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A diclofenac-β-cyclodextrin conjugate: from design as colonic pro-drug up to *in vivo* performance

António Manuel d'Albuquerque Rocha Gonsalves[1]

ABSTRACT

Design and synthetic study for a diclofenac cyclodextrin conjugate. Attempts to connect diclofenac through a covalent bond to cyclodextrin, full characterization of the product, and demonstration of the potential of such structure to be used for oral uptake and specific colon delivery of free diclofenac. The pharmacological and therapeutic reasons for such approach, market and industrial economic considerations.

Um conjugado de diclofenac-β-ciclo-dextrina: da conceção como pro-fármaco colónico ao desempenho in vivo

RESUMO

Conceção e estudo de síntese dum conjugado de diclofenac-β-ciclodextrina. Tentativas de formação de uma ligação covalente entre diclofenac e ciclo-dextrina, caracterização total do produto e demonstração do potencial dessa estrutura para ser administrada por via oral com absorção especifica de diclofenac no colon. Razões farmacológicas e terapêuticas para usar esta opção, considerações de mercado e economia industrial.

In our last communications to this Academy the presentation was addressed to basic developments in the area of basic synthetic Organic Chemistry though pointing to long range potential interest of the results and emphasizing specific objectives in relevant problems in areas of therapeutic, industrial, and environmental applications. This time we bring a lighter task from the point of view of chemical synthesis, but not necessarily easier to solve and of lower interest to the impact of science in ordinary common problems. In any case we paid attention and feel inserted into the motto of the Academy "*Nisi utile est quod facimus stulta est Gloria*".

As in many other occasions we were asked to find a place in our research group for a PhD student working with Prof. Francisco Veiga, Faculty of Pharmacy, University

[1] Academia das Ciências de Lisboa

of Coimbra, to develop the synthetic and analytical chemical studies having in mind a problem of reckoned interest from the pharmacological point of view.

The student Amélia C.F. Vieira started her work with us with follow-up of my then close assistant Doctor Arménio C. Serra, and of Doctor Alexandra C.A. Rocha Gonsalves for the Analytical Chemistry support and developments.

Our aim was to find the most convenient way for the drug to be protected from absorption and degradation in its journey through the upper gastrointestinal tract (GIT) and be selectively liberated and absorbed in the colon.

Absorption of drugs at the colon level is very often a favorable way to achieve high therapeutic efficiency while oral drug delivery is the most preferred and convenient route to drug administration for different reasons namely: high patient compliance; least sterility constraints; flexibility in the design of dosage forms; easy production, so cost effectiveness ratio. The different challenges faced after oral administration by both immediate dosage and modified dosage forms along the route of GI tract until reaching the blood system have to be considered to design the chemical answer to the problem of drug design.

Along the GIT pH goes from acidic values at level of stomach due to secretion of hydrochloric acid (HCl) but increases its value reaching the duodenum due to neutralization by alkaline pancreatic secretions and along the small intestine up to pH 7.5, Figure 1.

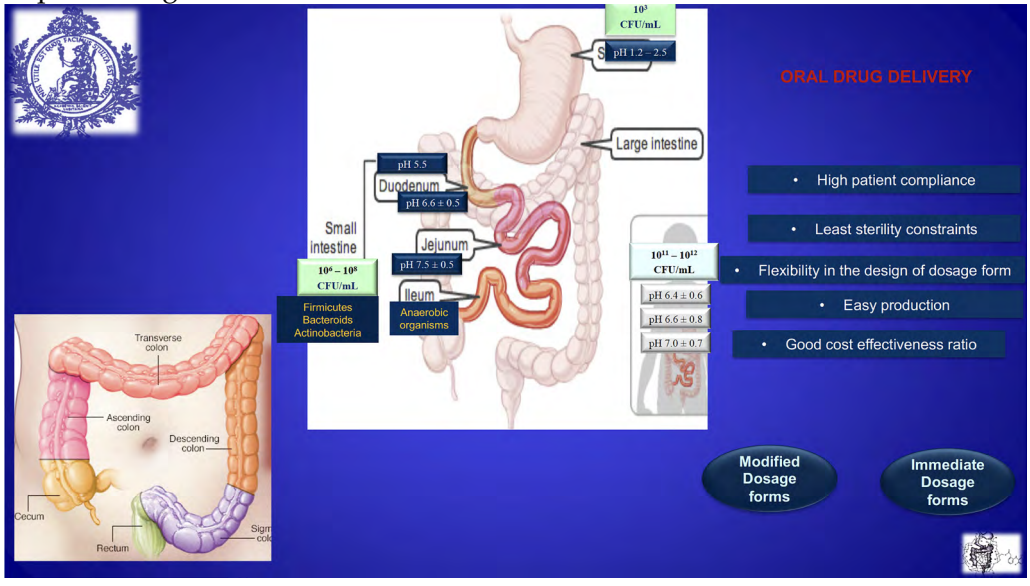


Figure 1. Schematic presentation of the gastrointestinal tract, GIT, sequential chemical environment and the approach of oral drug delivery.

Reaching the first part of large intestine, pH drops again due to short chain fatty acids originated from fermentation. Subsequently pH increases due to the lower carbohydrate fermentation and production of ammonia and urea resulting from protein metabolism.

Also, microbiota increases from stomach to the colon, being bacteria anaerobic in the lower GIT in contrast to the upper GIT where they were predominantly aerobic.

Transit time along the GIT tract is variable. At level of stomach, it can range from seconds to few hours and to cross the small intestine the range is between 1 to 9.5 hours. The presence or absence of food in the GIT influences the transit time of oral delivered drugs consequently affecting the corresponding bioavailability.

Colon-specific drug delivery as a method of targeting drug administration is often a solution for molecules that suffer degradation and or are poorly absorbed at level of the upper GIT. It has gained increasing interest in recent years for treating both local gastrointestinal diseases (Chron´s inflammatory and ulcerative colitis; constipation; diarrhea; colon rectal cancer; irritable bowel diseases and infections) as well as systemic disorders. In case of systemic application, disorders such as arthritis and asthma which are sensitive to the circadian rhythms have commonly a peak symptom at bedtime and can be managed by colon specific drug delivery, schematically illustrated in Figure 2 (and 3).

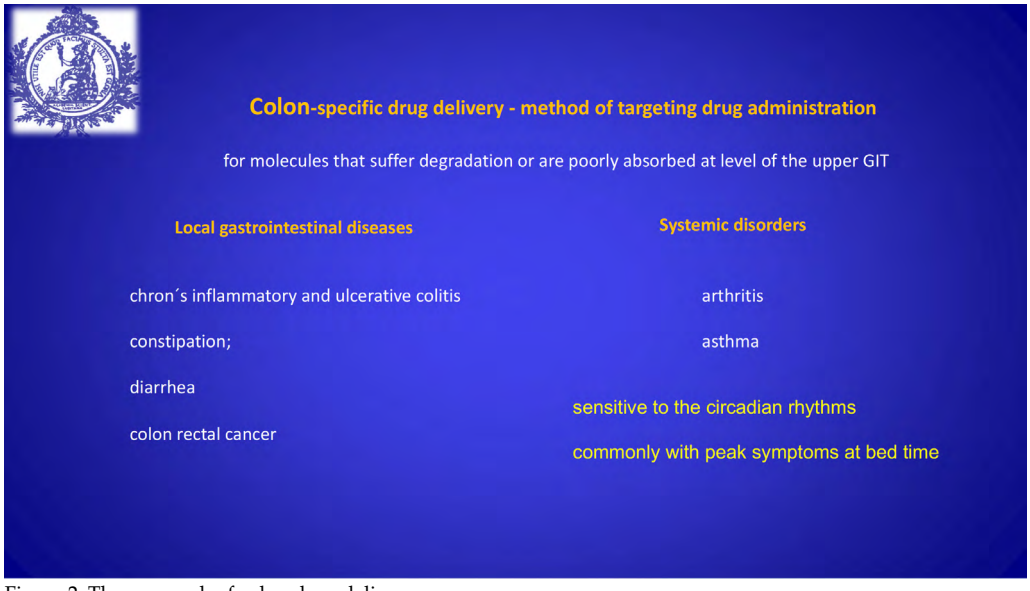


Figure 2. The approach of colon drug delivery.

To achieve the objective of protecting the drug molecule from the oral intake to the point where its liberation is required it can be linked in some way to another usually inactive molecule or molecular structure under conditions of being liberated at the appropriate point. This corresponds to an encapsulation at the molecular level.

To liberate the drug at the required point, different approaches range from taking advantage of pH changes in the gastrointestinal tract, exploit time dependent or pressure-controlled systems, and the metabolism by colonic microbiota.

The abrupt increase of bacteria at the colon associated with the unique enzymatic activity of the gut flora and their specific mechanisms of action has enabled the development of prodrugs targeted to this region as is the case of azo-bonded prodrugs, Figure 3.

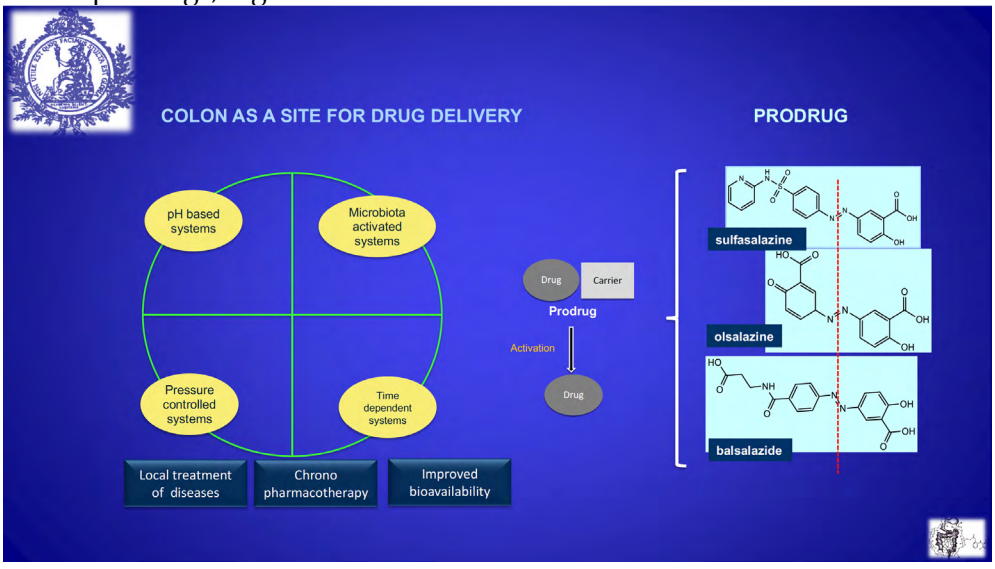


Figure 3. The liberation of a drug from the corresponding prodrug.

Diclofenac is a well absorbed non-steroidal anti-inflammatory drug used to treat painful conditions, pain suppression being considered nowadays one of the most important factors for life quality.

We thought that a likely interesting form for the desired diclofenac prodrug would be the preparation of a complex diclofenac-cyclodextrin.

Cyclodextrins are cyclic oligosaccharides, consisting of a macrocyclic ring of glucose subunits joined by α-1,4 glycosidic bonds. They are structures produced from starch by enzymatic conversion. The most common and useful cyclodextrin

structures are those of 6, 7, and 8 glucose units known as α, β, and γ-cyclodextrin respectively. For our study we selected γ-cyclodextrin whose structure and stereo model is illustrated in Figure 4. Cyclodextrin complexes are well documented as convenient to form prodrugs.

Relevant characteristics of the ring structure of the cyclo-dextrin molecules are the central inner cavity lined by the hydrophobic segments of the cyclic oligosaccharide while the hydroxyl groups give to the external face of the ring a hydrophilic behavior as well illustrated in the stereo model on Figure 4.

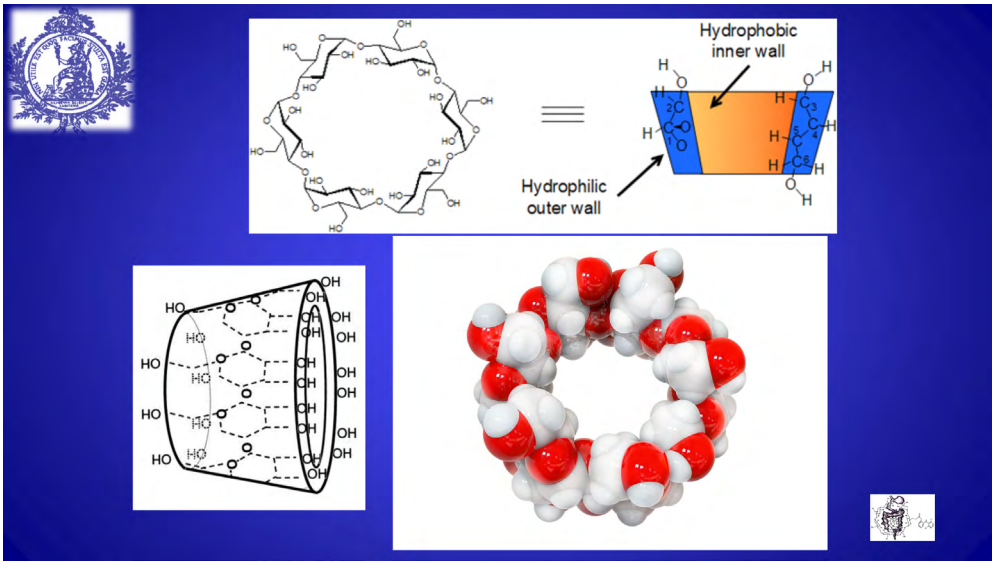
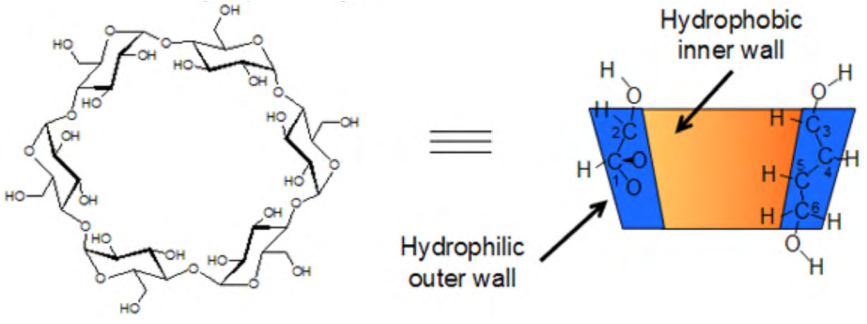


Figure 4. Structure of γ-cyclodextrin.

The above-mentioned characteristics are more clearly evidenced through the schematic figurative representation of Figure 5, illustrating the possibility of insertion of a drug into the hydrophobic pocket, whole molecule or part of it, or forming a chemical bond between functional groups existing in the two molecules.



Both possibilities have been exploited either to form prodrugs or, for a different role, to generate synthetic catalysts showing selectivity typical of enzymes.

Facing our specific case, cyclodextrin seemed a convenient choice to be used as a carrier for colonic delivery since it is not absorbed through biological membranes in the gastrointestinal tract, can pass intact through the upper GIT only being metabolized into small saccharides by the abundant microbiota of the colon.

Both the drug and cyclodextrin have convenient functionalities to form an ester from the carboxylic group in diclofenac and the hydroxy of the cyclodextrin.

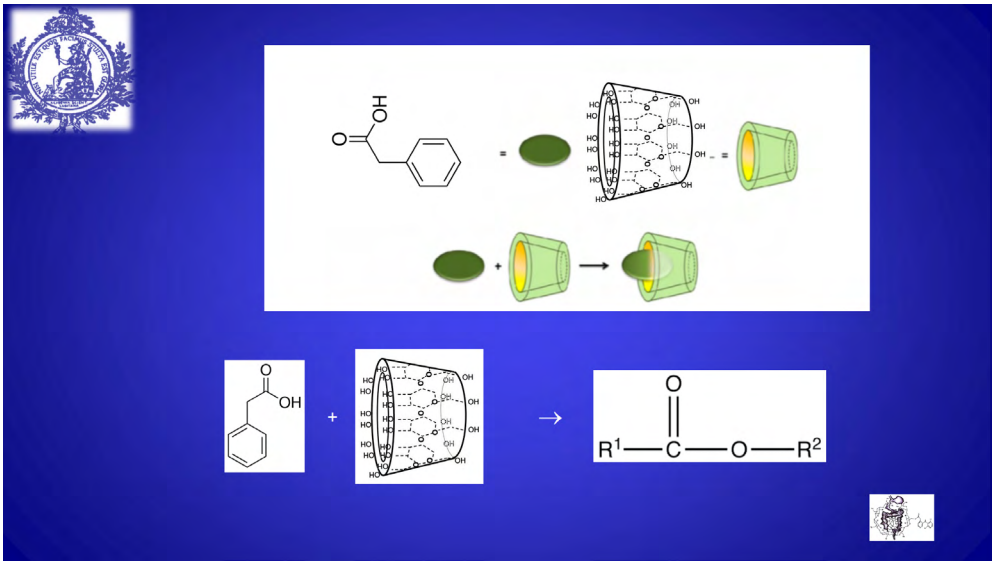


Figure 5. Figurative representation of the capacities of cyclodextrin to form prodrugs.

Such ester linkage seemed promising to be conveniently hydrolyzed at the required moment. Figure 6 illustrates the preceding rational.

At this point, we set up to attempt the required esterification reaction using standard reaction conditions for this well-known chemical reaction. But, contrary to expectations out of the simplistic primary approach relying plainly on classical reactions commonly described in texts as very easy to accomplish, they did not work in this case. Textbooks, books in general, are essential tools of knowledge but so are the lessons of the experience of walking and crossing the hurdles of life, and of Chemistry! This hard truth should always be, but frequently is not taken into account mind in the field of industrial property rights involving chemical synthetic methods.

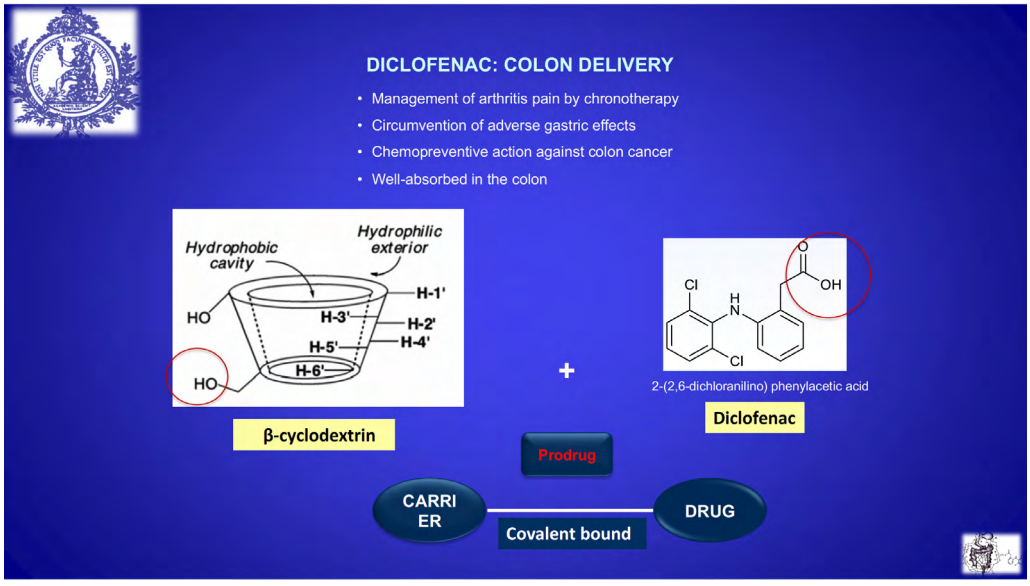


Figure 6. Structural and figurative formation of the prodrug cyclodextrin-diclofenac.

To solve our problem, we certainly looked in first place to the various classical known approaches to form an ester from two labile molecules one having a carboxylic and another an alcohol residue. The approaches included: activation of diclofenac using carbodiimides, and intermediary formation of an acid chloride or instead nucleophilic substitution of an alcohol activated derivative by a carboxylate with conventional heating. However, all these approaches proved unsuccessful. The only identified product obtained out of these was a cyclic species resulting from the internal reaction between the carboxylic group and the amino nitrogen of diclofenac. This undesired outcome could not even be avoided under simple very light warming conditions of diclofenac. The favorable intramolecular cyclization forming a five atoms lactam ring always prevails. With this observation, it became clear that our first approaches did not work due to the particular structure of diclofenac since the attempted methods proved successful in the preparation of many other cyclodextrin conjugates. The aforementioned experiments are summarized in Figure 7.

Despite of first unsuccessful attempts, gathered evidence about the origin of the failure led us to believe to be worth keeping the focus on overcoming the problem.

It was clear the need to avoid any extensive warming of diclofenac as in the case of approaches involving activation of its carboxylic group.

We thought that activation of the hydroxy group of the cyclodextrin followed by nucleophilic attack by diclofenac carboxylate though unsuccessful under classical warming could still be able to succeed if we could work under the novel milder approach of microwave irradiation work-up. We set-up then to an attempt using such warming conditions. For that approach it was necessary to consider the need of selectivity relatively to the two different types of hydroxyls occurring in the diclofenac molecule, primary and secondary. In this regard the softer the conditions used to promote the reaction the more likely the probability of being successful in our aim of obtaining the required chemo-selectivity and mono-tosylation of the primary hydroxyl and so to end- up with the corresponding monoester. A suitable API has to be a well-defined chemical structure.

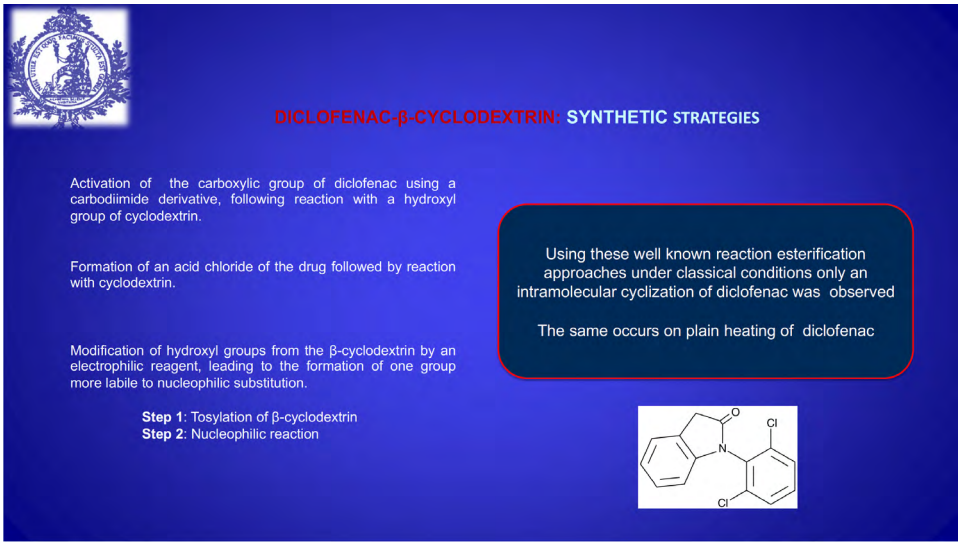


Figure 7. Synthetic strategies to prepare the prodrug.

Our developed approach consisted in adjusting experimental reaction conditions to allow a fairly high selectivity for mono-tosylation of cyclodextrin in a primary hydroxyl without significant amounts of di- or tri-tosylated species as shown by MALDI-TOF analysis. This unpurified tosylated product was directly used in the nucleophilic substitution reaction with diclofenac carboxylate during a very short reaction time under selected MW irradiation with the extra end bonuses of only needing a very small amount of solvent comparatively to conventional methods used for the preparation of other cyclodextrin conjugates. After chromatographic purification, about 20% yield of product was obtained.

This microwave approach to the synthesis of the conjugate, as illustrated in Figure 8 proved to be consistently reproducible.

The structure of our conjugate was fully identified and characterized by 1H NMR, and MALDI-TOF spectrometry, Figure 9.

The 1H NMR spectrum (C) of the conjugate is compared to those of the free cyclodextrin (A), and of the sodium diclofenac (B). Relevant data is as follows:

– we see that the ester bond is formed with one 6-hydroxyl of cyclodextrin since the protons of cyclodextrin secondary hydroxyl groups do not show any significant shift comparatively to the dramatic up field shift observed by the protons of primary hydroxyl (change from 4.60 ppm to 4.21 – 4.55 ppm);

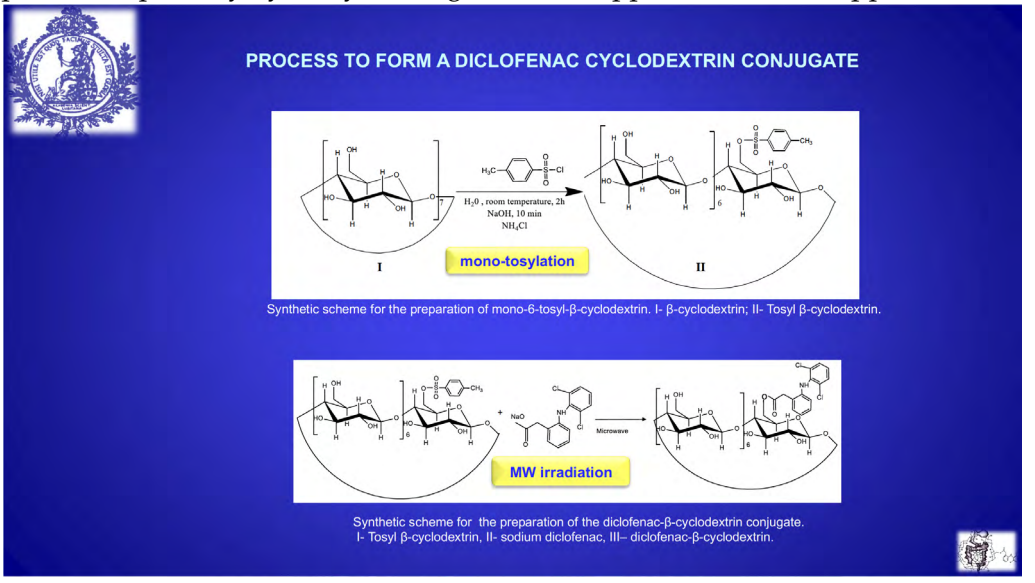


Figure 8. The two-step approach to synthesize the diclofenac-β-cyclodextrin conjugate from diclofenac and cyclodextrin under MW irradiation.

– in the conjugate, all the signals related to diclofenac suffer downfield shifts, except the H-2 protons characteristic of an ester bond, additionally, the 6-hydroxyl substitution is confirmed by the reduction in the integration of the 6-hydroxyl peak protons, the H-6 protons linked to diclofenac resonate downfield relatively to the unsubstituted H-6 protons, and show a characteristic multiplicity.

Mass spectrum (MALDI-TOF) of the product only shows the peak m/z 1434.308 corresponding to the [M+Na] adduct. No other relevant mass signal was observed as also shown in the Figure 8.

Overcome the problem of synthesizing a diclofenac-cyclodextrin ester complex, it was necessary to evaluate its stability along the GIT. In first place we

checked the chemical behavior of the complex under the typical pH conditions along the GIT. Firstly, we used appropriate buffer solutions. Plain solutions of hydrochloride pH 1.2, acetate buffer pH 4.5, phosphate buffer pH 6.8, phosphate buffer pH 7.4. Only for pH 1.2 and after 24 h degradation of about 25% of the conjugate was observed giving good evidence of a significant chemical stability to the pH environment found in the GIT.

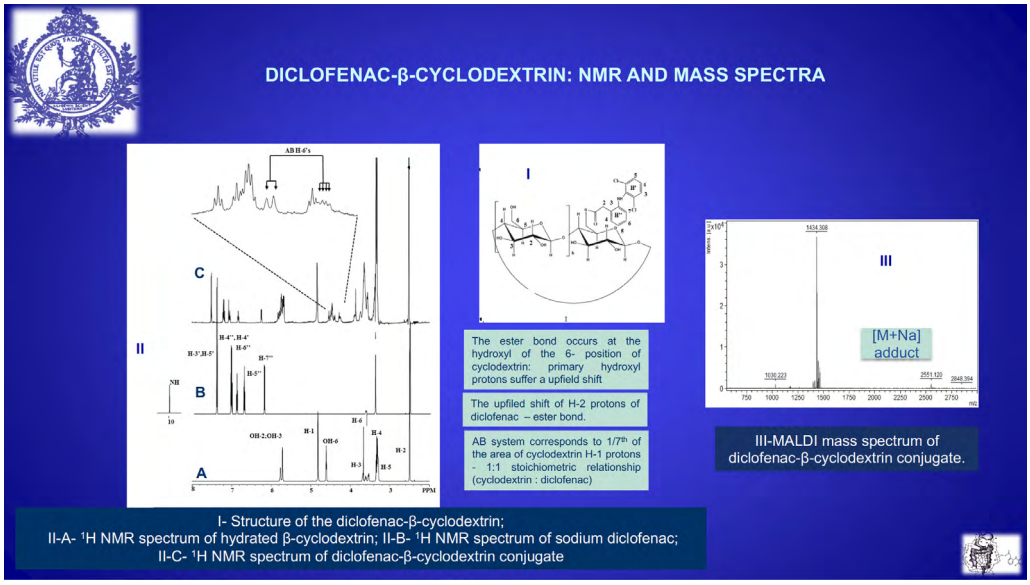
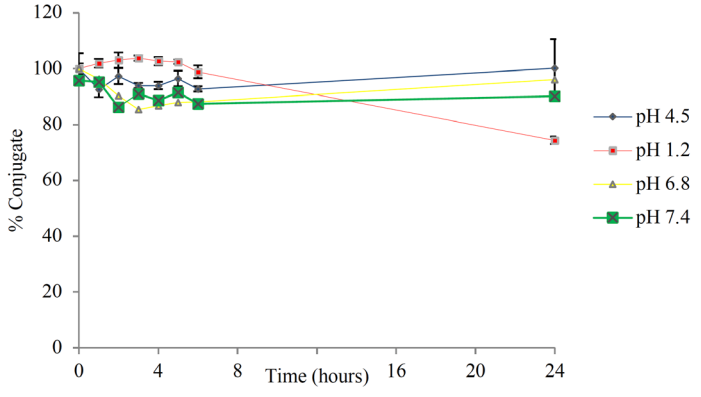


Figure 9. 1H NMR, and MALDI-TOF spectrum of the diclofenac-β-cyclodextrin conjugate.

Next step to assess the usefulness of our conjugate was to check its stability under conditions closer to those of GIT fluids. In first place simulated gastric and small intestine fluids were used. These hydrolysis studies proved that the conjugate is stable under such simulated fluids. Neither pepsin nor pancreatin can affect the stability of the conjugates. Thus, the conjugate passed this preliminary chemical laboratory test of stability in the upper gastrointestinal tract with detailed results illustrated in Figure 10.

Demonstrated the stability of the conjugate to the upper GIT environment conditions its behavior in the lower GIT was further studied. At this point we were going into an area a bit off plain chemistry requiring some specific expertise for experiments involving organic fluids, incubation and finally animal experiments. It was essential expert support, and this was obtained during a stay of our PhD student and use of facilities and supervision given by Prof. Abdul W. Basit in his Labs at the School of Pharmacy, University College of London.

To check the stability of the conjugate in the lower GIT, a simulated colonic fluid was used in the form of a fecal slurry prepared from human fresh feces. The conjugate was incubated in this slurry. A control experiment was also run in parallel in fecal slurry which was subject to autoclaving at 130 °C for 20 minutes, procedure intended to inactivate the bacterial enzymes. These experiments demonstrated that the conjugate was readily hydrolyzed in the human fecal slurry. Within 2 hours the conjugate is completely degraded, as evidenced by the rapid liberation of free diclofenac. By contrast, the conjugate was stable in the autoclaved fecal slurry confirming that the cleavage of the conjugate is due to bacterial enzymatic activity in the slurry.



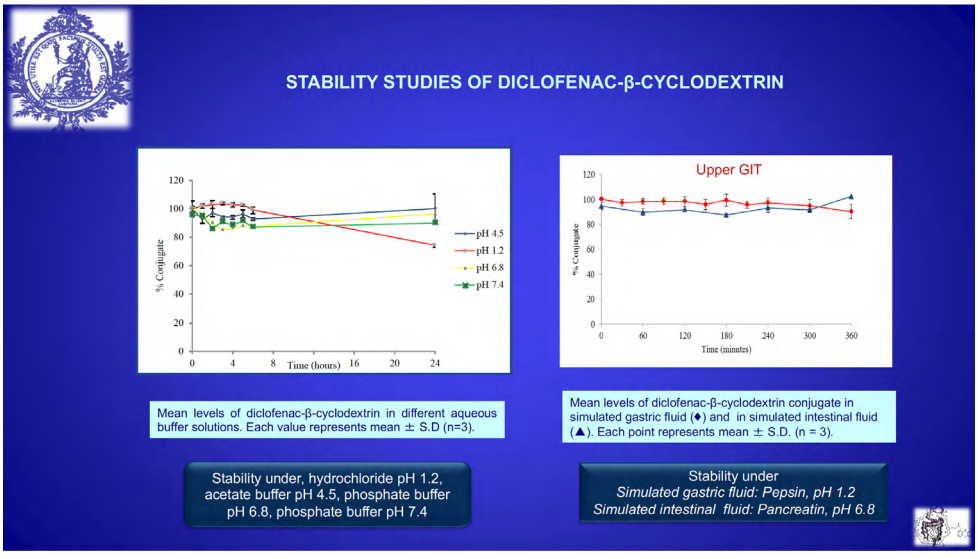


Figure 10. Stability of diclofenac-β-cyclodextrin conjugate under simulated GIT environments.

Release of the drug from the prodrug does not involve solely the hydrolysis of the ester linkage but also the integrity of the cyclodextrin, since release takes place under conditions where cyclodextrin is segmented into small saccharides by the colonic microbiota. These last results are illustrated in Figure 11.

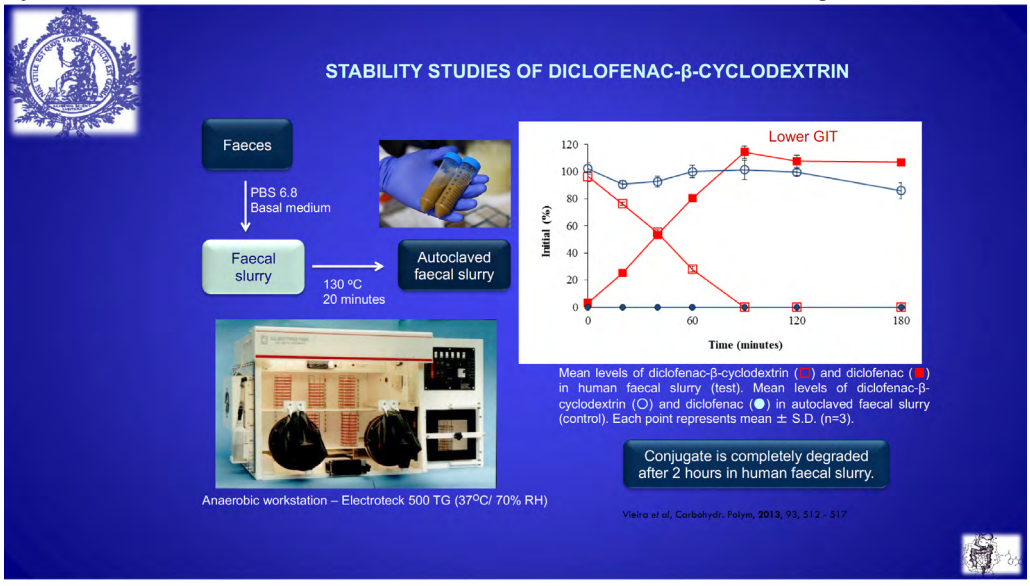


Figure 11. Behavior of diclofenac-β-cyclodextrin under lower GIT environmental conditions, *in vitro*.

At this point things were ready to start experiments with animals. Wistar rats were used to perform *"ex vivo"* and *"in vivo"* experiments. In these studies, we used not only our conjugate but also for control purposes the well-known market available model of colonic delivery, sulfasalazine. When orally administered, 90% of sulfasalazine reaches the colon as an intact molecule, and only their azo reductases cleave the azo bond liberating mesalazine and sulfapyridine. Sulfapyridine is therefore a good marker of transit time through the gastrointestinal tract and an indicator of colonic targeting.

"*Ex vivo*" studies were carried out in fluids collected from cecum and colon. Experiments were performed incubating sulfasalazine concomitantly with the conjugate in cecum and colonic fluids. From the experiments "*ex vivo*", samples were taken at selected time intervals and the stability of the conjugate diclofenaχ-cyclodextrin and sulfasalazine where subjected to analytical studies to assess the corresponding stabilities under the respective medium and conditions. In this study, the analytical method followed the main regulatory document from FDA for Bioanalytical Method Validation (FDA 2001).

"*In vivo*" studies were performed in two groups of rats. The first group received a suspension of both prodrugs. The second group received a suspension of diclofenac and sulfasalazine in equivalent concentration. These suspensions were administered orally by gavage. Blood samples were collected from the tail at predetermined time points and the concentration of diclofenac and sulfapyridine determined in both groups.

Figure 12 displays a schematic illustration of the performed "*ex vivo*" and "*in vivo*" experiments from where samples were collected.

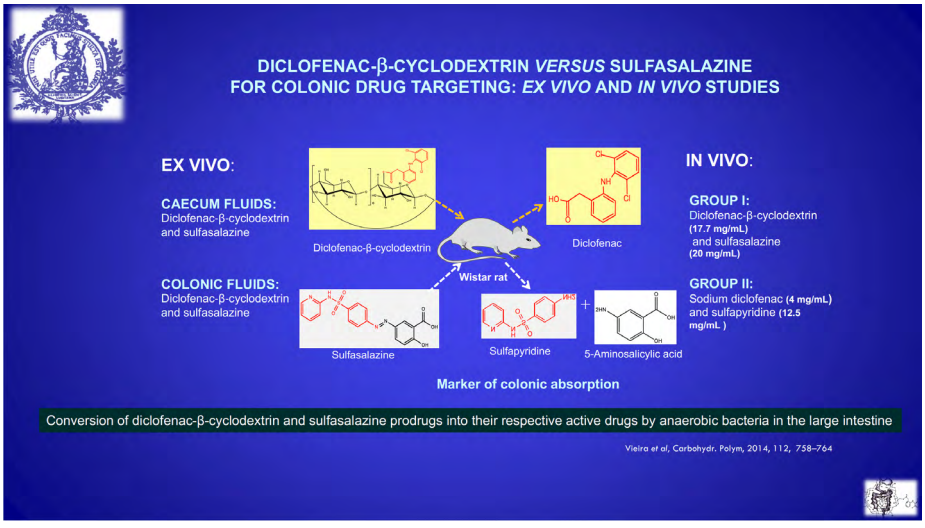


Figure 12. Colonic drug targeting of diclofenac-β-cyclodextrin vs sulfasalazine: *ex vivo* and *in vivo* studies.

Graphs in Figure 13 allow for a comparative assessment of the degradation of our conjugate diclofenac-β-cyclodextrin and sulfasalazine under the action of cecum and colon fluids. Graphs A and C show the decrease of the conjugate diclofenac-β-cyclodextrin and the increase of diclofenac along the time in both media while graphs B and D show the correspondent decrease of sulfasalazine. We can see that the disappearance of the conjugate diclofenac-β-cyclodextrin coincides with the appearance of free diclofenac demonstrating that the prodrug is able to liberate the drug in a colonic environment. From the graphs we also see that degradation of sulfasalazine is much faster than that of diclofenac-β-cyclodextrin.

This last observation can be interpreted on the basis that sulfasalazine is degraded by abundant azo reductases, enzymes produced by many different bacterial species in the large intestine. The supply of azo reductase is almost unlimited in the colon fluid.

In contrast, the cyclodextrin conjugate metabolism is less favored and likely to involve two types of enzymes, amylase and esterase. Probably the esterase can only act after the amylase has started degradation of the cyclodextrin carrier.

From the results in these graphs, we can also conclude that the degradation of both prodrugs is faster in colonic fluid comparatively to caecal contents and that the diclofenac-β-cyclodextrin conjugate shows potential as a sustained-release formulation to act in the colon.

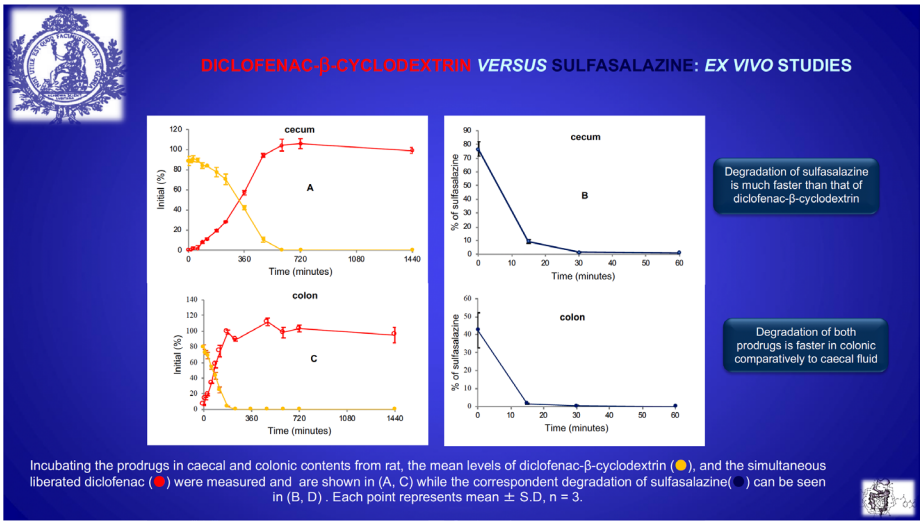


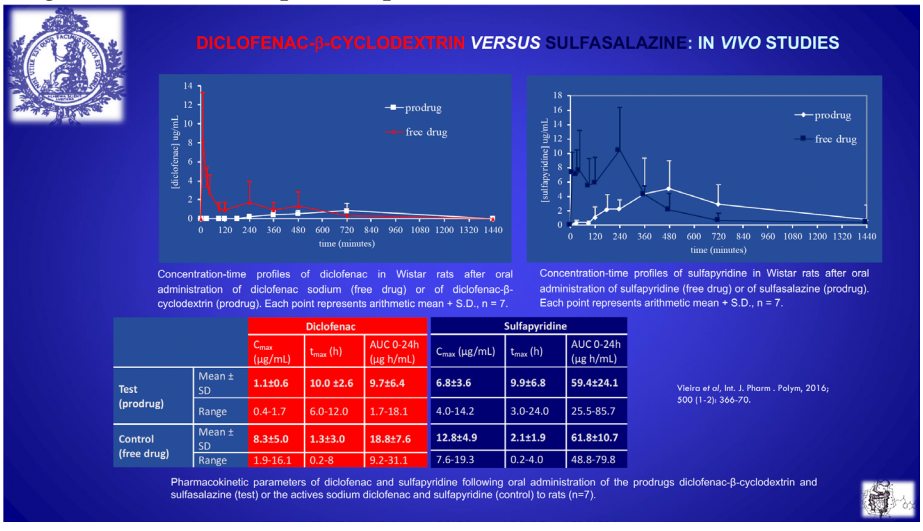
Figure 13. Colonic decomposition data for complexes diclofenac-β-cyclodextrin, and sulfasalazine.

The performance of our conjugate diclofenac-cyclodextrin were further assessed under "*in vivo*" conditions, matching again its behavior with that sulfasalazine. Blood samples were collected in the tail of the rat following gavage administration of samples of drug and prodrug. Graphs and tables in Figure 14 show how the administered samples reach the blood flow and how the liberation of the drugs from the corresponding complexes occurs. Graph A shows the concentration of diclofenac in blood samples at sequential time intervals for the cases of being orally administered as free drug or as prodrug. Graph B illustrates the same for the case of administration of sulfapyridine and sulfasalazine.

Although similar, there are clear differences for the two cases. In both, the complexes have a more extended absorption profile, being C_{max} achieved ca. 10 hours post- administration for the conjugates. In contrast rapid absorption occurs for the free drugs (t_{max} of diclofenac being 1.3 h, and that of sulfapyridine 2.1 h).

The higher tmax of diclofenac-β-cyclodextrin conjugate originates a lower value of C_{max} and lowest value of AUC (Area Under Curve). The same statistically tmax value for diclofenac-β-cyclodextrin and sulfasalazine confirms the colonic metabolism of diclofenac-β-cyclodextrin.

AUCs of the two drugs were affected to different extents following prodrug administration. While the AUC of the diclofenac prodrug was about half that of free diclofenac ($p<0.05$), the AUC of sulfapyridine was almost the same for the prodrug and the free compound ($p>0.05$).



		Diclofenac			Sulfapyridine		
		C_{max} (µg/mL)	t_{max} (h)	AUC 0-24h (µg h/mL)	C_{max} (µg/mL)	t_{max} (h)	AUC 0-24h (µg h/mL)
Test (prodrug)	Mean ± SD	1.1±0.6	10.0 ±2.6	9.7±6.4	6.8±3.6	9.9±6.8	59.4±24.1
	Range	0.4-1.7	6.0-12.0	1.7-18.1	4.0-14.2	3.0-24.0	25.5-85.7
Control (free drug)	Mean ± SD	8.3±5.0	1.3±3.0	18.8±7.6	12.8±4.9	2.1±1.9	61.8±10.7
	Range	1.9-16.1	0.2-8	9.2-31.1	7.6-19.3	0.2-4.0	48.8-79.8

Figure 14. Collected data of concentration of diclofenac and sulfapyridine in blood flow of Wistar rats following gavage administration as free drugs or as pro-drugs.

From the chemical perspective, this study was led to the point where it can be used by small to medium-sized enterprises interested in increasing their operation capacities. Actually, it is the kind of approach appropriate to be the core of development most suitable to be followed the majority of pharmaceutical companies existing all over and particularly in Portugal. However, this not only requires the chemical and biological studies as those we performed but a sensible information concerning economic and market data.

Interestingly, our study originated from an original request from a Pharmacy Faculty ending with the interest of a Pharmaceutical Company which patented the results. Further, they did not pay much attention to this subject considering apparently the low market price of diclofenac did not justify investment. This belongs to the kind of non- chemical aspects relevant to business decisions. Anyway,

it is apparently clear that our results are of potential applicable interest. Using adequately selection of drugs of interesting from the marketing point of view. The chemical and pharmacological results from this study are likely to be easily and successfully applied, conviction reinforced by the wide interest demonstrated by various demands after presentation of this work in scientific meetings.

Acknowledgements

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