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# **Oxidation of tamoxifen by human FMO1 and FMO3 to tamoxifen-N-oxide and its novel reduction back to tamoxifen by human cytochrome P450s and hemoglobin**

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**Running Title:** Tamoxifen-N-Oxide Formation by FMOs and Reduction by CYPs

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Dr David Kupfer passed away on 10th Dec 2004.

**Number of text pages:** 28 (*without* Figures and table); 33 (Total)

**Number of Tables:** 1

**Number of figures:** 4

**Number of references:** 39

**Number of words:** Abstract: 238

Introduction: 538

Discussion: 1077

**Abbreviations used:**

**CYP:** cytochrome P450; **ESIMS:** Electro Spray Ionization Mass Spectrometry;

**FMO:** flavin containing monooxygenase; **Hb:** Hemoglobin; **HLM:** human liver microsomes; **MTM:** methimazole; **OR:** NADPH-P450 oxidoreductase (P450 reductase); **RLM:** rat liver microsomes; **SERMs:** Selective Estrogen Receptor Modulators; **TAM:** tamoxifen; **TAO:** troleandomycin; **TNO:** tamoxifen N-oxide; **TPE:** triphenylethyleneamine;



## ABSTRACT

Tamoxifen(TAM), used as the endocrine therapy of choice for breast cancer, undergoes metabolism forming primarily N-desmethyltamoxifen, 4-hydroxytamoxifen,  $\alpha$ -hydroxytamoxifen and tamoxifen-N-oxide (TNO). Our earlier studies demonstrated that flavin-containing-monooxygenases (FMOs) catalyze the formation of TNO. The current study demonstrates that human FMO1 and FMO3 catalyze TAM N-oxidation to TNO and that cytochrome P450s (CYPs), but not FMOs, reduce TNO to TAM. CYPs 1A1, 1A2, 2A6, 2C8, 2C9, 2C19, 2D6, 2E1 and 3A4 all reduced TNO, with CYPs 2A6, 1A1, and 3A4 producing the greatest reduction. A portion of TAM formed by CYP3A4-mediated reduction of TNO was further metabolized, but not TAM formed by the other CYPs. TNO reduction by CYPs is extremely rapid with considerable TAM formation detected at the earliest time point that products could be measured. TAM formation exhibited a lack of linearity with incubation time, but increased linearly as a function of TNO and CYP concentration. TNO was converted into TAM by reduced hemoglobin (Hb) and NADPH-P450 oxidoreductase, suggesting involvement of the same heme-Fe<sup>2+</sup> complex in both Hb and CYPs. The findings raise the question of whether the reductive activity may be non-enzymatic. Results of this *in vitro* study demonstrate the potential of TAM and its N-oxide (TNO) to be interconverted metabolically. FMO appear to be the major enzymatic oxidants, while several CYP enzymes and even reduced hemoglobin are capable of reducing TNO back to TAM. The possibility that these processes may comprise a metabolic cycle *in vivo*, is discussed.



## INTRODUCTION

Tamoxifen (TAM), an anti-estrogenic triphenylethyleneamine derivative, is currently used extensively for breast cancer therapy and its use has been proposed as a prophylactic agent in healthy women that are considered to be at high risk for breast cancer (Jordan, 1995, Fischer et al., 1998). However, its potential prophylactic benefit is somewhat compromised by an infrequent incidence of TAM-mediated endometrial cancer (Fischer et al., 1998). The mechanism of the endometrial carcinogenic activity of TAM is not fully understood although it has been studied by several investigators, who proposed the formation of TAM-DNA adducts as the potential carcinogenic initiators. Indeed, Shibutani et al (Shibutani et al., 2000) demonstrated the formation of TAM-DNA adducts in women treated with TAM. DNA adduct formation seems to involve a reaction of “activated” TAM metabolites with DNA (Moorthy et al., 1996, Divi et al., 2001). TAM is metabolized by P450 enzymes (CYPs), predominantly into N-desmethyldamoxifen, 4-hydroxytamoxifen and  $\alpha$ -hydroxytamoxifen (Reunitz et al., 1984, Mani et al., 1993a, Lim et al., 1994) and into tamoxifen-N-oxide (TNO; Foster et al., 1980, McCague and Seago, 1986) by the flavin-containing monooxygenases (FMOs; Mani et al., 1993b). It has been proposed that the  $\alpha$ -hydroxylated-TAM metabolites which become O-sulfated are the reactive TAM intermediates that form DNA-adducts (Dasaradhi and Shibutani, 1997, Divi et al., 2001). Additionally, quinone methide metabolites of TAM have been suggested as the reactive compounds in DNA-adduct formation (Fan and Bolton, 2001, Fan et al., 2000). Despite these potential drawbacks, TAM is still considered to be the most useful triphenylethyleneamine (TPE) agent for the endocrine treatment of breast cancer and its safety as a therapeutic drug for up to 5 years treatment, has been established. Nevertheless, relatively novel TPEs, among these



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droloxifene, toremifene and idoxifene, that apparently do not form DNA adducts as avidly as TAM, are being investigated as potential 2<sup>nd</sup> generation endocrine therapeutic agents (Hasmann et al., 1994; Williams and Jeffrey, 1997; Pace et al., 1997).

Of additional interest is our earlier observation that a metabolite(s) of tamoxifen-N-oxide (TNO) binds covalently to hepatic microsomal proteins much less effectively than TAM metabolite(s) (Mani and Kupfer, 1991), suggesting that TNO, if used as a drug, may exhibit fewer undesirable side effects than TAM. That finding and the observations that TNO has similar anti-estrogenic potency to TAM in MCF7 proliferation assay (Bates et al., 1982) and that TNO can be easily reduced back to TAM (*see current investigation*), suggests that TNO could possibly be developed as an agent for treatment of breast cancer patients and/or for the prophylactic use instead of TAM *per se*.

Our earlier studies have shown that mouse FMO catalyzes the transformation of TAM into TNO (Mani et al., 1993b). In the course of that study, it was observed that rat and human liver microsomes incubated in the presence of methimazole (an FMO inhibitor) effectively reduced TNO back to TAM. In the current study, we investigated the role of human FMO enzymes in the conversion of TAM into TNO and the function of human CYPs and other heme-containing proteins in the reductive metabolism of TNO. Our findings that TNO is easily reduced to TAM by CYPs and by hemoglobin (Hb) and possibly by other heme-proteins, suggest that TNO could serve as a prodrug for generation of TAM.



## MATERIALS AND METHODS

**Chemicals.** NADPH, glucose 6-phosphate, glucose-6-phosphate dehydrogenase, methimazole, ketoconazole, troleandomycin, tranlycypromine, 1,2-Didodecanoyl-rac-glycero-3-phosphocholine (DL- $\alpha$ -dilauroyl phosphatidylcholine) and tamoxifen were purchased from Sigma-Aldrich (St. Louis, MO). [N-methyl- $^3\text{H}$ ]-TAM (85 Ci/mmol) was obtained from Amersham Biosciences, UK. Sources of other reagents were as follows: EcoLume, biodegradable scintillation fluid (for scintillation counting), from ICN (Costa Mesa, CA); individual and pooled human liver microsomes, male or female rat liver microsomes, control supersomes (containing “empty” vectors in insect cell line), recombinant human NADPH-P450 oxidoreductase (OR), cDNA-expressed human P450 reductase and cytochrome b<sub>5</sub>, human FMO1 and FMO3 supersomes and cDNA-expressed individual human cytochrome P450s supersomes (provided as microsomal preparations), from BD Biosciences (Woburn, MA); purified E.Coli-expressed recombinant human CYPs, purified recombinant human NADPH-P450 oxidoreductase and purified human cytochrome b<sub>5</sub> from PanVera (Invitrogen Life Technologies, Grand Island, NY), and hemoglobin (Hb) from Schwarz Mann (Orangeburg, New York). All other chemicals were of reagent grade quality.

**Incubation of [N-methyl- $^3\text{H}$ ]-TAM ( $^3\text{H}$ ]-TAM) with cDNA-expressed human FMO1 and FMO3 supersomes.** Incubation mixtures (1 mL) contained 0.6 mL sodium phosphate buffer (pH 8.6, 60  $\mu\text{mol}$ ), 1 mM NADPH, FMO1 or FMO3 and H<sub>2</sub>O with or without methimazole (MTM). After preincubation at 37 °C for 2 min, reactions were initiated with  $^3\text{H}$ ]-TAM (100,000 dpm, 100 nmol) added in 10  $\mu\text{L}$  ethanol and incubated at 37 °C for 30 or 60 min in a Dubnoff metabolic shaker bath



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under air. Controls lacked FMO. At the desired time point, 1 mL ice-cold ethanol was added and tubes were vortexed and centrifuged to eliminate the precipitated proteins at 14,000 rpm for 10 min. The resulting pellet was washed with 1 mL of ethanol and 1 mL methanol, successively. The alcohol supernatants were combined and evaporated at room temperature under nitrogen (N<sub>2</sub>). The dry residue was dissolved in 1 mL ethanol and centrifuged, the filtrate evaporated as above and the residue dissolved in 100 µl ethanol. Metabolites in 0.02 mL were separated by TLC using normal phase LK6DF Silica Gel 60Å. TLC plates containing a fluorescent indicator (Whatman, Inc., Clifton, NJ), developed in CHCl<sub>3</sub>:methanol:NH<sub>4</sub>OH (80:20:0.5) and were quantified by radioscanning as previously described (Mani et al., 1993 a, b). TAM and the corresponding TNO (see synthesis below) were chromatographed in adjacent lanes as standards. The plates were visualized under UV light (254 nm). The percentage of radioactivity in every UV absorbing compound in each lane was determined with System 200 Imaging Scanner (Bioscan, Inc., Washington, DC), and the amount of each metabolite was quantified.

**Synthesis of TNO:** The [N-methyl <sup>3</sup>H]-TAM substrate (70-85 Ci/mmol, Amersham Biosciences) was found to contain a contaminant on TLC chromatography. To purify the TAM, radiolabeled TAM was subjected to TLC and the silica gel zone containing TAM (detected by radioscanning) was scraped, eluted with 15 mL methanol, centrifuged at 3,000 rpm for 15 min at 4 °C, washed again with 15 mL ethanol and recentrifuged as above. The combined alcoholic supernatant containing the pure TAM was evaporated. TNO was synthesized as previously described (Foster et al., 1980; Mani et al., 1993b; Mani and Kupfer, 1991). Purified radiolabeled TAM was mixed with non-radiolabeled TAM to a concentration of 200,000 dpm, 100 nmol per 10 µl HPLC grade methanol. To 1 mL of this solution



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was added 30 % H<sub>2</sub>O<sub>2</sub> (0.5 mL / 15 mg TAM). Following storage in the dark at room temperature for 24 hr, the solution was evaporated to dryness under N<sub>2</sub>. The residual H<sub>2</sub>O<sub>2</sub> was eliminated by adding small amounts of ethanol and by evaporating the solution to dryness (3-times). The residue was dissolved in ethanol to obtain a concentration of 200,000 dpm, (100 nmol / 10 µl ethanol). After ascertaining by TLC that the purity of the TNO was close to 100 %, the TNO was used or stored in ethanol under argon at 4 °C until use.

***Metabolism of TNO by liver microsomes or FMO enzymes*** Incubations were as described for TAM except that a NADPH-regenerating system (glucose 6-phosphate, 10 µmol; NADPH, 0.5 µmol; glucose 6-phosphate dehydrogenase, 2 IU; MgCl<sub>2</sub> 10 µmol) was used for the liver microsomes. Reaction mixtures (1 mL) containing 1 mg of microsomal protein, FMO1 (50 pmol) or FMO3 (150 pmol) were tested to study the metabolism of TNO. The reactions were initiated with [<sup>3</sup>H]-TNO (200,000 dpm, 100 nmol) in 10 µl ethanol and incubated (in 1 mL final volume) at 37 °C for 60 min, unless stated otherwise. Controls contained the same constituents but without the liver microsomes or FMO enzymes. The incubations were terminated as described for incubations of TAM. When aerobic and anaerobic incubations were compared, both contained glucose (5mM), however, the anaerobic incubations were rendered hypoxic with a stream of argon prior to the addition of glucose oxidase (5 U) and catalase (30 U) to catalyze reactions that deplete the residual oxygen (Tscherret-Guth and Wood, 2003).

**Incubation of [<sup>3</sup>H]-Tamoxifen N-oxide with cDNA-expressed human cytochrome P450s supersomes.** Incubation mixtures (1 mL) contained supersomes for cDNA- expressed human cytochrome CYPs 1A1, 1A2, 2A6, 2C8,



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2C9, 2C19, 2D6, 2E1 or 3A4 (each at 100 pmol) and 2 mM NADPH in 60 mM sodium phosphate buffer, pH 7.4. After preincubation at 37 °C for 2 min, reactions were initiated with [<sup>3</sup>H]-TNO (200,000 dpm, 100 nmol) added in 10 µl ethanol and incubated at 37 °C for 20 or 60 min in a Dubnoff metabolic shaker bath. Potential contributions towards this reductive process by NADPH, NADPH-P450 oxidoreductase (OR) and cytochrome b<sub>5</sub> were determined using controls containing NADPH alone or with 100 pmol cDNA-expressed P450 reductase (OR) supersomes or cDNA-expressed OR containing coexpressed cytochrome b<sub>5</sub> supersomes (BD Biosciences, Woburn, MA). Additionally, a reconstituted system (devoid of supersomes) composed of lipid vesicles of 1,2-didodecanoyl-rac-glycero-3-phosphocholine (DL- $\alpha$ -dilauroyl phosphatidylcholine) containing various components was also used to investigate the potential contribution of OR and cytochrome b<sub>5</sub> to the reduction of TNO, as previously described for purified CYPs (Vatsis et al., 1982). Briefly, 150 pmol purified recombinant OR and 100 pmol cytochrome b<sub>5</sub> individually or in combination, were added to 30 µl of DL- $\alpha$ -dilauroyl phosphatidylcholine (1 mg / mL H<sub>2</sub>O), sonicated to form lipid vesicles and incubated with [<sup>3</sup>H]-TNO (100,000 dpm, 100 nmol) in 0.8 mL sodium phosphate buffer, (60 mM, pH 7.4. After preincubation at 37 °C for 2 min, reactions were started with 2 µmol NADPH in 0.2 mL sodium phosphate buffer (20 mM, pH 7.4) containing 10 µM MgCl<sub>2</sub> in a final volume of 1 mL and incubated at 37 °C. After 60 min, 1 mL ethanol was added and the metabolites formed were isolated and quantified by TLC chromatography and radioscanning as described above.

## Incubation of [<sup>3</sup>H]-TNO with HLM or with cDNA expressed human CYPs

**1A1, 2A6 and 3A4 and their respective inhibitors.** HLM or cDNA expressed



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human CYPs 1A1, 2A6 and 3A4 were incubated as described above in the presence or absence of their respective inhibitors [100  $\mu$ M  $\alpha$  naphthoflavone for CYP 1A1, 1  $\mu$ M tranlycypromine for CYP 2A6, and 1  $\mu$ M ketoconazole or 100  $\mu$ M troleandomycin (TAO) for CYP 3A4]. For incubations with TAO, the enzyme preparation (CYP 3A4) was preincubated with TAO in the presence of NADPH at 37 °C for 10 min (to generate the inhibitory species) before adding the substrate.

**Incubation of [<sup>3</sup>H]-TNO with hemoglobin.** Reaction mixtures (0.5 mL) containing [<sup>3</sup>H]-TNO (100,000 dpm, 50 nmol), hemoglobin (Hb) alone or with 75 pmol purified recombinant OR and/or 2 mM NADPH were preincubated for 2 min at 37 °C. The reaction was initiated with 100,000 dpm, 50 nmol [<sup>3</sup>H]-TNO. 1 mL ice-cold ethanol, was added after the preincubation but prior to adding [<sup>3</sup>H]-TNO for the zero time point, or after incubation at 37 °C for 30 min.

**HPLC-ESIMS of the metabolites of tamoxifen N-oxide reduction.** The extract components were separated using a Rheos 2000 solvent delivery system (Flux Instruments). Five microliters of the extract diluted 1:20 with 10 % ethanol were injected via a Rheodyne 8125 injector fitted with a 20  $\mu$ l loop. TNO and its metabolites were separated on a C-18 Hypersil column (3  $\mu$ m particle size, 100 x 1mm i.d.) by elution with 1 % ammonium acetate pH 5.7 in a gradient of 50 % methanol to 100 % methanol (in 7.5 min) and held at 100 % methanol (for 12.5 min). The mobile phase was at a flow rate of 50  $\mu$ l / min. The eluant leaving the HPLC column was directed into a ThermoFinnigan LCQ quadrupole ion trap mass spectrometer fitted with an electrospray ion source. Capillary and HV electrode



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potentials were 3 V and 4.5 kV, respectively. Data was acquired in the positive ion mode, over the mass range of  $m/z$  50-500.

**Data Analysis.** All data reported represent the mean  $\pm$  S.D. of triplicate determinations, unless stated otherwise. All experiments were conducted at least twice. Values were compared with unpaired 't' test and the difference was considered significant if the  $p < 0.05$ .



## RESULTS

**N-oxidation of TAM by human FMOs.** cDNA-expressed FMO1 and FMO3 oxidized TAM into TNO (Fig. 1). In our previous study, human FMO5 was found to be inactive towards TAM oxidation (Hodgson et al., 2000), and hence was not further investigated.

TAM N-oxidation was significantly elevated by increasing concentrations of cDNA expressed FMO1, at 30 min incubation time. Controls incubated in the absence of FMO1, did not generate TNO (not shown). Incubations of TAM with 50 and 100 nM FMO1, along with 0.2 mM MTM inhibited TNO formation by approximately 22 %; 1 mM MTM inhibited TNO formation by 52 and 53 %, respectively (Fig 1A). Increasing incubation time to 60 min increased TNO formation 1.5-fold over 25 nM FMO1 but did not alter the effect of 50 or 100 nM FMO1. The pattern and extent of inhibition in the presence of MTM remained essentially the same as that seen with 30 min incubation (data not shown).

FMO3 (Fig 1B) produced less TAM N-oxidation than FMO1. Nevertheless, as seen with FMO1, N-oxide formation progressively increased at FMO3 concentrations of 50, 100 and 200 nM. Whereas 0.2 mM MTM inhibited the catalysis by 60 – 90 %, in 30 min incubations, MTM at 1 mM entirely inhibited the N-oxidation catalyzed by 200 nM FMO3. Incubation for 60 min, did not enhance TNO formation further than was observed at 30 min incubation time (data not shown).

**Reduction of tamoxifen N-oxide (TNO) to TAM.** TNO was incubated with liver microsomes or with FMOs or CYP enzymes normally found in the hepatic endoplasmic reticulum. FMO1 and FMO3 did not catalyze the reduction of TNO back to TAM (Fig 2A). However, HLM (from a single human donor or from pooled human



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livers) and RLM (from male or female rats) increased TAM formation from TNO as compared to controls, indicating that enzymes other than FMOs present in these livers are involved in the reduction of TNO. To prevent the re-oxidation of TAM back to TNO by FMOs in the liver microsomes, the FMO inhibitor MTM was included in the incubations. The amount of TAM was substantially increased in the presence of MTM (Fig 2B, C, D); higher concentrations of MTM further increased TAM levels.

Heat treated RLM (50 °C for 90 sec) produced greater amount of TAM than that produced by RLM in the absence of heat, suggesting that heat inactivated the FMOs and thus prevented the regeneration of TNO from the newly formed TAM. However, MTM increased TAM accumulation even more than that seen with heat treated RLM, indicating that it was a more effective inhibitor of FMO activities than heat inactivation (Fig 3A). Additionally, more TAM was formed from TNO by HLM in 60 min than in 30 min incubation (Fig 3B). Surprisingly, depletion of oxygen from the incubation medium (depletion of oxygen is known to facilitate CYP reductive activity; Webster et al., 1985) did not significantly alter TAM formation by HLM, (Fig 3C). Control incubations (lacking RLM or HLM) yielded no TAM.

**Identification of CYPs reducing TNO into TAM.** cDNA-expressed human CYP 1A1, 1A2, 2A6, 2C8, 2C9, 2C19, 2D6, 2E1 and 3A4 were examined for their ability to reduce TNO. All of the CYPs examined catalyzed the reduction of TNO, although noticeably higher TNO reduction was observed with CYP1A1, 2A6 and 3A4 (Fig 4). In other experiments (data not shown), for all of the CYPs, the utilization of TNO could be entirely accounted for by TAM. However, for CYP3A4, TNO plus residual TAM accounted for only 70 to 77% of the products, indicating that other products were produced. Fig. 4 also shows that by 20 min, CYP1A2, 2A6, 2C8, 2C9,



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2C19 and 3A4 reduced TNO maximally, whereas CYP1A1, 2D6 and 2E1 continued to catalyze the reduction of TNO even after 60 min incubation.

NADPH alone (buffer without the CYP enzymes) did not reduce TNO to TAM, demonstrating that reducing equivalents alone were not sufficient to support that reduction (see control - Fig 4). However, a small but significant amount of TAM was generated from TNO in incubations containing NADPH and supersomes (vector only) –(data not shown) or by NADPH plus cDNA-expressed P450 reductase (OR) supersomes (without CYPs) in the presence or absence of cytochrome  $b_5$ , indicating some contribution to this process by the OR and possibly by cytochrome  $b_5$  (Fig 4). Indeed our experiments using purified recombinant OR and cytochrome  $b_5$  in a reconstituted system of lipid vesicles (described in Materials and Methods), suggest that supersomes probably contained some cytochrome  $b_5$  which catalyzed TNO reduction albeit to a small extent.

**Reduction of TNO by individual CYP enzymes in the presence of specific CYP inhibitors.** In a related experiment, selective CYP inhibitors,  $\alpha$ -naphthoflavone (CYP 1A1 inhibitor), tranlylcypromine (CYP 2A6 inhibitor), and ketoconazole and TAO (3A4 inhibitors), did not inhibit TNO reduction by their respective cDNA-expressed enzymes. By contrast, TAO and ketoconazole inhibited subsequent metabolism of the TAM formed by CYP3A4, indicating that these compounds are effective inhibitors only of the oxidative catalysis by the respective CYPs (data not shown). This could also mean that different “active” sites of CYPs (which are not amenable to the inhibitors) possibly in the vicinity of the heme or directly involving the heme, are involved in the TNO reduction. In turn, the likelihood of other heme-containing proteins, e.g., hemoglobin (Hb) being able to reduce TNO was also considered (see below).



**Incubation of [<sup>3</sup>H]-TNO with hemoglobin (Hb).** A small amount of TAM was formed by incubation of Hb with TNO (100,000 dpm, 50 nmol) and NADPH, which was evident at the earliest time point that could be examined (here designated zero incubation time), and did not increase at 30 min (Table 1, column III). Whereas reductase per se does not induce the reduction of TNO by Hb (Table 1, column II), Hb + NADPH + OR elicited in 30 min a dramatic increase in the amount of TAM formed (Table 1, column IV). As with the CYPs, NADPH is absolutely essential for this reaction.

**Kinetics of TAM formation from TNO by cDNA expressed CYP 1A1, 2A6 and 3A4.** TNO reduction by CYPs did not increase as a linear function of incubation time. The finding that there was considerable TAM formation at the ostensible 0-time points (no incubation) suggests that ethanol slowed rather than stopped the reaction. Nevertheless, reduction of TNO increased with increasing CYP concentration and increasing concentration of the substrate-TNO (not shown). The amount of TAM formed was 11.5, 20.2 and 29.1 nmol for CYP 2A6, 10.3, 11.7 and 20.8 nmol for CYP 1A1, and 15.5, 19.9 and 23.6 nmol for CYP 3A4 at enzyme concentrations of 25, 50 and 100 nM, respectively. For CYP 3A4, 100  $\mu$ M TAO was added to the incubations to block the further metabolism of the TAM formed.

The observation that there were marked differences in the extent of reduction of TNO by the individual human CYPs (i.e., CYP 2A6, 1A1 and 3A4 being the most active), suggested that the P450 apoproteins contribute to the rate of catalysis; the heme appears to be mainly responsible for the reaction.



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**HPLC-ESIMS analysis.** The product(s) generated by the P450 enzymes reduction of TNO were resolved by liquid chromatography (LC) and identified using mass spectrometry. LC-MS analysis of the metabolites identified a compound with the MW of TAM as the major metabolite. The retention times of TNO and TAM were 11 and 12.8 min, respectively. The  $MH^+$  ion from the product was observed at  $m/z$  372.2 indicating a MW of 371.2. This ion was also obtained with an authentic sample of TAM, thus confirming that the major metabolite was indeed TAM. The  $MH^+$  ion from authentic TNO was observed at 388.2 indicating a MW of 387.2.



## DISCUSSION

This study demonstrates that both human FMO1 and FMO3 oxidize TAM into TNO and that FMO1 is more potent than FMO3 in catalyzing that reaction (Fig 1). Moreover, FMO3 is substantially more sensitive to inhibition of TAM oxidation by the FMO inhibitor-methimazole (MTM). Other distinguishing characteristics of FMO1 and FMO3 have been previously reported. For example, whereas FMO1 N-oxygenates efficiently only *tertiary* amines, human FMO3 N-oxygenates the primary, secondary and tertiary amines (Cashman, 2000).

Earlier we had observed that TNO is metabolized *in vitro* to a much lesser extent than TAM (unpublished), and we speculated then, that TNO may function as a storage form for TAM in the body, yielding TAM as and when required. In the current study it was observed that incubations of TNO with rat or human liver microsomes supplemented with NADPH yield TAM as the major product, with TAM formation increasing further in the presence of the FMO inhibitor MTM (Fig 2B-D). We show here clearly that the microsomal reduction of TNO to TAM is catalyzed not by FMOs but by CYP enzymes.

Moreover, TNO can efficiently be reduced back to TAM by a variety of heme-containing proteins. It is of interest in this context that the ability of hemoglobin to facilitate CYP-like oxidations has been previously reported (Mieyal and Starke, 1994). Several aspects of the reduction indicate that it differs from a classic CYP monooxygenase reaction, in that it (a) occurs extremely rapidly; (b) is catalyzed by numerous CYPs without major selectivity; (c) is not significantly different in anaerobic vs aerobic conditions, and (d) is catalyzed by several heme containing compounds including hemoglobin and perhaps even cytochrome b<sub>5</sub>. These



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characteristics raise the possibility that the reductive reaction may be a chemical reaction involving heme, rather than a classic enzymic reaction. The evidence that reduction reaction rate for CYPs 1A1, 2A6 and 3A4 was not inhibited by their respective inhibitors, supports that idea. Thus the mechanism of the reduction reaction will require further study. The main point in this paper is that the reduction of TNO represents a novel type of reaction for CYP.

This investigation suggests that TAM may undergo oxidation and TNO reduction in the liver in a cyclic fashion. Thus, hepatic FMO1 and FMO3 oxidize TAM to TNO and in turn a certain portion of the TNO formed is reduced back to TAM by CYPs. Additionally, results with Hb *in vitro* suggest that TNO could possibly be reduced while circulating in body fluids by appropriate heme-proteins, e.g., by Hb in red blood cells or even by muscle myoglobin.

TNO has been detected in the plasma of breast cancer patients undergoing TAM therapy (Poon et al., 1993). However, there have not been any studies published as far as we are aware examining whether some of the TNO *in vivo* can be reduced back to TAM in either animals or humans. Of interest is a related study demonstrating a reduction of another amine N-oxide, the endogenous trimethylamine N-oxide in humans using a pharmacogenetic approach (Al-Waiz et al., 1987). In that study, involving individuals with an inherited deficiency of FMO3, approximately 50% of orally administered trimethylamine N-oxide was excreted as trimethylamine, indicating the presence of a functional reductive metabolism of N-oxides in humans; however, the nature of the catalysts and the mechanism involved in the *in vivo* reduction of trimethylamine N-oxide have not been identified. Nevertheless, on the basis of these observations and our *in vitro* findings, it can be



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speculated that the reduction of TNO to TAM in humans *in vivo*, would be a likely occurrence.

There is a tertiary amine N-oxide reduction system in rat liver (mitochondria, microsomes and cytosol) composed of quinone reductase/diophorase and heme (Kitamura et al., 1999). That reduction system was studied primarily with imipramine N-oxide as a substrate, however whether that system could also reduce TNO, has not been evaluated.

CYPs are involved in the reduction of certain classes of organic compounds. For instance, human CYP3A enzyme has been implicated in the reduction of the anticancer prodrug anthraquinone di-N-oxide, a tertiary amine-N-oxide (Raleigh et al., 1998). Our current study demonstrates the participation of human CYP enzymes, among these CYP3A4, in the reduction of the N-oxide of TAM. These findings and the observations that TNO forms far less DNA and protein adducts than does TAM (Umemoto et al., 2000, Dehal and Kupfer, 1999) suggest that TNO could be an attractive candidate for a prodrug of TAM. Other tamoxifen analogs undergoing clinical trials for anti breast cancer treatment, e.g. iodoxifene, toremifene and droloxifene have also been shown to form the corresponding N-oxides (McCague et al., 1990, Jones and Lim, 2002, John et al., 2002). Given the structural similarities between these TPE compounds and TAM, it seems reasonable that N-oxides of these SERM analogs and of related TPEs could also be reduced to their respective tertiary amines.

The concept of using bioreductively activated drugs in treatment of solid tumors has been gaining popularity. Tumors that are hypoxic due to malformed vasculature are thought to be indicators of more aggressive disease and enhanced levels of metastasis as they are resistant to radiotherapy and chemotherapy (Brizel et al.,



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1996, Hockel et al., 1996). As TNO is reduced to TAM in the presence or absence of oxygen, it can be expected that TNO will be reduced in both oxygenated or hypoxic tumors. Biotransformation of TNO by CYP forms expressed extra-hepatically, such as in breast and endometrium may be important in determining the tissue specific effects of TNO. For instance, normal breast tissue and breast tumors were found to contain mRNAs and proteins of certain CYPs (Huang et al., 1996, Warner et al., 1997). Since all CYPs examined by us as well as Hb, were active in TNO reduction, it is highly likely that CYPs and Hb in tumor or breast tissue will reduce TNO to TAM. Consequently to enhance transformation of TNO to TAM, vectors co-expressing for example, CYPs 2A6, 1A1 or 3A4 could be injected directly into breast tumors and thereby trigger these cells to over-express the respective enzyme. In turn, this procedure would likely increase the efficacy of TNO when administered as a prodrug. Such an approach has been successful in the past with cyclophosphamide treatment of tumors (Waxman et al., 1999). However, further studies are needed to explore the effectiveness of TNO as an anti breast cancer agent and the question of its potential toxicology *in vivo* needs to be ruled out before TNO could be aimed for clinical investigation.

## ACKNOWLEDGEMENTS

The authors are indebted to Dr James Evans of our institute for performing the LC-MS analysis and for his help in analysis of the products of TNO reduction. The assistance of Dr. Eszter Hazai with some of our studies and of Ms Elizabeth Euloth with all the secretarial help, is also sincerely acknowledged.



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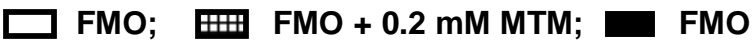


### FOOTNOTES

This study was supported by an NIH grant ES00834 from the National Institute of Environmental Health Sciences and its contents are solely the responsibility of the authors and do not necessarily represent the official views of the NIEHS.

Preliminary account of this investigation was presented at Experimental Biology 2004 Meeting in Washington D.C. (Abstract # 3429).



## Legends

**Fig 1. N-oxidation of tamoxifen by FMO1 and FMO3.** [<sup>3</sup>H] Tamoxifen (100,000dpm, 100nmol) was incubated for 30 min with **A**) 25, 50 or 100 pmol FMO1 or **B**) with 50, 100 or 200 pmol FMO3 in the presence of NADPH without or with 0.2 and 1 mM MTM. Incubation volume was 1 mL. Values of TNO formed are Mean  $\pm$  s.d. of triplicate determinations. Levels were considered significant at  $P \leq 0.05$ . \* indicates significance of **2a** with respect to **1a**, **2b** with respect to **1b** and **2c** with respect to **1c**; \*\* indicates significance of **3a** with respect to **2a**, **3b** with respect to **2b** and **3c** with respect to **2c**; ▲ indicates significance with respect to its 'FMO' group lacking MTM; ■ indicates significance compared to its 'FMO+0.2 mM MTM group; MTM: methimazole; TNO: tamoxifen N-oxide.  **FMO**;  **FMO + 0.2 mM MTM**;  **FMO + 1 mM MTM**

**Fig 2. Incubation of TNO with FMO, HLM and RLM (Formation of TAM).** [<sup>3</sup>H] TNO (200,000 dpm, 100 nmol) was incubated for 1 hr with **A**) 50 pmol FMO1 or 150 pmol FMO3, **B**) HLM (1 mg protein; single donor), **C**) HLM (1 mg protein; pool), **D**) male or female RLM (1 mg protein) in the presence of NADPH regenerating system without or with 0.2 or 1 mM MTM for 1h. Significance level set at  $P \leq 0.05$ . Bars represent the amount of tamoxifen formed. Controls lacked the enzyme preparations (FMO or liver microsomes) but otherwise were similarly treated. \* indicates



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significance with respect to control. \*\* indicates significance with respect to its preceding group. HLM:human liver microsomes; RLM: rat liver microsomes.

**Fig 3. Incubation of [<sup>3</sup>H] TNO (200,000 dpm, 100 nmol) with 1 mg RLM or HLM under various conditions (Formation of TAM).** Enzyme activity was studied under conditions of **A**) heat treatment of microsomes (50 °C for 90 s), **B**) incubation time (30 vs 60 min), and **C**) anaerobiosis vs aerobiosis. Bars represent the amount of tamoxifen formed. **Incubations in A and C panels were for 1 hr.** Respective controls were similarly treated but lacked the liver microsomes. P value of ≤ 0.05 was considered significant. \* represents significance with respect to RLM and \*\* represents significance with respect to preceding group in **A**; \* represents significance with respect to 30 min incubation in **B** and with respect to aerobic conditions in **C**.

**Fig 4. Reduction of tamoxifen N-oxide by CYPs and NADPH oxidoreductase (Formation of TAM).** [<sup>3</sup>H] TNO (200,000 dpm, 100 nmol) was incubated in the presence of only NADPH (control), NADPH+OR without or with coexpressed cytochrome b<sub>5</sub> or 100 pmol cDNA expressed human cytochrome P450 enzymes for 20 min (empty bar) or 60 min (filled bar) in a volume of 1 mL. Each bar represents the average value of TAM formed from two incubations.



**Table 1: Reduction of TNO by Hemoglobin (Hb) forming Tamoxifen**

Amounts of Hb per incubation	Period of incubation (min)	Hb (I)	Hb+reductase (II)	Hb+NADPH (III)	Hb+NADPH+reductase (IV)
50 pmol	0	1.1 ± 0.9	1.0 ± 0.9	6.3 ± 1.8	2.8 ± 0.3
	30	2.0 ± 0.2	1.1 ± 1.0	6.0 ± 0.9*	11.9 ± 1.4**
100 pmol	0	1.5 ± 0.0	2.3 ± 0.9	8.2 ± 1.3	4.5 ± 1.2
	30	1.1 ± 0.9	2.1 ± 0.4	8.1 ± 0.9*	28.2 ± 2.9**

**Conditions as in 'Materials & Methods'.** The incubations contained 2 mM NADPH and [<sup>3</sup>H]-TNO (100,000 dpm, 50 nmol) in a volume of 0.5mL. Data represents Mean values ± S.D. of TAM formed (nmol).

\* indicates significant difference compared to group 'I' and 'II' (p ≤ 0.05)

\*\* indicates significant difference compared to group 'I', 'II' and 'III' (p ≤ 0.05)



Fig 1

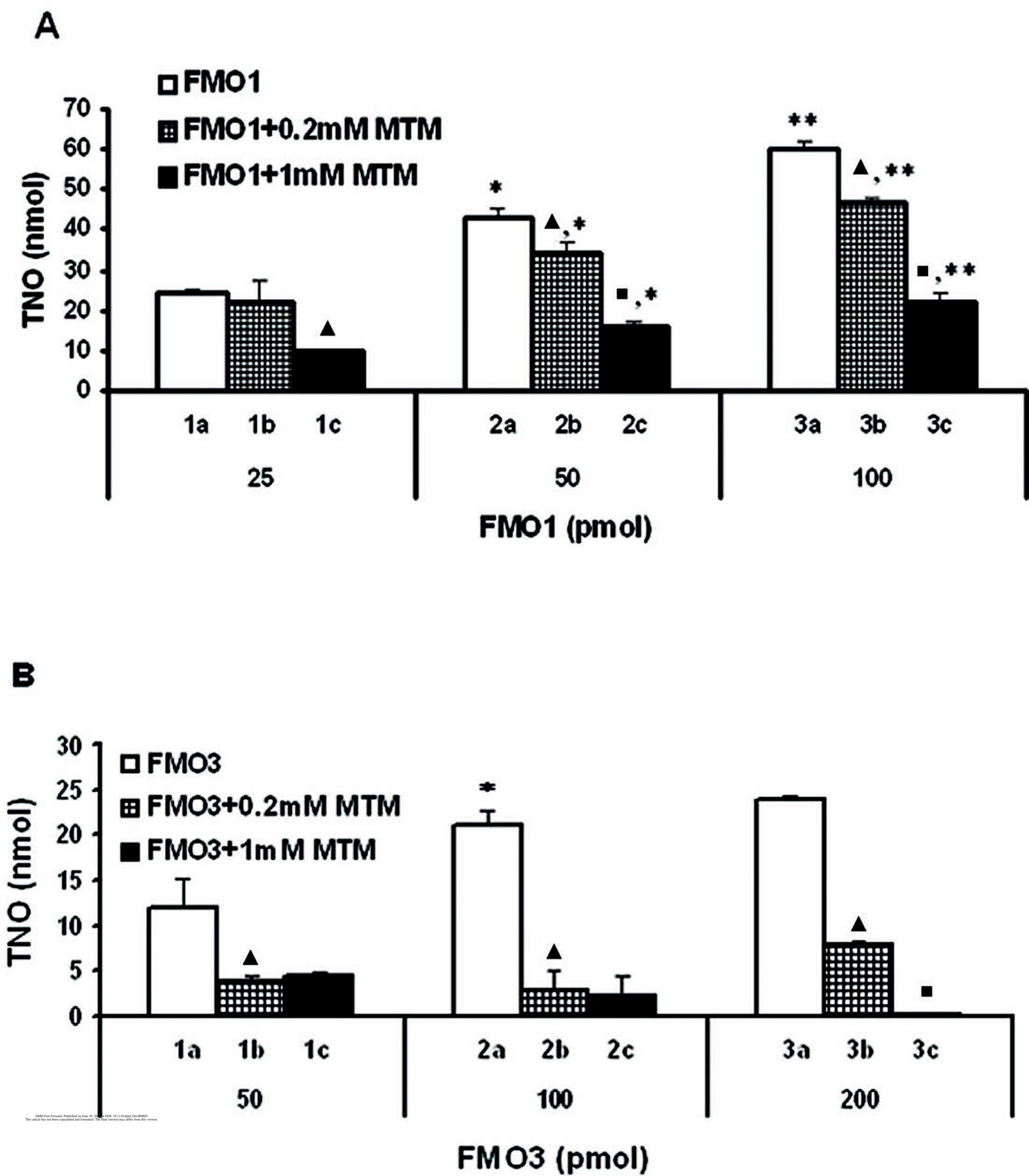
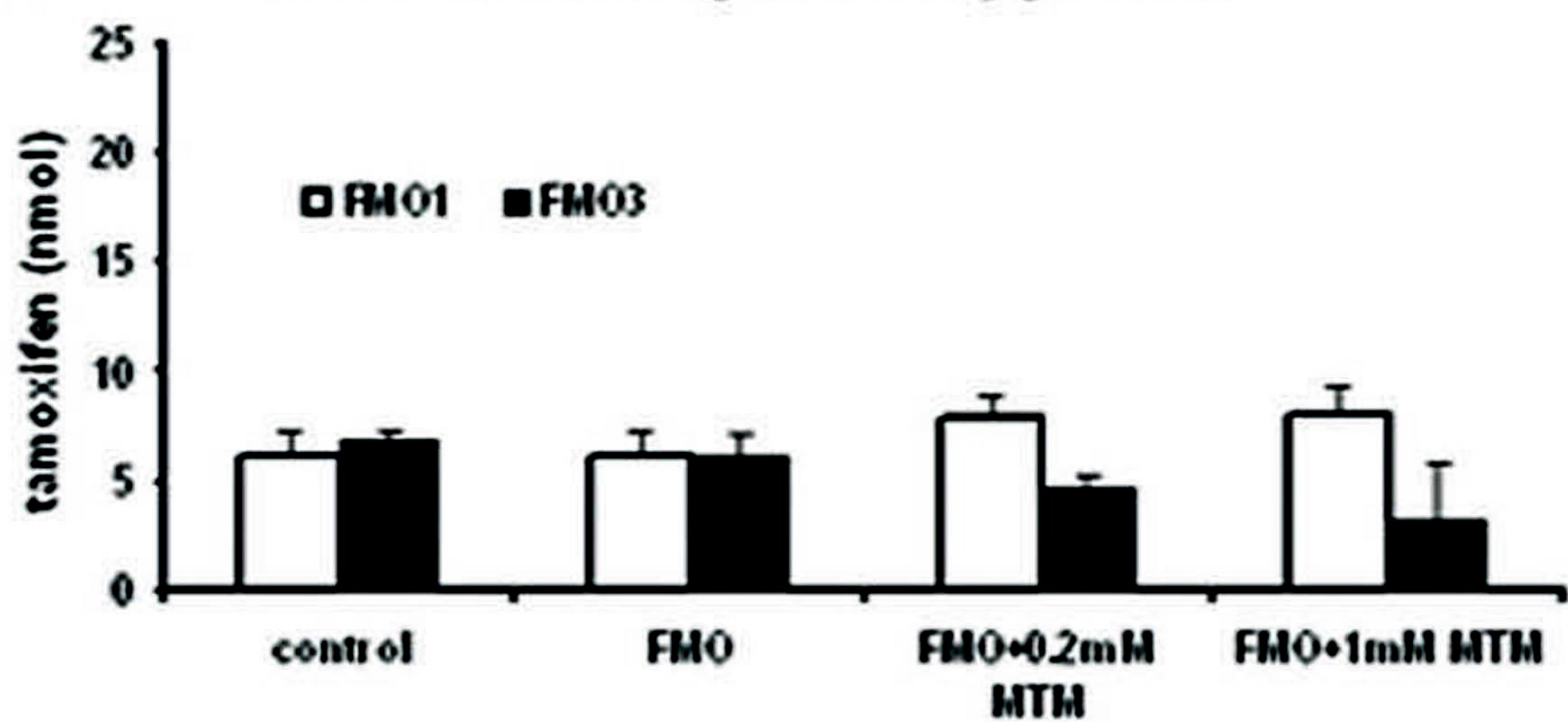


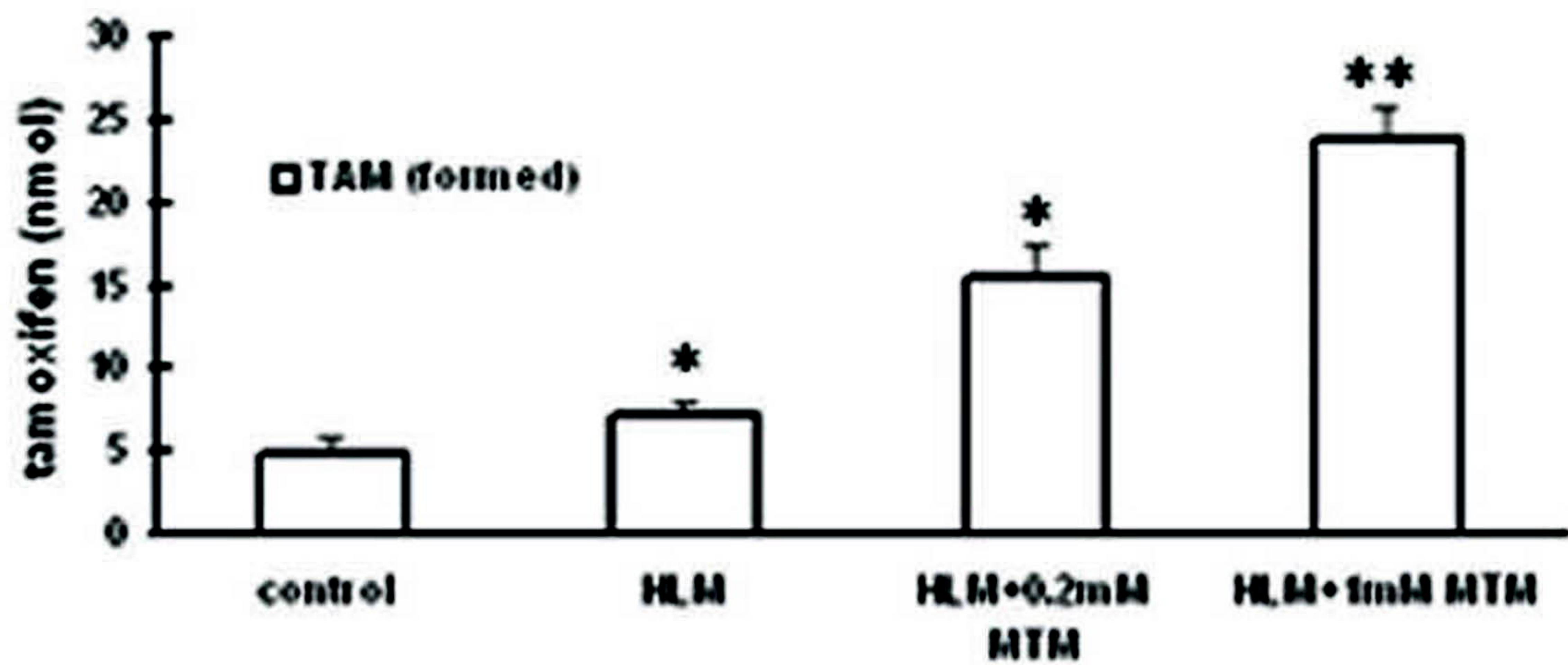


Fig 2

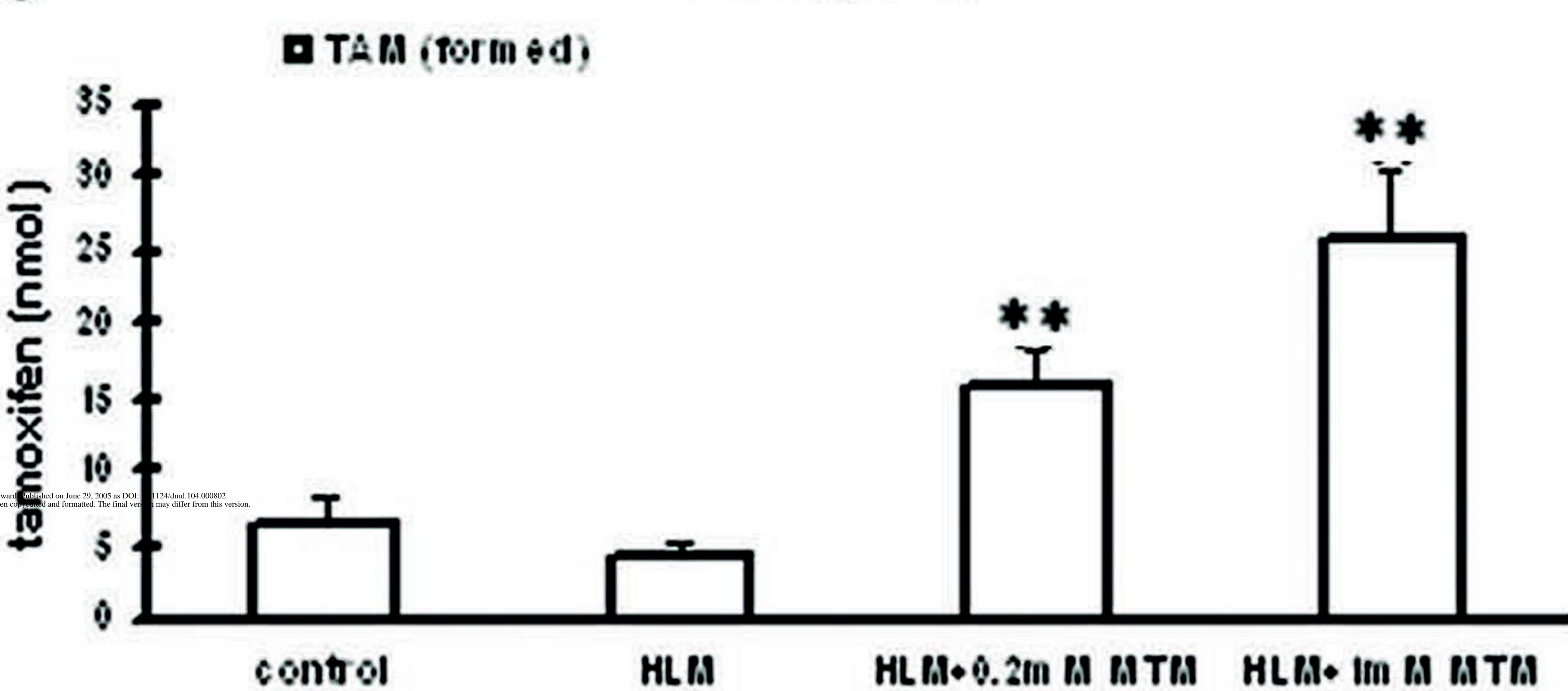
**A** Flavin-containing monooxygenases



**B** HLM (single donor)



**C** HLM (pool)



**D** RLM

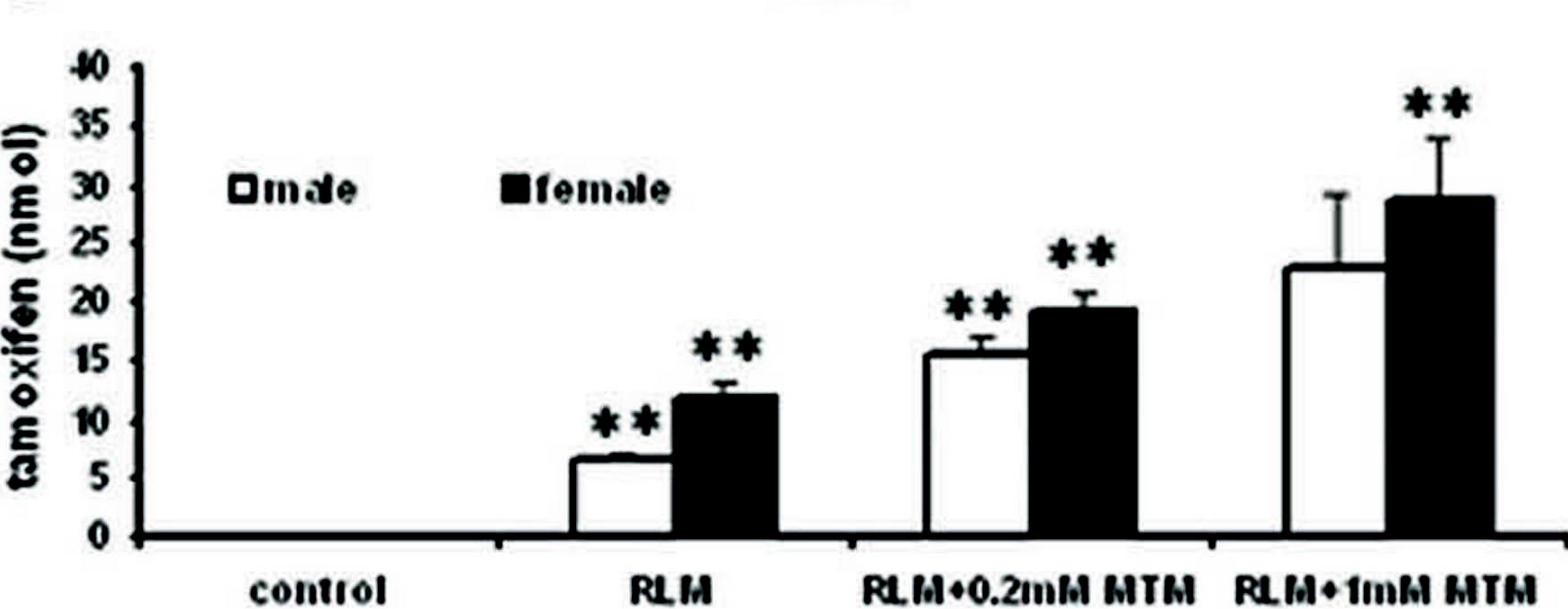




Fig 3

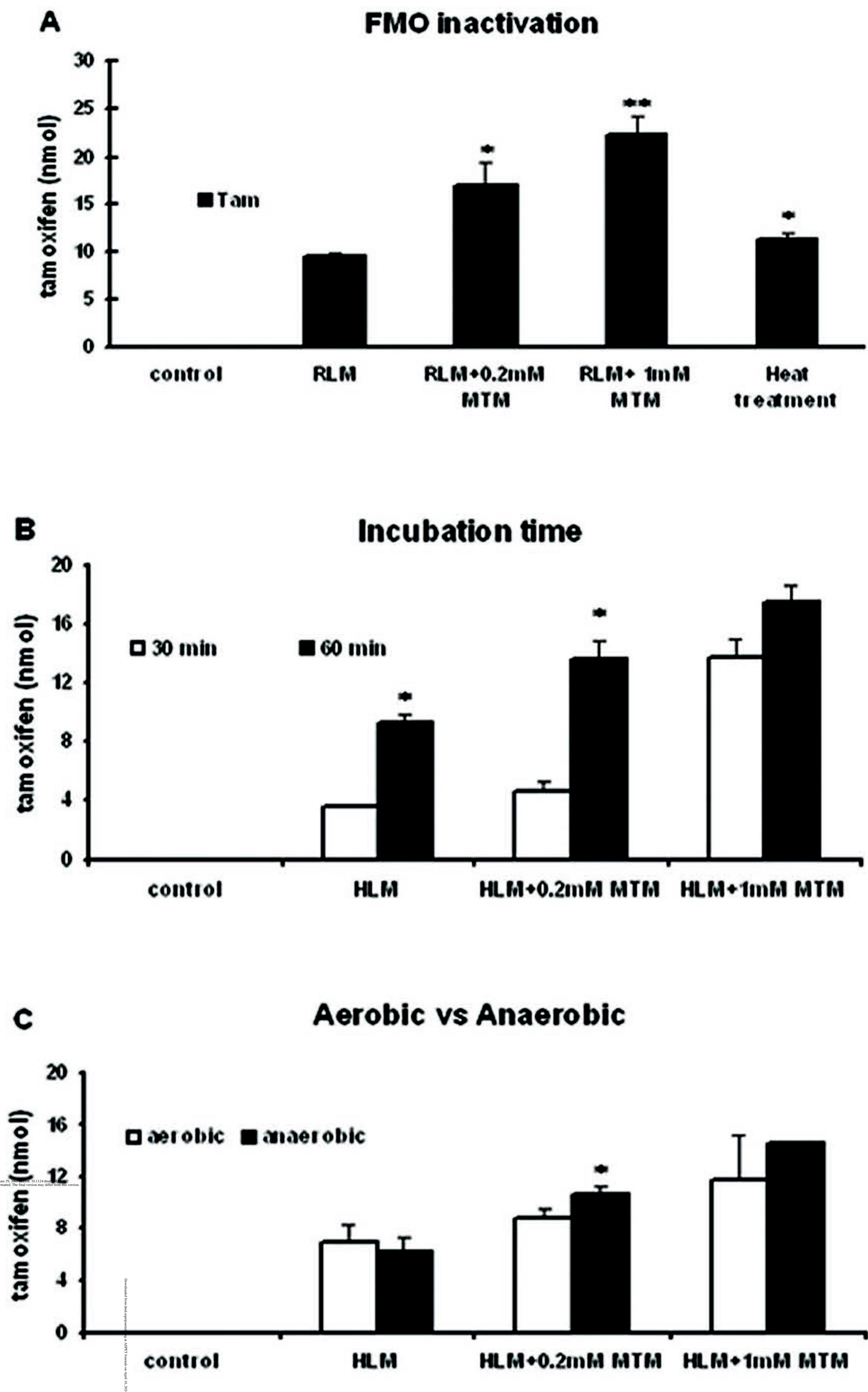




Fig 4

