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Unveiling the impact of morphine on tamoxifen metabolism in mice in vivo

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Keywords

Tamoxifen, Morphine, Morphine-3-beta-D-glucuronide, UDP-glucuronosyltransferase, drug-drug interactions, endoxifen-glucuronide, 4OH-tamoxifen-glucuronide, Metabolism, CYP = cytochrome P450

Abstract

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Background- Tamoxifen is used to treat breast cancer and cancer recurrences. After administration, tamoxifen is converted into two more potent antitumor compounds, 4OH-tamoxifen and endoxifen by the CYP3A4/5 and 2D6 enzymes in human. These active compounds are inactivated by the same UDP-glucuronosyltransferases isoforms as those involved in the metabolism of morphine. Importantly, cancer-associated pain can be treated with morphine, and the common metabolic pathway of morphine and tamoxifen suggests potential clinically relevant interactions.

Methods- Mouse liver microsomes were used to determine the impact of morphine on 4OH-tamoxifen metabolism in vitro. For in vivo experiments, female mice were first injected with tamoxifen alone and then with tamoxifen and morphine. Blood was collected, and LC-MS/MS was used to quantify tamoxifen, 4OH-tamoxifen, N-desmethyltamoxifen, endoxifen, 4OH-tamoxifen-glucuronide and endoxifen-glucuronide.

Results- In vitro, we found increased Km values for the production of 4OH-tamoxifen-glucuronide in the presence of morphine, suggesting an inhibitory effect on 4OH-tamoxifen glucuronidation. Conversely, in vivo morphine treatment decreased 4OH-tamoxifen levels in the blood while dramatically increasing the formation of inactive metabolites 4OH-tamoxifen-glucuronide and endoxifen-glucuronide.

Conclusions- Our findings emphasize the need for caution when extrapolating results from in vitro metabolic assays to in vivo drug metabolism interactions. Importantly, morphine strongly impacts tamoxifen metabolism in mice. It suggests that tamoxifen efficiency could be reduced when both drugs are co-administered in a clinical setting, e.g. to relieve pain in breast cancer patients. Further studies are needed to assess the potential for tamoxifen-morphine metabolic interactions in humans.

Contribution to the field

Our study has been designed to determine if morphine modulates tamoxifen metabolism in vitro and in vivo. Breast cancer is the most common and deadliest women cancer. Among anti-breast cancer drugs, tamoxifen decreases breast cancer recurrence and mortality rates. Tamoxifen is a pro-drug metabolized in the liver by enzymes leading to two more potent anti-tumour compounds, endoxifen and 4OH-tamoxifen (30-100 fold). Then, UDP-glucuronosyltransferase enzymes catalyze their inactivation into glucuronides. Cancer-associated pain (e.g. metastases, surgeries) represents a major problem solved by the use of analgesic drugs, including morphine. Morphine and tamoxifen share some catabolic pathway leading to morphine-glucuronide metabolites. Therefore drug-drug interactions could be expected and interfere with the expected therapeutical effect. As no data are available on tamoxifen-morphine potential interaction, we have focused our attention on such drug-drug interactions in vitro using mass spectrometry. To get rid of inter-individual variations, our in vivo protocol was designed to use each animal as his own control of tamoxifen metabolism. If a difference occurs, it may lead to rethink co-analgesic procedures in the case of breast cancer treatment.

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Unveiling the impact of morphine on tamoxifen metabolism in mice *in vivo*

Abbreviated title: Morphine increases tamoxifen-metabolite glucuronidation

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ABSTRACT

Background- Tamoxifen is used to treat breast cancer and cancer recurrences. After administration, tamoxifen is converted into two more potent antitumor compounds, 4OH-tamoxifen and endoxifen by the CYP3A4/5 and 2D6 enzymes in human. These active compounds are inactivated by the same UDP-glucuronosyltransferases isoforms as those involved in the metabolism of morphine. Importantly, cancer-associated pain can be treated with morphine, and the common metabolic pathway of morphine and tamoxifen suggests potential clinically relevant interactions.

Methods- Mouse liver microsomes were used to determine the impact of morphine on 4OH-tamoxifen metabolism *in vitro*. For *in vivo* experiments, female mice were first injected with tamoxifen alone and then with tamoxifen and morphine. Blood was collected, and LC-MS/MS was used to quantify tamoxifen, 4OH-tamoxifen, N-desmethyltamoxifen, endoxifen, 4OH-tamoxifen-glucuronide and endoxifen-glucuronide.

Results- *In vitro*, we found increased Km values for the production of 4OH-tamoxifen-glucuronide in the presence of morphine, suggesting an inhibitory effect on 4OH-tamoxifen glucuronidation. Conversely, *in vivo* morphine treatment decreased 4OH-tamoxifen levels in the blood while dramatically increasing the formation of inactive metabolites 4OH-tamoxifen-glucuronide and endoxifen-glucuronide.

Conclusions- Our findings emphasize the need for caution when extrapolating results from *in vitro* metabolic assays to *in vivo* drug metabolism interactions. Importantly, morphine strongly impacts tamoxifen metabolism in mice. It suggests that tamoxifen efficiency could be reduced when both drugs are co-administered in a clinical setting, *e.g.* to relieve pain in breast cancer patients. Further studies are needed to assess the potential for tamoxifen-morphine metabolic interactions in humans.

KEYWORDS

Tamoxifen, 4OH-tamoxifen, 4OH-tamoxifen-glucuronide, endoxifen, endoxifen-glucuronide, Morphine, M3G, metabolism, CYP, UDP-glucuronosyltransferase, drug-drug interactions.

BACKGROUND

Breast cancer is the most common and deadliest cancer diagnosed in women, even though major advances in screening and treatment have been made the last twenty years [1]. In estrogen receptor (ER)-positive breast tumors, the main strategy of breast anticancer drugs is to either antagonize ER signaling and/or decrease estrogen synthesis to prevent cancer cell proliferation. Among those drugs, tamoxifen is a selective estrogen receptor modulator (SERM) used for decades to decrease breast cancer recurrence [2]. Nowadays, , tamoxifen remains one of the major treatment for breast cancer, especially in countries with limited health care resources [3].

Tamoxifen is a pro-drug metabolized mostly in the liver by the phase I cytochrome P450 (CYP) 2D6 and 3A4/5 enzymes [4]. In human, hydroxylation of tamoxifen (CYP2D6) leads to 4OH-tamoxifen that can be further processed into endoxifen (via CYP3A4/5) through N-desmethylation. These two major metabolites are 30-100 fold more potent than tamoxifen itself and are responsible for its anti-tumoral activity.

In addition, N-desmethylation of tamoxifen generates the N-desmethyltamoxifen intermediate (CYP3A4/5) that is further metabolized into endoxifen through CYP2D6-mediated hydroxylation.

Endoxifen is the major metabolite of tamoxifen in humans. Alternatively, in mice, even though CYP2D6 isoform is absent, 4OH-tamoxifen is the main anticancer product of tamoxifen suggesting that other CYP2D isoforms, such as CYP2D22, could be involved in its metabolism [4; 5].

Phase II metabolizing enzymes including Uridine 5'-diphospho (UDP)-glucuronosyltransferases (UGT1A10, 1A4, 1A8, 2B7 and 2B15) convert active tamoxifen metabolites into inactive 4OH-tamoxifen-glucuronide and endoxifen-glucuronide (**Fig. 1**) [6]. Approximately 75% of a given dose of tamoxifen is excreted into the biliary tract as inactive glucuronides [7].

Cancer-associated pain resulting from metastases, anticancer treatment or surgery represents a major problem which is treated with analgesic drugs including morphine, codeine and/or paracetamol [8]. Morphine remains the gold standard for mild and severe pain relief despite side effects that limit its chronic use [9]. In humans, morphine acts on Mu opioid receptors (MORs) to produce analgesia. Its metabolism in the liver and brain leads mainly to the formation of morphine-3-glucuronide (M3G) [10] and morphine-6-glucuronide (M6G) [10; 11; 12]. In human, morphine-glucuronidation is catalyzed by UGT2B7 and to a lower extent by a number of other UGT isoforms (UGT1A10, UGT1A1, 1A3, 1A6, 1A8, 1A9, 2A1 and UGT2B21) [9; 13; 14]. However, in mice, UGT2B7, the major enzyme involved in morphine metabolism in human, is absent but its activity is rescued by UGT2B21 and UGT2B36 [14; 15; 16].

Drug-drug interactions, resulting in either enzyme inhibition or induction, are a major limitation for the use of co-treatments [17]. Usually, these drug-drug interactions are initially

studied *in vitro* and then *in vivo* [18]. While *in vitro* studies provide interesting results, their interpretation has proven to be complex when translated to *in vivo* drug metabolism [18].

Although anti-cancer agents share common catabolic pathways with many opiates, the impact of their co-administration on the metabolism and thus on the activity of anticancer drugs remains unexplored. These potential interactions between analgesic and anticancer drug metabolism could be used to treat more efficiently breast cancer. Therefore, as a proof of concept, we have investigated in mice whether morphine can alter tamoxifen metabolism.

METHODS

Animals

Experiments were performed with 11 to 29 weeks-old female C57BL/6J mice (23±4g; Charles River, L'Arbresle, France). Animals were housed according to a 12h light-dark cycle, at a temperature of 22°C±2°C and provided with food and water *ad libitum*. All procedures were performed in accordance with European directives (2010/63/EU) and were approved by the regional ethics committee and the French Ministry of Agriculture (license No. APAFIS#16827-2018092113192911 v4 to Y.G.).

Blood collection

The tail of the mouse was anaesthetized locally with a cutaneous application of lidocaine/prilocaïne 5% (Zentiva, Paris, France). After 5min, a small incision was performed at the end of the tail and 10µl of blood was collected using a calibrated capillary (Minicaps End-to-End10µl; Hischmann, Eberstadt Germany).

Tamoxifen and morphine injections

Female mice were injected intraperitoneally (i.p., calibrated Hamilton syringe) with 10mg/kg of tamoxifen (in 90% olive oil/10% ethanol, v/v; Sigma Aldrich, Lyon, France), and then with NaCl 0.9% at 0h, 1h and 2h following tamoxifen administration (**Fig. 2**). Blood was collected by tail vein sampling (see above) just before and at 1h, 2h, 4h, 8h, 24h and 48h after tamoxifen injection (**Fig. 2**). A second injection of tamoxifen was then performed at 48h and immediately followed by an injection of either 10mg/kg of morphine-HCl (diluted in 0.9% NaCl; Francopia, Paris, France) or saline solution (0.9% NaCl only). Mice then received two additional injections of morphine or saline at 1h and 2h after the second tamoxifen dose. Blood was collected at 1h, 2h, 4h, 8h, 24h and 48h after the second tamoxifen injection (**Fig. 2**).

Sample preparation

The blood was transferred from the capillary into a microtube containing 10µl of heparin and frozen at -20°C for later analysis. On the next day, blood was thawed and 10µl of an internal standard (IS; see below) and 100µl of ice-cold acetonitrile (ACN; Thermo Scientific, San Jose, USA) were added. The samples were next vortexed and centrifuged at 20 000g during 15min at 4°C. The supernatants were collected, dried under vacuum and suspended in 15µL of 50% methanol/0,1% formic acid (v/v; Sigma Aldrich) prior to LC-MS/MS analysis.

Microsome preparation

Liver tissues were collected from 10 week old male C57/BL6J mice. Samples were pooled and homogenized with an Ultra Turrax instrument (Ika, Staufen, Germany) in 10ml of extraction buffer (100mM Na3PO4 buffer pH 7.4, 0.32 M Sucrose, 1mM EDTA, 0.1mM DTT, protease inhibitor, cOmplete Mini, EDTA-free, Roche, Basel, Switzerland). The homogenate was then sonicated (2x10s, 100 W) and centrifuged for 12min at 2000g (4°C). The supernatant was transferred into polycarbonate ultracentrifuge tubes (Beckman Instruments, Palo Alto, USA), completed with extraction buffer and centrifuged 40min at 10 000g and 4°C in a type-70 Ti Rotor (Beckman Coulter, Brea, USA). The resulting supernatant was then centrifuged for 130min at 130 000g (4°C), and the pellet obtained was suspended in 800µl of storage buffer (0.1M Na3PO4 buffer pH 7.4, 0.5mM EDTA, 0.1mM DTT, 20% glycerol; Sigma Aldrich) and frozen. Protein concentration was determined using the Bradford method (Protein Assay, Bio-Rad, Marnes-la-Coquette, France).

Enzymatic Activity Assay

100µg of liver microsomes were used to perform 4OH-tamoxifen glucuronidation assays. First, increasing concentrations of 4OH-tamoxifen (10, 20, 40, 50, 60, 70, 80, 100, 125, 150, 200, 250 and 300µM; LGC Standard, Molsheim, France) with a fixed concentration of morphine (500µM) were dried under vacuum. Morphine was suspended in 4mM $MgCl_2$ adjusted with H_2O, and each 4OH-tamoxifen concentration was diluted with 69µl of the morphine-containing mix.

Microsomes were incubated for 15min at 4°C in the presence of alamethicin (30µg/mg of protein; Santa Cruz Biotechnology, Heidelberg, Germany) and Tris-HCl buffer (400mM) adjusted with H_2O. Then, 75µL of microsome were added to each 4OH-tamoxifen concentration and tubes were equilibrated at 37°C during 5min. The enzymatic reactions were started with the addition of 6µl of UDPGA to a final concentration of 5mM. Reactions were stopped 20s later with 900µl of cold 100% methanol. Samples were then diluted (1:5), and an IS (see below) was added to each sample. Samples were centrifuged for 15min at 20 000g, and 4°C and the supernatants dried under vacuum, then suspended in 45µl of 4% methanol/0.1% formic acid (v/v) prior to LC-MS/MS analysis. Km and Vmax were obtained with a Michaelis-Menten plot following a nonlinear curve fit with the least-squares method (Graphpad Prism 6 software).

LC-MS/MS instrumentation and analytical conditions

Analyses were performed with a Dionex Ultimate 3000 HPLC system (Thermo Scientific) coupled with a triple quadrupole Endura mass spectrometer. Xcalibur v2.0 software was used to control the system (Thermo Electron, Villebon Sur Yvette, France). Samples were loaded onto an Accucore RP-MS column (150 x 1 mm, 2µm, flow of 90µl/min; Thermo Electron) heated at 40°C. Buffer A was 1% ACN/ 98.9% H_2O/ 0.1% formic acid (v/v/v), whereas buffer B was 99.9% ACN / 0.1% formic acid (v/v). The gradient used is detailed in **Supplementary Table 1**.

Electrospray ionization was achieved in the positive mode with the spray voltage set at 3500 V. Nitrogen was used as the nebulizer gas, and the ionization source was heated to 250°C. Desolvation (nitrogen) sheath gas was set to 18 Arb and Aux gas was set to 7 Arb. Ion transfer tube was heated at 297°C. Q1 and Q2 resolutions were set at 0.7 FWHM, whereas collision gas (CID, argon) was set to 2 mTorr. Identification of the compounds was

based on precursor ion, selective fragment ions and retention times. Selection of the monitored transitions and optimization of collision energy and RF Lens parameters were manually determined (see **Supplementary Table 1** for details). Qualification and quantification were performed in MRM mode using Quan Browser software (Thermo Scientific).

Statistics

Statistical analysis was performed using GraphPad Prism 6 Software. Results were presented as mean values ± standard error of the mean (SEM). Groups were compared using multiple t tests.

RESULTS

Enzymatic study in vitro

As 4OH-tamoxifen is the major active metabolite of tamoxifen in mice, *in vitro* experiments were performed on mouse liver microsomes to study the impact of 500µM of morphine on the glucuronidation of 4OH-tamoxifen. Morphine was used at 500µM to determine the Km of the glucuronidation of 4OH-tamoxifen as this concentration corresponds to the Km previously determined for morphine glucuronidation in mice [12; 19]. As shown in **Figure 3**, morphine significantly affects the production of 4OH-tamoxifen-glucuronide. Specifically, morphine significantly reduced the production of 4OH-tamoxifen-glucuronide when 10 to 50 µM and 70µM of tamoxifen were used. Km values for the production of 4OH-tamoxifen-glucuronide in the absence and presence of morphine, as determined by the Michaelis-Menten equation, were 68µM and 98,6µM (+45%), respectively.

These results indicate that morphine reduces 4OH-tamoxifen glucuronidation *in vitro*.

Study of tamoxifen metabolism in vivo

First, we determined whether multiple injections of tamoxifen would alter its own metabolism (**Fig. 2 and Fig. 4A**). Blood was collected before and 1h, 2h, 4h, 8h, 24h and 48h after the first (**Fig. 4A, white part)** and the second injection of tamoxifen (**Fig. 4A, grey part**). Tamoxifen, 4OH-tamoxifen and endoxifen-glucuronide concentrations in the blood did not vary significantly at any time point between the two tamoxifen injections (**Fig. 4B**). In contrast, a significant increase in the concentrations of 4OH-tamoxifen-glucuronide, N-desmethyltamoxifen and endoxifen was observed. Accordingly, drug metabolic ratios (*i.e.* the concentration ratio of a metabolite compared to its parent molecule) were significantly altered at different time points (**Fig. 5A-F**). The ratio of endoxifen/N-desmethyltamoxifen was significantly elevated at 4h and 8h compared to the first injection suggesting an increase in endoxifen synthesis (**Fig. 5C**). In a more dramatic manner, 4OH-tamoxifen glucuronidation was increased by 1.5-2 fold at all time points compared to the first injection (**Fig. 5F**). Similarly, the t=2h ratio of endoxifen-glucuronide to its parent molecule endoxifen showed a 3-fold increase compared to the first injection (**Fig. 5E**). On the other hand, no difference was observed for 4OH-tamoxifen/tamoxifen (**Fig. 5A)**, N-desmethyltamoxifen/tamoxifen (**Fig. 5B**), and endoxifen/4OH-tamoxifen ratios (**Fig. 5D**). Together, these results indicate that tamoxifen metabolism is slightly potentiated following two subsequent injections of the drug.

As morphine has a short half-life in mice (30min), we have performed three injections of morphine to reach adequate concentrations in the blood (**Supplementary Fig. 1**). The highest concentrations of morphine and M3G in the blood were reached after 2h (1599±336 pmol/ml and 9773±1274 pmole/ml, respectively). Morphine was still present after 8h, allowing a long-lasting competition with tamoxifen metabolism.

Then, female mice were injected twice with tamoxifen (at 0h and 48h) in addition to morphine (at 48h, 49h and 50h) and blood samples were collected (**Fig. 6A**). Following morphine injections, the blood concentrations of tamoxifen, 4OH-tamoxifen, 4OH-tamoxifen-glucuronide, endoxifen and endoxifen-glucuronide were significantly increased compared to the first injection of tamoxifen (**Fig. 6B**). Only a tendency was observed for N-desmethytamoxifen. More importantly, ratios between 4OH-tamoxifen/tamoxifen (**Fig. 7A**) were significantly decreased by 1/2-1/5 fold 1h, 2h and 8h after the injection of morphine, suggesting that 4OH-tamoxifen was processed into its metabolites at a faster rate in the presence of morphine. Indeed, the ratios of 4OH-tamoxifen-glucuronide/4OH-tamoxifen showed a significant increase (2-3 fold) at every time point (**Fig. 7F**). Similarly, endoxifen-glucuronide/endoxifen ratios (**Fig. 7E**) were dramatically increased (1.5-4 fold) at 2h, 4h and 8h after the injection of morphine. On the other hand, the ratios of endoxifen/N-desmethyltamoxifen (**Fig. 7C**) and endoxifen/4OH-tamoxifen (**Fig. 7D**) were not altered by morphine administration. Together, these results indicate that the inactivation of tamoxifen and its active metabolites is exacerbated in the presence of equimolar amounts of morphine.

DISCUSSION

Repeated tamoxifen treatment potentiates glucuronide formation *in vivo*

Our results show that the blood formation pattern of N-desmethyltamoxifen and endoxifen is slightly modified *in vivo* after two subsequent tamoxifen treatments. Indeed, we observed a higher peak concentration in the case of N-desmethyltamoxifen and a slower elimination for endoxifen upon the second administration of tamoxifen. Furthermore, analysis of metabolic ratios revealed an increase in 4OH-tamoxifen-glucuronide and endoxifen-glucuronide formation compared to their parent drugs when animals received a second injection of tamoxifen. Such an increase of glucuronidation can be related to induction of the expression of UGTs present in the liver occurring 48h after the first injection of tamoxifen. Indeed, it has been described that several xenobiotics are able to promote UGT expression by acting on regulatory elements in the cell [20]. Tamoxifen acts as a selective modulator on the ER which in turn modulates the activity of numerous transcription factors implicated in the regulation of gene expression. Importantly, tamoxifen has been shown to increase the expression of CYP enzymes involved in its own metabolism, such as CYP3A4 [21]. In the same manner, one may hypothesize that the first injection of tamoxifen induced the expression of UGTs, resulting in a potentiation of 4OH-tamoxifen and endoxifen glucuronidation upon the second treatment.

Surprisingly, despite an increase in tamoxifen glucuronidation, we observed no concurrent decrease in the concentrations of 4OH-tamoxifen or endoxifen. The main degradation pathway of tamoxifen is glucuronidation, but significant amounts of its two active metabolites are eliminated through sulfation. Several sulfotransferase (SULT) isoforms (1A1, 1E1, 2A1) have been implicated in the degradation of 4OH-tamoxifen [22]. In

addition, it has been shown *in vitro* that tamoxifen metabolites are able to inhibit SULT2A1 through mixed or non-competitive inhibition [23]. Therefore, it is possible that our first tamoxifen administration inhibited SULT expression towards 4OH-tamoxifen and endoxifen. Thus, the balance between glucuronidation and sulfation could be modified without affecting 4OH-tamoxifen or endoxifen levels. Nevertheless, this hypothesis remains to be tested.

Morphine increases glucuronidation of tamoxifen active metabolites

Morphine was expected to reduce the glucuronidation of tamoxifen active metabolites through direct competition on the UGT-binding site as observed *in vitro*. Surprisingly, our results showed a dramatic increase in the levels of all active and inactive metabolites of tamoxifen when morphine was co-administered. The significant elevated levels of tamoxifen found in the blood after the coinjection with morphine may explain the increase observed for all compounds. This increase is likely to rely on differences of absorption due to drug-drug interactions with morphine rather than variability in tamoxifen injections. This point is strengthen by the fact that 19 mice were injected using a calibrated Hamilton syringe. Ratio between metabolites and their corresponding parent molecules were established to normalize the metabolite production with the tamoxifen injections. Analysis of the ratio revealed that morphine dramatically decreased the amount of 4OH-tamoxifen relative to that of its prodrug in the blood of tamoxifen-treated mice. This decrease is likely related to the concurrent massive increase of the glucuronidation of 4OH-tamoxifen and endoxifen.

It seems improbable that morphine would act as a cofactor of UGTs allowing faster glucuronidation since it did not occur in our *in vitro* experiments. A potential impact of morphine on the entry of tamoxifen in hepatocytes is also unlikely because tamoxifen is known to cross the cell membrane passively [7], whereas morphine influx relies on transporters including organic cation transporter 1 (OCT1) [24]. The last type of common molecular targets in the metabolism of tamoxifen and morphine are MRP and MDR transporters driving M3G, 4OH-tamoxifen, endoxifen, 4OH-tamoxifen-glucuronide and endoxifen-glucuronide out of the cell [7; 16; 25; 26]. One hypothesis involving those transporters may be that morphine decreases the efflux rate of tamoxifen active metabolites (and thus their glucuronidation rate). Additional studies are needed to decipher the molecular mechanism underlying this atypical change in tamoxifen metabolism.

In conclusion, co-administration of morphine in mice appears to promote the inactivation of the potent 4OH-tamoxifen and endoxifen metabolites. In light of these findings, we hypothesize that morphine could reduce the potency of tamoxifen anticancer treatment in mice. Further studies should determine if the impact of morphine on tamoxifen metabolism is sufficient to result in changes in anticancer activity at therapeutic doses.

Strengths and limitations

We chose to associate morphine with tamoxifen to develop our methodology as it was expected to be a simple model focusing primarily on the glucuronidation process. Morphine is mainly metabolized by UGTs and was not expected to impact CYP activity. Morphine and tamoxifen co-treatments are given after surgeries or in the case of severe cancer pain [27]. Otherwise, codeine and/or paracetamol are widely prescribed [8]. In human, these two

compounds are metabolized by the same CYPs (6D6/3A4) and UGTs (1A10, 1A4, 1A8, 2B7 and 2B15) [28; 29] as tamoxifen and might have a more complex impact on tamoxifen metabolic pathways [30; 31].

A main limitation of our study is that tamoxifen and morphine metabolisms differ in mice compared to humans. 4OH-tamoxifen is the major active mouse metabolite whereas endoxifen is found at greater concentrations in human serum. However, our approach using the isotopic dilution allowed us to observed non-negligible levels of both endoxifen and endoxifen-glucuronide in the blood of tamoxifen-treated mice. In mice, the Cyp2d gene cluster display nine functional genes (Cyp2d9, Cyp2d10, Cyp2d11, Cyp2d12, Cyp2d13, Cyp2d22, Cyp2d26, Cyp2d34 and Cyp2d40), whereas humans only have one (CYP2D6) [4]. Therefore, the presence of endoxifen suggest that CYP2D6 activity is rescued by an alternative CYP.

In addition, morphine is only metabolized into M3G in mice *versus* M3G and M6G in humans [32; 33]. Nevertheless, both species eliminate tamoxifen and morphine predominantly through glucuronidation. UGT2B7 [15], the main UGT involved in morphine metabolism in humans, is absent in mice. However, morphine and tamoxifen glucuronidation could be compensated by other enzymes including the mouse homologs of human UGT2B6, 2C9, 2C19, 3A4/5 [34], UGT2B36 and UGT2B21 [14; 15]. These differences lead to a tamoxifen half-life of 27h in humans and 6.8h in mice [4], as well as a morphine half-life of 30 min in mice and 2h in humans [32; 33]. Despite the existence of mouse equivalents to human CYP and UGT isoforms, major differences in isoform sequence and expression patterns limit the extrapolation of mouse data to humans. The development of humanized mouse models for CYP and UGT genes will allow to overcome such issues [34; 35].

Drug-drug interactions can lead to severe adverse effects and predicting these interactions *in vivo* is challenging. Thus, the Food and Drug Administration (FDA) and European Medicines Agency (EMA) are frequently publishing new guidelines regarding *in vitro* and *in vivo* drug-drug interaction studies [36]. We have used an *in vivo* methodology to monitor modulations of tamoxifen metabolism. Intraperitoneal injections of tamoxifen were used instead of oral administration (the typical route of administration in humans) in order to better control the given amounts of tamoxifen and morphine [37]. Indeed, the most used method is intraperitoneal injection, because the amount of administered compound can be better controlled, but delivery by oral gavage is also possible. However, oral administration suffers from significant first-pass metabolism [38], which limits absorption [39] and introduces inter-individual variability in drug metabolism [40]. The pharmacokinetics of tamoxifen were obtained by quantification of tamoxifen and its metabolites following an initial injection (10mg/kg). Then, a second injection was used to determine its pharmacokinetics in the absence or the presence of the competing drug morphine. Therefore, it was possible to accurately compare tamoxifen pharmacokinetics in the same animal to assess its potential interaction with morphine *in vivo*. It is however important to determine whether an injection of the drug of interest can induce adaptive processes responsible for differences in its metabolism following a second injection or chronic treatment.

Conclusions

In this study, we have investigated the effects of morphine on tamoxifen metabolism *in vitro* and *in vivo*. We have shown that *in vitro* morphine inhibits 4OH-Tamoxifen glucuronidation. Conversely, morphine reduced the blood levels of 4OH-tamoxifen in mice, while the inactivation of tamoxifen active compounds through glucuronidation greatly increased.

Our results suggest that morphine co-treatment could dramatically affect tamoxifen efficacy, and emphasize the need to test more common analgesics (*e.g.* codeine or paracetamol) in humans to re-evaluate the impact of pain treatments on anti-cancer drug metabolism and pharmacological activity.

DECLARATIONS

Ethics approval and consent to participate **-** All animal procedures were performed in accordance with European directives (2010/63/EU) and were approved by the regional ethics committee and the French Ministry of Agriculture (license No. APAFIS#16827-2018092113192911 v4 to Y.G.).

Consent for publication **-** All authors have approved the manuscript for submission.

Availability of data and materials **-** The authors declare that materials and data are available for readers.

Competing interests **-** The authors declare that they have no competing interests.

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ABBREVIATIONS

ACN, acetonitrile; ADME, absorption, distribution, metabolism, and/or excretion; AF, formic acid; AI, aromatase-inhibitors; CYP, cytochrome P450; ER, estrogen receptor; i.p., injected intraperitoneally; IS, internal standard; LC-MS/MS, liquid chromatography-mass spectrometry/mass spectrometry; M3G, morphine-3-glucuronide; M6G, morphine-6-glucuronide; MOR, Mu opioid receptors; SEM, standard error of the mean; SERM,

selective estrogen receptor modulator; TDM, therapeutic drug monitoring; UGT, UDP-glucuronosyltransferases; SULT, sulfotransferase.

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FIGURES

Fig. 1- Simplified metabolic pathway of tamoxifen in humans. Compounds in green and red are the active and inactive metabolites of tamoxifen, respectively.

Fig. 2- Protocol used to study tamoxifen-morphine drug-drug interactions.

Fig. 3-. Morphine (500µM) inhibits the formation of 4OH-tamoxifen-glucuronide *in vitro*. A t test using the Holm-Sidak method was performed to compare each concentration of 4OH-tamoxifen. n=7 for tamoxifen alone and n=5 in the presence of morphine; *, $p<0.05$. Values are means ± SEM.

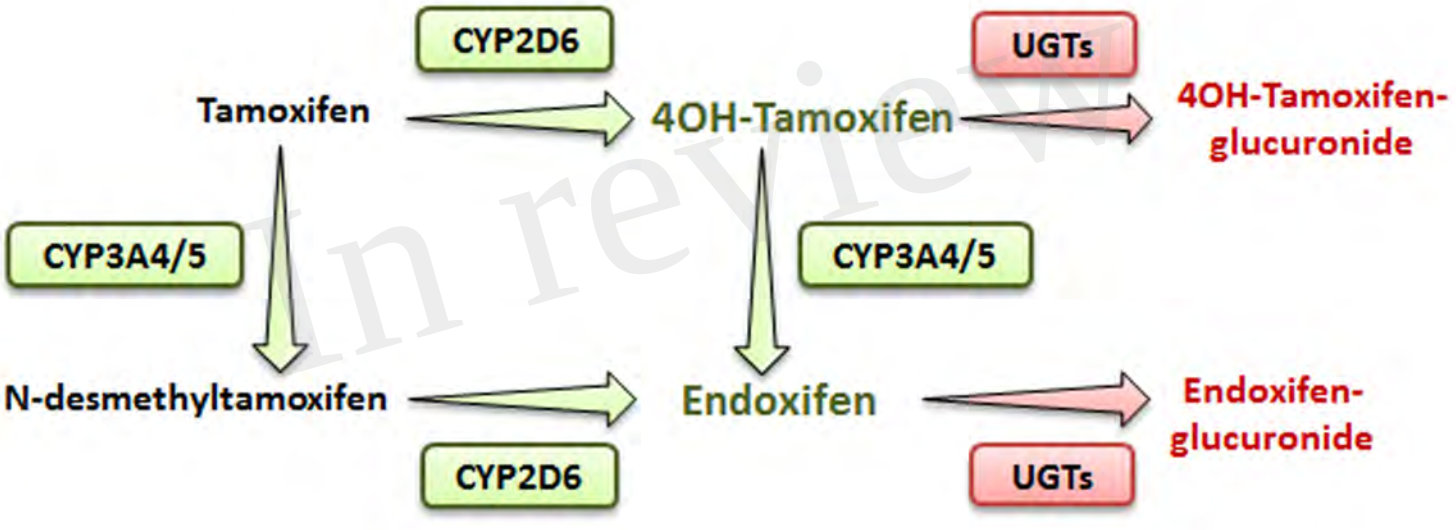
Fig. 4- Tamoxifen metabolism is affected by a prior injection. Effect of 2 subsequent injections of tamoxifen (10 mg/kg i.p.) on the levels of tamoxifen and its metabolites. **A**, Protocol. Injections of NaCl 0.9% at 0h, 1h and 2h are not represented. **B**, Left panels, levels of tamoxifen, 4OH-tamoxifen, N-desmethyltamoxifen, endoxifen, 4OH-tamoxifen-glucuronide and endoxifen-glucuronide during 96h. Right panels correspond to the superimposition of the first 0-48h (white area) and last 48-96h (grey area). The grey area corresponds to an increase in the quantity of the corresponding molecule after the second injection (48-96h). Multiple t tests with the Holm-Sidak correction were applied. Values are means ± SEM. *, $p<0.05$.

Fig. 5- Tamoxifen potentiates its own metabolism. Ratio between metabolites and parent compounds. (**A**) 4OH-tamoxifen/tamoxifen, (**B**) N-desmethyltamoxifen/tamoxifen, (**C**) endoxifen/N-desmethyltamoxifen, (**D**) endoxifen/4OH-tamoxifen, (**E**) endoxifen-glucuronide/endoxifen and (**F**) 4OH-tamoxifen-glucuronide/4OH-tamoxifen. N are indicated within columns. Values are means ± SEM. T tests; *, $p<0.05$; **,$p<0.001$.

Fig. 6- Morphine increases tamoxifen metabolism. Effect of 3 injections of morphine (10 mg/kg i.p.) on the blood concentrations of tamoxifen, 4OH-tamoxifen, N-desmethyltamoxifen, endoxifen4OH-tamoxifen-glucuronide and endoxifen-glucuronide. **A**, Protocol. Injections of NaCl 0.9% at 0h, 1h and 2h are not represented. **B**, Left panels, levels of tamoxifen, 4OH-tamoxifen, N-desmethyltamoxifen, endoxifen, 4OH-tamoxifen-glucuronide and endoxifen-glucuronide during 96h. Right panels correspond to the superimposition of the first 0-48h (white area) and last 48-96h (grey area). Grey area corresponds to an increase of the quantity of the corresponding molecule after the second injection (48-96h). Values are means ± SEM. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$

Fig. 7- Morphine promotes the inactivation of tamoxifen through increased glucuronidation. Effect of 3 injections of morphine (10 mg/kg i.p.) on the ratio between metabolites and parent compounds. (**A**) 4OH-tamoxifen/tamoxifen, (**B**) N-desmethyltamoxifen/tamoxifen, (**C**) endoxifen/N-desmethyltamoxifen, (**D**) endoxifen/4OH-tamoxifen, (**E**) endoxifen-glucuronide/endoxifen and (**F**) 4OH-tamoxifen-glucuronide/4OH-tamoxifen. N are indicated within columns. T tests; *, $p<0.05$; ***, $p<0.001$; ****, $p<0.0001$.

Figure 1.TIF



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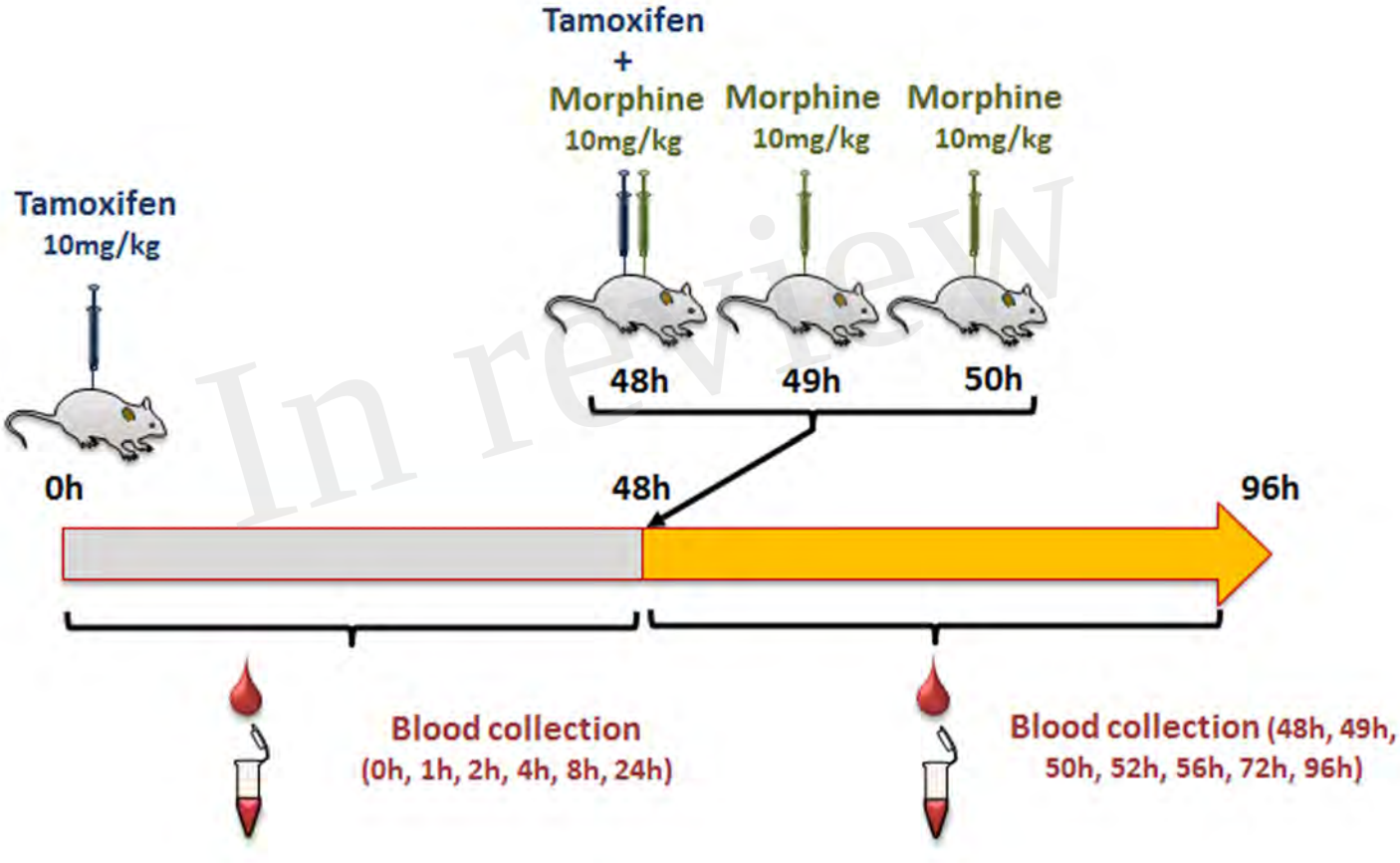

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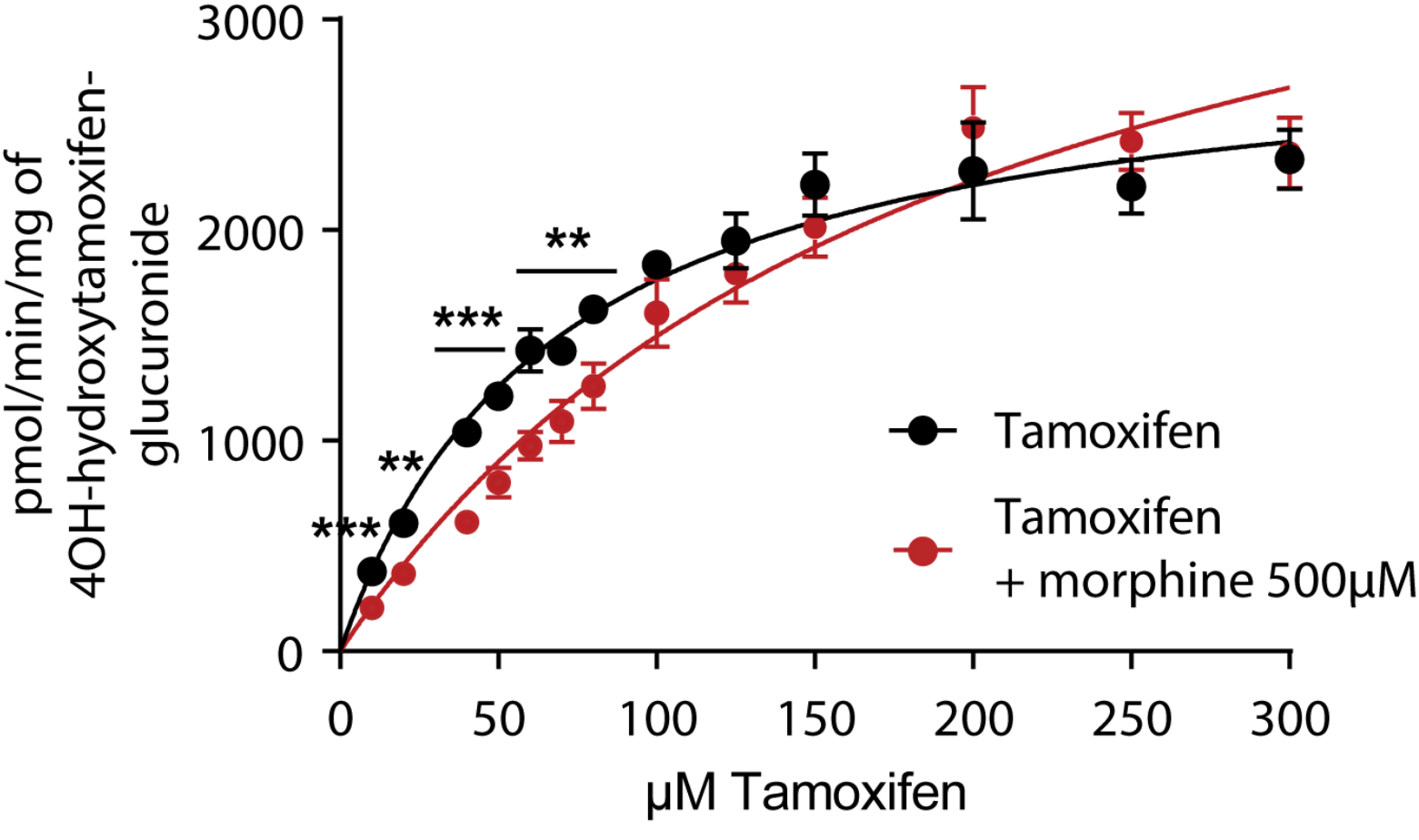


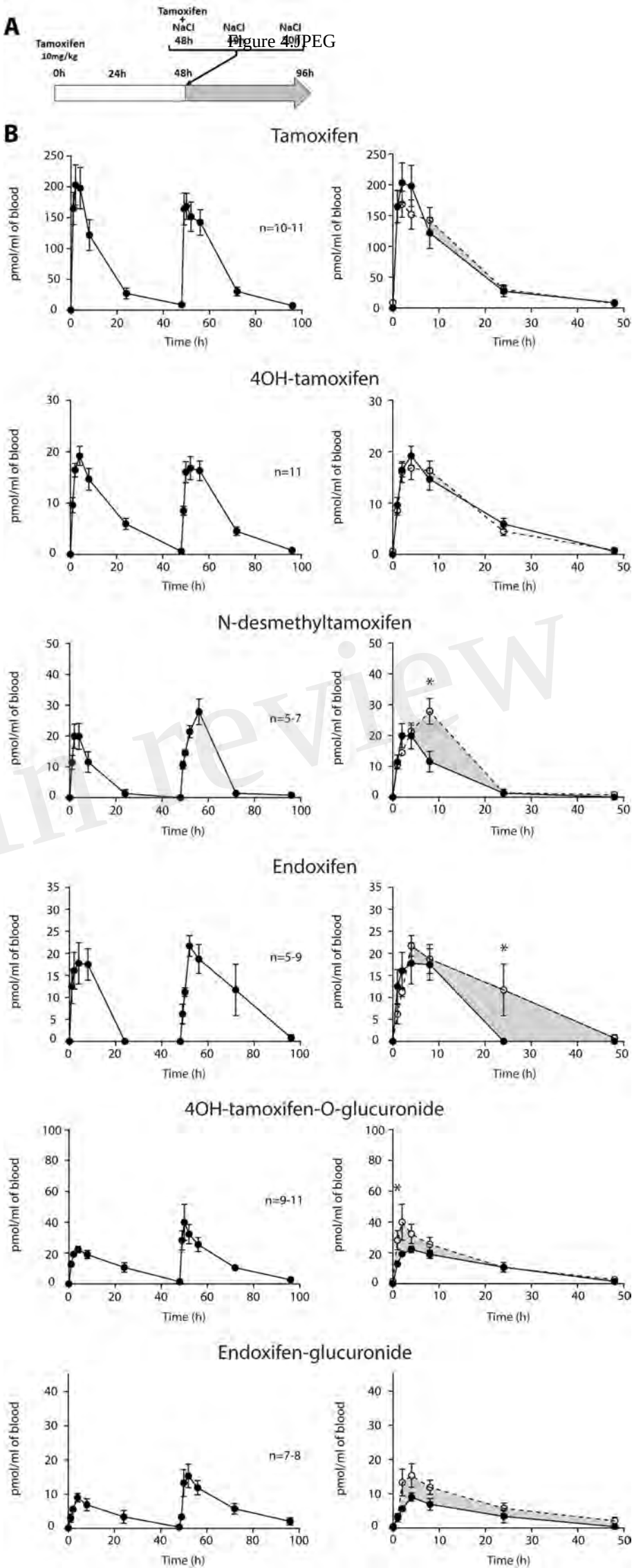
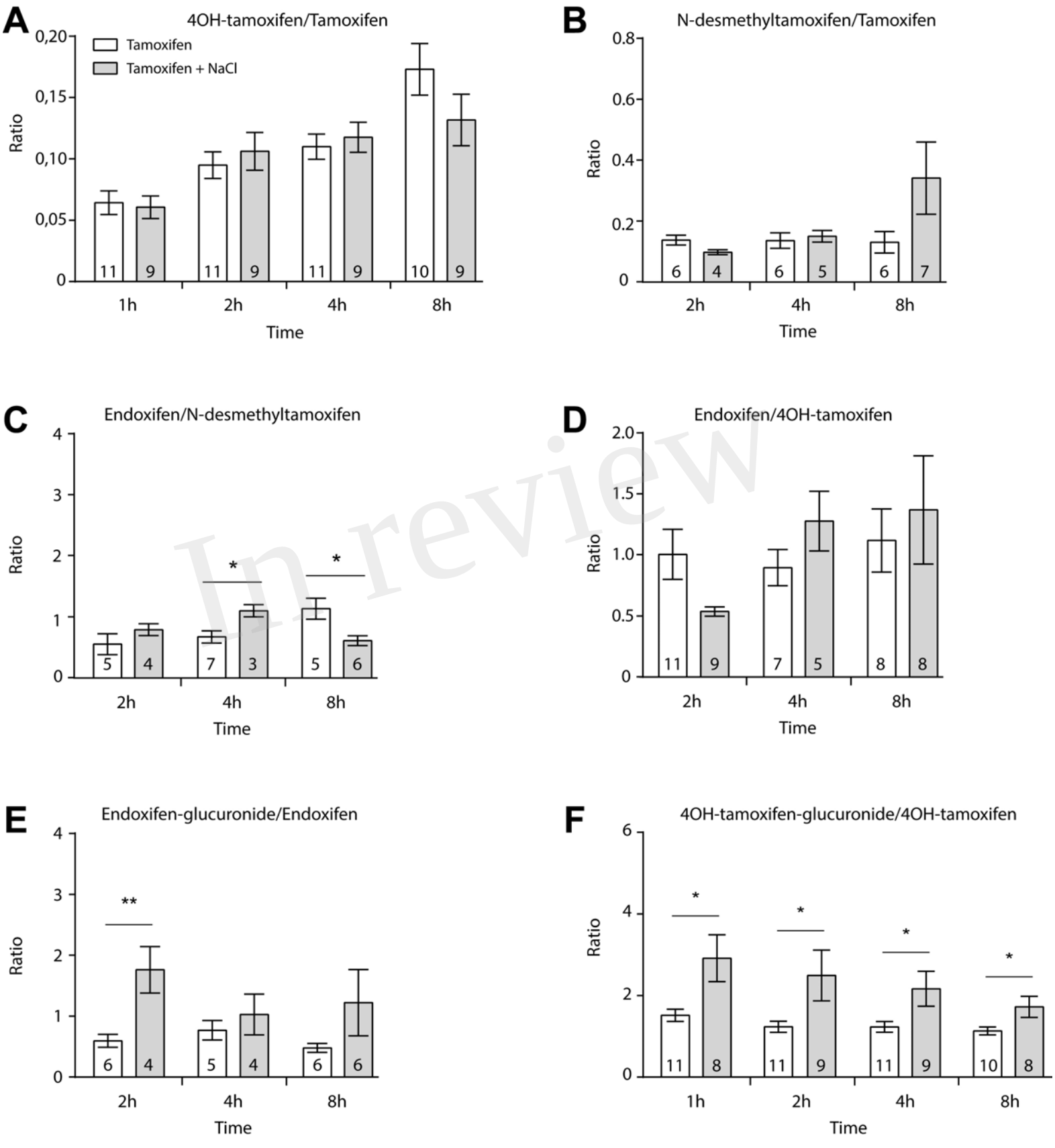


Figure 5.JPEG



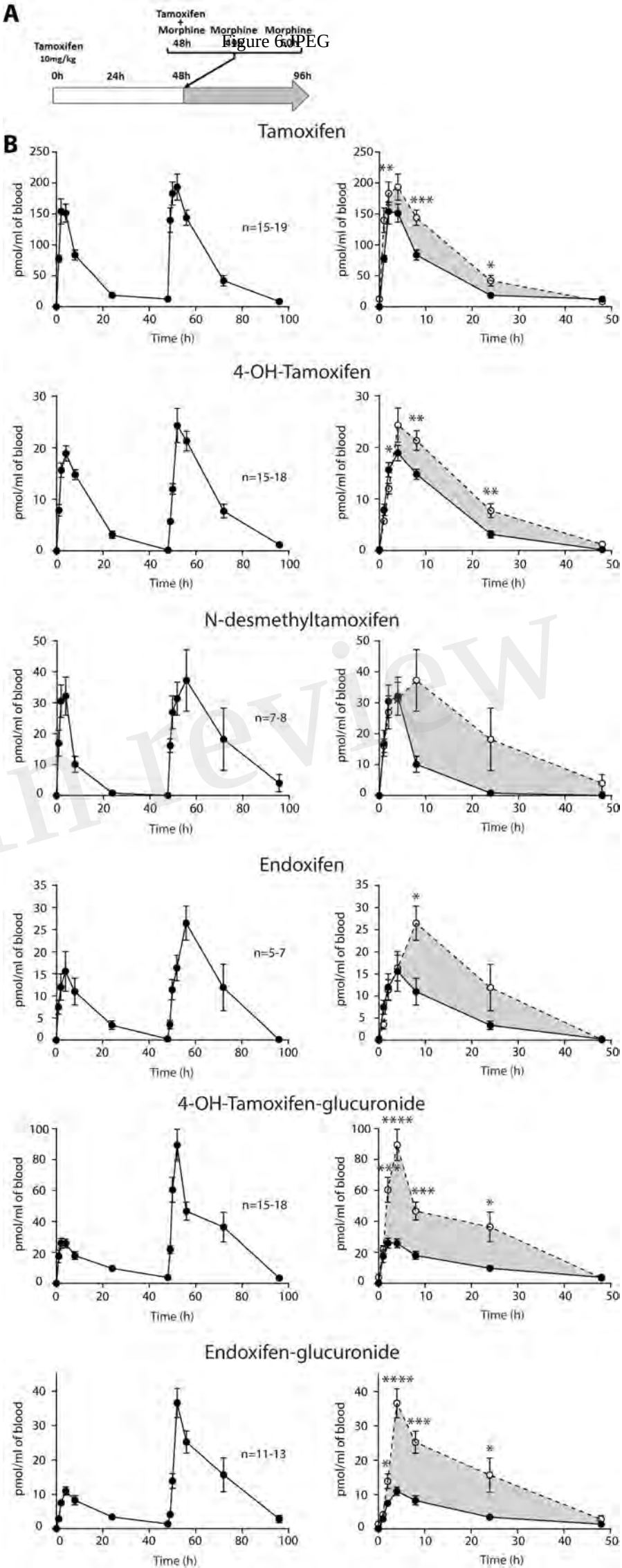


Figure 7.JPEG