Drug-like properties and ADME of xanthone derivatives: the antechamber of clinical trials

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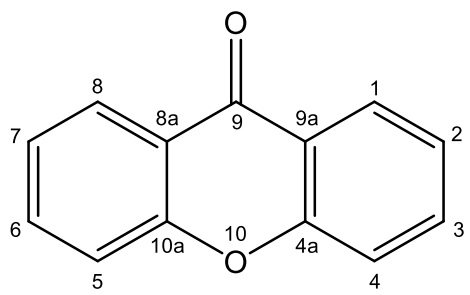
Abstract

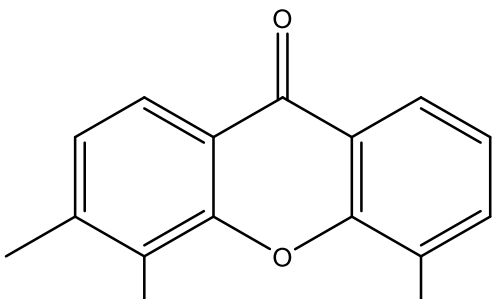
Xanthone derivatives have been described as compounds with privileged scaffolds that exhibited diverse interesting biological activities, such as antitumor activity, directing the interest to pursue the development of these derivatives into drug candidates. Nevertheless, to achieve this purpose it is crucial to study their pharmacokinetics and toxicity (PK/tox) as decision endpoints to continue or interrupt the development investment. This review aims to expose the most relevant analytical methods used in physicochemical and PK/tox studies in order to detect, quantify and identify different bioactive xanthones. Also the methodologies used in the mentioned studies, and the main obtained results, are referred to understand the drugability of xanthones derivatives through *in vitro* and *in vivo* systems towards ADME/tox properties, such as physicochemical and metabolic stability and biovailability. The last section of this review focus on a case-study of the development of the drug candidate DMXAA, which has reached clinical trials, to understand the paths and the importance of PK/tox studies. In the end, the data assembled in this review intends to facilitate the design of potential drug candidates with a xanthonic scaffold.

Xanthone; analytical method; drug-like; pharmacokinetics; toxicity; drug development; preclinical; metabolism; chromatography.

1. Introduction

The xanthone nucleus or 9*H*-xanthen-9-one (dibenzo-γ-pirone **1**, **Fig. 1**) comprises an important class of oxygenated heterocycles and is considered a privileged structure [1]. It includes relevant secondary metabolites which are present in commercialized extracts with human health promotion properties [2]. Considering synthetic derivatives, one compound emerged as drug candidate, 5,6-dimethylxanthenone-4-acetic acid (DMXAA, Vadimezan, ASA404, **2**, **Fig. 1**) and reached phase III clinical trials towards antitumor activity [3]. Due to a great number of studies in isolation, synthesis and biological/pharmacological properties of xanthone derivatives, several reviews [1,4-16] have gathered important information to guide the design of these compounds as potential drug candidates. Nevertheless, little focus was given to drug pharmacokinetics (PK), toxicity studies, and structure-properties relationships of xanthone derivatives.





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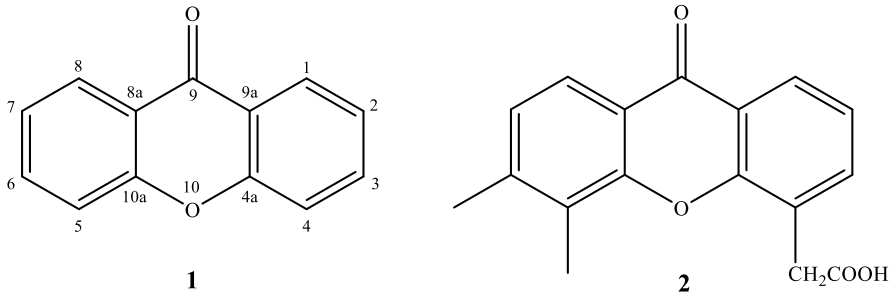

Fig. 1: Xanthone (**1**) and DMXAA (**2**).

Nowadays it is recognized that in addition to pharmacological properties, PK and toxicological properties are crucial determinants of the ultimate clinical success of a drug. This recognition has led to the early introduction of absorption, distribution, metabolism, excretion and toxicity (ADME/Tox) screening during the drug discovery process, in an effort to filter drugs with problematic ADME/Tox profiles. Prior to the early 1980s, for example, PK studies in the pharmaceutical industry were largely detailed and were primarily focused on clinical development efforts in support of product registration, with little attention being devoted to the PK characteristics of new chemical entities and to mechanistic insight into the fate of candidate drugs in biological systems. The fact that the primary reason for failure of drug candidates in clinical development during the 1960s and 1970s was judged to be due to inappropriate human PK led to the progressive integration of PK studies as a key component of the overall drug discovery process [17]. This shift was relatively easily accomplished using existing methodologies and, currently, development attrition due to PK properties is no longer the bottle-neck in drug discovery and development [18].

The optimization of a large number of variables is required early in the design and evaluation of the chemical entities to increase the probability of finding clinical candidates not only of complex parameters related to toxicity and bioavailability but also the most favorable physicochemical properties (i.e., molecular weight, solubility, polar surface area, lipophilicity, among others). The importance of these properties in drug design has always been recognized in an implicit manner in structure-activity relationship studies; nevertheless, it is established that analyzing the data of these properties makes more probable the success in drug discovery [19].

The physicochemical properties of some investigated xanthone derivatives suggest that they might exhibit high bioavailability, an important factor contributing to their efficacy and success as drug candidates. However, studies involving drug-like properties of xanthone derivatives are sparse. This review provides an opportunity to organize the emerging data concerning the methods used to investigate PK and physicochemical properties of bioactive xanthone derivatives. Firstly, the most relevant analytical methods and conditions are described for xanthones from different sources (natural, metabolic, or synthetic) and classes (prenylated, glycosylated, carboxylated). Following, methodologies and results involved in drug-like properties characterization are revised. At the end of this review, a case-study of the drug DMXAA (**2**) highlights the rational of PK studies in the drug discovery and development pipeline. The methodologies and data gathered in this review may pave the way for the design of potential drug candidates with a xanthonic scaffold.

2. Chromatographic methods applied to xanthones in preclinical and clinical studies

Chromatographic methods have been used to study drugs and their PK behavior, namely gas chromatography – mass spectrometry (GC-MS) and liquid chromatography (LC) with ultraviolet (UV), fluorescence, radioactivity and MS detection systems, being the late one probably the most applied nowadays, both in *in vitro* and *in vivo* studies [20,21]. In the case of LC-MS, the LC (usually HPLC – High Performance Liquid Chromatography - or UPLC – Ultra Performance Liquid Chromatography) allows the separation of the different components present in the sample and the MS enables the identification of the compounds previously separated in the chromatographic method [22].

The establishment of quantitative structure-property relationships for xanthone derivatives is a major focus of interest for PK studies. The use of reversed-phase HPLC coupled with a UV detector has been developed in order to describe the behavior of xanthone derivatives according to a model in which both the molecular structure parameters and mobile phase properties are taken into consideration. Appling this method, the chromatographic behavior of xanthone derivatives are dependent of the solvent properties (polarity/polarizability parameter, hydrogen-bond basicity) and solute properties (most positive local charge, the sum of positive charges on hydrogen atoms contributing in hydrogen interaction and lipophilicity index (Log P)) [23]. Another chromatographic technique that can be applied in the evaluation of lipophilicity of xanthone derivatives is micellar electrokinetic chromatography, since the separation selectivity of xanthones can be modulated by changing the micellar phase or aqueous phase between which the analytes are partitioned, being this behavior closely associated with the hydrophobicity of the compounds [24]. Techniques such as capillary zone electrophoresis (CZE), measuring the mobility of a given solute as a function of pH, is a reliable method to determine dissociation constants (pKa) for pharmacologically active xanthones [25].

2.1. Natural xanthones

Due to the wide distribution of xanthonic compounds in many natural products used in folk medicine, in the last decade the study of methods to purify, isolate, identify and analyze these derivatives has become an important goal in Medicinal Chemistry [5,6,24]. Several natural xanthones are currently undergoing preclinical studies.

One example of these compounds is mangiferin (**3**, **Fig. 2**) an hydroxylated and glycosylated xanthone that was originally isolated from *Mangifera indica* L. and has already been reported to have several pharmacological activities, such as antitumor, antiviral, antidiabetic, among others [1]. For all these reasons, many research groups have found great interest in studying the PK behavior of this compound, through chromatographic methodologies (**Table 1**).

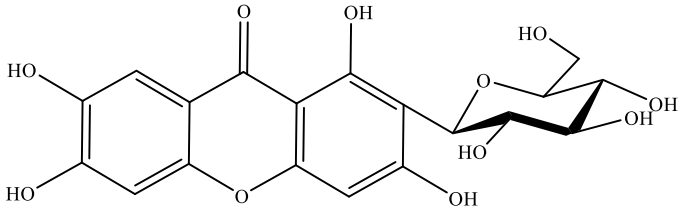

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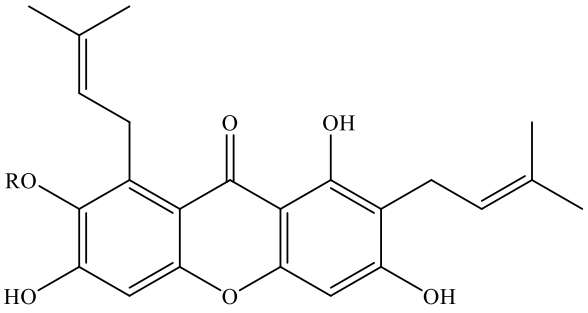
Fig. 2: Chemical structure of mangiferin (**3**).

Table 1: Chromatographic methods applied to PK studies of mangiferin (**3**).

PK study	Sample	Chromatographic method	Chromatographic conditions	Ref.
Quantification after oral administration of mangiferin-hydroxypropyl-β-cyclodextrin inclusion	Rat plasma	HPLC-DAD (258 nm)	MP – methanol:0.6% glacial acetic acid solution (27:73) SP – C_{18} FR – 1 mL/min	26
Blood-ocular barrier permeability after oral administration of Mangiferin-hydroxypropyl-β-cyclodextrin inclusion	Rat eye fluid	HPLC-DAD (258 nm)	MP – methanol:0.6% glacial acetic acid solution (27:73) SP – C_{18} FR – 1 mL/min	26
Simultaneous determination of **3**, neomangiferin and other active components after oral administration of Zi-Shen Pill extract	Rat plasma	LC-ESI-MS/MS	MP - acetonitrile:0.1% formic acid solution in gradient program SP – C_{18} FR – 0.25 mL/min	27
Chemical profile of **3** and other active components after oral administration of Shengxian Decoction	Rat plasma	UPLC-Q-TOF-MS	MP – 0.01% formic acid solution with 0.1 mM ammonium acetate: acetonitrile (85:15) SP – C_{18} FR – 0.25 mL/min	28
Glucuronidation of **3**	Rat liver	HPLC-DAD (320 nm) and LC-MS^2	MP – acetonitrile:0.1% formic acid solution in gradient program SP – C_{18} FR – 1 mL/min	29
Metabolism of **3** after oral administration	Rat urine, plasma, feces, liver, kidneys, heart, spleen, lung and intestinal tract contents	LC-ESI-IT-MS^n	MP - acetonitrile:0.5% formic acid solution in gradient program SP – C_{18} FR – 0.8 mL/min	30
Identification of **3** and other bioactive components of Zhimu-Huangqi herb-pair extract and their metabolites after oral administration	Rat plasma and urine	LC-ESI-MS^n	MP - acetonitrile:0.2% formic acid solution in gradient program SP – C_{18} FR – 1.0 mL/min	31

MP – mobile phase; SP – stationary phase; FR – flow rate; DAD – diode array detector; TOF – time of flight; Q – quadrupole; LC – liquid chromatography; ESI – electrospray ionization; IT – ion trap; MS – mass spectrometry.

Other important representatives of natural xanthones with biological interest are *α*-mangostin (**4**, **Fig. 3**) and γ-mangostin (**5**, **Fig. 3**), prenylated xanthones present in high concentrations in the pericarp of *Garcinia mangostana* L. These compounds have shown antioxidant, anti-inflammatory and antitumor activities, among others [1,14,32-34]. Due to the increasing interest and knowledge of the mechanisms behind the biological activities of mangosteens, several studies have been carried out over the last years to understand the PK proprieties of these compounds, using chromatographic methods (**Table 2**).



4 - R = CH_3
5 - R = H

Fig. 3: Chemical structures of *α*-mangostin (**4**) and γ-mangostin (**5**).

Table 2: Chromatographic methods applied to PK studies of *α*-mangostin (**4**) and γ-mangostin (**5**).

PK study	Sample/ Matrix	Chromatographic method	Chromatographic conditions	Ref.
Quantification of **4** and **5** after oral and intravenous administration of Mangosteen extract	Mouse plasma	HPLC-MS/MS	MP – acetonitrile containing 0.1% formic acid:0.1% formic acid solution (70:30) SP – C_{18} FR – 0.35 mL/min	35
Tissue distribution of xanthones and their phase II metabolites	Mouse liver, tumor, feces and serum	HPLC-DAD and HPLC-MS	MP – acetonitrile containing 2% acetic acid solution: *n*-butanol in a gradient program SP – C_{18} FR – 0.5 mL/min	32,36
In vitro study of the intestinal uptake, retention, transport and metabolism of **4**	Caco-2 human intestinal cells	HPLC-DAD (254 nm)	MP – acetonitrile: 2% acetic acid solution in a gradient program SP – C_{18} FR – 0.8 mL/min	37
Study of absoption, tissue distributon and tissue metabolism of **4** after oral and intravenous administration	Mouse plasma, liver stomach, small and large intestines, lung, heart, kidney, fat, mesentery, muscle and brain	HPLC-MS/MS	MP – acetonitrile containing 0.1% formic acid:0.1% formic acid solution (70:30) SP – C_{18} FR – 0.35 mL/min	38
PKs of **4** after oral and intravenous administration	Rat plasma	LC-MS/MS	MP – acetonitrile containing 0.05% formic acid:0.05% formic acid solution (80:20) SP – C_{18} FR – 0.5 mL/min	39

MP – mobile phase; SP – stationary phase; FR – flow rate; DAD – diode array detector; LC – liquid chromatography; MS – mass spectrometry.

Several other xanthones, isolated from natural sources have been investigated by chromatographic methods to obtain information about their PK proprieties (**Table 3**): the cytotoxic gambogic acid (**6**, **Fig.**

4), a polyprenylated xanthone isolated from the resin of several *Garcinia* species [40]; the vasorelaxing and antihypertensive 1-hydroxyl-2,3,5-trimethoxy-xanthone (**7**, **Fig. 4**) isolated from the a Tibetan medicinal herb *Halenia elliptica* D. Don [41], the xanthone glycoside sibiricaxanthone F (**8**, **Fig. 4**), isolated from *Polygala sibirica* L., which has shown *in vitro* effect on peroxisome proliferator-activated receptors (PPARs) and in accelerating the differentiation of 3T3-L1 preadipocytes cells [42]; the promising antitumor compound cudratricusxanthone B (**9**, **Fig. 4**), an isoprenylated xanthone isolated from *Cudrania tricuspidata* (Carr.) Bur. [43].

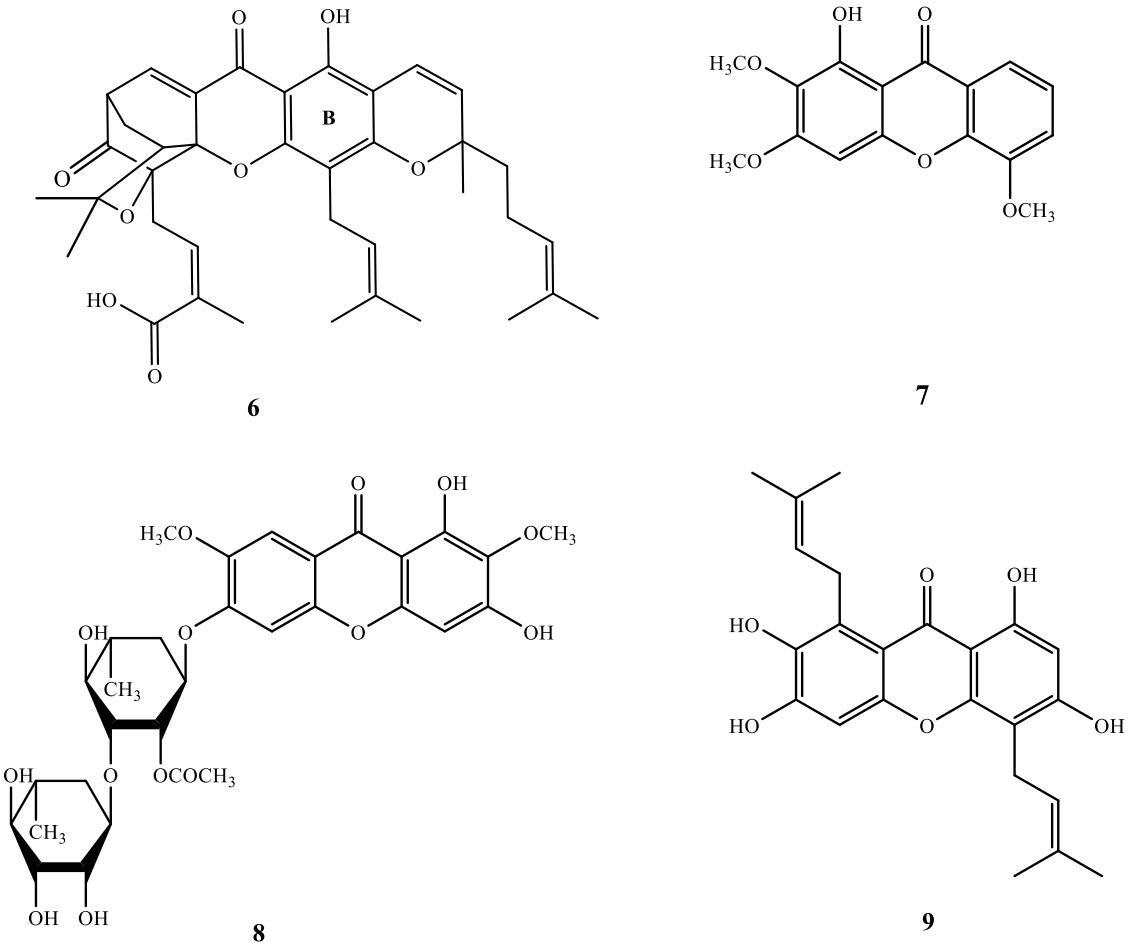


Fig. 4: Chemical structures of gambogic acid (**6**), 1-hydroxy-2,3,5-trimethoxy-xanthone (**7**), sibiricaxanthone F (**8**), and cudratricusxanthone B (**9**).

Table 3: Chromatographic methods applied to PK studies of other natural xanthones (**6-9**).

PK study	Sample	Chromatographic method	Chromatographic conditions	Ref.
Quantification of **6** after intravenous administration	Dog plasma	HPLC-UV detector (360 nm)	MP – methanol:0.05% phosphoric acid solution (94:6) SP – C_{18} FR – 1.0 mL/min	44
Identification of metabolites of **6** after intravenous	Rat bile	LC-TOF-IT-MS	MP – acetonitrile:0.05% acetic acid solution in gradient program	45

administration			SP – C_{18} FR – 0.2 mL/min	
Structure elucidation of metabolites of **6** after intravenous administration	Rat bile	HPLC-UV (280 nm), LC-ESI-IT-MS^n and LC-NMR	MP – methanol:water (85:15) SP – C_{18} FR – 1.0 mL/min	[46]
In vitro identification of cytochrome P450 isoforms responsible for the metabolism of **7**	Human liver microsomes	LC-IT-TOF-MS^n	MP – acetonitrile:0.5%acetic acid solution in gradient program SP – C_{18} FR – 0.8 mL/min	[47]
In vitro metabolite characterization of **7** and other bioactive xanthones	Rat liver microsomes	LC-IT-TOF-MS^n	MP – acetonitrile:0.5%acetic acid solution in gradient program SP – C_{18} FR – 0.8 mL/min	[41]
Quantification of **8** after oral and intravenous administration	Rat plasma	LC-ESI-MS/MS	MP – acetonitrile containing 0.01% formic acid:0.01% formic acid solution in gradient program SP – C_{18} FR – 0.4 mL/min	[48]
In vitro metabolite characterization of **8**	Human and rat liver microsomes	HPLC-EMS-IDA-EPI	MP – acetonitrile containing 0.1% formic acid:0.1% formic acid solution in gradient program SP – C_{18} FR – 0.3 mL/min	[42]
Quantification of **9** after intravenous administration	Rat plasma	HPLC-ESI-MS/MS	MP – 0.5% formic acid in methanol SP – C_{18} FR – 0.3 mL/min	[43]

MP – mobile phase; SP – stationary phase; FR – flow rate; TOF – time of flight; LC – liquid chromatography; NMR – nuclear magnetic resonance; ESI – electrospray ionization; IT – ion trap; EMS - enhanced mass spectrum; IDA - information dependent acquisition; EPI - enhanced product ion ; MS – mass spectrometry.

2.2. Synthetic xanthone derivatives

Due to the pharmacological interest in xanthone derivatives, several research groups work in the synthesis of xanthonic compounds in an attempt to find new drug candidates with better pharmacodinamic and PK properties. **Table 4** shows different classes of synthetic derivatives, namely thioxanthones, in preclinical studies, and the applications of chromatographic methods in their PKs proprieties studies. It also displays the PK study of the 9*H*-xanthen-9-one (**1**) - in an *in vivo* model [49].

The development of an HPLC methodology to determine the amount of a thioxanthone drug candidate (**10**, **Fig. 5**) and its metabolites in plasma was applied to determine effective and toxic doses, as well as to predict and quantify the metabolic profile of the drug candidate in *in vivo* tests [50].

The intestinal absorption of a xanthonolignoid (**11**, **Fig. 5**), a protein kinase C inhibitor, was studied in our group, using Caco-2 cell lines as a model to predict the absorption of the drug candidate, using a HPLC to quantify the cell permeability [51].

A pyranoxanthone derivative (**12**, **Fig. 5**) with potential antitumor activity intestinal absorption was also studied applying the same chromatographic technique. Another HPLC methodology was developed

to evaluate the improvement of absorption and therefore enhancement of biological activity at lower doses of compound **12** by inclusion into drug delivery systems, namely PLGA nanoparticles [51,52].

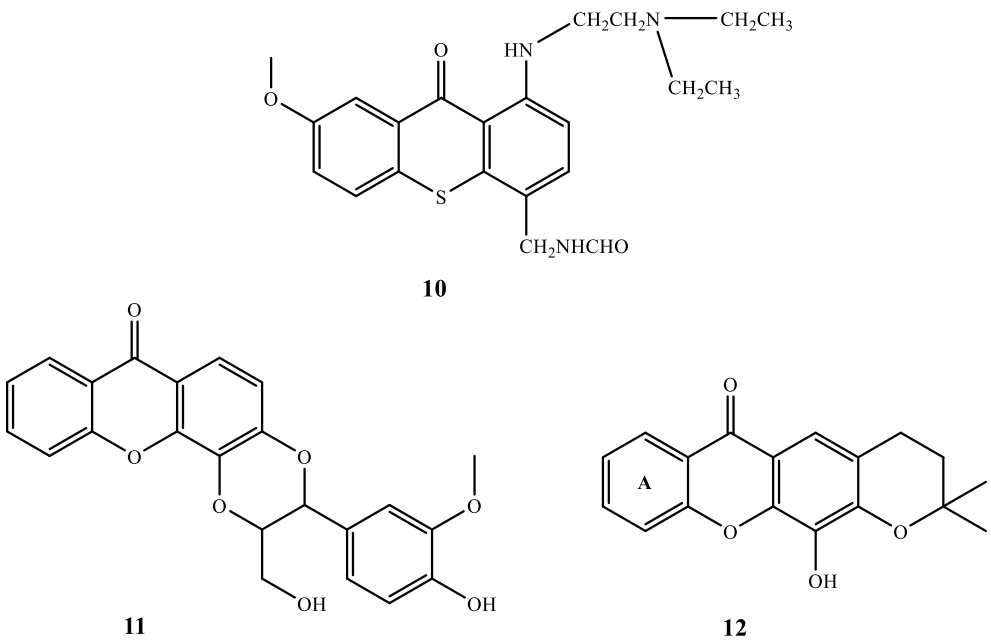


Fig. 5: Chemical structures of a thioxanthone derivative **10**, a xanthonolignoid **11** and a pyranoxanthone **12**.

Table 4: Chromatographic methods applied to PK studies of synthetic xanthones (**1**, **10-12)**.

PK study	Sample	Chromatographic method	Chromatographic conditions	Ref.
Determination of **1** after oral and intravenous administration	Rat plasma	HPLC-DAD (254 nm)	MP – acetonitrile containing 0.1% trifluoroacetic acid:0.1% trifluoroacetic acid solution in a gradient program SP – C_{18} FR – mL/min	49
Quantification of **10** and its metabolites	Mouse plasma	HPLC-DAD (266 nm)	MP - methanol:10 mM phosphate buffer pH 3.5 (45:55) SP - C_{18} FR - 0.8 mL/min (10'), 1.4mL/min (15')	50
Prediction of intestinal absorption of **11**	Caco-2 cell monolayers as an intestinal model	HPLC-DAD (237 nm)	MP - methanol:water (85:15) SP - C_{18} FR - 1 mL/min	51
Prediction of intestinal absorption of **12**		HPLC-DAD (254 nm)	MP - methanol:water (80:20) SP - C_{18} FR - 1 mL/min	
Study of the intracellular delivery of **12** entrapped in PLGA nanoparticles	MCF-7 culture cell lines exposed to the xanthone derivative included in nanocapsule formulation	HPLC-DAD (254 nm)	MP - methanol:water (85:15) SP - C_{18} FR - 1 mL/min	52,53

MP – mobile phase; SP – stationary phase; FR – flow rate; DAD – diode array detector.

The introduction of a drug candidate in human trials is a highly demanding and expensive process. Therefore, only the drug candidates with best results in preclinical studies achieve this level in the pipeline of drug development [22]. DMXAA (**2**, **Fig.1**) is a drug candidate that reached clinical trials and the chromatographic methods and conditions applied to PK studies are highlighted in **Table 5**.

Table 5: Chromatographic methods applied to PK studies of DMXAA (**2**).

PK study	Sample	Chromatographic method	Chromatographic conditions	Ref.
Determination of major metabolites of **2**	Human liver microsomes	HPLC-fluorescence detector (345 and 409 nm)	MP – acetonitrile:10 mM ammonium acetate buffer (24:76 or 19:81, according to the metabolite) SP - C_{18} FR – 1.5 or 2.5 mL/min	54,55
Plasma disposition, metabolism and excretion of **2** after intravenous administration	Mouse, rat and rabbit plasma and urine	HPLC-fluorescence detector (for quantification of DMXAA in plasma and urine)	MP – water:acetonitrile:acetic acid (60:40:2) SP - C_{18} FR – 1.8 mL/min	56-58
		HPLC-UV detector (254 nm) (for isolation of urinary metabolites)	MP – 14 mM diammonium hydrogen phosphate buffer:isopropyl alcohol (80:20) SP - C_{18} FR – 1 mL/min	
Gender differences in metabolism and PKs of **2**	Human, rat and mouse plasma and liver microsomes	LC-MS	MP – acetonitrile:10 mM ammonium acetate buffer (24:76) SP - C_{18} FR – 1.0 mL/min	59,60
		HPLC-fluorescence detector (345 and 409 nm)	MP – acetonitrile:10 mM ammonium acetate buffer (24:76) SP - C_{18} FR – 2.0 mL/min	
Influence of other anticancer drugs on the metabolism of **2**	Human liver microsomes	HPLC-fluorescence detector (345 and 409 nm)	MP – acetonitrile:10 mM ammonium acetate buffer (24:76) SP - C_{18} FR – 2.5 mL/min	61
Identification of the human liver cytochrome P450 isoenzyme responsible for the 6-methylhydroxylation of **2**	Human liver microsomes	LC-MS (Identification of the metabolite)	MP – acetonitrile:10 mM ammonium acetate buffer (24:76) SP - C_{18} FR – 1.0 mL/min	59
		HPLC- fluorescence detector (345 and 409 nm) (Quantification of the metabolite)	MP – acetonitrile:10 mM ammonium acetate buffer (24:76) SP - C_{18} FR – 2.5 mL/min	
Quantification of unbound **2** in plasma	Human plasma	HPLC- fluorescence detector (345 and 409 nm)	MP – acetonitrile:10 mM ammonium acetate buffer (24:76) SP - C_{18}	62,63

			FR – 2.5 mL/min	
Identification and reactivity of the major metabolite of **2**	Rat, mouse, rabbit and human plasma and human urine	HPLC-fluorescence detector (345 and 409 nm)	MP – acetonitrile:10 mM ammonium acetate buffer (24:76 and 19:81, depending on the metabolite) SP - C_{18} FR – 2.5 mL/min and 2.0 mL/min (depending on the metabolite)	54,56,59,64
		HPLC-fluorescence detector (345 and 409)	MP – water:acetonitrile:acetic acid (60:40:2) SP - C_{18} FR – 1.8 mL/min	
Interspecies differences in the metabolism of **2**	Rat, mouse, rabbit and human liver microsomes	LC-MS (Identification of the metabolite)	MP – acetonitrile:10 mM ammonium acetate buffer (24:76) SP - C_{18} FR – 1.0 mL/min	54,59,65
		HPLC- fluorescence detector (345 and 409 nm) (Quantification of the metabolite)	MP – acetonitrile:10 mM ammonium acetate buffer (24:76) SP - C_{18} FR – 2.5 mL/min	
Interspecies differences in the plasma protein binding and blood cells distribution of **2**	Rat, mouse, rabbit and human plasma	HPLC- fluorescence detector (345 and 409 nm)	MP – acetonitrile:10 mM ammonium acetate buffer (24:76) SP - C_{18} FR – 2.5 mL/min	56,66
Strain differences in the metabolism of **2** and its correlation with the maximum tolerated dose	Five different mouse strains liver microsomes	HPLC-MS-APCI/ESP (Identification of the metabolite)	MP – acetonitrile:10 mM ammonium acetate buffer (24:76) SP - C_{18} FR – 1.0 mL/min (for HPLC-MS-APCI) or 0.5 mL/min (for HPLC-MS-ESP)	54,59,64,67
		HPLC- fluorescence detector (345 and 409 nm) (Quantification of the metabolite)	MP – acetonitrile:10 mM ammonium acetate buffer (24:76) SP - C_{18} FR – 2.5 mL/min	
Impact of preclinical factors in the clearance of **2**	Human plasma and liver microsomes	LC-MS (Identification of the metabolite)	MP – acetonitrile:10 mM ammonium acetate buffer (24:76) SP - C_{18} FR – 1.0 mL/min	68
		HPLC- fluorescence detector (345 and 409 nm) (Quantification of the metabolite)	MP – acetonitrile:10 mM ammonium acetate buffer (24:76) SP - C_{18} FR – 2.5 mL/min	
Quantification of unbound **2** in plasma	Human plasma	LC-MS/MS	MP – acetonitrile containing 0.1% formic acid:0.1% formic acid solution in gradient program SP - C_{18} FR – 0.5 mL/min	69

Determination of plasma and urine concentration of **2** and its metabolites after intravenous administration	Human plasma, red cells, urine and tumor samples	HPLC- fluorescence detector (345 and 409 nm)	MP – acetonitrile:10 mM ammonium acetate buffer (25:75) SP - C_{18} FR – 2.0 mL/min	[70,71]
Determination of [^{14}C] ASA404 and its metabolites after intravenous administration	Human plasma and feces	HPLC - off-line radioactivity detector	MP – gradient elution consisting of solvent A (5 mM ammonium formate with 0.1% formic acid, pH~3) and B (acetonitrile with 0.1% formic acid) SP - Waters Xbridge column FR – 1.0 mL/min	[72]
Determination of **2** and its acyl glucuronide Caco-2 cell monolayers for transport studies	Caco-2 cell monolayers	HPLC- fluorescence detector (345 and 409 nm)	MP – acetonitrile:10 mM ammonium acetate buffer, pH 5.0 (24:76) SP - C_{18} FR – 1.2 mL/min	[73]

MP – mobile phase; SP – stationary phase; FR – flow rate; LC- liquid chromatography; MS – mass spectrometry; APCI/ESP - Atmospheric pressure chemical ionization/electrostatic precipitator.

According to all these data, it is possible to verify that the most commonly applied chromatographic method is LC, especially HPLC, using a C_{18} stationary phase in reversed phase, using mixtures of water and an organic solvent, most commonly acetonitrile or methanol. The detection method applied depends on the nature of the analyte and the aim of the PK work, being fluorescence and diode array detectors widely used for quantitative analysis and identification of compounds, especially when standards are available. In metabolism studies and identification of xanthone derivatives and its metabolites, mass spectrometers detectors appear to be the most applied, due to the sensitivity, reliability, specificity and information that can be assessed.

3. Physicochemical properties and preformulation studies of xanthone derivatives

The discovery of new drugs needs a thorough investigation for its safety and efficacy before their release into the market. The old paradigm of drug discovery process has changed due to technology innovation. Rational drug discovery requires an early appraisal of all factors impacting on the likely success of a drug candidate in the subsequent preclinical, clinical and commercial phases of drug development. Important preformulation considerations include solubility, stability, pka, all properties that affect drug permeability and distribution. The next sections highlight the main structure-properties relationships established from the analysis of xanthone derivatives.

3.1. Solubility

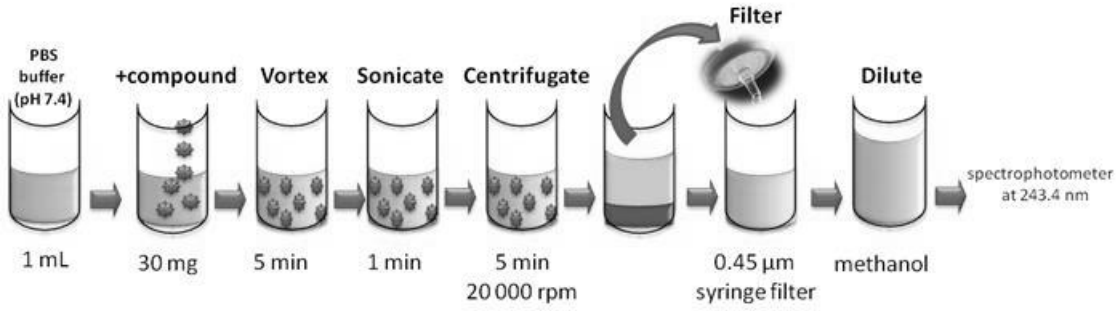
Solubility is emerging as one of the major issues in drug discovery and development of new chemical entities [74]. Compounds with low solubility not only cause problems for *in vitro* and *in vivo* assays, but also have a higher risk of attrition in the drug discovery pipeline [75].

Lipinski has classified the solubility ranges (high is > 60 g/mL, moderate is 10-60 g/mL, low is < 10 g/mL) to provide a general guideline for achieving acceptable human absorption for compounds with average potency and permeability. This classification is different from the Biopharmaceutics

Classification Systems whereas, for example, for a 1 mg/kg intravenous (I.V.) dose in rat with an ideal dosing volume of 1-5 mL/kg, the required concentration is 0.2 – 1 mg/mL [76].

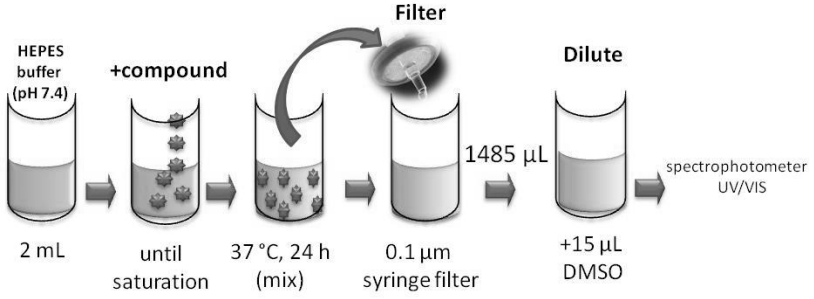
Different analytical tools (i.e., light scattering/turbidity, UV plate reader, LC-UV and LC–MS/LC-CLND - chemiluminescence nitrogen) can be used for detection of the maximum amount of a compound that can remain in solution at a certain volume of solvent, temperature, and pressure, under equilibrium conditions. The pH of the buffer in solubility methods is typically 6.5 (intestine) or 7.4 (blood).

α-Mangostin (**4**) showed poor aqueous solubility and low oral bioavailability, hindering its therapeutic application. The aqueous solubility of free *α*-mangostin (**4**) was found to be 0.2 ± 0.2 µg/mL [77]. Briefly, an excess amount from the samples was added to phosphate-buffered saline (PBS) at pH 7.4 (**Scheme 1**). The mixtures were mixed and then centrifuged. The supernatant was collected, filtered and diluted in methanol. The samples were then analyzed using a spectrophotometer at 243.4 nmto determine *α*-mangostin (**4**) concentration [77].



Scheme 1: Schematic representation of the solubility assay.

Other study investigated the thermodynamic solubility in water at pH 7.4 HEPES (*N*-2-hydroxyethylpiperazine-*N*-2-ethane sulfonic acid) buffer of 12-hydroxy-2,2-dimethyl-3,4-dihydropyran[3,2b]xanthene-6(2*H*)-one (**12**) and five of its analogues bearing substituents on ring A [75]. All compounds showed low solubility (0.1-0.6 µM) which was explained by the high planarity and rigidity of these compounds. Each compound was added to 2 mL of HEPES buffer until a saturated solution was obtained (**Scheme 2**). The suspension of each compound was agitated at 37 °C for 24 h. Each sample was filtered through a 0.1 µm filter and 1485 µL of each filtrate was added to another eppendorf containing 15 µL of DMSO. The UV/Vis spectrum was traced in a double-beam spectrophotometer and the concentration was determined according to the standard calibration curve for each compound.



Scheme 2: Schematic representation of the thermodynamic solubility assay.

Oxo-9*H*-xanthene-3,6-diyl bis(3-chlorobenzoate), 9-oxo-9*H*-xanthene-3,6-diyl bis(4-*tert*-butylbenzoate) and 9-oxo-9*H*-xanthene-3,6-diyl bis(4-methoxybenzoate) also showed poor water solubility. This limitation was successfully overcome with the formulation of these derivatives into cyclodextrins [78].

The intrinsic aqueous solubility of 40 structurally diverse *Garcinia* natural-product-like xanthones based on gambogic acid (**6**) was determined. All the synthetic compounds displayed aqueous solubility that was improved over that of gambogic acid. Among all the tested compounds, the most soluble exhibited an intrinsic solubility of 10.37 mM, almost 1000 times more soluble than gambogic acid. Some structure-properties were drawn: hydrophilic amine substitution, a hydroxyl group at position 1, and removal of the *gem*-dimethyl group provides improved water solubility, while bulky hydrophobic groups such as a pyran ring or prenyl, and hydroxyl in B ring decrease water solubility [79].

3.2. Lipophilicity

Lipophilicity is one of the first physicochemical properties to be evaluated in the early phases of a drug discovery program and has been correlated with several other physicochemical and PK properties, for example, solubility, permeability, plasma protein binding, metabolism, blood-brain barrier (BBB) penetration, volume of distribution and clearance. A compound with moderate lipophilicity has a good balance between solubility and permeability and is optimal for oral absorption, cell membrane permeation in cell-based assays, and is generally good for BBB penetration (optimal distribution coefficient logarithm -log D- ~2). Consequently, it may extensively influence the success or the failure of a drug discovery program. In fact, compounds with high lipophilicity have shown an increased risk of attrition during the clinical trials.

Lipophilicity is commonly evaluated by the logarithm of the partition coefficient (log P) of an un-ionized solute in a biphasic system using the so-called shake-flask method. Different solvent systems have been used for estimating this property, but the octanol/phosphate buffer pH 7.4 system remains the most common, and mimics, respectively, the biomembranes and the blood *in vivo*. The determination of the log P of a solute in a biphasic octanol–water system by the shake-flask method is described in the OECD (Organization for Economic Co-operation and Development) guidelines for testing chemicals [80]. The solute is simply partitioned between the two liquid phases of the proposed solvent system in a test vessel. Both solvents should be mutually saturated at room temperature (20 to 25 ºC) before the log P determination. After equilibrium and separation, usually achieved by centrifugation, the relative solute concentration in each layer is determined using a variety of analytical techniques. These include spectroscopic methods, HPLC, GC, thin-layer chromatography (TLC), among others.

The octanol–water distribution of several hydroxylated and/or methoxylated xanthones was systematically investigated in a biphasic octanol–water system and their log P values lie in the range 2.90–3.80 [81]. Optical density of the aqueous phase and of the organic phase was measured on a spectrophotometer. Stock solutions of compounds (concentration 10^{-2} M) were prepared in octanol and shaken with a known volume of extractant (doubly distilled water saturated with octanol). The aqueous phase was acidified with HCl (1 N) to pH 2-3 in order to avoid dissociation for the study of the

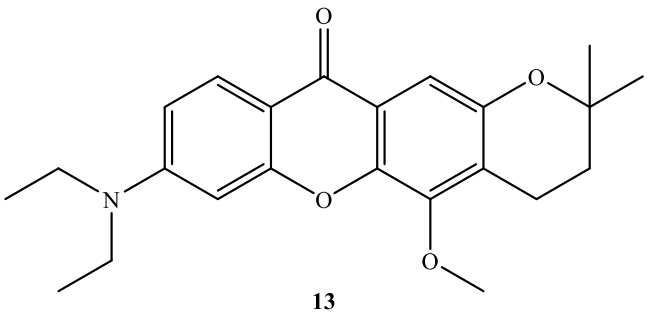
distribution of compounds containing free phenolic groups. Experiments were conducted with varying phase (octanol–water) ratios (1:20, 1:30, 1:40, 1:50, 1:60) to cover a wide range of lipophilicity values, at 20–22°C [81].

The octanol–water system has some limitations since it fails to create the anisotropic media that is found on biomembranes and encode some important interactions that take place between the solute and the membranes. Therefore, other models have been developed such as liposomes and micelles which have proved to be advantageous when compared to octanol–water. The lipophilicity of several prenylated and pyranoxanthones was determined as the partition coefficient (P) of the solute between buffer (pH 7.4) and micelles or liposomes, and calculated without a phase separation [75,82]. The tested compounds showed a moderate to high lipophilicity. A good correlation between the two models, micelles and liposomes was observed, which implies that there is no clear tendency for the tested compounds to increase the affinity for either of the membrane models with an increase in hydrophobicity. The results obtained led to the observation that micelles can be used as a surrogate for liposomes for the studied compounds with the advantage of not having the light scattering limitation (wavelengths below 300 nm) as well as a higher applicability and easier preparation [75,82].

All the pyranoxanthones showed a Log Kp in liposomes and micelles superior to 3 but below the critical 5 (upper limit referred in the Lipinski "rule of five"). The presence of a hydroxyl *ortho* to the carbonyl leads to a dramatic increase in the lipophilicity. Considering the relationship between the hydroxyl or methoxyl group with the oxygen of the fused pyran ring, the compounds with an *ortho* relationship seem in most of the cases to have a lower partition coefficient than in *meta* position. Comparing the linear with the angular arrangement of the molecule of pyranoxanthones, it can be observed that in general, the latter have a less partition coefficient than the former. Regarding the presence or absence of the double bond in the fused ring, there is not a clear tendency among the different set of compounds [82].

Considering the effect of ring A (see **Fig. 5**) substituents on the Kp of compound **12**, it was observed that chlorine and methyl were associated with an increase in Kp. In the case of the methoxyl, the introduction of this group in position 8 did not interfere with the Kp while the introduction in position 10 was associated with an increase of the Kp. For the hydroxyl group an unexpected result was observed and no decrease in Kp was determined. In fact, for assays with micelles, an increase in Kp was observed. Considering the analogues of compound **12** with a methoxyl in position 12, it can be observed that this substituent was associated with an increase in the Kp. Comparing with the analogues with a different ring D orientation, it can be observed that most of the compounds had a Kp near or higher than 4 with the chlorine and the diethylamine derivatives representing the compounds that showed a higher Kp [75].

Among the xanthones tested, pyranoxanthone **13** (**Fig. 6**) was the most lipophilic [75,82].



13

Fig. 6: Pyranoxanthone (**13**) with the higher Kp.

HPLC method is also described in the OECD guidelines for testing chemicals [83]. With the development of reversed phase HPLC in many laboratories, readily accessible retention factor of a new compound, compared to the standard but time consuming shake-flask method, made the method extremely popular and widely used [84]. Reversed phase HPLC is performed on analytical columns packed with a solid phase containing long hydrocarbon chains chemically bound onto silica. The chemicals are retained in the column in proportion to their hydrocarbon-water partition coefficient, with hydrophilic chemicals eluted first and lipophilic chemicals last [83].

Using HPLC methods, the log P values of a series of xanthone derivatives - obtained by cationic modification of the free C3 and C6 hydroxyl groups of *α*-mangostin with amine groups [85] - ranged between 5.42 and 15.01. In this study the log P and molecular hydrophobicity parameters were found less predictive than pKa for improving the antimicrobial properties of xanthone analogues.

Most solutes are weak bases or acids which become partially ionized when dissolved on water. This has raised the need to develop the distribution coefficient (D) which is used to determine the ratio of the sum of the concentrations of all forms of the solute (pH dependent mixture of ionized plus un-ionized forms) in each of the two phases. The logarithm to the base 10 of the distribution coefficient (log D) is normally used to express results [86].

The log D at pH 7.4 of a set of 40 compounds obtained by propargylation of hydroxyxanthone substrates were determined according to the method of Avdeef and Tsinman on a Gemini Profiler instrument (pION) by the "gold standard" Avdeef−Bucher potentiometric titration method. Log D values were between 0.44-6.0 [79].

3.3. Acid dissociation constant (pKa)

It is well known that ionized compounds are more soluble in water than the neutral forms, but less permeable. Ionization is determined by the pKa and aqueous pH; thus, pKa has a major effect on PK properties. Buffer solutions with different pH values have to be prepared by mixing appropriate volumes of the stock solutions and then diluting to ionic strength I = 0.03, which were controlled by calculation and by acid–base equilibrium theory. The pH should be measured at 25 °C by a pH meter calibrated in advance with standard buffers at the pH range.

Mangiferin (**3**) pKa values were determined in aqueous solution by UV–Vis spectroscopic studies coupled with nuclear magnetic resonance (NMR) detector. The NMR study complemented the pKa

values assignment and evidenced a hydrogen interaction at C-1 with the carbonyl group. Results showed that the proton at hydroxyl in position 1 is the less acidic, assigning the last pKa to this proton. This result contrasted with the predicted by the ACD/pKa DB version 5 (Advanced Chemistry Development, Inc., Toronto, Ont., Canada, www.acdlabs.com, 2003) that estimated this proton as the most acidic one [87].

Capillary zone electrophoresis (CZE) method, which relies on measuring the mobility of the solute as a function of pH, has been proved to be an effective and convenient technique for determining the pKa values being considered as an universal technique for determining pKa values in a wide pH range. The accuracy of pKa values obtained from CZE has been confirmed to be in agreement with those from potentiometric methods and, in general, better than those from single chromatographic or spectrophotometric methodologies. [25]. Compared to spectroscopy, potentiometry, conductivity or other techniques, CZE has advantages concerning sensitivity (only small amounts of sample are required), analysis time (relatively short), the result is independent of the solute purity (high purity is not necessary), it is applicable to samples that are weakly soluble in water (can be performed in aqueous or nonaqueous solvents), and the procedure does not require solute measurement or titrant concentrations, but only the migration times of solutes [25]. Another important advantage of CZE method is that the pKa values of solutes based on both their migration times and UV absorbance measured from online spectra can determine, when online diode array detection (DAD) is used [25].The pKa values of 10 hydroxylated and/or methoxylated xanthones, isolated from herbal medicine *Securidaca inappendiculata*, have been determined by CZE based on linear and nonlinear regression models and the results were confirmed by UV absorbance from online DAD [25]. The results showed that the precision of the two methods, expressed in terms of the acceptable repeatability and reproducibility of the migration time, mobility and pKa values, is acceptable [25].

In another study, prediction of pKa values was highlighted as a new way to improve “hits” for the development of membrane-active antibiotics that target drug-resistant pathogens [85]. Results showed that the antimicrobial activities of cationic xanthone derivatives can be generally predicted based on the pKa values of the corresponding amines. Pyrazole and triazol moieties which led to low pKa values reduced the antimicrobial activity of the derivatives when compared with the parent compound [85].

3.4. Permeability

With the development of drug discovery, methods that can screen *in vitro* permeability of compounds rapidly received significant attention. The Caco-2 cells represent a well-known and interesting cell culture-based model for enterocytes which has been used in several *in vitro* bioavailability assays to evaluate the intestinal absorption rate of a broad range of drugs. The first report on xanthones bioavailability and metabolism was performed using the coupled *in vitro* digestion/Caco-2 human intestinal cell model [88]. Optimal bioavailability of α- (**4**) and γ-mangostin (**5**) were dependent on incorporation into bile salt mixed micelles. In addition, α-mangostin (**4**) was transported across the apical surface of enterocyte-like Caco-2 cells and partially converted to phase II metabolites [37]. Both unconjugated α-mangostin (**4**) and its phase II metabolites were effluxed across the basolateral membrane suggesting that they were absorbed. Transepithelial transport was enhanced by addition of products of lipid digestion in the apical compartment, suggesting that absorption was dependent on the assembly and

secretion of chylomicrons. *α*-Mangostin metabolites also were retro-transported across the apical membrane into the simulated gut luminal compartment [37]. An HPLC-UV analysis from the *in vitro* permeation assay with Caco-2 cells monolayer was performed to predict the intestinal permeability of different xanthonic derivatives (oxygenated, prenylated, xanthonolignoids) [89] which revealed high apparent permeability coefficients (Papp) similar to other well absorbed drugs [51].

Parallel artificial membrane permeability assay (PAMPA) has been applied early in drug development. Most drugs are absorbed through passive diffusion across the lipid bilayer membrane of the epithelial cells into systemic circulation. The process is driven by concentration gradient. The PAMPA assay is designed to measure passive diffusion through an artificial lipid membrane. Permeability coefficients of a set of 40 propargylxanthone derivatives were determined using a standard PAMPA [79]. The results suggested that the trends of permeability of propargylxanthone derivatives are similar to their behavior in solubilities (see section 3.1): compounds with smaller frameworks and hydrophilic groups are also likely to have better permeability.

3.5. Modulation of ABC Transporters

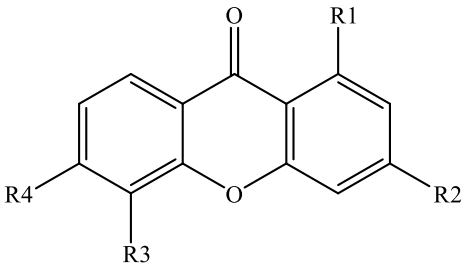
ATP-binding cassette (ABC) transporters are present in all cells of all organisms and use the energy of ATP binding/hydrolysis to transport substrates across cell membranes. Several membrane proteins belonging to the ABC family of proteins act by preventing the absorption of orally ingested or airborne xenobiotics or drugs. Among these proteins, P-glycoprotein (P-gp) and multidrug-resistance protein 1 (MRP1) are the most extensively studied [90,91]. These ABC transporters are attractive therapeutic targets since their inhibition could overcome multidrug resistance to anticancer drugs while its induction/activation could limit the toxicity of substrates such as pesticides. Thus, investigation on ABC transporters modulation by xanthone derivatives is not limited to permeability studies and aims mainly these pharmacological perspectives.

3.5.1. MRP1 modulation

MRP1 promotes drug efflux in which is considered phase III metabolism in two possible ways: conjugation with glutathione (GSH) or by co-transport with free GSH [92]. In the search of compounds with cytotoxicity towards cancer cells overexpressing MRP1, Genoux-Bastide *et al.* [93] studied a library of xanthones and compared the results with a well-known activator of massive GSH efflux by MRP1, the calcium channel blocker verapamil [94,95].

The total cellular GSH determination was measured using the enzymatic method described by Tiezte [96], as modified by Anderson [97] to the microtiter plate. From the library of xanthones evaluated, derivatives with more than 40% of GSH efflux scores were investigated to determine their cytotoxicity against a sensitive tumor cell line (NCI-H69) and a MRP1-overexpressing human resistant cell line (H69AR/MRP1 derived from NCI-H69). In order to evaluate if the most active compounds for selective killing of MRP1-overexpressing resistant cells activity was correlated with MRP1 expression, cytotoxicity of the two best compounds was tested on three HEK-293 cell lines, (one with wild-type Pgp, one with ABCG2 and one with pcDNA3.3) and on two MDCK2 cell lines (one with wild-type MRP2 and one without it). Since the achieved IC_{50} value was greater than 100 µM to the five cell lines, it allowed to conclude that the

analyzed compounds were selective killers of resistant cells overexpressing MRP1 [93]. Structures of the most promising compounds are shown on **Fig. 7**.



Compound	R1	R2	R3	R4
14	OH	CH_3	OCH_3	H
15	OH	OH	OCH_3	H
16	OH	OH	H	H
17	OH	OH	H	OCH_3
18	OH	OH	H	CH_3
19	OH	OH	H	OH
20	OH	OCH_3	H	OCH_3
21	OCH_3	O-Bz	H	OCH_3
22	OH	OH	H	NH-$COCF_3$
23	OH	OH	H	NH_2

Fig. 7: Chemical structures of xanthones with GSH efflux of more than 40%.

The results allowed inferring that cell death was not dependent just by the GSH efflux, since some xanthones induced efflux of GSH by more than 50% and show no significant cytotoxicity towards the tested cell line. Therefore, the selective cytotoxicity presented by compound **17**, **Fig. 7** is more likely due to a MRP1 activation followed by apoptosis through an acceleration of GSH efflux from the cell [93].

3.5.2. P-gp modulation

P-gp is responsible for the transmembrane transport of neutral or positively-charged hydrophobic substrates, namely anticancer agents. Over the past decades, this target has drawn a lot of interest in several research areas, such as Medicinal Chemistry and Toxicology. First, because its inhibition can lead to increase cytotoxicity and therefore increase therapeutic efficacy of anticancer agents and, on the other hand, due to its broad substrate specificity and ubiquitous presence in excretory and barrier tissues, its activation can play a crucial role in limiting the absorption and distribution of toxic xenobiotics [98-100].

In our group, to improve the efficacy in sensitizing resistant P-gp overexpressing cell lines, (thio)xanthones P-gp modulators were obtained based on structure-based design approaches [101]. The methods applied to validate a library of dual inhibitors of P-gp and tumor cell lines are summarized in **Table 6** [101].

Table 6: Methods applied in biological studies of a library of thioxanthones.

Aim	Methods	Description
Cell growth study	Sulphorhodamine-B assay	Serial dilutions of tested compounds were applied on K562 and K562Dox cell lines to determine de GI_{50} values.
	Trypan blue exclusion assay	Investigation of cellular viability in MRC-5 cell line, using the compounds in their GI_{50} concentration achieved to the K562 cell line.
P-gp modulation study	Rhodamine-123 accumulation assay	K562 and K562Dox cell lines were incubated with the thioxanthones and rhodamine-123, followed by flow cytometry analysis.

	ATPase assay	ATPase activity of P-gp was determined using the luminescent ATP detection kit (P-gp-Glo Assay Kit, Promega, Germany). The remaining ATP was detected as a luciferase-generated luminescent signal.
	Sulphorhodamine-B assay	Evaluation of the ability of thioxanthones to decrease doxorubicin GI_{50} in K562 and K562Dox cell lines. Serial dilutions of tested compounds and a solution of doxorubicin were applied on K562 and K562Dox cell lines to determine de GI_{50} values.

K562 and K562Dox cell lines – human chronic myelogenous leukemia cell line and doxorubicin-induced P-gp overexpression K562 cell line; GI50 – drug concentration that exerts a growth inhibition rate to 50% of the maximum rate; MRC-5 cell line – human fetal lung fibroblast-like cell line; ATPase – adenosine triphosphatase; P-gp – permeability glycoprotein.

In this study, both inhibitors and activators of P-gp were found and in **Fig. 8** are highlighted the hit compounds concerning P-gp noncompetitive and competitive inhibitors [101].

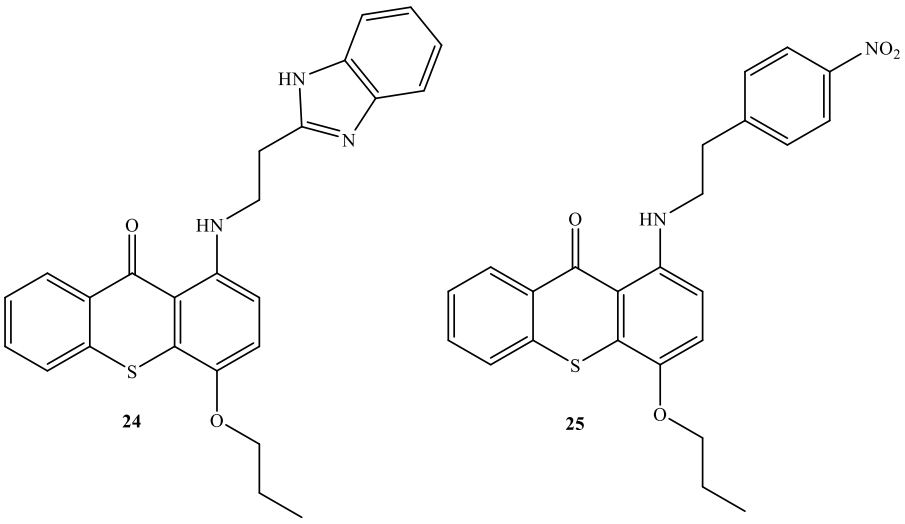


Fig. 8: Thioxanthone noncompetitive **24** and competitive **25** inhibitors of P-gp.

Further studies to evaluate the selectivity profile of thioxanthones as inhibitors of MDR were performed, by studying their interaction with other ABC transporters, namely MRP1, MRP2, MRP3 and BCRP. Another important assessment was the evaluation of the interaction of thioxanthones with P450 3A4 (CYP3A4), as well as the prediction of their binding conformations and metabolism sites [102]. The results allowed to conclude that compound **25** (**Fig. 8**) is a promising multi-target inhibitor of P-gp, BCRP and MRP1. On the other hand, 1-[2-(diethylamino)ethyl]amino-4-propoxy-9*H*-thioxanthen-9-one (**26**, **Fig. 9**) is a multi-target inhibitor of P-gp, BCRP, MRP1 and MRP3.

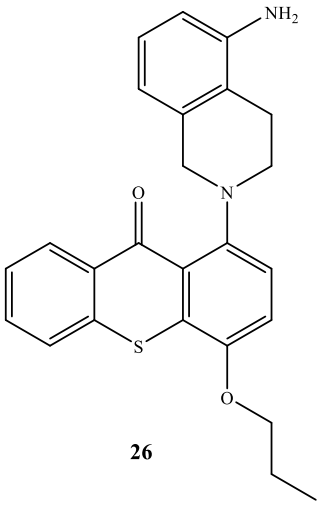


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Fig. 9: Multi-target inhibitor of P-gp, BCRP, MRP1 and MRP3 thioxanthone **26**.

Nowadays, strategies to increase P-gp expression and/or activity are considered as a potential detoxification pathway to prevent the harmfulness mediated by toxic P-gp substrates, by reducing their intracellular accumulation [100,103,104]. Thioxanthones that revealed an effect compatible with P-gp activation were further validated as P-gp activators for their effect in protecting against Caco-2 cells from PQ-induced toxicity [105]. A library of dihydroxylated xanthones (**27**-**30**, **Fig. 10**) was also evaluated on their effect towards P-gp activation and induction. **Table 7** shows the methods applied in these investigations [100].

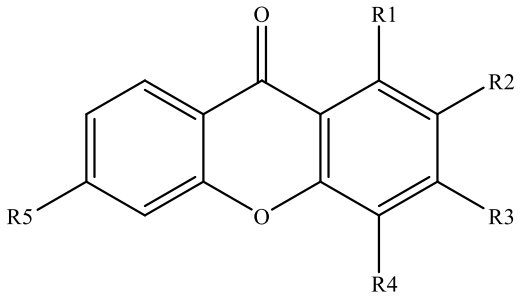
Table 7: Methods applied for the study of the effect of (thio)xanthones on P-gp.

Aim	**Method**
Cytotoxicity evaluation	MTT reduction assay
	Neutral red uptake assay
P-gp expression evaluation	Flow cytometry
P-gp transport activity evaluation	Rhodamine-123 efflux assay in Caco-2 cells pre-exposed to xanthones for 24h (Flow cytometry)
	Rhodamine-123 efflux assay in Caco-2 cells in the presence of xanthones (Flow cytometry)
P-gp ATPase activity evaluation	ATPase screening assay
Paraquat cytotoxicity evaluation	Neutral red uptake assay

P-gp – permeability glycoprotein; ATPase – adenosine triphosphatase; Caco-2 cells – human epithelial colorectal adenocarcinoma cell line.

Fig. 10 shows structures of the tested dihydroxylated xanthones. The most active compound was **30**, followed by **27**, **29**, **16**, and **28** was the least active. The overall results allowed to conclude that a

hydroxyl group on position 3 of the xanthonic scaffold seems to increase the P-gp activation, while when in position 1, the activation was decreased [100].



Compound	R1	R2	R3	R4	R5
27	H	H	OH	OH	H
28	OH	OH	H	H	H
16	OH	H	OH	H	H
29	H	OH	OH	H	H
30	H	H	OH	H	OH

Fig. 10: Dihydroxylated xanthones tested for their P-gp activation/induction activity**.**

3.6. Plasma protein binding

For studying the interaction between small molecules as drugs and proteins, several techniques have been applied, such as electrochemistry, chromatography, NMR and spectral analysis. Spectral analysis is applied widely because of the easy operation, low cost, and abundant theory foundation [106].

A simple HPLC method with fluorimetric detection for the determination of the free DMXAA (**2**, **Fig. 1**) concentration in human plasma was reported [62]. Sample preparation involves the ultrafiltration of plasma and extraction with an acetonitrile:methanol mixture. The HPLC method has been used for the analysis of preclinical studies [62]. DMXAA (**2**) exhibited high plasma protein binding which was concentration-dependent and with significant variation between animal species [62].

Binding experiments with BSA were performed for mangiferin (**3**) [106]. BSA exhibits an intrinsic fluorescence when excited at 280 nm, which gives valuable information regarding both the macromolecule structure and dynamic complex state [107]. In the fluorescence assays is possible to observe a continuous decrease in tryptophan quenching of BSA with the binding. Mangiferin (**3**, **Fig. 2**) strongly absorbs wavelengths in the UV range due to its glycosidic residue at position 2 [107]. Complex formation is evident by both the hypochromic and bathochromic effects in the presence of BSA, where the bathochromic effects can be attributed to structural changes that are induced by the bound ligand [107]. Synchronous fluorescence spectra indicated that there are conformational changes in the polypeptide backbone of BSA upon a ligand binding [106]. In a different study performed by cyclic voltammetry, results indicated that there is an irreversible charge transfer between mangiferin (**3**) and BSA that is modulated by diffusion on the electrode surface, where two electrons are transferred [107].

3.7. Stability

Stability of drug conjugates - as metabolic and chemical stability- is a matter of great concern to be investigated during the drug development process, once it affects the safety and efficacy of the final drug product [108], being compounds containing highly reactive functional groups less stable [109]. The methods generally used to assess the stability result of guidelines published by ICH (International Conference on Harmonization). This guidance state the requirement of stability testing data to understand how the quality of a drug substance and drug product changes with time under the influence of environmental

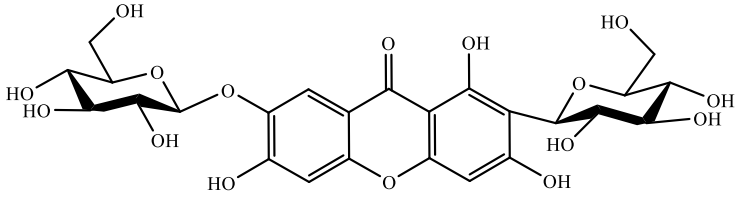
factors and specific experimental conditions [108]. Investigations on stability include titration, spectrophotometric and chromatography methods. They are relatively inexpensive and easy techniques, being HPLC the most commonly used. To study stability it is necessary to analyze the structure and its physicochemical properties to understand which compounds can derive from the degradation of the drug candidate and identify them by a simple and reproducible chromatographic method [22,110].

Studies performed with xanthone derivatives in biological and in non-biological samples considered different stress factors as highlighted in the following sections.

3.7.1. Stability in biological samples

3.7.1.1. Extreme temperatures stability

The study of stability in extreme temperatures involve cycles of freezing at -20ºC for hours to days and thawing at room temperature for hours, repeating three times. These kind of studies were developed for some xanthone derivatives, namely mangiferin (**3**) [27,111,112], neomangiferin (**31**, **Fig. 11**) [27], gambogic acid (**6**) [44], and its major metabolite 10-hydroxygambogic acid (10-OHGA) [113], polygalaxanthone (**32**, **Fig. 11**) [114], sibiricaxanthone F (**8**) [48], α- (**4**) and γ-mangostins (**5**) [35], and DMXAA (**2**) [69].. Analysis were conducted with comparison with QC (Quality Control) samples replicates, where a standard solution was added to a blank matrix sample submitted to the same conditions [27,44,112]. Afterwards, the samples were analyzed in LC-ESI-MS/MS [27,35,69], UFLC-MS/MS [112] or HPLC-UV [44]. Results obtained revealed that these compounds are stable [27,44,69,111,113,114] or did not exhibited significant degradation [7,35,112] under the studied conditions.



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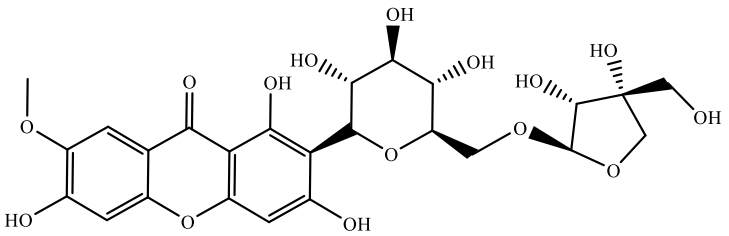

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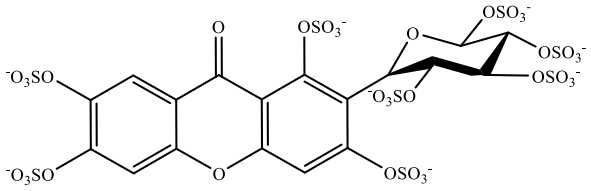
Fig. 11: Strucures of neomangiferin (**31**) and polygaloxanthone (**32**).

3.7.1.2. Short-term stability

The short- term stability usually corresponds to the storage time of samples before analysis from a few hours (i.e. 4h) to 24h at room temperatures [10,35,44,111,113-115]. Analysis were conducted with comparison with QC samples and analyzed in LC-ESI-MS/MS or HPLC-UV as mentioned in freeze/thaw studies.

A polysulfated derivative of mangiferin (**33**, **Fig. 12**) was submitted to stability evaluation in human plasma for 3h at 37ºC and showed no significant difference from the standard solution (**Scheme 3**) [116]. Also, DMXAA (**2**) showed stability in human plasma at 37 ◦C (in water bath) for at least 2 h through LC-MS/MS analysis [69].



33

Fig. 12: Structure of the polysulfated derivative of mangiferin (**33**).

For assessing the stability of biological samples there is necessary to a pre-treatment process before analysis. To fulfill such purpose, some stability studies were performed by leaving prepared samples at 4ºC for 8 h [112,114]. Other studies were conducted storing pre-treated samples in the auto-sampler for 4h [111], 12h [27,35] at room temperature or 6 days at 2-8º C before re-injection in order to control reproducibility [69]. No significant degradation occurred when the extracted samples were kept in the autosampler at room temperature for 12 h [27] or 6 days at 2-8º C [69].

3.7.1.3. Long-term stability

For the assessment of long-term stability, samples of *α*-mangostin (**4**) and γ-mangostin (**5**) were subjected to a storage time of 21 days [35], for one month to samples of sibiricaxanthone F (**8**) [7] and for two months to samples of 10-OHGA (hydroxylated derivative of gambogic acid in position 10) [113] at -20º C and no significant alterations after HPLC MS/MS [7,35] or LC-ESI-MS [108] analysis were observed.

3.7.2. Stability in non-biological samples

3.7.2.1. Short-term stability

Gambogic acid (**6**, **Fig. 4**) was dissolved in different extraction solvents and the sample solutions were stored for a week before being tested and analyzed by HPLC. The stability of gambogic acid (**6**) was dependent of the solvent used, once when extracted and stored in methanol appeared another peak in the chromatogram, further identified corresponding to10-OHGA [117].

3.7.2.2. Long-term stability

Polysulfated xanthone **33**, analyzed by HPLC-DAD, was found to be chemically stable for 15 days under different temperature storage conditions, namely room temperature, 4º C and -20º C [116].

3.7.2.3. Accelerated stability

Forced degradation of *α*-mangostin (**4**) (**Fig. 3**) was carried out under thermolytic, photolytic, acid/base hydrolytic, and oxidative stress conditions and the analysis was performed in HPLC-UV [118]. Thermal (in a controlled-temperature oven at 80º C for 3 h) and photo-degradation (under UV at 254 and 366 nm for 6 h) were performed in solid state. For hydrolytic and oxidative degradation, solutions were prepared by dissolving *α*-mangostin (**4**) extract in a small volume of methanol, and later dropped with 3% hydrogen peroxide, 3N HCl, or 3N NaOH solution and heated at 80º C for 3 h. All sample solutions used for for acid/base hydrolysis and oxidative stress were kept in a dark to prevent the effect of light. It was found that *α*-mangostin (**4**) was stable under light, heat, and basic hydrolytic conditions. Nevertheless, the *α*-mangostin (**4**) demonstrated decomposition in acidic and oxidative conditions.

3.7.2.4. pH stability

To assess pH stability of polysulfated xanthone **33** the following buffers were used: HCl (pH 1.0), sodium acetate (0.05 M, pH 5.0), potassium phosphate (0.1 M, pH 6.8), PBS (pH 7.4), and sodium boric acid (0.05 M, pH 9.1). Solutions were left at 37º C for further analysis in HPLC-DAD at 0, 1, 2 and 3 h. This compound did not degrade at the range of pH values selected [116].

Regarding mangiferin (**3**), different solutions were prepared at acid, neutral and basic pH and analyzed after 24 h, revealing that a small variation within the experimental interval through UV/Vis spectroscopy occurred. These data leaded to the decision to not use mangiferin (**3**) solutions for more than 12 h after preparation in order to assure they will remain stable [119].

3.8. Clearance

The lack of data concerning bioavailability of interesting xanthone derivatives impelled the study of PK parameters including clearance which evaluates the rate of a compound that is cleared from the blood after administration. For mangiferin (**3**, **Fig. 2**) [27,120,121], neomangiferin (**31**) [27], cudratricusxanthone B (**9**) [43] and *α*-mangostin (**4**) [37] some *in vivo* studies have been carried out in rats after intravenous and/or oral administration. Blood samples were collected in different time periods to achieve a concentration *versus* time relationship. Samples were centrifuged to obtain plasma and after that pre-treated to eliminate proteins for chromatographic analysis; further PK parameters were calculated using a software [27,39,120]. Regarding to mangiferin (**3**) [27,120,121] and cudratricusxanthone B (**9**) [43] these compounds have a low rate of clearance but neomangiferin (**31**) being a more hydrophilic compound has a higher clearance [27]. Also differences in clearance may be found after an oral or intravenous treatment with mangiferin (**3**) [120].

4. Xanthone derivatives metabolism

Genetic aspects of both drug metabolism and susceptibility to drug toxicity have become widely assessed in an early stage of drug development aiming to diminish the attrition rate during development phases due to poor PK [17,122,123]. Further, the study of the metabolic fate of a compound is interesting not only in a bioavailability and in a toxicity perspective, but also to discover possible active metabolites and lead compounds for optimization [124,125].

There are different approaches to assess drug metabolic profile that could be categorized in three major models: *in silico*, *in vitro* and *in vivo* [122]. Regarding to *in vitro* models, beyond other methodologies, it is possible to assess drug metabolism (phase I and II) with rat and human liver microsomes and with recombinant isoforms of CYP450 enzyme (taking into consideration genetic polymorphisms). Also, it is important to search for glutathione conjugates allowing to search for electrophilic metabolites [122]. In the presence of an inhibitor of CYP450 it is also possible to understand potential drug-drug interactions [17,122]. Additionally, it is important to understand the metabolism by the intestinal flora that could be assessed *in vitro*, regarding a future oral bioavailability [30,42,126].

The study of drug metabolism with *in vivo* models usually is conducted by analyzing plasma, urine and feces. The investigation of bile appears to be interesting in order to complement the study of the main metabolic and disposition pathways in animals. It allows metabolic profiling across species and offers insight into the extent of *in vivo* formation of metabolites [122]. Usually the analysis, metabolites detection and identification, is made through LC-MS/MS [122].

Besides diverse and potential activities of xanthone derivatives have been well documented [1,4-6,15,16] the understanding of their metabolism is an area in exploration to realize the possible metabolic pathways and characterize their metabolites [30,127]. Pre-clinical metabolism studies of xanthones have been carried out through *in vitro* (**Table 8**) and *in vivo* models (**Table 9**) in a quantitative and comparative way. Further, it is valuable to take into account the knowledge of the major CYP450 enzymes involved in the metabolism of potential drug candidates to predict possible drug-drug interactions, as well as genetically based individual variation in drug metabolism. *In vitro* models (**Table 8**) with rat and human liver microsomes and CYP450 isoforms allowed to discover and to identify metabolites of DMXAA (**2**), mangiferin (**3**), *α*-mangostin (**4**), gambogic acid (**6**), and other natural xanthone derivatives.

Table 8: *In vitro* models carried out for xanthones metabolism studies.

PK study	Model	Sample Pre-preparation	Chromato-graphic method	Ref.
To investigate intestinal flora metabolism of mangiferin (**3**)	Rats intestinal flora homogeneized with anaerobic cultural solution. Filtration. Incubation with mangiferin (37 ºC. Reaction terminated by cooling them at 4º C, after 2, 6, 10 and 24 h).	SPE column. Dryness by nitrogen gas. Dissolution in methanol. Centrifugation.	LC-ESI-IT-MS	30
To investigate phase II conjugation of **3**	Subcellular rat liver fractions (microsomal and cytosolic). Glucuronidation –microsomes (with UDPGA, 37 ºC, 60 min. Reaction terminated with ice-cold methanol). Sulphation – cytosolic fraction (with PAPS, same conditions as above). Enzymatic hydrolysis of glucuronidated metabolites (37º C, 120 min).	Centrifugation. Extraction with methylene chloride and centrifugation. Dryness by nitrogen gas. Dissolution in water followed by SPE column.	HPLC-UV/Vis	29
To study the metabolism of *α*-mangostin (**4**) by different human	Human cell cultures: THP-1, HepG2, Caco-2 HTB-37, HT-29 and MDM (37 º C, 95% humidity and 5% CO2, 24 h). Medium and cells were	w/i *	RP-HPLC-UV	33

cells' types in normal and pro-inflammatory environments	collected. Previously treatment with LPS for the pro-inflammatory environment.			
To investigate the metabolism of gambogic acid (**6**). To identify the major CYP450 isoform involved the metabolism and potential DDI	Rat liver microsomes (with NADPH, 37 ºC, 30 min. Reaction was terminated with HCl 1 mol/L). For DDI the same study was performed with chemical inhibitors.	Extraction. Centrifugation. Dryness. Dissolution in mobile phase.	HPLC-UV	[128]
To clarify the final disposition and metabolic profile of **6**	Liver microssomes: - NADPH-dependent CYP450-mediated and non-P450-mediated metabolism - UDPGA-dependent UGT-mediated metabolism - GSH-dependent non-GST-mediated metabolism Reactions terminated with ice-cold acetonitrile.	Centrifugation. Dryness by nitrogen gas. Dissolution in mobile phase.	LC-MS/MS	[127]
To characterize metabolism and predict possible *in vivo* metabolites of sibiricaxanthone F (**8**) and aglycone .	i) Rat intestinal flora (homogeneized with culture medium). Centrifugation. Incubation at 37 ºC, for 0.17, 0.5, 1, 1.5, 2, 3, 4, 6, 8, 12, 18 or 24 h. Reactions were terminated with ice-cold *n*-butanol). ii) Microssomal proteins (with NADPH, 37 ºC, 60 min. Reaction terminated with ice-cold methanol). iii) Glucuronidation – Rat and Human liver microsomes (with UDPGA, 37º C, 15 min. Reaction terminated with ice-cold methanol).	i) Internal standard was added. Centrifugation. Dryness by nitrogen gas. Dissolution in methanol and filtered (0.45 µm membrane). ii) and iii) Centrifugation.	HPLC-EMS-IDA-EPI	[42]
To identify the CYP450 isoform responsible for metabolism and interindividual variability of DMXAA (**2**)	HLM and cDNA-Expressed Human Microsomes containg recombinant human liver CYP isoforms (with NADPH, 37 ºC, 40 min. Reaction terminated with ice-cold acetonitrile:methanol (3:1)).	Addition of internal standard. Centrifugation. Dryness by nitrogen gas. Dissolution with mobile phase.	HPLC-SF250 flourescence detector	[59]
To study the metabolism of **2** by Caco-2 cells	Human intestinal Caco-2 cells (37º C in triplicate. Medium aliquots were collected at 0, 5, 10, 15, 20, 30 and 60 min. At the end cells were harvested and digested with 0.02N HCl/methanol (1:1).	w/i *	HPLC-SF250 flourescence detector	[129]
To study the metabolism and DDI of mangosteen extract	Pooled HLM[25] and recombinant CYP450 supersomes (with NADPH, 37 º C, 20 min. (HLM) and 0, 5, 10, 15, 30 and 45 min (CYP450). Reaction terminated with ice-cold acetonitrile).	Addiction of internal standard. Centrifugation.	LC-MS/MS	[130]
To illustrate metabolic pathways of 1-hydroxyl-2,3,5-	Pooled HLM and recombinant CYP450 isoforms (with NADPH, 37 ºC, 60 min, 800 rpm. Reaction terminated with ice-cold acetonitrile).	Addition of internal standard. Extraction with	LCMS-IT-TOF Isolation of metabolite with by	[47]

trimethoxy-xanthone (HM-1) purified from *Halenia elliptica* D. Don. To identify CYP450 isoforms responsible for the metabolism and potential DDI	For DDI the same study was performed with chemical inhibitors.	ethyl acetate. Centrifugation. Organic layer dried by nitrogen gas. Dissolution in methanol.	preparative HPLC and its identification by ^{1}H NMR and ^{13}C NMR.	
To study Phase I metabolism of Xanthones isolated from *Halenia elliptica* D. Don	Rat liver microsomes (with NADPH, 37º C, 60 min. reaction terminated with ice-cold acetonitrile).	Centrifugation. Extracted with ethyl acetate. Dryness by nitrogen gas. Dissolution in methanol.	LCMS-ESI-IT-TOF	[41]

SPE - solid phase extraction; LC – liquid chromatography; ESI – electrospray ionisation; IT – ion trap; MS – mass spectrometry; UDPGA - uridine 5'-diphospho-glucuronic acid; PAPS – phosphoadenylylsulphate; THP-1 - monocyte-like leukemia; HepG2 - hepatocelular carcinoma; HTB-37 - colorectal adenocarcinoma cells that spontaneously differentiate to enterocyte-like phenotype; HT-29 - colorectal adenocarcinoma; MDM - monocyte-derived macrophage; LPS – lipopolysaccharides; RP- reversed phase; CYP450 – cytochrome P450; DDI – drug-drug interaction; NADPH - nicotinamide adenine dinucleotide phosphate; UGT - Uridine 5'-diphospho –glucuronosyltransferase; GSH – glutathione; GST - glutathione S-transferase; EMS – enhanced mass spectrum; IDA – information dependente acquisition; EPI – enhanced product ion; HLM – human liver microssomes; cDNA – complementary DNA; SF - synchronous fluorescence; TOF – time of light.

* w/i – without information.

Further, *in vivo* models (**Table 9**) allowed to attain more complete insights of what occurs to xanthones during metabolic phases as well as they take in consideration other PK properties [32,126]. Also *in vivo* assays enable the discovery confirmation of some hypothetic metabolites identified through *in vitro* models as it occurred for gambogic acid **6** [127,128]. In the case of mangiferin (**3**) the aglycone metabolite was detected in *in vitro* and *in vivo* models due to intestinal flora activity [30,126].

Table 9. *In vivo* models carried out for xanthones metabolism studies.

PK study	Model	Samples	Sample Pre-preparation	Chroma to-graphic method	Ref.
To study the metabolism of mangiferin (**3**)	SD rats. Single dose of 200 mg/kg of mangiferin by oral gavages.	Urine and faeces 0-48h period. Blood samples via angular vein of one hour period from 1-11h. Organs (heart, liver, kidneys, spleen, lung) and contents of intestinal tract at 2, 4, 7 and 10h.	Blood in heparinized Eppendorf was centrifuged obtaining plasma. Organs were weighted and homogeneized with PBS buffer. Centrifugation. Contents of intestinal tract were diluted with saline solution. All samples were stored at -80 º C. Urine, tissue and intestinal homogenates were pre-treated in a SPE column. Faeces were extracted with methanol. Plasma was precipitated with acetonitrile:acetic acid (9:1) and centrifuged. All extracting solutions were dried by	LC-ESI-IT-MS	[30]

			nitrogen gas and reconstituted in methanol.		
To study the metabolism of **3**	Conventional rats, pseudo-germ-free rats, STZ-induced diabetic rats. Single dose of mangiferin 400 mg/kg by oral administration.	Blood samples were collected via the angular vein at 20 min, 40 min, 70 min, 2, 3, 4, 5, 6.5, 8, 9.5, 11, 13 and 24h. Urine and feces were collected during the period of 0-48h.	Samples were pre-treatment according to the method described by Liu et al., 2011 (1st entry).	HPLC-DAD	126
To isolate and identify metabolites in rat urine of **3**	SD rats. Single dose of mangiferin 120 mg/kg by oral administration.	To isolate the metabolites - Urine samples were collected at times of 6, 12, 24, 30, 36, 48 and 60h. For the cumulative study - Urine samples were collected at times of 1, 2, 4, 6, 10, 12, 24, 36, 48 and 60h.	Metabolites isolation - Urine samples were concentrated. Residues were suspended in methanol. The supernatant was removed and evaporated to dryness, then the residue was dissolved in water and partitioned with n-butanol three times and then extracted with D101 (H_20/EtOH) and Sephadex LH-20 (MeOH:H20; 8:2). After that eluted fractions were submitted to a silica gel chromatography column (cyclohexane-2-propylalcohol system). Finally a semi-preparative HPLC was performed.	HPLC-UV/Vis	115
To investigate conjugates of *α*-mangostin (**4**)	Athymic Balb/c nu/nu mice – HT-29 colon cell xenografts model. Diet with 900mg/kg of *α*-mangostin for two weeks.	Samples of liver, tumor, blood and feaces.	Clotted blood was centrifuged to isolate serum – storage at -80 ºC. Samples of liver, tumor and feaces were weighted and homogeneized in ice-cold PBS. Acetonitrile was added to aliquots, and centrifuged. Supernatant was dried under nitrogen gas. Film was re-solubilized in acetate buffer (pH 5.5) and incubated at 37 ºC in the absence or presence of glucuronidase/sulphatase mixture from *Helix pomatia*. SPE using C_{18} cartridges.	HPLC-DAD and HPLC-MS.	32
To study the metabolism of gambogic acid (**6**) and identify metabolites in rat bile	SD rats with a bile cannula. 6.0 mg/kg of GA was injected into the vena caudalis.	Bile was collected on ice during 12h.	Bile samples were acidified with 0.1% H_3PO_4 solution and extracted. Dryness by nitrogen gas. The residue was dissolved in mobile phase and centrifuged.	HPLC-UV/Vis, LC-ESI/MS, LC-NMR	46
To clarify the final disposition and metabolic profile of **6**	SD rats. IV administration of 4 mg/kg of GA through tail vein.	Blood samples were collected from tail vein in heparinized tubes at 0.033, 0.067, 0.15, 0.3, 0.75, 1, 2, and 4h after administration. Bile samples were collected through canulla during 24h. Urine and feaces samples were	Plasma samples were extracted. The organic layer was separated and evaporated under stream of nitrogen in a water bath of 40º C. The residue was reconstituted in mobile phase. Fecal samples were homogeneizedin acetonitrile:water (5:5) and centrifuged (3000g, 10 min) after ultrasonication for 10 min. the supernatant was filtered through precut membranes (0.45 µm). the filtrate was centrifuged and extracted as described for plasma samples.	LC-MS/MS	127

		collected during 24h.			
To identify major constituents of Zhimu-Huangqui Herb-pair extract and their metabolites in rat urine	18 male SD rats. Single dose treatment via oral administration.	Blood samples collected at 0.5, 1, 2, 4, 6 and 8 h from the abdominal aorta. Urine samples collected for 24 h.	Blood was centrifuged (12000g, 10 min). Aliquots from both plasma and urine were loaded on a SPE cartridge, washing it with water and eluting with methanol. The eluted solution was evaporated under nitrogen gas, 37 ºC, and residue was reconstituted in methanol.	LC-ESI-MS	[31]

SD - Sprague-Dawley; PBS - Phosphate buffered saline; LC – liquid chromatography; ESI – electrospray ionisation; IT – ion trap; MS – mass spectrometry; STZ – streptozotocin; DAD – diode array detector; HT-29 - colorectal adenocarcinoma; GA – Gambogic Acid.

Mass spectrometry was found to be crucial in the identification of putative metabolites in both *in vitro* and *in vivo* analysis. For analysis with LC there is a need to perform samples pre-preparation what usually involves protein precipitation, centrifugation and extraction with an organic solvent, afterwards lead the extract to dryness and dissolution in an appropriate solvent or in the mobile phase to be used in the analytical process. Also, it could be used a SPE column with methanol extraction to pre-treat samples, but when mangiferin (**3**) was the analyte this method revealed as a setback once an extremely low amount of mangiferin was detected by HPLC-DAD and LC-MS analysis [29]. In order to investigate the conjugated metabolites is important to consider the use of glucoronidase and/or sulphatase to detect their presence [30].

These metabolic studies gave new insights of hypothetic metabolic pathways of the above mentioned xanthone derivatives namely mangiferin (**3**) and gambogic acid (**6**), once they could be metabolized by a variety of biochemical routes, including oxidation, hydration, demethylation, glutathionylation, glucuronidation, and glucosidation depending on the xanthone scaffold moiety; some examples are represented in **Fig. 13** [115,127]. These findings, from assays with intestine and liver of rats, provide information on the major metabolic soft spot of different xanthone derivatives, which is not only useful in the future human metabolic study of these compounds but also for other xanthones analogues [127].

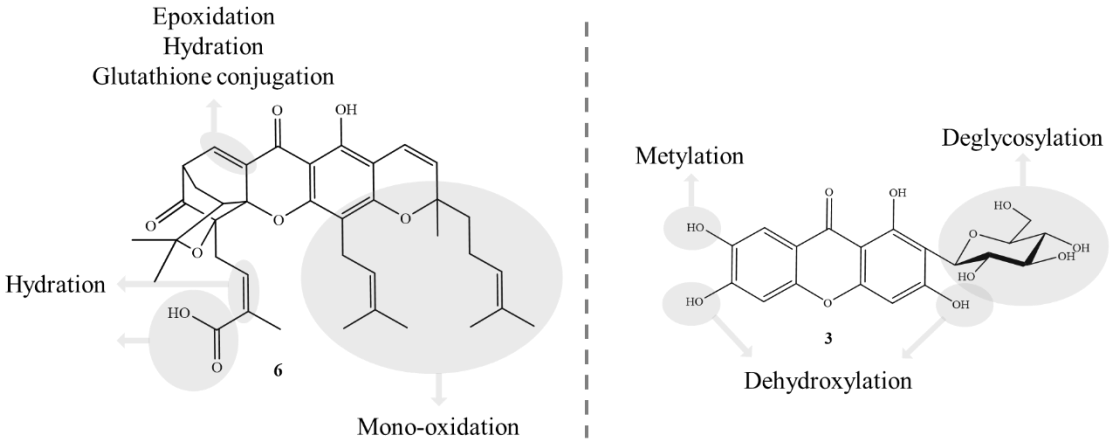


Fig. 13. Putative metabolic soft spots for Gambogic Acid (**6**) and Mangiferin (**3**).

5. Drug candidate DMXAA (5,6-dimethylxanthenone-4-acetic acid or ASA404 or Vadimezan): a case-study

A notable example among the large number of natural and synthetic xanthone derivatives is DMXAA (**2**), a simple carboxylated xanthone (5,6-dimethylxanthenone-4-acetic acid or ASA404 or Vadimezan) [1,14]. This compound has attracted scientific interest due to its excellent pharmacological profile since its discovery [131].

Several reviews have been devoted on DMXAA (**2**), focusing mainly on its antitumor activity [132-141]. DMXAA (**2**) is a novel tumor vascular-disrupting agent discovered in a structure-activity relationship study involving a series of xanthenone-4-acetic acids related to the parent drug flavone acetic acid [131]. It was selected as a clinical candidate from several xanthone and flavone derivatives, based on its superior potency and activity against solid murine tumors. Although its molecular target(s) is(are) not yet known, the mechanism of action as well as toxicological and pharmacological profiles are remarkably different from most conventional cytotoxic drugs. DMXAA (**2**) reveals immune modulatory activities inducing cessation of tumor blood flow and rapid vascular collapse, leading to ischemia and tumor necrosis [142,143]. The anti-vascular and immune-modulating effects of DMXAA (**2**) have been attributed to its ability to induce a panel of cytokines, particularly tumor necrosis factor (TNF)-α [123,144-150], interferons (IFNs) [123,151-154] and interleukins (ILs) [152]. It also possesses inductive effects in serotonin [155-157], nitric oxide (NO) [155,158,159], nuclear factor κB (NF-κB) [145,151,160], and apoptosis of endothelial cells of tumor blood vessels [161-163]. DMXAA (**2**) has additional antiangiogenic effects [153], and the ability to promote an influx of macrophages [164] and neutrophils [165] into the tumor. All these effects may contribute to its antitumor activity.

Interestingly, DMXAA (**2**) interacts productively with radiotherapy [166-169], immunotherapy [170,171], radioimmunotherapy [172,173], photodynamic therapy [174,175], antibody-directed enzyme prodrug therapy [176], hyperthermia [169,177], and several drugs, such as chemotherapeutic [178,179], bioreductive cytotoxic [180,181], non-steroidal anti-inflammatory [182] and thalidomide [183,184].

DMXAA (**2**) has been evaluated in Phase I/II clinical trials as a single agent [63,71,185,186] and in combination with standard chemotherapy agents [187-191] in patients with advanced cancer. In DMXAA (**2**) plus chemotherapy studies, an improvement of tumor response, median time to disease progression, and median overall survival were observed, in both patients with squamous and nonsquamous non-small-cell-lung cancer (NSCLC) [187-189]. These promising clinical studies led to, large-scale, randomized Phase III placebo-controlled trial of DMXAA (**2**) with or without paclitaxel and carboplatin in NSCLC [3]. It is important to highlight that DMXAA (**2**) was the first of its class to enter Phase III trials. Although, normally well tolerated, the addition of DMXAA (**2**) to paclitaxel and carboplatin failed to improve frontline efficacy, indicating a lack of utility [3]. Consequently, Phase III trials were stopped [192].

The future clinical development of DMXAA (**2**) as an anticancer drug is currently uncertain, however additional efforts aimed to identify analogues with greater activity are under investigation [193,194]. Moreover, while originally developed as an antitumor agent, alternatively other activities have been described for DMXAA (**2**), namely antiplatelet and antithrombotic [195], or even antiviral [196] (**Fig. 14**).

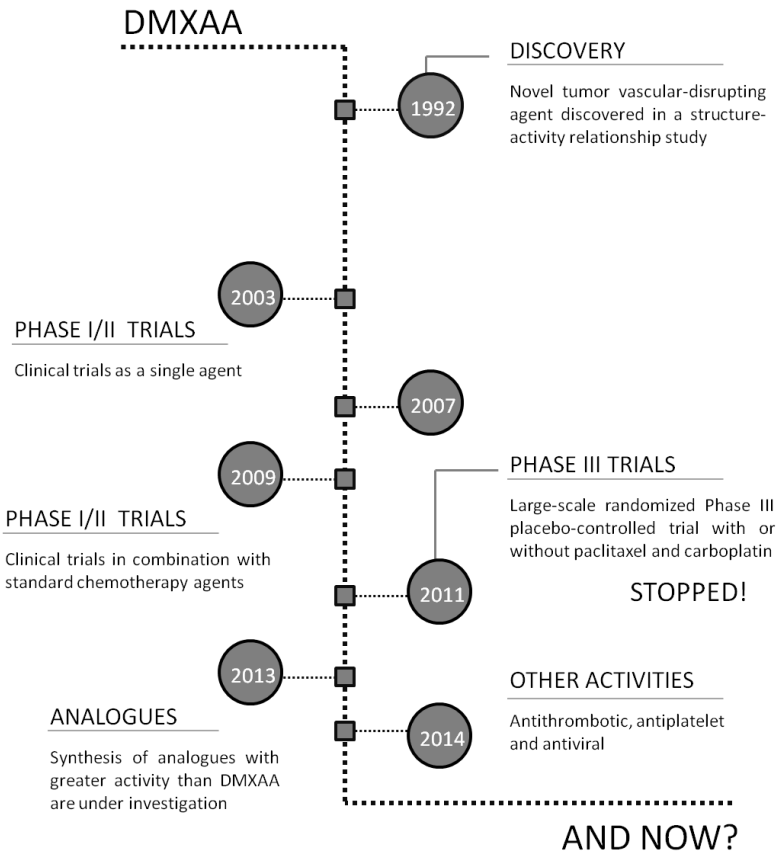


Fig. 14. Timeline of the most important developments for DMXAA (**2**).

The distribution-metabolism-elimination profile and PK properties of DMXAA (**2**) have been extensively investigated, by *in vitro* and *in vivo* studies using different species of animals and humans [138,197]. All those features will be emphasized in the next section.

5.1. Distribution

DMXAA (**2**) was extensively bound in plasma from various species, with concentration-dependence and considerable interspecies differences [56,57,62,66,198]. For example, the unbound fraction of DMXAA (**2**) (500 μM) was 2.07 ± 0.23 (human), 4.61 ± 1.10 (mouse), 2.59 ± 0.32 (rat) and 2.02 ± 0.48 % (rabbit); with DMXAA (**2**) concentrations ≥ 1000 μM the unbound fraction in the plasma from all species increased significantly [66]. Considerable alterations in DMXAA (**2**) distribution into blood cells also occurred with increasing concentrations of DMXAA (**2**). For example, the human blood:plasma concentration ratio (CBL/Cp) of DMXAA (**2**) was 0.673 ± 0.103 over the range 50-1000 μM, but increased significantly at higher concentrations of DMXAA (**2**) [66].

Plasma binding of DMXAA (**2**) is also altered by the environment of disease, since in plasma from cancer patients there was no significant relationship between bound:free DMXAA (**2**) concentration ratio and albumin concentration; in opposition, in healthy human plasma a linear correlation was observed [66]. Further study demonstrated that there is a 2- to 3-fold variation in the binding of DMXAA (**2**) by plasma proteins in healthy humans and cancer patients. Unbound DMXAA (**2**) fraction in healthy humans was significantly less than that observed in cancer patients [68]. Negligible gender differences occurred in

plasma protein binding of DMXAA (**2**) in mice, rats and humans [60]. Albumin was the principal binding protein, with minor binding to human γ-globulin and α1-acid glycoprotein [66]. Similar to preclinical studies, highly albumin-bound of DMXAA (**2**) and saturation of protein binding at higher doses were also described in clinical trials [63,71,187]. For example, in a Phase I trial, at doses up to 320 $mg.m^{-2}$, more than 99% of DMXAA (**2**) was plasma protein bound; at higher doses (4900 $mg.m^{-2}$) the percentage of free drug increased to a maximum of 6.9% [63].

5.2. Metabolism and Elimination

In vitro studies using isolated perfused rat liver, human liver microssomes as well as *in vivo* animal studies have revealed that conjugation of the acidic side chain with glucuronic acid and 6-methylhydroxylation are the major metabolic pathways of DMXAA (**2**) [54,56,198,199]. In all species studied, hepatic and renal DMXAA (**2**) acyl glucuronidation and 6-methylhydroxylation followed Michaelis-Menten kinetics [56]. Hepatic isozymes responsible for the metabolism of DMXAA (**2**) were identified, demonstrating that DMXAA (**2**) glucuronidation is catalyzed by uridine diphosphate glucuronosyltransferases (UGT1A9 and UGT2B7) [200], and 6-methylhydroxylation by cytochrome P450 (CYP1A2) [201]. The involvement of flavin-containing monooxygenase (FMO) in the 6-methylhydroxylation of the DMXAA (**2**) was investigated by use of human liver microsomes and microsomes containing cDNA-expressed FMOs. The results confirmed that human FMO3 had the capacity to metabolize DMXAA (**2**), although with less significant role than CYP1A2 [202].

Additional studies were conducted in order to determine interindividual variations in the expression and activity of the major liver enzymes that metabolize DMXAA (**2**), indicating that there was a 6- and 5-fold interindividual variation in the enzyme levels of CYP1A2 and UGT2B7, respectively [68]. Using an established human liver bank, the relative contribution of each pathway to the metabolism of DMXAA (**2**) demonstrated to be subject not only to the protein levels of enzymes involved but also to the substrate concentration [203]. For example, at 5 μM DMXAA (**2**), 6-methylhydroxylation and acyl glucuronidation contributed 26 and 76%, respectively, to its metabolism; at 350 μM of DMXAA (**2**) the relative contribution of each pathway to the metabolism were 7 and 93%, respectively. Furthermore, gender differences in the metabolism of DMXAA (**2**) in mice, rats and humans have been reported [60]. In fact, using human liver microssomes, the DMXAA (**2**) was mainly metabolized by 6-methylhydroxylation in men; in contrast, a notably higher rate of DMXAA (**2**) glucuronidation occurred in women.

Significant strain differences in DMXAA (**2**) acyl glucuronidation and 6-methylhydroxylation in mousse liver microssomes were also observed [67].

The resultant metabolites, namely DMXAA acyl glucuronide (DMXAA-G) (**36**, **Fig.15**) and 6-hydroxymethyl-5-methylxanthenone-4 acetic acid (6-OH-MXAA) (**37**, **Fig.15**), have been successfully identified and quantified in different biological matrixes including animal and human plasma, urine, faeces and microsomes during PK, particularly metabolism studies [54,56,60,63-65,67,68,72,198,200,202-204]. Accordingly, DMXAA (**2**) and its major metabolites (DMXAA-G, **36** and 6-OH-MXAA, **37**) are excreted in bile and urine [56,198,205]. Using the isolated perfused rat liver model, DMXAA-G (**36**) was identified as the major biliary metabolite, and its presence in bile accounted for >50% of the DMXAA (**2**) dose. A total of 28% of DMXAA (**2**) was recovered unchanged in the perfusate, liver and bile [198]. In urine of patients

receiving DMXAA (**2**), at least five metabolites of DMXAA (**2**) were observed, with up to 60% of the total dose excreted as DMXAA-G (**36**), 5.5% as 6-OH-MXAA (**37**) and 4.5% as the glucuronide of 6-OH-MXAA (**37**) [205]. This study also indicated that the major metabolite (DMXAA-G, **36**) is chemically unstable under physiological conditions, undergoing enzymatic and non-enzymatic hydrolysis, molecular rearrangement and covalent binding to plasma proteins [205].

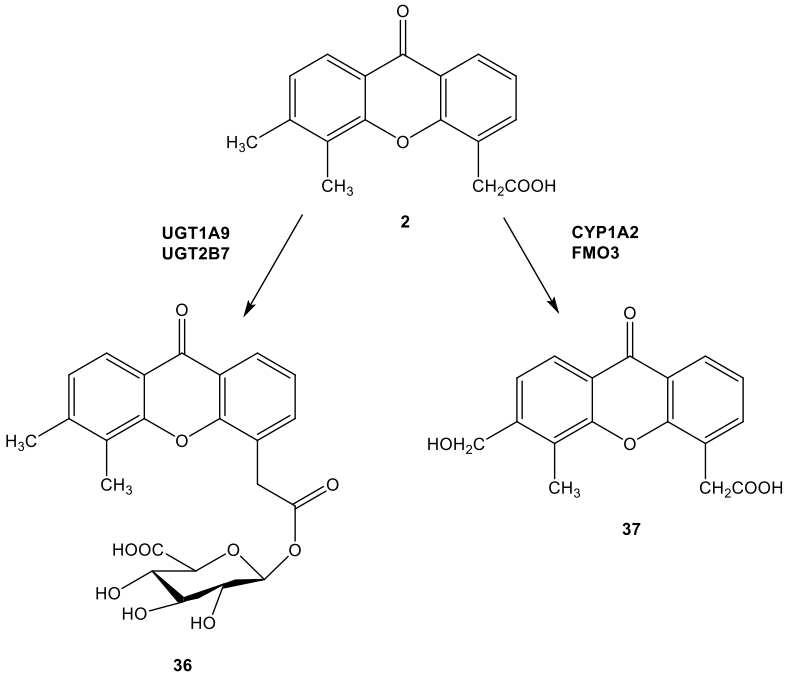


Fig. 15. Chemical structures of the major metabolites of DMXAA (**2**): DMXAA-G (**36**) and 6-OH-MXAA (**37**).

In a recent Phase I study using radioactive technique, two novel metabolites were identified, a DMXAA dimer (**38**, **Fig. 16**) and a DMXAA dimer glucuronide conjugate (**39**, **Fig. 16**). Both metabolites were detected in plasma and feces, and the dimer glucuronide conjugate was also detected in urine [72]. Once again, DMXAA-G (**36**) was the major metabolite excreted in urine and accounted for the elimination of between 32 and 48% of the administered dose. The 6-OH-MXAA (**37**) was the major metabolite excreted in the feces and accounted for the elimination of between 7 and 27% of the dose. The mean contributions of the urinary and fecal routes to the overall elimination of DMXAA (**2**) were 53.9% and 33.3% of the administered dose, respectively. Unchanged DMXAA (**2**) was the major radioactivity component detected in plasma within the first 24 h after dosing. However, it was eliminated mainly as metabolites. Actually, urinary and fecal excretion of unchanged DMXAA (**2**) accounted for only 10 and 20% or less of the dose, respectively [72]. Additionally, studies of DMXAA (**2**) metabolism by human intestinal Caco-2 cells revealed that DMXAA (**2**) was metabolized to a very small extent (up to 5%), suggesting that no considerable intestinal metabolism would take place when DMXAA (**2**) is orally administered [129].

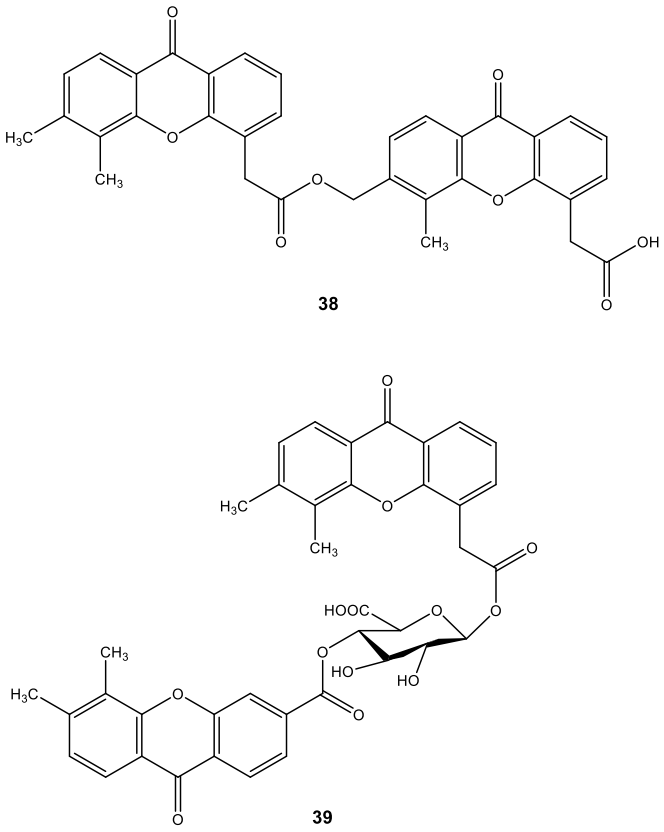


Fig. 16. Chemical structures of two novel metabolites of DMXAA (**2**): DMXAA dimer (**38**) and DMXAA dimer glucuronide conjugate (**39**).

Various anticancer drugs inhibited the major metabolic pathways of DMXAA (**2**) in human liver microsomes [55,61]. Specifically, vinblastine, vincristine and amsacrine (500 µM) inhibited DMXAA (**2**) glucuronidation (inhibition constants (Ki) = 319, 350 and 230 µM, respectively), but not 6-methylhydroxylation. On the other hand, daunorubicin and *N*-[2-(dimethylamino)-ethyl]acridine-4-carboxamide (100 and 500 µM) showed inhibition of DMXAA (**2**) 6-methylhydroxylation (Ki = 131 and 0.59 µM, respectively), but not glucuronidation. It is important to emphasize that some drugs, such as 5-fluoroucacil, paclitaxel, tirapazamine and methotrexate, exhibited insignificant inhibition of the metabolism of DMXAA (**2**) [61]. These studies are fundamental in order to predict potential *in vivo* DDI as well as to establish doses of DMXAA (**2**) when used in combination with other common anticancer drugs.

5.3. Pharmacokinetic parameters

Prior to clinical evaluation of DMXAA (**2**), studies revealed differences among species in PK showing nonlinear plasma PK at MTD (maximum tolerated dose) [56,57]. The PK of DMXAA (**2**) in cancer patients was also dose dependent, being the non-linearity remarkably consistent over a large dose range [63,71,185]. For example, peak plasma concentrations and area under the curve (AUC) level increased from 4.8 µM and 3.2 µM h, respectively, at the lowest dose (6 $mg.m^{-2}$) to 1290 µM and 7600 µM h at the MTD (3700 $mg.m^{-2}$), while clearance declined from 7.4 to 1.7 $l\ h^{-1}.m^{-2}$ over the same dose range. The terminal elimination half-life for all patients was 8.1 ± 4.3 h [63].

The saturation of elimination mechanisms and plasma protein binding were the most plausible source for the non-linearity PK of DMXAA (**2**) [71,185,206].

Based on the findings of dose-escalation Phase I trials, doses in the range of 1200 $mg.m^{-2}$ of DMXAA (**2**) were selected for further combination studies [185]. Specifically, at 1200 $mg.m^{-2}$, the Cmax and the area under the concentration-time curve over 24 h for total and free DMXAA (**2**) plasma concentrations were 315 ± 25.8 $\mu g.mL^{-1}$, 29 ± 6.4 $\mu g.mL^{-1}$ day, 8.0 ± 1.77 $\mu g.mL^{-1}$, and 0.43 ± 0.07 $\mu g.mL^{-1}$ day, respectively [185]. Subsequently randomised phase II study in patients with NSCLC demonstrated that when DMXAA (**2**) (1200 $mg.m^{-2}$) was co-administered with carboplatin and paclitaxel, there was little change in the systemic exposure or disposition of either total or free carboplatin or paclitaxel as well as total DMXAA (**2**). Nevertheless, the concentration of free DMXAA (**2**) was increased, suggesting that the chemotherapy drugs or excipients changed the partitioning of DMXAA (**2**) within plasma [188]. Further clinical studies corroborated the feasibility of combining DMXAA (**2**) (elevated the dose to 1800 $mg.m^{-2}$) with carboplatin and paclitaxel, emphasizing the absence of adverse PK interactions, in addition to safety profile and improvements in efficacy variables, and survival [187,189].

However, some drugs such as thalidomide [70], diclofenac [207], cyproheptadine [208] have been shown to modulate the PK of DMXAA (**2**).

DMXAA (**2**) exhibited good oral bioavailability (73%) in mice, but revealed low antitumor activity when given as a single dose [209]. However, after multiple oral doses it gave a 90% cure rate of colon 38 tumors [210]. To better understand its oral PK properties, the intestinal absorption of DMXAA (**2**) and its major metabolite was characterized using human intestinal cell line Caco-2 monolayers [73,129].

DMXAA (**2**) can be considered as a weak acid with a pKa of 5.5, being mainly present as ionized form at pH 7.4, whereas DMXAA-G (**36**) is a high hydrophilic conjugate with an approximate pKa of 3.5. The permeability coefficient (Papp) values of DMXAA (**2**) over 10-500µM were 4×10^{-5} cm/s to 4.3×10^{-5} cm/s for both apical (AP) to basolateral (BL) and BL-AP transport, regardless of concentration. On the other hand, the Papp values for the BL to AP flux of DMXAA-G (**36**) were significantly greater than those for the AP to BL flux, with net efflux ratio (Rnet) values of 4.5-17.6 over 50-200. The transport of DMXAA-G (**36**) was energy and Na^{+-} dependent and inhibited by MK-571, a multidrug resistance associated protein (MRP) 1/2 inhibitor. These results indicated that DMXAA (**2**) was passively transported by intestinal cells, whereas the transport of DMXAA-G (**36**) was mediated by multidrug resistance associated protein (MRP) 1/2 µM [73,129]. The effect of other drugs on DMXAA (**2**) and DMXAA-G (**36**) transport, namely thalidomide, diclofenac, probenecid, verapamil, cimetidine were evaluated, but none showed any significant effect [129].

Perspectives and conclusion

For the physico-chemical and PK studies of xanthonic compounds, the chromatographic and spectrometric methods have been widely used as analytical tools for compounds detection, quantification, and metabolites identification.

Currently, it is not only compounds biological activities that are important to become drug candidates, with physico-chemical and PK properties representing relevant roles to achieve drug-likeness.

Thus, at first it is desirable to study compounds physico-chemical properties, such as lipophilicity to predict a good balance between its aqueous solubility and permeability through the cells membranes. Preclinical studies should be directed in order to investigate the PK and toxicity early in the process of hit-to-lead optimization since they give valuable information in the analogue-based design. It is relevant to understand the bioavailability, exposure, distribution volume, plasma protein binding, metabolism and clearance. These key properties and their intrinsic parameters are interlinked and complement each other for a detailed characterization of the compound.

The most investigated naturally-occurring xanthones are compounds with low aqueous solubility (range of 10^{-6} M) due to their planar scaffold and presence of prenyl and other hydrophobic groups. This affects absorption and volume distribution, and consequently compromises bioavailability. Nevertheless, chemical synthesis has allowed to introduce structural alterations in order to increase their solubility, such as: hydrophilic amine substitution, salt formation, introduction of hydroxyls and elimination of hydrophobic and/or bulky substituents. Such examples of these chemical alterations were highlighted for gambogic acid (**6**) and its synthetic derivatives and for pyranoxanthones. Studies with Caco-2 cell line indicated the high permeability of xanthones and predicted the observed good *in vivo* distribution. Mangiferin (**3**) and DMXAA (**2**) have been reported to extensively bind to plasma proteins.This potential limitation opens paths to explore DDI and dosing to guarantee further safety. Moreover, mangiferin (**3**), being poorly soluble in aqueous solutions and suffering dehydroxylation, metylation or deglucosydation as main metabolic reactions, shows a low rate of clearance.

To perform all of these physico-chemical and PK assays it is important that compounds are stable during experiments and storage in biological and non-biological samples. In regards to stability, the studied xanthones are stable at different temperatures, time and solution pH values, excepting *α*-mangostin (**4**) and mangiferin (**3**) in acidic solutions and the former also in oxidative environments.

Overall, this physico-chemical and ADME/tox characterization has abled to dig underlying valuable information about xanthonic derivatives in order to guide the design and development of new chemical entities and further studies. The characterization of the metabolism is important to understand the metabolic pathways, possible DDI, the extent that xanthonic derivatives are metabolized (clearance) and further which metabolites could be active or are associated with toxicity. Once the structural soft spots for metabolism are identified they could be also valuable for further chemical synthesis design.

In sum, having in mind the potential biological activities of xanthone derivatives and a deeper knowledge about their structure-properties relationships, it impels to the rational design of new derivatives aiming to reach their clinical application.

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