containing 1 % of pancreatin during 48 h.

The presence of several enzymes of pancreatic juice leads to a partial degradation of the nanoparticles, allowing the release of the drug. In the Ringer solution, nanoparticles degradation did not occur and the drug remained associated to the nanoparticles. Nevertheless, the presence of pancreatin was not enough to achieve the complete release of

the Dtx loaded in 24 h. In an independent prolonged experiment on a 48 h period (Fig. 2.b) it could be shown that Dtx release reached a plateau after 36 h, corresponding to 90 % of the initial load.

The use of pancreatin in the release experiments is common and different works in the literature use to add this mix of digestive enzymes in order to mimic the intestinal fluids, not only to evaluate drug release kinetics, but also to investigate drug stability in the intestinal fluids [14, 48, 49]. The concentration used (1 % w/v) was conforming to the USP XXXI standards. However, the composition of the physiological intestinal juice is more complicated and other components could play an important role in the fate of the drug delivery system. In the case of PIBCA nanoparticle, the most important ones are the esterases [50]. In the literature, it was reported that three different enzymes (amylase, pepsin and esterase) had an influence on the stability of PIBCA nanoparticles. It was found, that esterase had the most significant effect on the stability of the nanoparticles, the activity of which was optimum at pH 7 [21, 51, 52]. For this reason we are inclined to think that Dtx release under *in vivo* conditions can occur faster and may reach 100 % before 48 h. If the US Pharmacopeia reported the concentration of pancreatin used to simulate intestinal medium but does not mention the esterase concentration. The studies, mentioned above, tested different concentrations in order to understand the nanoparticle stability and the degradation mechanism.

3.4. Permeability studies in Ussing Chamber

The Ussing chamber is an *ex vivo* technique in which intestinal tissue is collected and immediately mounted as a flat sheet between two half-chambers, establishing a luminal and a serosal sides [49]. Lennernäs [53] has reported that transport properties for different compounds were highly correlated between rat and human when using rat intestinal specimens in the Ussing Chamber model. Different authors chose to study drug absorption by

the Ussing Chamber technique instead of monolayers of the human colonic adenocarcinoma-derived cell line Caco-2, the most frequently used [49, 54, 55]. Caco-2 cell line is well accepted as a model to investigate the relationship between the molecular structure, the physico-chemical properties, and the absorption potential of drugs. However, cultured epithelial cell layers lack a variety of different intestinal cell types, such as goblet cells, therefore they do not produce mucin molecules forming a mucous layer. From the viewpoint of the contribution of enzymatic/diffusional barriers in the mucous/glycocalyx layers to drug permeation, the lack of these barriers in the cultured epithelial Caco-2 cells might lead to the constitutional difference in the permeation behavior between conditions *in vitro* and *in vivo* (in physiological state), especially in the case of drug substances susceptible to enzymatic degradation in the mucous/glycocalyx layer [56].

The permeation of Dtx-loaded nanoparticles was then investigated by using Ussing Chamber technique which is commonly used for studying tissular permeation of substances in solution, but recently we proposed its use for studying the effects of formulations on drugs intestinal permeability [49]. Indeed, it is particularly well adapted for studying the effect of drug-loaded delivery system formulations on drug fluxes across the intestinal barriers. For example, mechanisms by which drug-loaded nanoparticle suspensions could enhance intestinal absorption have been clarified because it is possible to reproduce in static conditions some of the events underwent by the formulation in the intestine. For these reasons, we chose the Ussing Chamber technique to evaluate the intestinal transport and permeability of Dtx from nanoparticle formulations. In order to reproduce as much as possible the conditions prevailing during release experiments, 1 % (w/v) of pancreatin was added in the Ringer solution. The possible effect of this medium on tissue integrity was checked by measuring the permeability of paracellular and transcellular markers (radiolabelled mannitol and testosterone, respectively).

The apparent permeabilities (P_{app}) of mannitol were $(4.8 \pm 3.1)x10^{-6}$ cm.s^{-1} and $(11.6 \pm 4.1)x10^{-6}$ cm.s^{-1}in presence of pancreatin in the medium or not (control), respectively (Fig. 3.a). Even if statistically different ($p<0.005$), apparent permabilities P_{app} values were obtained, which fall within the range of values found in the literature for these molecules [42, 57-59]. Further, the P_{app} of testosterone were $(20.1 \pm 2.2)x10^{-6}$ cm.s^{-1}, in the medium with 1 % (w/v) of pancreatin and $(22.0 \pm 5.0)x10^{-6}$ cm.s^{-1}, in the Ringer solution (Fig. 3.b). These values were very similar and even in this case confirmed the previously determined apparent permeability data [58, 59].

Therefore, it could be concluded that 1 % (w/v) pancreatin could be added to the Ringer solution in the Ussing chamber experiment without altering the permeability of the fresh intestinal tissue to compounds, which was of interest in order that to mimic the *in vivo* luminal enzymatic conditions.

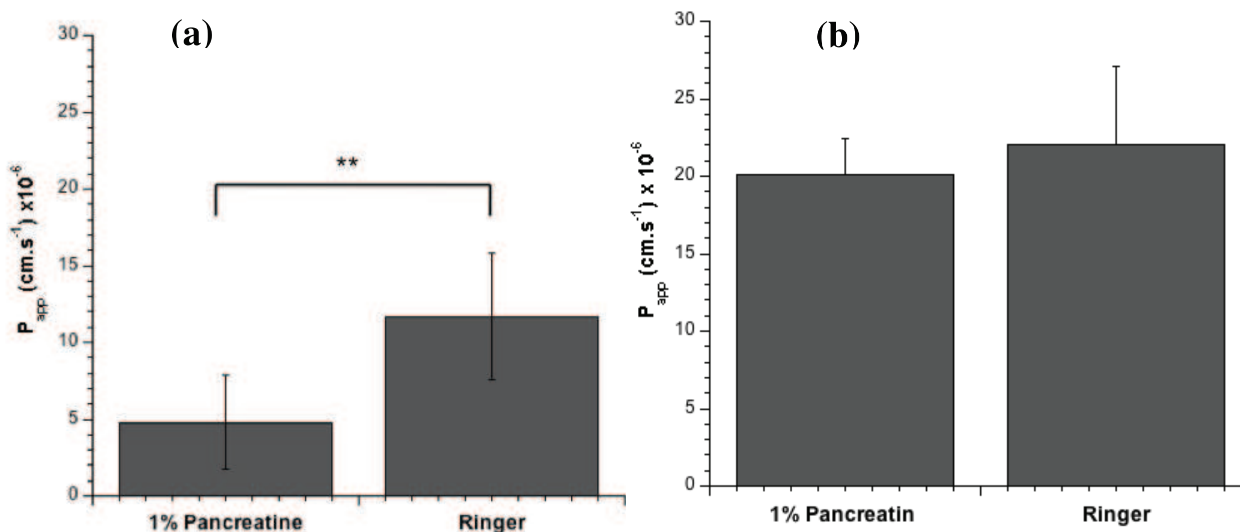

Figure 3. Effect of pancreatin on the apparent rat intestinal jejunum permeabilities in the Ussing chamber technique, of (a) mannitol and (b) testosterone. ** $p < 0.005$.

3.4.1. Intestinal permeability of Dtx in solution and released from nanoparticles

The effect of the of Me-ß-CD/PIBCA nanoparticles coated with Chito-TBA on the intestinal permeation of Dtx, has been studied by introducing these suspensions in the mucosal side of Ussing chambers under different experimental conditions (temperature, co-administration of efflux pumps inhibitors and avoiding the nanoparticles contact with intestinal tissue).

A first series of experiments aimed to assess the intestinal permeability of Dtx to determine the importance of the serosal to mucosal secretion of Dtx. For these experiments, a 2 % v/v ethanolic Ringer solution of Dtx has been used. The very low water solubility of this compound necessitated the addition of 2 % v/v ethanol to the Ringer medium for being able to observe experimentally detectable Dtx fluxes to the receiver. Permeation fluxes were determined over a 3 h period and permeability coefficients in the two directions: mucosal-to-serosal (M-S, absorptive direction) and serosal-to-mucosal (S-M, secretory direction) were determined from the absorption profiles. As can be seen, these profiles in Figure 4 were not

linear, but rather exponential. This aspect was reproducible whatever the conditions. The reasons for this trend have not been yet determined, although a progressive down regulation of multi drug resistance pumps by unidentified mechanisms cannot be excluded. Whatever, apparent permeability coefficients were calculated between 30 and 90 min and tabulated in Table III.

In jejunum tissue, in the M-S direction, Dtx dissolved in a 2 % v/v ethanolic Ringer solution exhibited a P_{app} of $(5.82 \pm 1.24)x10^{-6}$ cm.s^{-1}, which falls in the range of 10^{-6} cm.s^{-1} commonly encountered for various lipophilic substances in rats [49]. On the contrary, when the apparent intestinal permeability of Dtx was studied from the S-M direction, i.e. in the secretory direction, the drug permeability was much higher than observed in the absorptive direction (M–S) as showed in Table III.

Table III. Apparent rat intestinal jejunum permeabilities of a 2 % v/v ethanolic Ringer solution of Dtx under different experimental conditions in the Ussing chamber technique. Each value represents the mean ± S.D. (n = 4). M–S: mucosal-to-serosal direction; S–M: serosal-to-mucosal direction. R: Absorption enhancement ratio.

Experimental conditions	**P_{app} x10^{-6}(cm.s^{-1})**	**R**
M-S: 37 °C	5.82 ± 1.24	1
S-M: 37 °C	31.3 ± 12.4*	5.4
M-S: 4 °C	26.1 ± 4.73*	4.5
M-S: 37 °C + Verapamil	26.0 ± 5.19*	4.5
M-S: 37 °C + empty Nps	1.00 ± 0.27	0.2

* $p < 0.05$ all groups *vs* the 2 % v/v ethanolic Ringer Dtx solution M-S. (*U* Mann-Whitney test).

The existence of differences in permeation rates through rat jejunal intestinal membrane for Dtx in M–S and S–M directions typically indicated that efflux pumps were involved in the transport of drug across the intestinal tissue [7]. It has been showed for Dtx this efflux would

be mediated by efflux pumps localized at the surface of enterocytes [10]. This phenomenon was corroborated by experiments of co-administration of verapamil, which is a well known inhibitor of multi drugs efflux pumps [60]. In this case, the apparent permeability coefficient $(26.0 \pm 5.29) \times 10^{-6}$ $cm.s^{-1}$ of Dtx in M–S direction was found to be very close to the apparent permeability coefficient of Dtx in S-M direction. Similar values were found when experiments were performed at 4 °C. Under those conditions, the activity of the ATP dependent efflux pumps was disabled and Dtx would be only absorbed by passive diffusion [61].

A second set of experiments consisted in introducing the nanoparticle suspensions in the donor chamber and determining the corresponding permeation profiles (Fig. 5). In this situation, an evaluation of P_{app} would not be appropriate, since Dtx concentration available in the donor chamber was not know precisely, due to continuous Dtx release in the medium from the nanoparticles (contrarily to the situation for Dtx solutions in Ringer). However, it was possible to make a simple comparison of the permeated profiles in Figure 5 for the different formulations and under different experimental conditions.

The flux of Dtx absorbed from nanoparticles increased progressively during the 3 h duration of the experiments. The highest fluxes were observed for the nanoparticles coated with thiolated chitosan, and which were much higher than the ones observed for Dtx solution in 2 % v/v ethanolic Ringer. From a practical point of view, it shows that nanoparticles could be effective for enhancing Dtx oral delivery, which of course should be confirmed *in vivo*. This could be explained by different reasons: first, the presence of thiolated chitosan in the corona of the particles might have an inhibitory activity of efflux pumps, which has been reported in the literature [25, 62-64]. To test this hypothesis, we measured the permeability of the intestinal tissue to Dtx in presence of a physical mixture of a 2 % v/v ethanolic solution of Dtx in Ringer and empty Me-ß-CD/PIBCA Chito20/Chito20-TBA (75/25) % nanoparticles.

Surprisingly, the permeation profile of this physical mixture was lower than the Dtx solution alone (Fig. 4). Although an interaction between free Dtx and the nanoparticles could not be excluded, thus limiting the amount of available Dtx, it was more likely related to the fact that without association of Dtx to the mucoadhesive nanoparticles, the concentration of Dtx in the vicinity of the epithelium was less, which could not result in any significant flux modification. This result suggests equally that the empty nanoparticles did not inhibit the efflux pumps.

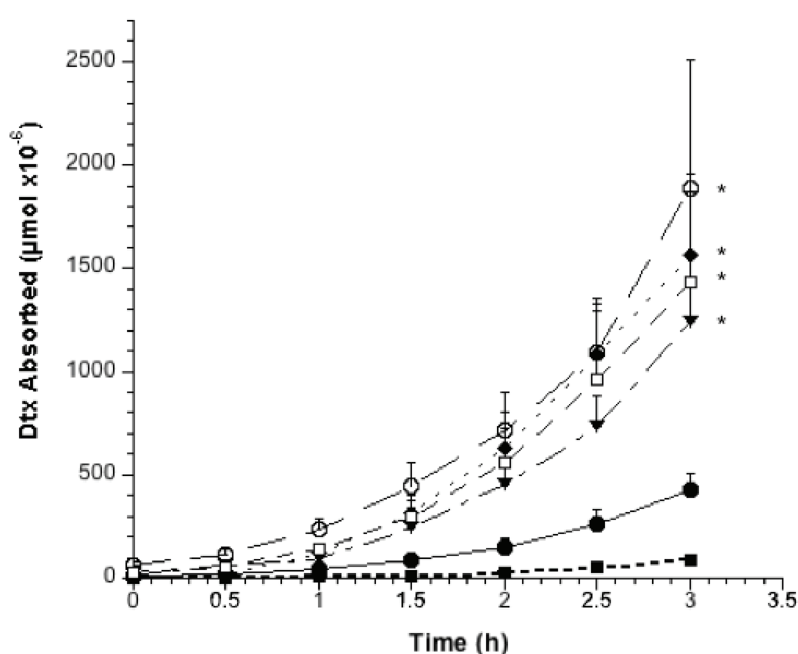

Figure 4. Amount of Dtx absorbed across the jejunum tissue in Ussing chamber experiments when formulated in ethanolic solution or Me-ß-CD/PIBCA Chito20/Chito20-TBA (75/25 %) nanoparticles. Data expressed as the mean ± S.D. (n = 4). The experiment was carried out under the following experimental conditions: (■) Dtx solution mucosal-to-serosal direction (M–S) 37 °C in presence of empty Nps ; (•) Dtx solution (M–S) and 37 °C; (▾) Dtx nanoparticles (M-S); (□) Dtx solution M–S, presence of verapamil (0.2 mM) as inhibitor of the P-gp, 37 °C; (◆) Dtx solution (M–S) and 4 °C; (O) Dtx solution serosal-to-mucosal direction (S–M), 37 °C. * $p < 0.05$ all groups *vs* the ethanolic Dtx solution M-S. (*U* Mann-Whitney test).

To further investigate a possible effect of mucoadhesion on Dtx intestinal permeation, an experiment has been conducted during which any direct contact of thiolated PIBCA

Chito20/Chito20-TBA nanoparticles with the epithelium was avoided by using a semi-permeable cellulose membrane placed in the donor chamber while ensuring an easy diffusion of Dtx (M_w = 807.85 g.mol^{-1}) across the dialysis membrane. As previously reported [23], the presence of positively charged chitosan in surface of the particles normally results in enhanced adhesion of the particles at the intestinal surface. This characteristic could be enhanced due to the presence of thiolated chitosan. Because of the presence of the thiol groups, disulfide bonds with the cysteine-rich sub-domains of glycoproteins in the mucus layer could be formed [65]. For this reason, we checked the influence of mucoadhesion capacity of nanoparticles on the Dtx absorption. As can be seen in Figure 5 avoiding the direct contact of the nanoparticles with the intestinal tissue, resulted in a decrease of Dtx flux to the level observed for the control 2 % v/v ethanolic Ringer solution, which confirmed the major role of mucoadhesion. In fact, the nanoparticle immobilization at the mucosal surface resulted in prolonged residence time close to the enterocytes, which increases considerably the drug concentration gradient at the absorption site, compared to a solution homogenously distributed in the donor chamber [24].

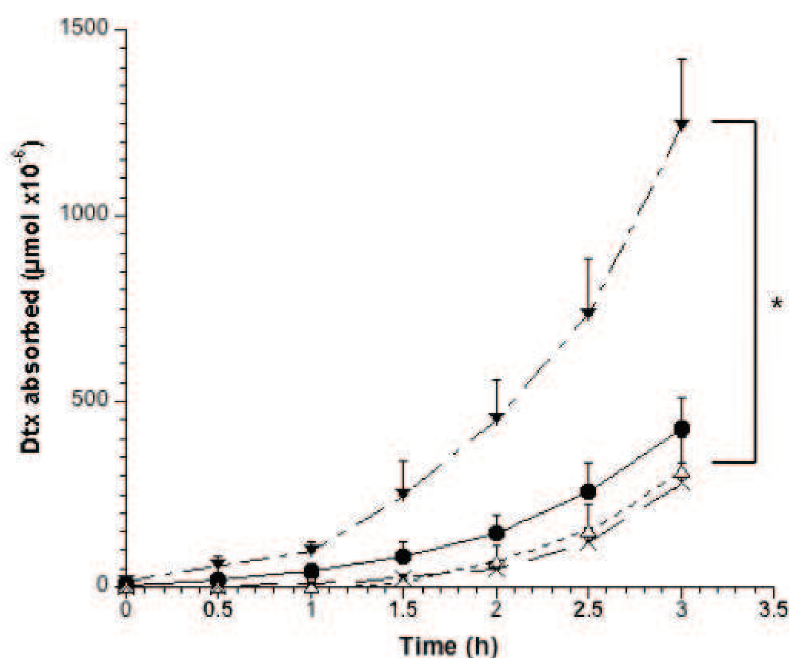

Figure 5 Influence of mucoadhesion in the Dtx absorption across the jejunum tissue in Ussing chamber experiments when formulated in Me-ß-CD/PIBCA Chito20/Chito20-TBA nanoparticles. Data expressed as the mean ± S.D. (n = 4). (x) Me-ß-CD/PIBCA Chito20/Chito20-TBA (100/0 %) (M–S) 37 °C; (△) Me-ß-CD/PIBCA Chito20/Chito20-TBA (75/25 %), addition of a dialysis membrane (cut-off 100,000 mw) to avoid the interaction between nanoparticles and the mucosa, 37 °C; (•) Dtx ethanolic solution M-S, 37 °C; (▼) Dtx loaded Me-ß-CD/PIBCA Chito20/Chito20-TBA (75/25 %) nanoparticles (M-S). * $p < 0.05$ all groups *vs* the Dtx nanoparticles (*U* Mann-Whitney test).

Moreover, this experience helped to highlight the effect of the thiol groups in the permeation of Dtx. The absorption profile of Dtx formulated in PIBCA nanoparticles only coated with 100 % of chitosan 20,000 $g.mol^{-1}$ and 0 % of thiolated chitosan was overlapped to the profile in which the mucoadhesion was avoided. This could suggest that thiol groups at the nanoparticle surface enhanced mucoadhesion in comparison with the native chitosan as demonstrated by previous works [67]. They found the presence of reduced thiol groups on the nanoparticle surfaces should ensure the development of covalent disulphide bonds with the mucus cysteine groups.

4. Conclusion

In summary, the association of Dtx in the form of an inclusion complex with Me-ß-CD in mucoadhesive PIBCA Chito20/Chito20-TBA nanoparticles resulted in enhanced Dtx intestinal permeation using the Ussing model. This results likely due to a local increase in Dtx concentration in front of the absorptive membrane, which could represent an interesting strategy to improve the oral bioavailability of Dtx. Further, under *in vivo* conditions, the time-controlled release of Dtx thanks to the slow degradation of nanoparticles in the intestinal fluids, which could match the duration of persistence of the particles in the gastro-intestinal tract due to mucoadhesion. The further step will be to confirm by *in vivo* studies if this kind of nanoparticles is able to enhance the bioavailability of Dtx allowing to display an anti-tumor activity.

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Supplementary information

Determination of Dtx concentration

Samples containing Dtx were analysed by reversed phase HPLC using a symmetric C_{18} column (250 x 4.6 mm) and UV detection at 231 nm (Waters system, France). The mobile phase was an isocratic mixture of methanol/water (70:30 % v/v). HPLC analyses were performed at 35 °C at a flow rate of 1 $mL.min^{-1}$. The validated range of concentrations (1.0 - 50 $\mu g.mL^{-1}$) was prepared from a standard solution of the inclusion complex Me-ß-CD/Dtx at 1 $mg.mL^{-1}$ and diluted with the mobile phase. Preliminary studies were carried out to check the possibility to use the same validated range of concentrations for all experiments. First of all, the repeatability of the dilution method was checked. A standard solution of inclusion complex at 0.8 $mg.mL^{-1}$ was prepared and diluted 1/10 with the Me-ß-CD or mobile phase. The obtained solution was diluted again 1/10 with mobile phase. The best resultant chromatograms were obtained with the mobile phase or Me-ß-CD/mobile phase dilution (Fig.1). Moreover the pick areas were exactly the same, indicating that both dilutions could be used for the preparation of standards solutions. The experience was repeated 6 times and as showed in Figure 2 there was no significant difference between the 6 standard solutions. Then, different validation curves necessaries for each experiment were analyzed after dilution with DMSO or with Ringer solution. DMSO is generally used to hydrolyse the nanoparticles for the determination of the total drug contained in the sample, while Ringer solution was used in the release experiments. The obtained curves were graphically compared with the one obtained with the mobile phase. The three linear least square lines were superimposed (Fig. 3).

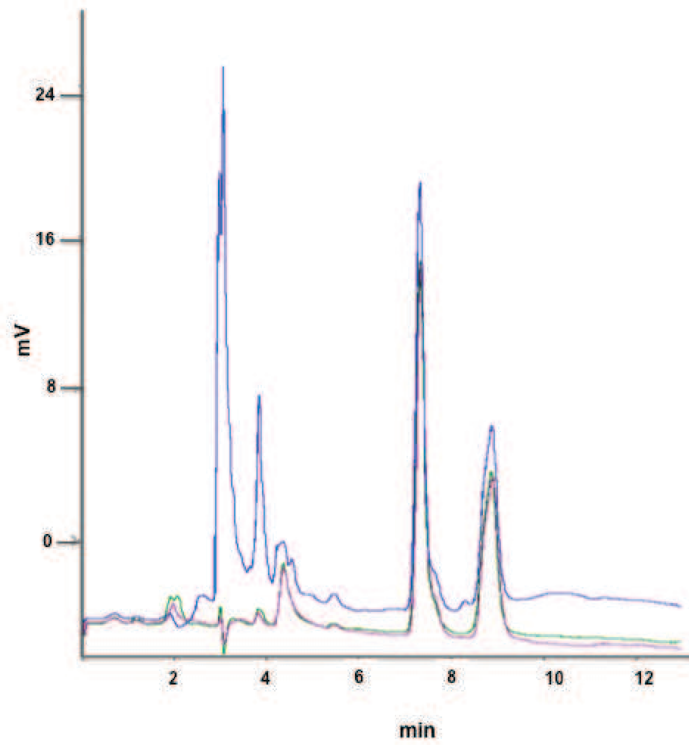


Figure 1. Chromatograms obtained with Me-ß-CD (blue), Me-ß-CD/mobile phase (red) and mobile phase (green) dilutions.

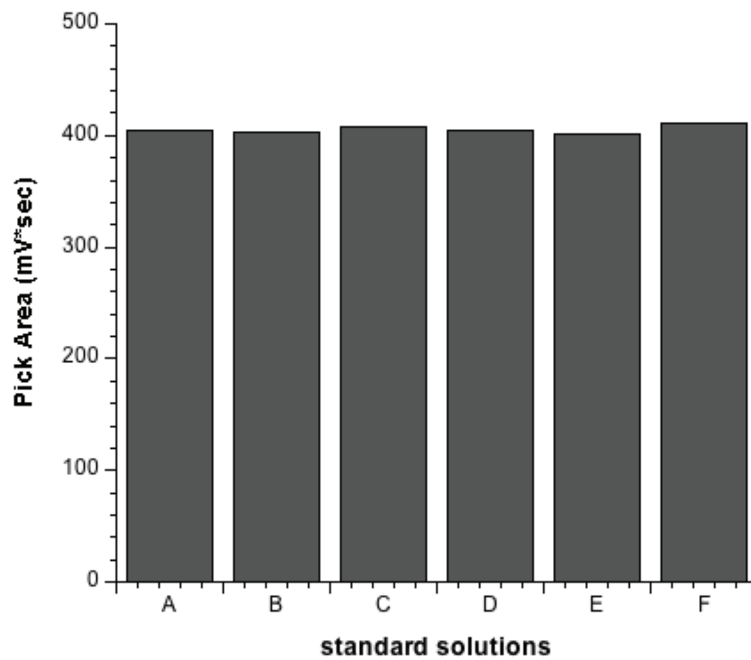


Figure 2. Repeatability of 6 standard solutions.

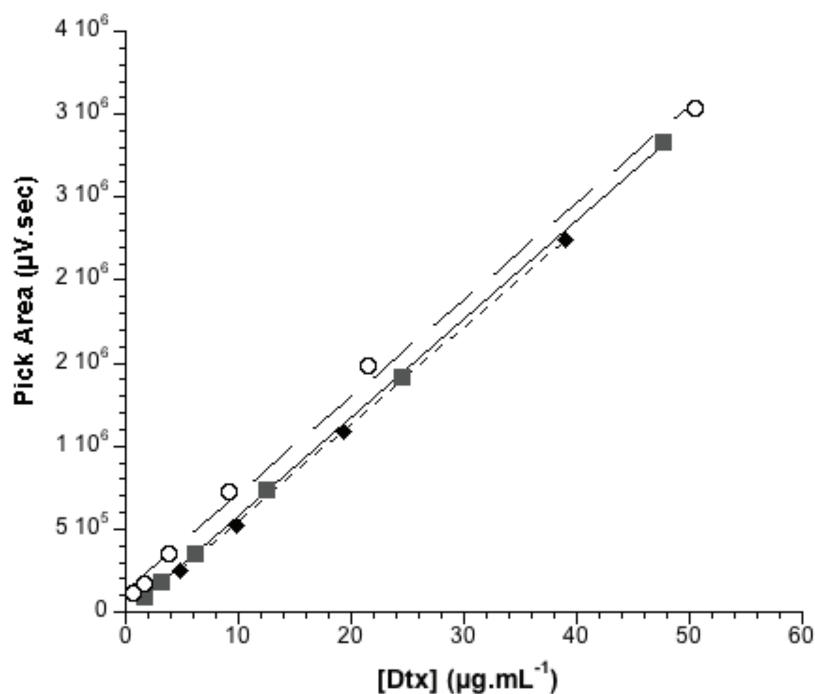


Figure 3. Three linear least square lines correspondent to mobile phase (**O**), ringer solution (◆) and DMSO (■) dilutions. The equation were y = 58564x + 127780, y = 58702x + 40931 and y = 59160x + 7448 respectively.

Investigation of the stability of Dtx under different experimental conditions

The stability of Dtx complexed to Me-ß-CD was first studied in the acidic polymerization medium. Me-β-CD/Dtx inclusion complexes were prepared according to the protocol previously described [1]. A solution of Me-ß-CD/Dtx complex (5 mL at 6.5 $mg.mL^{-1}$) containing nitric acid (0.2 M) was stirred for 1 h at 40 °C to mimic the polymerization conditions. Secondly, the stability of Dtx was studied under the conditions of NP storage. The same solution of inclusion complex was adjusted at a pH of 4.5 with NaOH (1 M). Finally, the stability of the inclusion complex was investigated under the conditions of NP solubilization. This process was necessary for the determination of the total amount of Dtx in the NP suspension in the yield of encapsulation experiment. A solution complex (0.1 mL at 6.5 $mg.mL^{-1}$) was added to 0.5 of DMSO for 12 h at 40 °C to mimic NP solubilization conditions. At the end of each stability experiment, the concentration of Dtx remaining in the samples

was determined by HPLC after dilution with mobile phase, as following described. All experiments were performed in triplicate from which the average and standard deviation were calculated. The percentage of lost Dtx in the sample (Dtx_{lost}) was calculated using Eq.1 from the percentage of Dtx found after incubation in the different conditions ($Dtx_{after\ incubation}$):

$$Dtx_{lost} = [100 - Dtx_{after\ incubation}]100 \quad \textbf{Eq.1}$$

Results in Table I showed that a small amount of Dtx was lost in all media since 87, 69 and 85 % w/w of the drug was recovered in the media by HPLC analysis performed after incubation at pH 1.2, 4.5 and in DMSO, respectively.

The instability of Dtx in different stressed conditions was already reported in the literature. Different studies showed the degradation of Dtx under high stress conditions of heat, acid and base [2, 3]. Acidic medium at 40 °C is fundamental to obtain nanoparticles by the process of emulsion polymerization techniques, and these conditions cannot be changed. In the same way it is necessary to increase the pH for the NP to 4.5 during storage. To solve this problem, one solution might be to chang the NP preparation method in order to avoid acid-base stress conditions. Anyway, we decided to go ahead with the study with this kind of NPs because the concentration of Dtx obtained in the final formulation is particularly high and it could be interesting to understand if a mucoadhesive system as chitosan-coated PIBCA NPs, could improve his intestinal permeability.

Concerning the stability in DMSO, the ideal solution is to replace it by another solvent to dissolve NPs. However, this not an easy task because the NPs are composed of an amphiphilic poly(alkylcyanoacrylate)-polysaccharide copolymer which is particularly difficult to dissolve. Bertholon *et al.* [4] have succeeded in dissolving similar nanoparticles composed of dextran-PIBCA copolymer either in hot DMSO or after partial hydrolysis of the

PIBCA part of the copolymer using sodium hydroxide. However, the nanoparticle dissolution is a technical requirement to determine the yield of encapsulation. At the end of experiment we can consider the amount of Dtx lost (15 % w/w) in the estimation of Dtx concentrations.

Table I. Stability of Dtx determined after incubation in polymerization, storage and NPs dissolution medium. (n=3)

Stability Medium	[Dtx] before incubation ($mg.mL^{-1}$)	[Dtx] after incubation ($mg.mL^{-1}$)	% Dtx recovered after incubation (w/w)	% Dtx lost during incubation (w/w)
pH 1.2	6.5 ± 0.11	5.6 ± 0.01	87	13
pH 4.5		4.5 ± 0.03	69	31
DMSO		5.5 ± 0.33	85	15
MilliQ water		6.5 ± 0.11	100	0

Stability of Dtx loaded into nanoparticles

The stability of the Dtx into PIBCA/Me-ß-CD/Chito20/Chito20-TBA (75/25 %) nanoparticles was checked, by determining the yield of encapsulation experiment during 15 days (n=4) (Fig.4).

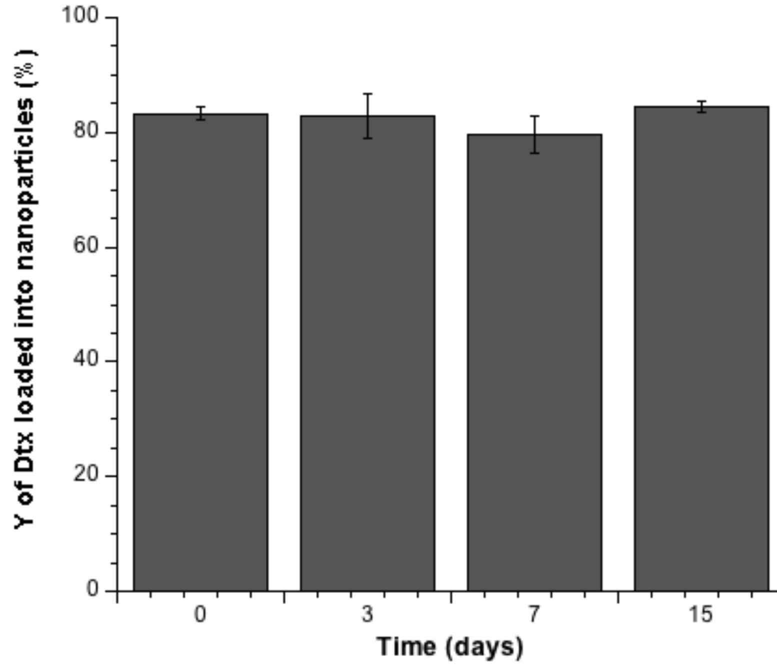


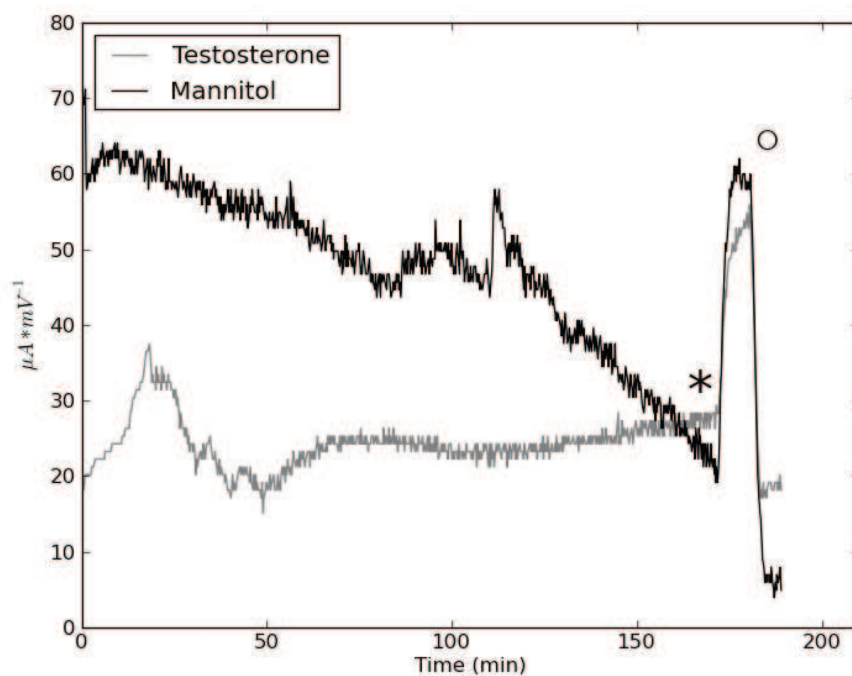
Figure 4. Stability of Dtx loaded into Me-ß-CD/PIBCA nanoparticles coated with Chito20/Chito20-TBA (75/25) % stored at pH 4.5 at 4 °C.

Measurement of electrical parameters

Electrical parameters were recorded to determine the tissue viability during the experiments. Transmucosal potential difference (PD) was continuously recorded between two KCl saturated agar bridges connected to a voltage clamp (Biomecatronics, Ruiz, France) via calomel electrodes filled with saturated KCl solution. Potential difference was short-circuited through the experiment by a short-circuit current (I_{SC}) via platinum electrodes connected to an automatic voltage clamp (Biomecatronics, Ruiz, France). At the beginning of t he experiment, I_{SC} was corrected by subtracting intrinsic fluid resistance. Only tissues showing PD > 2×10^{-3} V and $I_{SC} > 40 \times 10^{-6}$ A/cm^2 after 30 min equilibration were retained for the study. As a test of the viability of the tissues, at the end of the experiment 50 μL of a 2 mM forskolin ethanol solution in the serosal compartment was added. Forskolin increases Cl^- secretion by the cells and therefore the I_{SC} [5]. If there was no increase in I_{SC}, damages in the tissue were suspected and all samples collected from the corresponding chambers were discarded. After the increase

phenomena of I_{SC}, bumetanide ethanol stock-solution (10 mM) may be added in the serosal compartment to bring back the I_{SC} to the normal level. In fact, bumetanide is a specific inhibitor of the [Na^+ K^+ $2Cl^-$] co-transporter, therefore, it lead to a decreases of ion secretion by the cells and therefore the I_{SC} [6]. In Figures 5.a we reported the I_{SC} profile when mannitol and testosterone passage were studied. We can see the increase of the I_{SC} due to the increment of the ions Cl^- secretion under the forskolin effect. This increment was followed by an I_{SC} decrease after the addition of bumetanide, which lead to due to the inhibition of the [Na^+ K^+ $2Cl^-$] co-transporter. The same profiles were observed in the Dtx experiences formulated both as ethalonic solution and PIBCA NPs (Fig. 5.b).

(a)



(b)

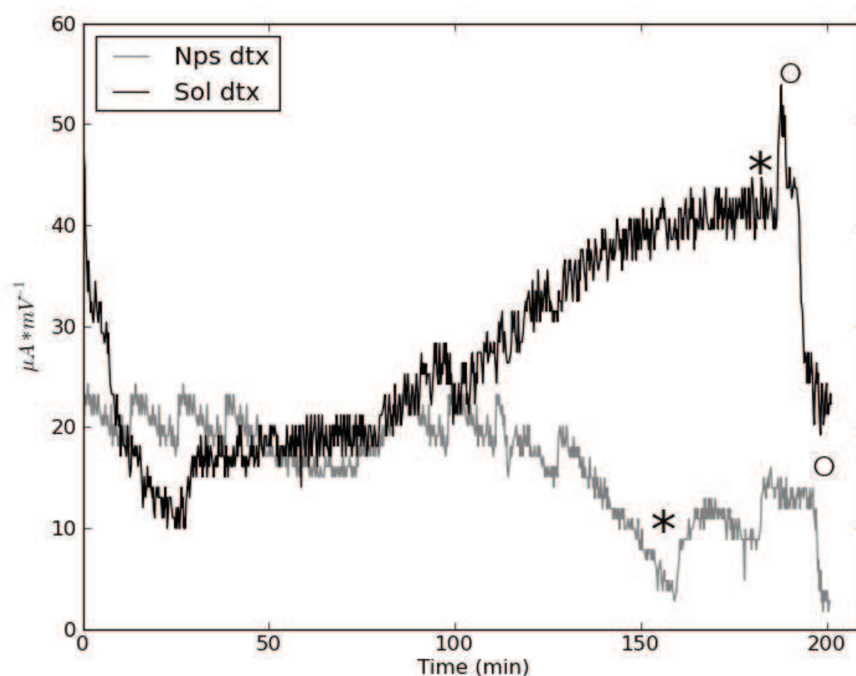


Figure 5. Evaluation of the intestinal tissue viability during Ussing chamber experience. (a) Testosterone (grey) and mannitol (black) passage. (b) Dtx passage formulated as ethanolic solution (black) and loaded PIBCA Chito20/Chito20-TBA NPs (grey). (∗) Addition of forskolin; (**O**) addition of bumetanide.

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CHAPITRE IV: Encapsulation of docetaxel in Me-ß-CD/PIBCA mucoadhesive nanoparticles coated with thiolated chitosan reduces local intestinal toxicity in a mouse xenograft model

Encapsulation of docetaxel in Me-ß-CD/PIBCA mucoadhesive nanoparticles coated with thiolated chitosan reduces local intestinal toxicity in a mouse xenograft model

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Abstract

The present study suggests that the local intestinal toxicity of docetaxel (Dtx) can be reduced when encapsulated in mucoadhesive nanoparticles composed of methyl-ß-cyclodextrin (Me-ß-CD)/poly(isobutyl cyanoacrylate) coated with thiolated chitosan. The experiments were carried out using intestinal specimens collected from mice with a subcutaneous tumor at an advanced stage in order to obtain challenging pathological conditions. Surprisingly, the Dtx-loaded nanoparticles and unloaded-nanoparticles when administered orally induced a lower level of intestinal and colonic inflammations and ulcerations compared to intravenous (i.v) Dtx injection. Both small intestine and colon sections showed a preserved architecture, normal thickness of the mucosa, and normal viability. Furthermore, Dtx-loaded nanoparticles and the unloaded-nanoparticles allowed decreasing the release of the lactate dehydrogenase (LDH) enzyme of 22 % and 43 % respectively in comparison with the i.v. Taxotere® administration. These results indicated clearly that: *(i)* Me-ß-CD/poly(isobutyl cyanoacrylate) nanoparticles coated with thiolated chitosan reduced the local intestinal toxicity of Dtx, *(ii)* unloaded mucoadhesive nanoparticles had a protective effect allowing the preservation of intestinal integrity in mouse xenograft model.

Keywords: docetaxel, PIBCA nanoparticles, mucoadhesion, intestinal toxicity, LDH, Ussing chamber, histological analysis.

1. Introduction

Docetaxel (Dtx) is a semi-synthetic analogue of paclitaxel, which is widely used in oncology to treat different solid tumors, especially ovarian, lung, breast, head and neck cancers. However, severe allergic reactions and peripheral neurotoxicity are caused by the intravenous (i.v) delivery of the commercial formulation Taxotere®, requiring thus the oral administration of dexamethasone and antihistamine before infusion. In this context, there is an urgent need to design better tolerated Dtx formulations to reduce these side effects and improve the patient's quality of life. We already designed an oral Dtx drug delivery system consisting in mucoadhesive nanoparticles composed of methyl-ß-cyclodextrin (Me-ß-CD) combined with poly(isobutyl cyanoacrylate) (PIBCA) and coated with thiolated chitosan. This formulation allowed a dramatic increase of the intestinal permeability when investigated *ex vivo* in rats (See Chapter III).

However, whatever the administration route, the delivery of many anti-cancer drugs represents a great challenge in chemotherapy due to the toxicity induced by their unspecific mechanism of action. Indeed, the most part of anti-cancer drugs attacks the rapidly proliferating cells of the body, in particular the gastrointestinal (GI), the bone marrow and the hair follicle cells, causing strong side effects such as nausea, vomiting, stomatitis, diarrhea, severe myelosuppression and neutropenia. Additionally, the side effects of chemotherapy can be so severe, that they can escalate beyond what is tolerable for the patient inducing the suspension of the therapy until the side effects can be controlled. For this reason, when foreseeing chemotherapy, it is mandatory to consider the toxic aspects.

Particularly, a great concern related to the oral administration of anti-cancer drugs is the eventual increase of the local intestinal toxicity, which could be due to the direct contact of the anti-cancer drugs with GI-cells. However, contradictory data were reported in the literature concerning the local intestinal toxicity of oral chemotherapy. Most of research

works are clinical trials, in which the side effects of the oral *vs* the i.v administration of different anti-cancer agents such as vinorelbine, topotecan, etoposide and cisplatin, were compared. Among the non-hematological side effects, GI toxicities, i.e. nausea, diarrhea and vomiting, were the predominant adverse effects and they were only slightly higher after oral than i.v. administration [1-5]. However, lower incidence of these side effects was reported after oral administration of capetabicine and UFT versus i.v. administration of 5-FU and leucovorin for the treatment of colorectal cancer [4, 6].

In this context, the main objective of the present study was to evaluate the intestinal mucosa damages induced by the oral administration of Dtx encapsulated into PIBCA mucoadhesive nanoparticles coated with thiolated chitosan. We chose to conduct this study using mice with a subcutaneous xenografted carcinoma at an advanced stage, in order to obtain the most challenging pathological conditions.

Testing cell damages may be easily achieved by measuring the release of the lactate dehydrogenase enzyme (LDH). This enzyme located in the cell cytosol is released when cells are damaged or dead. Consequently, any compound that causes acute damage to cells will lead to an immediate measurable increase of LDH in the medium. Generally, LDH test was selected to evaluate the local toxicity of the drugs by several studies carried out *in vitro* (by using Caco-2 cell line) and *ex vivo* (by using animal intestines) [7-13]. In these studies, the drug is put directely in contact with the cells or the intestines. In the present work, the evaluation of the LDH will be done by using intestinal specimens collected from xenografted mice after oral *in vivo* administration of the formulations. Ussing chamber technique was used for the LDH evaluation and simultaneously the electrical conductibility of the membrane, which is one indicator of the intestinal viability, was determined. This protocol is believed to be closer to the *in vivo* situation than previous models, including cellular models, reported in the literature.

Furthermore, qualitative histological analysis provided information about the state of the intestinal wall. The relevant histological parameters are the changes of the general architecture of the mucosa, its thickness, the presence of ulceration and the infiltration of the mucosa by inflammatory cells eventually observed in treated *vs* control untreated animals. We examined the mice's intestinal tissue after oral administration of Dtx nanoparticles. The results were compared with those obtained after i.v. administration of the commercial formulation Taxotere®.

2. Material and methods

2.1. Reagents

Anhydrous Dtx, 98.5 % was purchased from Chemos GmbH (Germany). Me-ß-CD Rameb® was purchased from Cyclolab (Budapest, Hungary). Isobutylcyanoacrylate (IBCA) was kindly provided as a gift by Henkel Biomedical (Dublin, Ireland). Chitosan M_w 400,000 $g.mol^{-1}$ was purchased from Fluka (Saint-Quentin-Fallavier, France). 2-iminothiolane HCl (Traut's reagent) was synthesized in the Department of Organic Chemistry (Biocis UMR CNRS 8076, Faculté de Pharmacie, Université Paris Sud, Châtenay-Malabry, France). Calcium chloride, monobasic potassium phosphate, dipotassium phosphate, sodium chloride, sodium hydroxide, sodium nitrite, bumetanide and forskolin were provided by Sigma Aldrich (France). Magnesium chloride and sodium hydrogen carbonate were purchased from Prolabo (France). Taxotere® was a gift from Paul Brousse Hospital (Villejuif, France). Human prostate cancer cell lines (PC_3) were a gift from Dr. Fariba Nemati of Institut Curie (Paris, France). BD Matrigel™ Matrix was purchased from BD-Bioscience (Le Pont de Claix, France). FineFix was purchased from Milestone (Italy). LDH colorimetric assay kit was purchased from Abcam (Paris, France). Solutions were prepared by weight using MilliQ® water (Millipore, France). All chemicals were of analytical grade and used as received.

2.2. Chitosan depolymerization and characterization

Chitosan was selectively hydrolyzed following the method developed by Huang et al. [14] previously described (see Chapter III section 2.2). The hydrolysis was performed using reaction with sodium nitrite at concentrations of 9 $g.L^{-1}$. The average molecular weight of the depolymerized chitosan was 20,000 $g.mol^{-1}$ (Chito20) as evaluated by capillary viscosimetry (viscosimeter AVS400, Schott Geräte) by using the Mark–Houwink Sakurada equation, $\eta = KM_w^a$ with K= 1.81×10^{-6} and a = 0.93 [15, 16]. The percentage of deacetylation of chitosan was 60 % as determined by ^{1}H-NMR analysis (Bruker MSL-400 spectrometer, Bruker Instrument Inc., Wissembourg, France) according to the method of Hirai et al. [17]. The inclusion of thiol groups in the hydrolyzed chitosan was carried out following the method developed by Bernkop-Schnürch et al. [18-21]. Thiolated polymer was chitosan-4-thiol-butylamidine, named Chito20-TBA according to the original molecular weight of the unmodified polymer.

2.3. Preparation and characterization of Dtx-loaded nanoparticles

Nanoparticles were prepared by anionic emulsion polymerization according to the method of Bertholon et al. [22]. In order to achieve the maximum concentration of Dtx in the nanoparticles, a modification of the protocol was introduced. Briefly, 0.069 g of mixture of hydrolyzed and thiolated chitosan (Chito/Chito-TBA 75/25% w/w) was dissolved in 5 mL of inclusion complex Me-ß-CD/Dtx (10 % w/w / 6 $mg.ml^{-1}$) [23] solution containing nitric acid (0.2 M), in a glass tube at 40°C, under gentle stirring and argon bubbling. After 10 min, 0.250 mL of IBCA were added under vigorous magnetic stirring. Argon bubbling was kept for additional 10 min and stopped. The reaction was allowed to continue at 40 °C under vigorous stirring for 50 min. After ice cooling for 5 min, pH was adjusted to 4.5 with NaOH (1 M). Blank nanoparticles were obtained according to the same protocol. A solution of Me-ß-CD (10 % w/w) was used instead of the Dtx inclusion complex.

The hydrodynamic diameter of the nanoparticles (~ 200 nm) and the size distribution were determined at 20 °C by quasi-elastic light scattering using a Zetasizer Nanoseries (Malvern Instruments Ltd. UK). The scattered angle was fixed at 90° and 60 μL of each sample was diluted in 2 mL of acetic acid 0.16 μM and filtered thought 0.8 μm membrane filters (Millex, SLAP 0225, Millipore, France). The positive zeta potential (+45 mV) of nanoparticles was measured using Zetasizer Nanoseries (Malvern Instruments Ltd. UK). Dilution of the suspensions (1:33 (v/v)) was performed in NaCl (1 mM).

2.4. *In-vivo* evaluation of intestinal Dtx toxicity after oral administration

Xenograft model

The human prostate cancer cell line PC-3 was cultured in RPMI medium with 1 % of streptomycin and penicillin and 10 % of bovine serum media in a monolayer. When cells reached 80 % confluence, they were washed with phosphate buffer solution, trypsinized and centrifuged at 500 rpm for 10 min at 4 °C. Thereafter, the cells were resuspended in RPMI media, to a reach concentration of 2×10^6 million cells per 100 μL.

Female athymic nude mice from Harlan Laboratories (Gannat, France), 3-week-old at arrival, were kept on a week light–dark cycle with access to food and water ad libitum. Experimental procedures were carried out according to the guidelines by the Malmö-Lund Ethical Committee for use and care of laboratory animals. The mice were subcutaneously injected with approximately 2×10^6 PC-3 cells into the right flank. Tumors were established 10 days after injection. The dimensions of the tumors were estimated in two perpendicular directions (length and width) using a caliper and the volumes were calculated by $0.5 \times$ length $\times$ width^2 (mm^3).

A total of 12 mice were used, with a mean bodyweight of 20 ± 3 g at the day of first injection. The animals were divided into 3 treated groups: Dtx/Me-ß-CD/PIBCA

chito20/chito20-TBA (2 mice); empty Me-ß-CD/PIBCA chito20/chito20-TBA nanoparticles (placebo, 2 mice); reference product Taxotere® (2 mice). Further 4 mice were divided in two groups, 2 mice were kept without tumor (control A) and 2 mice were subcutaneously injected with PC_3-cells and they received no further treatment (control B). Dtx nanoparticles (45 $mg.kg^{-1}$) and empty nanoparticles were given p.o. for 5 consecutive days. Taxotere® (20 $mg.kg^{-1}$, unique dose) was administered by i.v. At the sixth day the mice were sacrificed by cervical dislocation and intestines were collected for the following experiments.

LDH Leakage

To evaluate epithelial cell membrane damage, the release of LDH from the jejunum membrane was measured using the Ussing chamber technique. LDH is a cytosolic enzyme, and its presence in the apical compartment is generally regarded as evidence of cell membrane damage [24]. Jejunum from fresh small intestine of sacrificed mouse was excised, rinsed with chilled physiological saline solution (NaCl 0.9 %) and cut into segments of 1 cm length. After visual examination of the tissue, sections containing Peyer's Patches were discarded. Jejunum portions were mounted in Ussing chambers bathed with Ringer's solution at pH 6.8 containing glutamine 0.2 M. The system was maintained at 37 °C and continuously oxygenated with O_2/CO_2 (95/5 %). For the LDH studies, 100 μL of samples were withdrawn from the donor site at the beginning of the experiments. The amount of LDH released from the intestinal membrane was determined with a LDH colorimetric kit (Abcam, Paris-France).

Evaluation of the intestinal viability

Another strategy to assess the membrane damage is the evaluation of the intestinal viability, recording the electrical parameters during the Ussing chamber experiment. Transmucosal potential difference (PD) was continuously recorded between two KCl saturated agar bridges connected to a voltage clamp (Biomecatronics, Ruiz, France) via

calomel electrodes filled with saturated KCl solution. Potential difference was short-circuited through the experiment by a short-circuit current (I_{SC}) via platinum electrodes connected to an automatic voltage clamp (Biomecatronics, Ruiz, France). Delivered I_{SC} was corrected for fluid resistance and recorded during the entire experiment. After 1 h, 50 μL of a 2 mM forskolin ethanol solution was added in the serosal compartment in order to provoke a secretion of Cl^- ions and therefore an increase of I_{SC}. After the increase phenomena of I_{SC}, bumetanide ethanol stock solution (10 mM) may be added in the serosal compartment to bring back the I_{SC} to the normal level.

Histological examination of intestinal wall

Intestines of sacrificed mice were excised, rinsed with chilled physiological saline solution (NaCl 0.9 %). Four specimens were sampled from ileum and colon. After prior fixation in FineFix, (Milestone, Italy), embedding in paraffin, deparaffinized 4 μ thick slides were stained with hematoxilin, eosin and saffranin. The light micrographs of specimens are obtained with a Microscope Axiophot Zeiss (Germany) connected to a digital camera (PCO, Germany). Whole specimens were scanned with a slide scanner (Nikon Super Coolscan 8000) customized for histopathology (Groupe Régional d'études sur le cancer, Caen, France).

3. Results

LDH Leakage

Figure 1 showed the LDH activity observed after different treatments. In the untreated mice, the tumor itself (control B) provokes an important release of the enzyme, in comparison with the healthy mice (control A). The i.v. administration of the commercial formulation Taxotere® led to higher release of the LDH enzyme, while in the mice treated with both empty and Dtx-loaded nanoparticles, respectively, a decrease of LDH activity of 43 % and 22 % in

comparison with untreated mice (control B) and a decrease of 52 % and 34 % in comparison with the i.v. Taxotere® administration were observed.

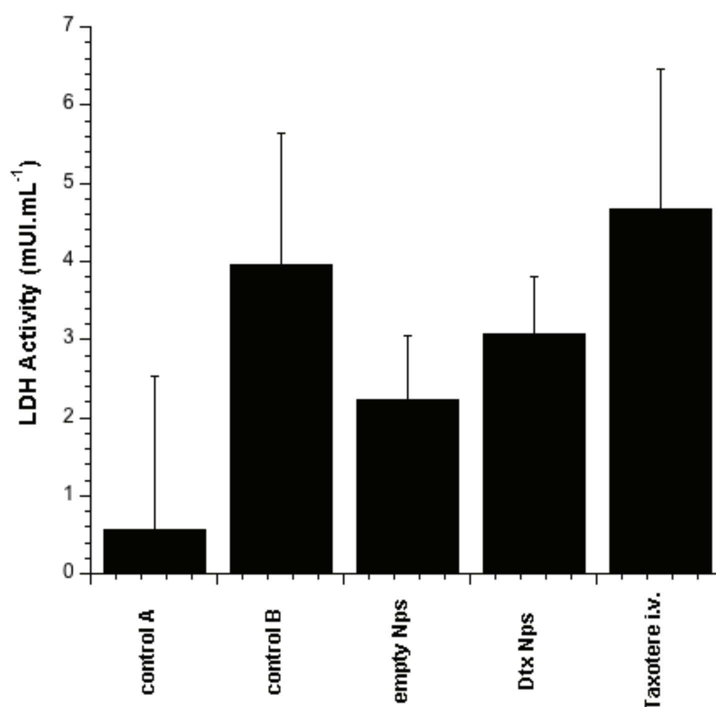

Figure 1. LDH Activity (mUI.mL^{-1}) released from the jejunum tissue at the beginning of the Ussing chamber experiment. (control A: healthy mice; control B: untreated mice with tumor)

Evaluation of the intestinal viability

The intestinal tissue viability was investigated through the measurement of the electric conductibility of the membrane installed in the Ussing chamber experimental setup. The evaluation of the intestinal viability is a current practice during intestinal permeation experiments [25, 26]. This technique was previously used by Kamsu-Kom et al. [27] to study the eventual toxicity of the oxaliplatin in direct contact with the intestinal mucosa. Intestinal tissue was collected from mouse and immediately mounted as a flat sheet between two half-chambers, establishing the luminal and the serosal sides. As a test of the viability of the tissues, forskolin ethanol solution was added at the end of experiments in the serosal compartment. Forskolin increases Cl^- secretion by the cells and therefore the I_{SC} [28]. If there

is no increase in I_{SC}, damages in the tissue have to be suspected. After the increase of I_{SC}, an ethanolic solution of bumetanide may be added in the serosal compartment to bring back the I_{SC} to the normal level. In fact, bumetanide is a specific inhibitor of the [Na^+ K^+ $2Cl^-$] co-transporter, leading to a decrease of ion secretion by the cells and, in turn, I_{SC} [29].

The viability of the intestinal tissue was verified evaluating I_{SC} during 1 h of the Ussing chamber experiment. In Figure 2 we reported the I_{SC} profile of the different jejunum tissues collected from mice treated in different ways. In all cases, an increase of the I_{SC} occurred due to an increased secretion of Cl^- anions after the forskolin addition in the serosal side. This increase of I_{SC} could be reversed after addition of bumetanide, which inhibits the [Na^+ K^+ $2Cl^-$] co-transporter. All tissues from the different mice groups responded to these two compounds, suggesting a healthy and active status of the intestinal membrane.

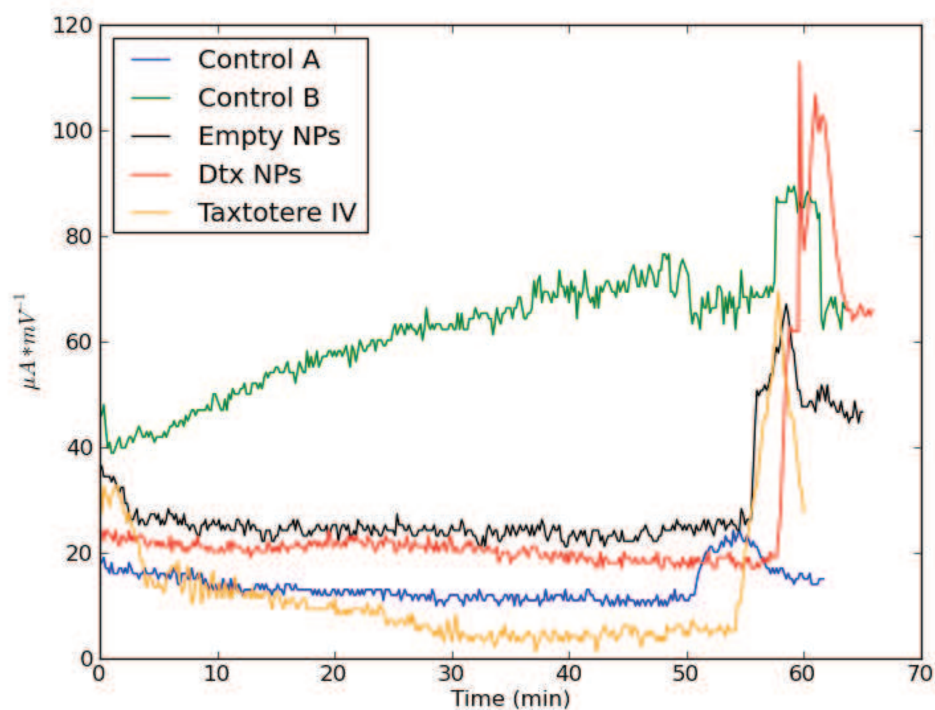

Figure 2. Evaluation of the intestinal viability after oral administration of Dtx-loaded nanoparticles (red), empty nanoparticles (black); i.v. administration of Taxotere® (orange); untreated mice with (control B) or without tumor (control B) (green and blue respectively).

Histological examination of intestinal wall

Histological examination of different intestinal sections (small intestine and colon) was carried out in order to observe the mucosa and the presence of any inflammations or ulcerations, compared with normal pattern in control animals (healthy mice (control A) and untreated mice with tumor (control B)). Figure 3 shows the light micrographs of the intestinal mucosal membranes in the ileum and colon of mice after different treatments. As we can see, among the different groups, there were no significant changes in the architecture of the intestinal mucosa. The only tissues exhibiting initial slight lesions were the ones taken from the untreated mice with tumor (Fig. 3.b) and from the mice treated with a single injection of Taxotere® (Fig. 3.c). Particularly, both specimens presented a mild adhesion of peritoneal adipose tissue to the muscular wall (Fig. 3.b; 5.a-c). An occasional and focal decrease of thickness of the colon or ileum mucosa was observed in i.v. injected mice (Fig. 3.b).

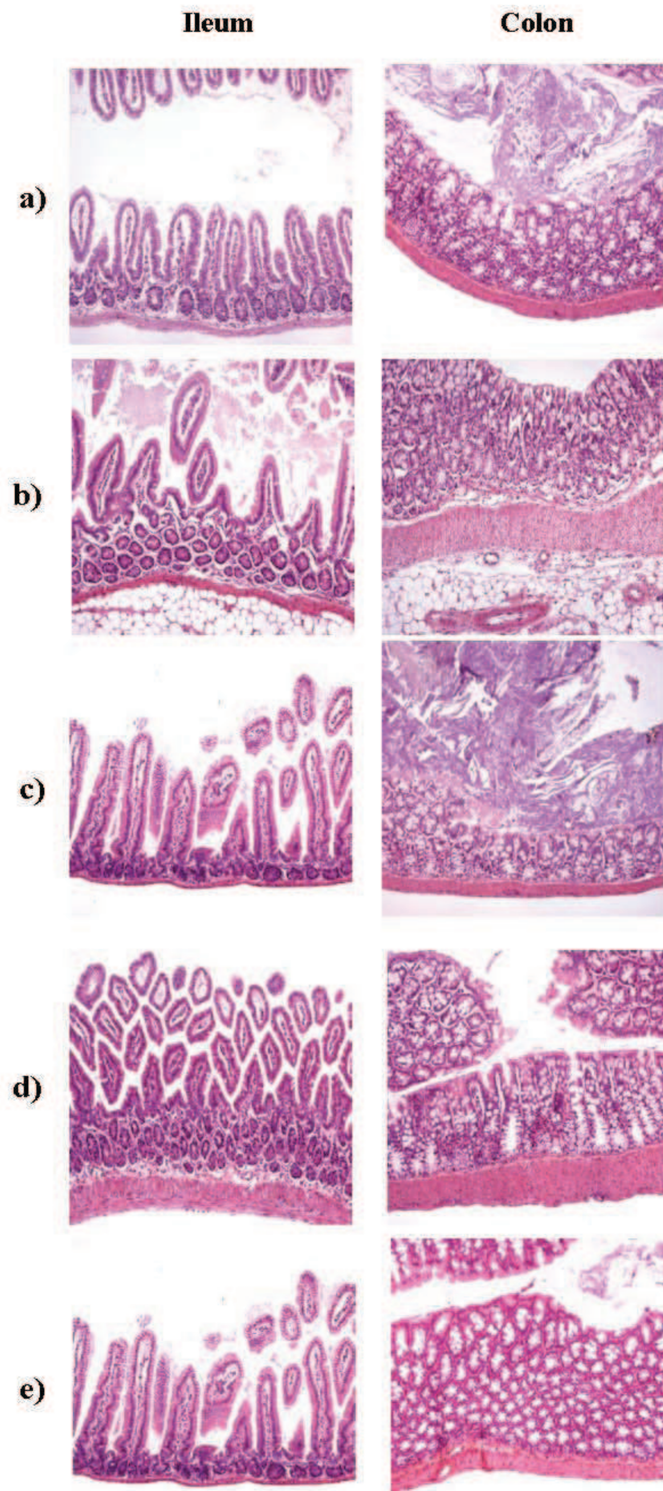

Figure 3. Light micrographs of ileum and colon specimens taking from different mice groups: healthy mice (a) (Control A); untreated mice with tumor (b); i.v. injected with Taxotere® (c); treated p.o. with Dtx Nps (d) and empty NPs (e). Among the different groups, no significant changes are observed. A mild adhesion of peritoneal adipose tissue to the muscular wall was observed in the intestinal wall of the untreated mice with tumor (b) (Control B). An occasional decrease of thickness of the colon and ileum mucosa was observed in i.v. injected mice (c). (Magnification x100).

Moreover, in the colon mucosa of the mice treated with Taxotere® the presence of some inflammatory infiltrate was found (Fig. 4). Inflammatory cells were either scattered through the mucosa (Fig. 4) or localized in foci (Fig. 5.a). On the contrary, for each small intestine and colon specimen from the mice treated with the unlaoded and Dtx-loaded nanoparticles, no apparent histological damages were found (Fig. 3.d-e).

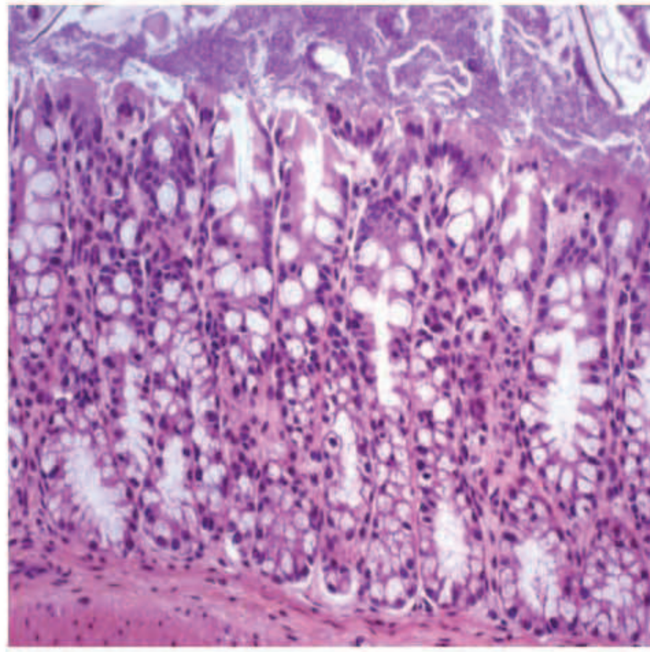

Figure 4. Presence of a moderate inflammatory infiltrate within the colic mucosa in a Dtx i.v. injected mouse. (Magnification x200).

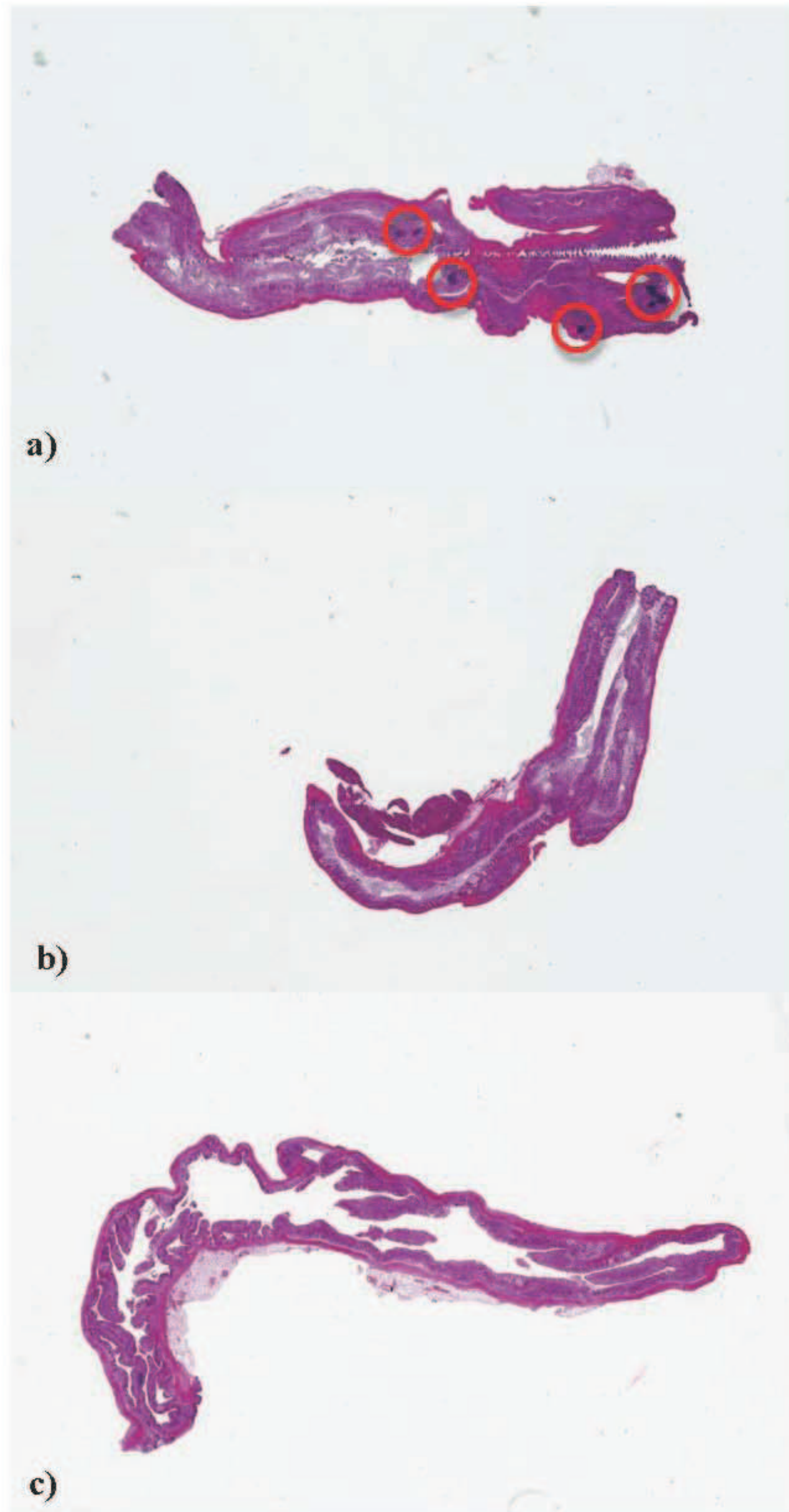

Figure 5. Scan images of the colic wall: (a) i.v. injected mice; (b) mice treated with Dtx-loaded nanoparticles; (c) control B (untreated mice with the tumor). A mild adhesion of peritoneal adipose tissue to the muscular wall was observed in the intestinal wall of i.v. injected (a) and untreated mice with tumor (c). The presence of lymphoid inflammatory infiltrates localized in foci, could be observed in the colonic mucosa of the mice Dtx i.v. injected (a).

4. Discussion

The aim of this study was to evaluate the local intestinal toxicity after oral *in vivo* administration of Dtx/Me-ß-CD/PICBA nanoparticles coated with thiolated chitosan. The toxicity was evaluated by studying three different parameters: *(i)* the release of the LDH enzyme, *(ii)* the global intestinal viability electrically evaluated and *(iii)* the histological status of the intestinal tissue. We compared the effect on the intestinal mucosa of orally administered Dtx-loaded nanoparticles to an i.v. administration of the commercial formulation Taxotere®. Furthermore, we evaluated the intestinal tissues from mice treated with oral empty nanoparticles and untreated mice with or without tumor, as controls.

We decided to use an aggressive model of tumor to check the local intestinal toxicity of Dtx-loaded nanoparticles in the most challenging pathological conditions. In fact, the elevated number of PC-3 cells injected and the use of Matrigel™ containing the growth factors, allowed having a tumor at an advanced stage (~ 400-500 mm^3). The tumor was so aggressive that even the i.v. administration of Taxotere® did not lead to a decrease of the tumor volume (data not shown). As can be seen from Figure 1, xenograft mice intestine was much fragile as showed by an elevated LDH leakage (control B) in comparison with healthy mice (control A).

Interestingly, the toxicity of the orally-administered encapsulated Dtx was lowered in comparison with the i.v administration. The results of the quantification of the released LDH experience (Fig. 1) and the histological examination of the intestinal wall (Fig. 3-4-5), suggested a protective effect of the nanoparticles on the intestinal mucosa. The highest levels of intestinal damages were observed with the untreated xenograft animals and those treated with i.v. Taxotere®. While results obtained with the i.v. administration of Dtx are not surprising because the GI toxicity of Taxotere® including nausea, vomiting, stomatitis and diarrhea is well reported in previous works [30], the decrease of Dtx intestinal toxicity

encapsulated into PIBCA nanoparticles coated with thiolated chitosan has never been reported in the literature yet. The only data relating the local GI toxicity of drugs after encapsulation in nanoparticles concerned anti-inflammatory drugs such as indomethacin formulated as amphiphilic β-cyclodextrin nanocapsules [31], naproxen and acetylsalicylic acid diclofenac, and indomethacin [32-34]. These research works demonstrated that the anti-inflammatory drug side effects ranging from GI irritation, gastric ulceration and hemorrhages were significantly reduced after their chemical pre-association with phosphatidylcholine [32-34]. It has been suggested that the mechanism of the phosphatidylcholine protective effect on GI tract could be related to their ability to inhibit cyclooxygenase (COX) [32]. The reduction of local toxicity of the anti-inflammatory drugs could be also related to a decrease of their direct contact with the mucosa. Indeed, GI ulceration and mucositis of anti-inflammatory drugs were lowered when coated with pharmaceutical excipients (i.e. surfactants, lipids or hydrophilic) [35]. In the same way, the oral administration of meloxicam-loaded Eudragit EPO nanoparticles, resulted in a reduced ulcerogenicity in comparison with meloxicam suspension, indicating that nanoparticles can decrease the adverse effects associated with meloxicam treatment [31].

However, the explanation of the protective effect of empty PIBCA nanoparticles mechanism coated with thioaled chitosan constitutes a surprising result, which is not fully understood. Earlier studies of the direct toxicity of PIBCA nanoparticles and its degradation products on Caco-2 cell line [36-38] showed that the toxicity of poly(alkyl cyanoacrylate) nanoparticles was relatively mild and does not hinder the use of this system by the oral route. Indeed, the poly(alkyl cyanoacrylate) polymers induced cellular damages but only when high nanoparticles concentrations were added in the cell culture medium. The toxicity was rather due to the release of the degradation products (isobutylic alcohol and cyanocrylic acid [37]), not only in the degradation medium but also close to cell membranes when the particles are

adhering to the cell surface [38]. Furthermore, it was proved that the association of cyclodextrins with these nanoparticles led to a decrease of their toxicity on Caco-2 cells compared to blank nanoparticles. This effect could be attributed to the capacity of cyclodextrins to mask the cytotoxic effects of the degradation products of the polymers [36]. In the work presented here, no intestinal toxicity was found in the mice treated with empty nanoparticles, confirming the sufficiently large safety margin for the dosing of PIBCA nanoparticles by the oral route.

5. Conclusion

This study aimed to evaluate an eventual local intestinal toxicity after repeated oral administration of Dtx-loaded mucoadhesive Me-ß-CD/PIBCA nanoparticles coated with thiolated chitosan. Interestingly, no intestinal damages were observed in the group of mice treated with the Dtx-loaded nanoparticles. Both small intestine and colon sections showed a preserved architecture, a normal thickness of the mucosa, and a normal viability. On the contrary, slight intestinal damages, such as focal atrophies of the mucosa, were recognized in the mice treated with a single dose of Taxotere®, injected intravenously and in the untreated mice with tumor. These data suggest that further studies including pharmacokinetic and *in vivo* antitumor activity of Dtx formulated as oral nanoparticles, can be foreseen in order to understand their potential properties in oral chemotherapy of Dtx.

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DISCUSSION GENERALE

Bien que la voie orale soit la voie d'administration préférée, cette voie d'administration est limitée à certaines molécules dont les propriétés physico-chimiques permettent une absorption suffisante sous forme active dans la circulation générale. Dans le domaine de la cancérologie, cette voie est assez peu utilisée pour diverses raisons historiques. Du point de vue de leur formulation, les molécules anticancéreuses ne se distinguent pas des autres dans le sens où elles partagent diverses propriétés physico-chimiques et biologiques défavorables à l'absorption avec d'autres molécules actives. C'est notamment le cas du docétaxel (Dtx), un des agents anticancéreux les plus utilisés en thérapeutique, dont nous avons cherché à permettre l'administration orale par la recherche d'une formulation adaptée. Ainsi, à ce jour différentes stratégies ont été proposées pour résoudre ces problèmes d'absorption, telles que la synthèse de prodrogues, l'administration simultanée avec des inhibiteurs des différents mécanismes du métabolisme entérocytaire ainsi que l'emploi de formulations variées, qui ont été amplement présentées et discutées dans la première partie décrivant les travaux antérieurs, et qui permettent de contrecarrer telle ou telle propriété défavorable.

Parmi les différentes possibilités pharmacotechniques, l'encapsulation des principes actifs dans des nanoparticules s'avère une solution prometteuse lorsque ceux-ci sont mal absorbés après administration orale du fait d'une faible solubilité dans l'eau et d'une absorption lente liée à une faible perméabilité intestinale. C'est le cas du Dtx pour lequel, nous proposons l'emploi des formes nanoparticulaires bioadhésives destinées à : *(i)* assurer la dispersion du principe actif, *(ii)* prolonger l'absorption du principe actif et *(iii)* à le protéger vis-à-vis d'un environnement défavorable. Assurer une absorption par voie orale du Dtx implique en effet de faire face à différents verrous tels que sa faible solubilité aqueuse, son passage limité à travers l'épithélium intestinal et le protéger vis-à-vis du métabolisme entérocytaire. De plus, le mécanisme d'action aspécifique des agents anticancéreux conduit à

une toxicité sur les cellules en prolifération rapide telles que les cellules du tractus digestif, de la moelle osseuse et du follicule pileux. Lorsque une administration orale est envisagée, il faut se demander si les effets secondaires gastro-intestinaux peuvent augmenter à cause du contact direct des molécules anti-cancéreuses avec les entérocytes, ce qui pourrait alors conduire à une toxicité locale.

1. Différentes stratégies pour l'amélioration de la biodisponibilité orale des agents anti-cancéreux.

1.1. Augmentation de leur solubilité aqueuse

Beaucoup des agents anti-cancéreux appartiennent à la Classe IV du Système de Classification Biopharmaceutique, dans lequel les molécules ont une faible solubilité aqueuse et une faible perméabilité. Habituellement, ces molécules ne sont pas bien absorbées au niveau de la muqueuse intestinale. De plus, une grande variabilité entre les patients et d'une dose à l'autre, peut être attendue. Différentes stratégies pour améliorer la solubilité des ce type de molécules peuvent être envisagées, allant de l'utilisation des excipients pharmaceutiques à l'élaboration de nouvelles formulations [1] ou à la synthèse de prodrogues [2].

Une des stratégies très utilisées dans ce domaine s'avère être l'utilisation des cyclodextrines (CDs), car elle nous permet d'éviter l'utilisation de co-solvants et de tensioactifs. De plus, les CDs sont biocompatibles et non immunogéniques, protègent parfois les substances du milieu extérieur et favorisent généralement leur biodisponibilité et leur libération au niveau du site de résorption [3].

Les CDs (Figure 1.a), découvertes par Villiers en 1891 [4] sont des oligosaccharides cycliques obtenues par la dégradation enzymatique de l'amidon. On distingue trois CDs naturelles appelées α-, β- et γ-CDs qui sont formées respectivement de 6, 7 et 8 unités de D-(+) glucopyranose. Le nombre d'unités de D-(+) glucopyranose détermine le diamètre de la cavité (4.7-5.3, 6.0-6.5 et 7.5-8.3 Å) (Figure 1.b) et ainsi leur volume (174, 262 et 427 Å pour α-, β-, et γ-CD respectivement). Malheureusement, la β-CD qui est la plus utilisée pour l'incorporation de principes actifs est la moins soluble dans l'eau (18.5 $g.L^{-1}$ à 25 °C). L'alkylation des groupements hydroxyles de la β-CD a permis la synthèse de dérivés plus

solubles dans l’eau tels que la méthyl-β-CD (Me-β-CD), l’hydroxypropyl-β-CD (HP-β-CD) et la sulfobutylether-β-CD (SBE-β-CD) [5].

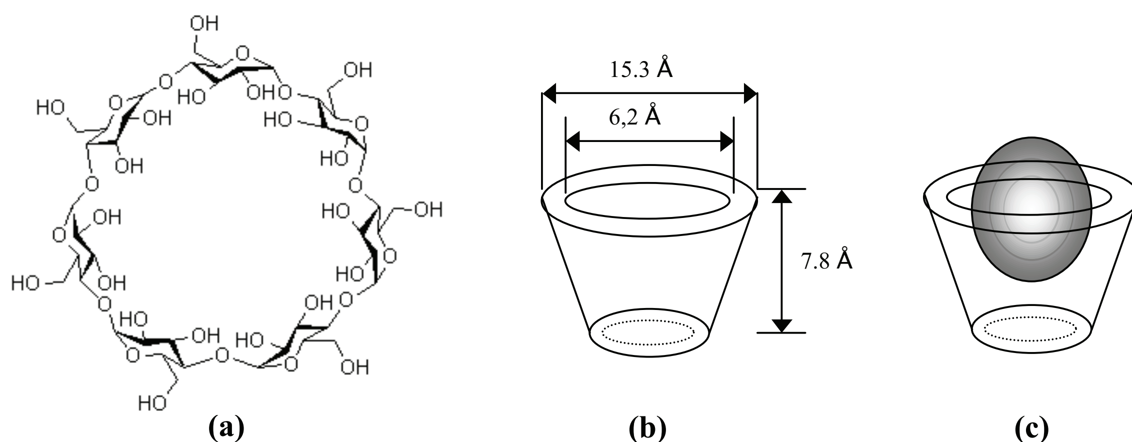

Figure 1: Formule chimique de la ß-cyclodextrine (a) de taille caractéristique (b) formant un complexe d’inclusion avec un principe actif (c).

Les unités de D-(+) glucopyranose sont liées entre elles par une liaison α-(1,4)-glucosidique, conférant aux CDs la forme d’un tronc cônique creux, fortement hydrophile à l’extérieur et relativement hydrophobe à l’intérieur de la cavité. Cette structure particulière à double polarité confère aux CDs la capacité d’inclure dans leur cavité des molécules lyophiles en formant un complexe d’inclusion (Figure 1.c) permettant ainsi d’améliorer leur solubilité apparente en milieux aqueux [6].

En ce qui concerne les agentes anti-cancéreux, cette stratégie a été explorée pour la doxorubicine [7], l’acide usnique [8], le tamoxifène [9] et les taxanes [10-18]. La plupart des données de la littérature à propos de la complexation des taxanes avec les CDs, concernent principalement le paclitaxel. Très peu de recherches décrivent l’utilisation de CDs pour la solubilisation du Dtx. En particulier, un travail récemment présenté, s’est intéressé à l’amélioration de la solubilité du Dtx en utilisant des CDs modifiées chimiquement [18]. Cette étude a permis d’obtenir une concentration maximale du Dtx égal à ~ 1,3 $mg.mL^{-1}$ (~1,6 mM) [18]. Durant nos travaux de recherches nous avons réussi à montrer que la Me-β-CD (à

une concentration de 10 % m/m) peut augmenter la solubilité apparente du Dtx d'environ 5000 fois (de 0,0019 mg.mL^{-1} à 9,98 mg.mL^{-1} (~ 11 mM)), ce qui jusque là n'avait jamais été rapporté dans la littérature. De plus, nous avons étudié de façon plus approfondie le complexe d'inclusion obtenu. A l'aide de différentes techniques telles que la ^{1}H-RMN, la microcalorimétrie par titration isotherme (ITC), le dichroïsme circulaire, ainsi que la modélisation moléculaire, nous avons identifié une stœchiométrie de complexation de type 2:1, selon laquelle les deux molécules de Me-β-CD interagissaient avec une molécule de Dtx selon un mode « séquentiel » [19]. Ce type de modèle, dans lequel une première molécule de CD interagit avec un ou plusieurs groupes de la molécule suivie de l'interaction d'une deuxième CD avec un deuxième groupement, a été rapporté pour différents composés avec les CDs natives [20, 21], les dimères de CDs ainsi que l'association des CDs avec les polymères [22-24], mais jusqu'ici il n'avait pas été rapporté pour les complexes entre le Dtx et les CDs, en particulier la Me-β-CD.

1.2. Systèmes nanoparticulaires pour l'administration orale d'agents anti-cancéreux.

La faible solubilité aqueuse de certains agents anti-cancéreux ne représente qu'une première problématique à résoudre pour améliorer la biodisponibilité du Dtx une fois administré par voie orale. La molécule doit atteindre l'intestin où a lieu l'absorption de 90 % des composés [25-27].

Pour atteindre cet objectif, nous avons pensé qu'agir au niveau de la formulation pouvait s'avérer une stratégie intéressante, en particulier avec la conception de formes nanoparticulaires mucoadhésives. Récemment, au sein de notre équipe nous nous sommes intéressés aux propriétés des systèmes constitués par des nanoparticules (NPs) cœur-couronne composées de poly(isobutyle cyanoacrylate) (PIBCA) recouvertes de chitosane thiolé.

Différentes études ont montré la capacité des NPs de poly(alkyle cyanoacrylate) (PACA) à encapsuler différentes molécules actives telles que les peptides [27], en particulier l'insuline [28-30], les vaccins [31], les agents cytotoxiques ainsi que d'autres composés hydrophobes [32, 33]. Aujourd'hui, les NPs de PACA sont considérées comme des systèmes colloïdaux polymériques intéressants pour la délivrance de médicaments pour le traitement du cancer [33, 34]. Dans le cas spécifique de l'administration orale, les propriétés bioadhésives et biodégradables des PIBCA font de ce polymère un candidat idéal pour la voie orale [35, 36]. Ainsi, la présence de chaînes de chitosan chargées positivement anis que la présence de groupements thiols sur la surface permettent aux NPs d'adhérer à la couche de mucus au niveau intestinal [37, 38].

Le chitosane est l'un des polysaccharides les plus utilisés dans le domaine biomédical et pharmaceutique grâce à ses propriétés intéressantes telles que la biodégradabilité, la biocompatibilité et la faible toxicité [35]. Le chitosane est un polysaccharide obtenu par déacétylation d'un polysaccharide naturel, la chitine qui est le deuxième polysaccharide le plus important et le plus abondant dans le monde après la cellulose. La chitine est principalement extraite de la carapace de crustacés tels que le homard ou le crabe, mais elle est aussi présente dans les champignons et chez les insectes. Le chitosane a montré en tant qu'excipient, des caractéristiques intéressantes et une grande versatilité dans ces emplois. Ses propriétés mucoadhésives dues à la présence des charge positives et sa capacité d'améliorer le passage des molécules à travers les barrières biologiques, notamment pour l'administration orale ou nasale de principes actifs [39-41]. Une autre caractéristique importante du chitosane est la présence des groupements amine libres. Ces fonctions ont permis la synthèse de plusieurs dérivés tels que les conjugués chitosane-EDTA, utiles pour surmonter la barrière enzymatique en vue de l'administration orale de peptides thérapeutiques [42]. Parmi les différents chitosanes modifiés, les chitosanes thiolés ou thiomers ont un intérêt particulier. En

effet, la présence de groupements thiols, leur permet de former une liaison disulfure avec les sous-domaines des glycoprotéines du mucus riches en cystéine [43]. Différents dérivés thiolés du chitosane ont été synthétisés : les conjugués avec l'acide thioglycolique, la cystéine, le 4-butyl-thioamidine (chitosane-TBA) [44, 45], et les dérivés chitosane-thioethylamidine (Chito-TEA) [46]. Par ailleurs, différentes études ont montré que le chitosane thiolé possédait la capacité d'inhiber les pompes d'efflux P-gp, ce qui facilite encore davantage le passage intestinal des molécules concernées par ce mécanisme d'efflux [47, 48].

Dans ce contexte, un de nos objectifs a été d'examiner la possibilité d'utiliser les NPs de PIBCA recouvertes de chitosane thiolé afin d'améliorer la biodisponibilité de principes actifs mal absorbés par voie orale, tells que les agents anti-cancéreux. La technique choisie pour la préparation des NPs était la polymérisation anionique en émulsion. Cette technique est particulièrement intéressante car les NPs composées de copolymères amphiphiles sont obtenues en une seule étape. La polymérisation est rapidement et spontanément initiée par les fonctions hydroxyle et pour qu'elle se fasse préférentiellement avec les ions hydroxyle du chitosane plutôt que ceux de l'eau, la réaction est conduite à pH acide (pH ~ 1) [49]. Le fait d'avoir augmenté la solubilité apparente de notre principe actif, le Dtx, à l'aide de Me-ß-CDs implique la participation de ces dernières à la polymérisation. En effet, afin d'obtenir un rendement d'encapsulation maximum, nous avons conduit la synthèse des NPs dans une solution contenant le complexe d'inclusion Me-ß-CD/Dtx. Il a était prouvé que l'incorporation des CDs dans des NPs peut apporter une amélioration du rendement d'encapsulation des composés lipophiles tels que les stéroïdes [50, 51] ainsi que le saquinavir utilisé comme molécule antivirale [52].

Toutefois, nous avons constaté une certaine instabilité du complexe d'inclusion en mimant les conditions pour la préparation des NPs. La perte totale de Dtx a été de 31 %. Cependant, le taux d'encapsulation du Dtx dans les NPs reste assez élevé, d'environ 70-80 %

et stable au cours du temps. Nous avons décidé alors de continuer le travail en évaluant la capacité de ce système à améliorer l'absorption intestinale du principe actif afin d'avoir une preuve de concept.

Une propriété primordiale des ce type de NPs est leur mucoadhésion. Par exemple, il a été démontré dans le cas du paclitaxel que les propriétés mucoadhésives des formulations permettaient le maintien de la concentration plasmatique du médicament au niveau thérapeutique pour une période prolongée dans les temps en améliorant sa biodisponibilité [53, 54]. Une étude du passage intestinal du Dtx au moyen de chambres d'Ussing nous a confirmé l'importance de cette propriété.

La chambre d'Ussing est une technique très intéressante qui permet d'évaluer le passage d'une molécule à travers un tissu intestinal de rat fixé entre deux demi-chambres (Figure 4) de manière à définir un compartiment luminal et un compartiment sérosal.

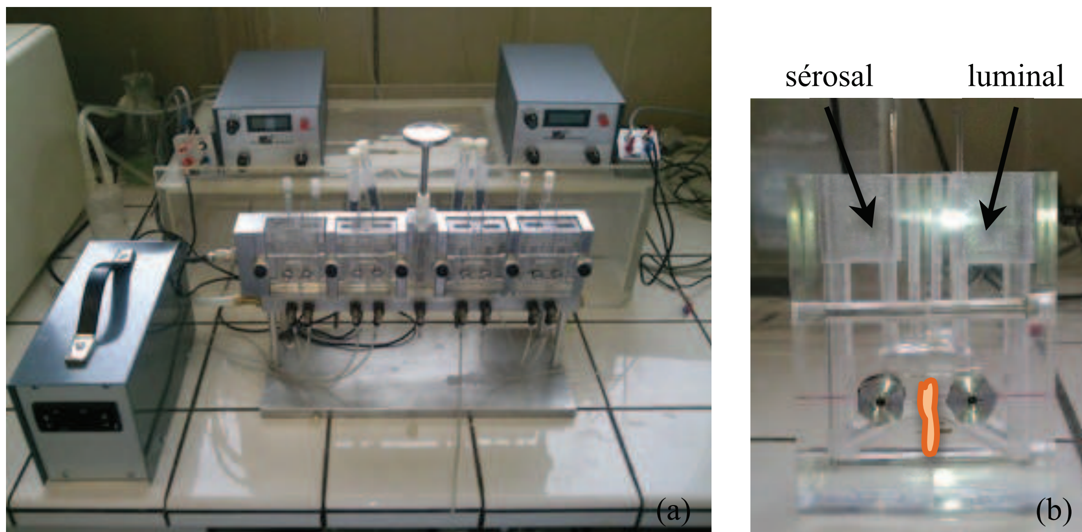

Figure 4. L'ensemble de l'appareillage de la chambre d'Ussing (a). Chambre où l'intestine est fixé (b).

L'utilisation d'un tissu intestinal plutôt qu'une monocouche cellulaire obtenue par culture présente l'avantage de pouvoir mimer de manière plus proche le devenir du médicament une fois arrivé au niveau intestinal en comparaison avec les modèles de culture cellulaires. Ainsi, différents auteurs préfèrent l'utilisation de la chambre d'Ussing à l'emploi de monocouches cellulaires issues de la lignée cellulaire Caco-2 ou HT29 couramment utilisées [55-57]. Ces lignées cellulaires, acceptées comme modèle pour étudier la relation entre la structure moléculaire, les propriétés physico-chimiques, et le potentiel d'absorption des médicaments, ne permet pas de reproduire la variété cellulaire de l'épithélium, telles que les cellules caliciformes nécessaires pour la production de mucine. L'absence du mucus est un facteur important dans l'étude de l'absorption de substances médicamenteuses sensibles à la dégradation enzymatique [58] ou pour dans l'étude des interactions entre la formulation et les glycoprotéines présentes dans la couche de mucus. Les résultats de cette expérience, ont montré que les NPs élaborées dans ce travail, sont capables d'améliorer l'absorption du Dtx par rapport à celles obtenues avec une formulation contrôle (solution de Ringer à 2% d'éthanol). Nous avons étudié différentes conditions et en particulier nous avons évalué le rôle de la mucoadhésion qui joue un rôle décisif dans le passage du principe actif. Ainsi, si nous empêchons le contact des NPs avec le tissu intestinal, le profil d'absorption du Dtx redescend à un niveau de celui obtenu avec la solution de Dtx et confirme donc que l'adhésion directe des particules à la surface de la muqueuse est indispensable pour observer un passage important de la molécule. L'importance de cette propriété a été mise en évidence dans d'autres travaux. Le même phénomène a été mis en évidence par Bravo-Osuna *et al* [37-59], puis par Aguëros *et al*. dans un récent travail avec des NPs chargées en paclitaxel [55]. Ce dernière a calculé les coefficient de perméabilité apparente (P_{app}) pour chaque formulation et condictions testées, et a montré en particulier que quand le paclitaxel était encapsulé dans les NPs mucoadhésives la perméabilité apparent P_{app} était augmentée de 12 fois par rapport à

la formulation contrôle (le Taxol® par voie orale). En même temps, elle était diminuée de 25 fois si le développement des interactions mucoadhésives entre les NPs et la muqueuse était évité. Il est utile de rappeler que, comme nous l'avons précédemment évoqué dans le chapitre III de ce mémoire, la perméabilité apparente d'une molécule, P_{app} (Eq.1*)* est un coefficient qui dépend de la concentration initiale du composé :

$$P_{app} = \left(\frac{dQ}{dt}\right) x \left(\frac{1}{AC_0}\right) \qquad \textbf{Eq.1}$$

où $\frac{dQ}{dt}$ représente le flux du principe actif traversant la membrane intestinale mesuré dans des conditions expérimentales données, A représente la surface impliquée dans l'absorption et C_0 représente la concentration initiale à l'interface avec la muqueuse intestinale. Cette constante est généralement exprimée en $cm.s^{-1}$. Dans nos études, nous avons choisi de ne calculer les coefficients P_{app} quand dans le cas où le principe actif était en solution (Ringer + 2% d'ethanol) et non quand il était formulé dans des NPs. En effet, dans ce dernier cas la concentration du composé change de façon continue dans le temps, en raison de la libération contrôlée assurée par la formulation. Dans ce contexte, nous avons trouvé plus correct de confronter les profils d'absorption des différentes formulations plutôt que de calculer des coefficients de perméabilité P_{app} basés sur des concentration nondirectement mesurées expérimentalement.

Les avantages de l'utilisation du chitosane pour le développement de nouvelles formulations pour l'administration orale des molécules actives, ont été rapportés par d'autres auteurs. Il a été prouvé que des conjugués de chitosane de faible masse molaire avec le paclitaxel ou le Dtx permettait d'améliorer l'absorption intestinale de ces molécules en augmentant leur solubilité, en prolongeant le temps de rétention du principe actif dans le tractus digestif grâce aux propriétés mucoadhésives des conjugués et en évitant les

mécanismes du métabolisme entérocytaire [60, 61]. De plus, d'autres formes pharmaceutiques mucoadhésives telles que les NPs composées d'acide polylactique (PLA) et de vitamine E TPGS [62] ou des micelles polymériques composées de le Pluronic® (F61 ou F127) et l'acide polyacrylique (PAA) [63] ont donné des résultats encourageants pour l'administration orale des agents anti-cancéreux.

Nous avons déjà mentionné que le chitosane thiolé, peut exercer une action inhibitrice sur le mécanisme du métabolisme entérocytaire en bloquant les pompes d'efflux [47, 48, 60, 61, 64-66]. Nos études sur le passage à travers la muqueuse intestinale en utilisant la chambre d'Ussing nous ont montré que le profil d'absorption du Dtx formulé au sein de NPs était exactement superposable aux profils obtenus dans les conditions où l'effet des pompes d'efflux et du métabolisme entérocytaire était contourné. Nous rappelons que cet effet est contourné de trois façons différentes : *(i)* lors du passage du Dtx de coté serosal vers le compartiment luminal (basolatéral-apical), *(ii)* en bloquant tous les mécanismes du métabolisme en opérant à 4 °C, et *(iii)* en co-administrant le verapamil qui est un inhibiteur des pompes d'efflux. Il s'agit d'un résultat très intéressant qui nous permet de conclure que la formulation que nous avons conçue, est capable de surmonter les différents verrous qui s'opposent à l'administration orale des agents anti-cancéreux. En particulier, avec ce système il n'y pas besoin de co-administrer des inhibiteurs des pompes d'efflux ou du CYP450 pour augmenter l'absorption du principe actif. Nous avons voulu approfondir cet aspect et nous avons réfléchi sur la possibilité que les NPs en tant que telles puissent avoir un effet anti-pompes d'efflux, en particulier grâce à la présence du chitosane thiolé à leur surface. Par conséquence, nous avons testé la perméabilité du tissu intestinal du mélange physique de la solution éthanolique du Dtx et des NPs vides. D'une façon étonnante, le profil d'absorption résultant était plus bas que la solution éthanolique du Dtx seul en indiquant que, non seulement les NPs n'ont pas un effet inhibiteur des pompes, mais rendent le passage du

médicament plus difficile. Cette expérience suscite donc des questions relatives au mode d'action de la formulation chargée en Dtx qui parvient à améliorer au maximum l'absorption de cette molécule. Une hypothèse qu'on pourrait avancer est que grâce à la mucoadhesion, les NPs sont capables d'amener une concentration très importante du principe actif au plus près du site d'absorption, ce qui pourrait être suffisant pour obtenir une saturation des mécanismes du métabolisme de manière à laisser libre le passage aux autres molécules du médicament.

2. Effet protecteur des nanoparticules vis-à-vis de la muqueuse intestinale

Nous avons déjà amplement traité ce problème dans la première partie de travaux antérieurs et mentionné ci-dessus que les effets toxiques et/ou indésirables sont des aspects fondamentaux à évaluer lorsque nous envisageons une chimiothérapie. En particulier, le mécanisme d'action aspécifique des agents anti-cancéreux, rend ces molécules très dangereuses pour l'organisme car elles touchent également les cellules saines et en particulier les cellules en prolifération continue telles que les cellules du tractus gastro-intestinal. Ce sont en particulier les cellules situées à la pointe des villosités qui sont en reproduction rapide et qui devraient donc être touchées en cas de toxicité cellulaire. Il faut donc se demander si l'administration orale d'un agent anti-cancéreux pourrait augmenter la toxicité au niveau intestinal en endommageant localement les cellules entérocytaires.

Très peu de travaux, dans la littérature ont évalué de façon approfondie la toxicité locale au niveau intestinal d'une chimiothérapie par voie orale. La plus part des études, sont des essais cliniques dans lesquels sont évalués les effets secondaires d'une administration orale d'un agent anti-cancéreux *vs* une administration intraveineuse (i.v.) [67-71]

Dans ce contexte, nous avons décidé d'étudier l'effet d'administrations orales répétées du Dtx formulé dans les NPs, au niveau de la muqueuse intestinale sur un modèle de souris xénogreffées. Nous avons choisi d'utiliser un modèle de tumeur très agressive, de manière à conduire l'étude dans les conditions pathologiques les plus graves. Nous avons comparé l'état

des intestins de différents animaux après un traitement par chimiothérapie orale avec les NPs chargées ou non en Dtx durant 5 jours successifs, avec un dose unique de Taxotere® par voie i.v. Les résultats ont été comparés à ceux obtenus chez les animaux seins ou atteints de tumeur n'ayant reçu aucun traitement. Nous avons exploré différentes techniques parmi lesquelles l'analyse de l'activité des lactates déshydrogénases (LDH) et l'observation histologique du tissue se sont relevées les plus intéressants. La LDH est une enzyme localisée dans le cytosol des cellules qui est libérée au moment où elles sont endommagées ou lorsque se produit la mort cellulaire. En conséquence, tout composé qui cause des dommages graves aux cellules conduira à une augmentation immédiate mesurable de la LDH dans le milieu [72]. Différentes études conduites soit sur les cellules Caco-2, soit sur les intestins des animaux, ont évalué la libération de cette enzyme pour estimer la cytotoxicité des composés administrés [73-79].

Les résultats obtenus au cours des différentes expériences que nous avons réalisées, ont montré que les NPs semblent avoir un effet protecteur de la muqueuse intestinale vis-à-vis de la toxicité intestinale inhérente au Dtx. En effet, nous avons noté une diminution de l'activité des LDH par rapport aux intestins des souris atteintes de la tumeur mais non traitées, et les images histologiques montrent un tissu sain, sans aucun signe de toxicité. L'effet protecteur des nanoparticules administrées par voie orale est un concept déjà rapporté dans la littérature. En effet, il a été prouvé pour les anti-inflammatoires, que l'utilisation des formes pharmaceutiques innovantes telles que les microparticules et les nanoparticules, pouvait protéger le tractus gastro-intestinal contre les ulcérations et les mucosités [80-84]. De plus, des études préliminaires ont montré que le contact direct des nanoparticles de PIBCA ou leur produits de dégradation tels que l'alcool isobutylique et l'acide cyanoacrylique avec les cellules Caco-2, engendrait une faible toxicité [85-87]. De plus, il a été prouvé que la

présence de CDs dans la formulation pouvait masquer les effets cytotoxiques des produits de dégradation des PIBCA [85].

3. Conclusions

La formulation nanoparticulaire imaginée a montré sa pertinence dans l'objectif d'améliorer le passage intestinal du Dtxl, tout en étant dénuées de toxicité locale importante. Plusieurs problèmes technologiques devront toutefois encore être résolus avant de pouvoir envisager une future application clinique, notamment l'instabilité partielle du Dtx dans le milieu de polymérisation des nanoparticules. Toutefois, il est surtout nécessaire de confirmer *in vivo* le potentiel de cette formulation car seules des études pharmacocinétiques et d'activité sur un modèle tumoral chez l'animal pourront permettre d'établir la capacité de ces formulations nanoparticulaires à augmenter l'absorption du Dtx de manière à ce qu'il puisse exercer son action antitumorale. Un succès à e niveau ouvrirait de grandes perspectives dans le cadre de l'utilisation clinique de cette molécule anticancéreuse majeure.

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CONCLUSION ET PERSPECTIVES

Le nombre toujours croissant de travaux scientifiques ayant pour objectif l'amélioration de la biodisponibilité orale des agents anticancéreux montre l'intérêt porté au développement de traitements de chimiothérapie administrables par la voie orale. Néanmoins, beaucoup de problèmes doivent être résolus avant de disposer de formulations sûres et efficaces et leur potentiel en oncologie reste à établir. D'un point de vue pharmaceutique, les problèmes posés par l'administration orale des médicaments anticancéreux ne sont pas différents de ceux posés par d'autres molécules thérapeutiques. Comme pour toute molécule, il est nécessaire que la formulation permette d'obtenir un profil pharmacocinétique pertinent, caractérisé par une biodisponibilité suffisante. S'agissant de substances anti-cancéreuses, leur toxicité importante pourrait s'exercer localement au niveau digestif et représenter une réelle limitation au développement de telles formulations.

Au cours de nos recherches, nous nous sommes intéressés à l'administration orale du docétaxel. Cette molécule majeure en cancérologie est extrêmement mal absorbée par voie digestive, ce qui justifie de rechercher des formulations originales, capables d'améliorer son absorption. Ainsi, nous avons montré que l'utilisation de suspensions colloïdales composées de nanoparticules mucoadhésives contenant des cyclodextrines pouvait constituer une stratégie intéressante. En effet, ces nanoparticules polymères comportent plusieurs fonctionnalités leur permettant de lever plusieurs des verrous qui s'opposent à l'absorption du docetaxel. Nous avons notamment explorés trois pistes principales, avec en premier lieu l'utilisation des cyclodextrines pour augmenter la solubilité apparente du principe actif dans l'eau lors de la phase d'encapsulation du docétaxel dans les nanoparticules, ensuite l'utilisation de dérivés mucoadhésif du chitosan afin simultanément d'améliorer l'absorption du principe actif au niveau intestinal et de contourner le mécanisme du métabolisme entérocytaire. Au cours d'expériences de perméation conduites au moyen de chambres de

Ussing, les formulations ont montré leur capacité à améliorer le passage intestinal du docétaxel, tout en étant dénuées de toxicité locale importante. Il faut préciser que, si les résultats sont très prometteurs, ils sont encore aussi préliminaires. Pour pouvoir envisager une future application clinique, il faudra d'abord résoudre les problèmes techniques rencontrés au cours de la formulation du système (telle que l'instabilité partielle du docétaxel dans le milieu de polymérisation des nanoparticules) et surtout il est nécessaire de confirmer *in vivo* le potentiel de cette formulation. En effet, seules des études pharmacocinétiques et d'activité sur un modèle tumoral chez l'animal pourront permettre d'établir la capacité de ces formulations nanoparticulaires à augmenter l'absorption du médicament de manière à ce qu'il puisse exercer son action antitumorale. En cas de succès, une telle formulation orale dans laquelle l'agent anticancéreux est dispersé au sein de nanoparticules, offrirait en outre la possibilité de moduler très simplement la dose administrée, donc d'ajuster finement la posologie et finalement d'offrir au corps médical et aux patients les bénéfices d'une thérapie personnalisée.

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2011 S. Mazzaferro, K. Bouchemal, J-F. Gallard, B.I. Iorga, M. Cheron, C. Gueutin, C. Steinmesse, G. Ponchel. Bivalent Sequential Binding Model of Docetaxel to Methyl-β-cyclodextrin. *Int J Pharmaceut.* 416 (171-180)

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RESUME

Rendre possible l'administration orale du docétaxel (Dtx), un de puissant agent anticancéreux administré par voie intraveineuse, représente un défi important en cancérologie. Disposer de formulations administrables par voie orale, moins toxiques et mieux tolérées, représenterait une avancée majeure au plan clinique. Toutefois, plusieurs études ont montré que la très faible biodisponibilité du Dtx par voie orale résulte simultanément de : ***(i)*** sa faible solubilité aqueuse, ***(ii)*** son faible passage transépithélial au niveau intestinal, ***(iii)*** son efflux par les pompes d'efflux (P-gp) et son métabolisme par le cytochrome P450. Nous avons conçu une formulation capable de répondre simultanément à ces différents problèmes. Ainsi, nous avons tout d'abord fait appel aux cyclodextrines (CDs) pour augmenter la solubilité apparente du Dtx. La complexation du Dtx avec la méthyl-β-CD a permis d'augmenter la solubilité apparente du Dtx d'environ 5000 fois. Ce complexe a ensuite été associé à des nanoparticules (NPs) polymères composées d'un cœur de poly(cyanoacrylate d'alkyle) et recouvert en surface de chitosan thiolé afin de leur conférer des propriétés mucoadhésives et de diminuer localement le métabolisme. Ces NPs ont montré *in vitro* et *ex vivo* leur capacité à arriver intactes au niveau de l'intestin, d'y adhérer et de libérer le Dtx de manière contrôlée dans le temps, et finalement d'améliorer son absorption intestinale. Une évaluation de la toxicité de cette formulation vis-à-vis de la muqueuse intestinale suggère que l'encapsulation du Dtx dans les NPs assure une certaine protection de la muqueuse. Au final, la formulation orale proposée offre en perspective la possibilité de moduler la dose administrée, donc d'ajuster finement la posologie et finalement d'offrir au corps médical et aux patients les bénéfices d'une thérapie personnalisée.

MOTS CLES : voie orale, docetaxel, cyclodextrines, nanoparticules polymériques, absorption intestinale.

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