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Strong Inhibition of Neuronal Nitric Oxide Synthase by the Calmodulin Antagonist and Anti-Estrogen Drug Tamoxifen

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ABSTRACT. The anti-estrogen drug tamoxifen (TMX) was found to act as a strong inhibitor of purified neuronal nitric oxide synthase (nNOS) (IC_{50} = 2 ± 0.5 μM), whereas it was inactive toward inducible macrophage NOS (IC_{50} > 100 μM). TMX affected the activation of NOS by calmodulin, as it not only inhibited L-arginine oxidation to nitric oxide and L-citrulline but also NADPH oxidation and calmodulin-dependent cytochrome *c* reduction catalyzed by nNOS. These results suggest that TMX could exert some of its biological effects by interfering with constitutive NOS-dependent formation of nitric oxide and/or superoxide ion in various tissues. BIOCHEM PHARMACOL **54**;10:1109–1114, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. tamoxifen; calmodulin; constitutive nitric oxide synthase; inhibitor; peroxynitrite

Tamoxifen (TMX)§ (Fig. 1) is a nonsteroidal triphenylethylene anti-estrogen drug widely used for the treatment of hormone-sensitive breast cancer [1–3]. The mechanism of action of TMX is related to its competition with the natural hormone estradiol for binding to the estrogen receptor [4, 5]. Apart from its primary effect on breast cancer cells, it has a significant action in reducing serum cholesterol and helping to limit the development of osteoporosis [6]. As adjuvant therapy for breast cancer, TMX has few undesirable side effects. However, concerns about its potential safety were raised when it was found that long-term administration of TMX to rats at high dose levels gave rise to hepatocellular carcinomas [7]. TMX itself is not genotoxic but can be activated in rat liver into genotoxic intermediates [8]. The molecular mechanisms of action of TMX seem to be the result of antagonism of the mitogenic action of estradiol, the cellular effects including inhibition of protein kinase C, calmodulin-dependent cAMP phosphodiesterase, and modulation of growth factors including transforming growth factor β [9, 10]. Some of these actions likely contribute to the antiproliferative activity of TMX via their involvement in the signaling pathways maintaining cellular viability. In particular, the inhibitory action of TMX on tumor growth seems to correlate with inhibition of the calcium-dependent regulatory protein calmodulin (CaM) [11, 12], and a synergistic induction of apoptosis in breast cancer cells by TMX and CaM inhibitors has been reported [13]. The binding of TMX to CaM has been examined in detail [14, 15], and the inhibition of the Ca^{2+}/CaM signaling pathway has been shown to be responsible for the estrogen receptor-independent cytotoxicity of TMX [11, 12].

Recently, nitric oxide (NO) has emerged as an important signal and effector molecule in mammalian physiology [16, 17]. Nitric oxide is produced by constitutive, Ca^{2+}/CaM-dependent, neuronal and endothelial nitric oxide synthases (nNOS and eNOS, EC 1.14.13.39), as well as by inducible NOSs (iNOS) that are weakly affected by Ca^{2+} but induced by lipopolysaccharides (LPS) and cytokines [18]. NOSs are heme-thiolate proteins that catalyze the five-electron oxidation of L-arginine (L-Arg) to NO and L-citrulline (L-Cit). All NOSs contain two domains separated by a CaM binding site: a C-terminal reductase domain that binds NADPH, FAD, and FMN and exhibits a high sequence identity to cytochrome P450 reductases, and an N-terminal oxygenase domain that binds heme, tetrahydrobiopterin (BH_4), and L-Arg [19]. In fact, NOSs catalyze three kinds of reactions: 1) the reduction of electron acceptors such as cytochrome *c* (Cyt *c*) by NADPH, which only requires the reductase domain and Ca^{2+}/CaM for maximal activity; 2) the reduction of dioxygen to O_2^- by NADPH; and 3) the oxidation of L-Arg to NO and L-Cit by

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§ *Abbreviations:* BH_4, tetrahydrobiopterin; CaM, calmodulin; L-Cit, L-citrulline; Cyt *c*, Cytochrome *c*; DMTMX, *N*-desmethyl-tamoxifen; DTT, dithiothreitol; L-Arg, L-arginine; LPS, lipopolysaccharide; NO, nitric oxide; NO_2Arg, N^{ω}-nitro-L-arginine; n, i, or eNOS, neuronal, inducible, or endothelial nitric oxide synthase; OHTMX, 4-OH-tamoxifen; TMX, tamoxifen.

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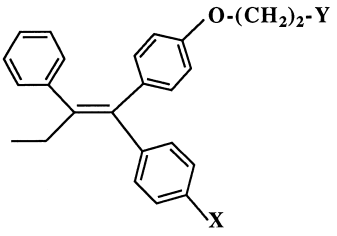

FIG. 1. Structures of tamoxifen and two of its major human metabolites.

Tamoxifen (TMX)	**X = H**	**Y = N(CH3)2**
4-OH-tamoxifen (OHTMX)	**X = OH**	**Y = N(CH3)2**
N-desmethyl-tamoxifen (DMTMX)	**X = H**	**Y = NHCH3**

NADPH and O_2. The latter two reactions are mediated by the heme moiety and require complete NOS [20–23]. Contrary to nNOS and eNOS, the activity of iNOS is almost independent of Ca^{2+} and CaM, as iNOS binds CaM with a very high affinity [18].

In this paper, we report that TMX and two of its major human metabolites, 4-hydroxy-TMX (OHTMX) and *N*-desmethyl-TMX (DMTMX) (Fig. 1) [24, 25], are strong inhibitors of nNOS. This inhibition of nNOS seems to be mainly related to the effects of TMX on the activation of nNOS by the Ca^{2+}/CaM system. Accordingly, TMX is a much less potent inhibitor of murine macrophage iNOS.

MATERIALS AND METHODS

Chemicals

[2,3,4,5-^{3}H]L-Arginine hydrochloride (300 mCi/mmol) was purchased from Dupont-NEN (Les Ulis, France). NADPH came from Boehringer Mannheim Biochemicals (Meylan, France) and tetrahydrobiopterin from Alexis Biochemicals (COGER, Paris, France). Dowex 50W-X8 was from Aldrich Co. (Saint Quentin Fallavier, France). L-Citrulline, L-arginine hydrochloride, N^{ω}-nitro-L-arginine (NO_2Arg), dithiothreitol (DTT), calmodulin, tamoxifen, and all other common chemicals were purchased from Sigma Chemical Co. (Saint Quentin Fallavier, France). 4-Hydroxy-tamoxifen and *N*-desmethyl-tamoxifen were gifts from ICI Pharmaceuticals (Macclesfield, U.K.).

Enzyme Preparation

RECOMBINANT RAT BRAIN NOS (nNOS). *Yeast strain and plasmid. Saccharomyces cerevisiae* strain WR *fur1* derived from strain W303-1.B (*leu-2*, *his-3*, *trp-1*, *ura-3*, *ade2-1*, *can*^{R}, *cyr*$^+$) has been previously described [26, 27]. pYeDP60 (V60) yeast expression vector contains both *URA3* and *ADE2* selection markers and carries an expression cassette consisting of a *GAL10-Cyc1* promoter and *PGK* terminator sequences [28]. The A379 plasmid carrying the rat brain NOS cDNA was a generous gift from Prof. P. O. De Montellano [29] (University of California, San Francisco, CA, USA).

Subcloning of rat brain NOS cDNA into V60. An *Eco*RI digest of A379 plasmid releasing the rat brain NOS open reading frame was ligated to the V60 *Eco*RI-digested expression vector leading to the nNOS-V60 plasmid. Rat brain NOS expression was achieved upon transformation of the WR*fur 1* yeast strain by the nNOS-V60 plasmid as previously described [30].

Purification of rat brain NOS. The culture procedures used to achieve nNOS expression in yeast were identical to those previously described for the inducible NOS [30], and nNOS was purified as described previously [29]. Typically, 4 liters of high density cell culture were pelleted for 10 min at 7,000 × *g*. The pellet was resuspended in buffer A (50 mM Tris HCl, pH 7.4, 1 mM L-Arg, 150 mM NaCl, 3 mM DTT, 5 μM BH_4, and 2 mM $CaCl_2$) containing 20% glycerol, and the cells were mechanically disrupted in the presence of protease inhibitors as described previously [30]. The crude extract was centrifugated for 1 hr at 100,000 × *g* at 4°, and the supernatant was loaded onto a 2-mL calmodulin agarose column (Sigma Chemical Co.) equilibrated with the same buffer. The column was washed with 100 mL of buffer A containing 10% glycerol. The enzyme was eluted with 10 mL of buffer A without $CaCl_2$ but containing 10% glycerol and 5 mM EGTA. The fractions displaying significant absorbance at 400 nm were pooled and concentrated by ultrafiltration on Centricon 50 (Amicon Grace Co., Beverly, MA, USA). The protein was quickly frozen in liquid nitrogen after addition of an equal volume of buffer A containing 40% glycerol and stored at −80°. Under these conditions, approximately 2 mg of nNOS could be routinely purified from 1 liter of culture. Its V_m and K_m values for oxidation of L-Arg to L-Cit and NO by NADPH were 350 ± 50 nmol min^{-1} mg^{-1} and 10 ± 2 μM (toward L-Arg).

RECOMBINANT iNOS. Recombinant iNOS was obtained from yeast *S. cerevisiae* transfected with a gene encoding the murine macrophage isoform and was purified through 2′,5′-ADP-agarose (Sigma) affinity column chromatography, as described previously [30]. nNOS and iNOS were

buffer-exchanged through a Sephadex G-50 column to 50 mM HEPES, pH 7.5, just before use and appeared 95% pure from SDS-PAGE stained with Coomassie Blue. Protein concentrations were determined by the Bradford protein assay kit (Bio-Rad, Ivry sur Seine, France) using bovine serum albumin as standard.

NOS Activity Assays

[^{3}H]L-CITRULLINE FORMATION. NOS-dependent oxidation of L-Arg to L-Cit was determined according to a previously described technique [31]. Briefly, enzymatic reactions were conducted at 37° for 5 min in 50 mM HEPES (pH 7.5) containing 5 mM DTT, 50 μM L-Arg, ca. 500 000 cpm [2,3,4,5-^{3}H]L-Arg, 1 mM NADPH, 1 mM $CaCl_2$, 10 μg/mL CaM, 20 μM BH_4, 4 μM FAD, 4 μM FMN, and the tested agents as indicated. Final incubation volumes were 100 μL. The reactions were started by the addition of protein and terminated by the addition of 500 μL of cold stop buffer (20 mM sodium acetate, pH 5.5, 1 mM L-Cit, 2 mM EDTA, and 0.2 mM EGTA). Samples (500 μL) were applied to columns containing 1 mL of Dowex AG 50W-X8 (Na^+ form, prepared from the H^+ form) preequilibrated with stop buffer, and a total of 1.5 mL of stop buffer was added to eluate [^{3}H]L-Cit. Aliquots were then counted on a Packard Tri-Carb 2300 liquid scintillation spectrometer (Packard Instrument, Rungis, France). Control samples without NOS, CaM, or NADPH were included for background determinations. Activity of iNOS was similarly determined, but $CaCl_2$ and CaM were omitted.

NADPH CONSUMPTION BY nNOS. The initial rates of NADPH oxidation were quantitated spectrophotometrically at 340 nm using an extinction coefficient of 6.2 mM^{-1} cm^{-1} [21]. Cuvettes contained 1 mL of 50 mM HEPES, pH 7.4, 5 mM DTT, 1 mM $CaCl_2$, 500 μM NADPH, 4 μM FAD, 4 μM FMN, 20 μM BH_4, and, when required, L-Arg (1 mM), CaM (10 μg/mL), and the tested compounds. Incubations were run for 5 min at ambient temperature in a Kontron 941 spectrophotometer (Kontron Instrument, Saint Quentin en Yvelines, France) and were initiated by the addition of nNOS.

CYTOCHROME C REDUCTASE ACTIVITY OF nNOS. Initial rates of flavin-dependent reduction of Cyt *c* by nNOS were quantitated spectrophotometrically at 550 nm, using an extinction coefficient of 21 mM^{-1} cm^{-1} [20]. The reaction mixture (final volume, 1 mL) contained 50 mM HEPES, pH 7.5, without DTT, 1 mM $CaCl_2$, 100 μM NADPH, 4 μM FMN, 4 μM FAD, and 40 μM Cyt *c*. Some assays contained CaM (10 μg/mL) and the studied compounds as indicated. The reactions were run for 5 min and were initiated by the addition of nNOS.

TABLE 1. Effect of TMX, OHTMX, and DMTMX on the formation of [^{3}H]L-Cit from [^{3}H]L-Arg catalyzed by purified recombinant nNOS and iNOS from activated murine macrophages

	Activity (% complete system)	
	nNOS	**iNOS**
Complete system	100	100
−CaM	<1	ND
+NO_2Arg (25 μM)	<1	14 ± 4
+TMX (10 μM)	9 ± 4	85 ± 6
+OHTMX (10 μM)	13 ± 6	88 ± 6
+DMTMX (10 μM)	10 ± 2	82 ± 6

[^{3}H]L-Cit formation from [^{3}H]L-Arg was measured as described under "Materials and Methods." Specific activities for purified nNOS and iNOS were 350 ± 50 and 80 ± 20 nmol L-Cit min^{-1} mg $protein^{-1}$, respectively, for the complete system. ND, not determined. Results are expressed as mean ± SEM from three to four experiments.

NITRITE FORMATION FROM ACTIVATED MURINE MACROPHAGES. Murine macrophages were obtained from C3H/HeN mice injected intraperitonally with thioglycollate broth (Institut Pasteur, Paris, France) 3 days before the cells were harvested. Adherent macrophages (3.10^6/mL in RPMI supplemented with 10% fetal calf serum) were incubated for 18 hr at 37° with 10 ng/mL LPS under a 5% CO_2 humidified atmosphere. Activated macrophages were washed twice and put in fresh medium containing 2 mM L-Arg and various concentrations of the studied compounds. Nitrite production [32] and cell viability [33] were measured 24 hr later according to previously described methods.

RESULTS

Recombinant nNOS purified from yeast expressing rat brain NOS catalyzed the oxidation of [^{3}H]L-Arg by NADPH and O_2 to [^{3}H]L-Cit. As expected, this nNOS activity was suppressed in the absence of CaM or in the presence of 25 μM N^{ω}-nitro-L-arginine (NO_2Arg), a well-known NOS inhibitor [17, 18] (Table 1). TMX exerted a concentration-dependent inhibition of the reaction (Fig. 2) with an IC_{50} value of 2.0 ± 0.5 μM. Interestingly, OHTMX and DMTMX inhibited nNOS with similar IC_{50} values (4.0 ± 1.0 and 2.0 ± 1.0 μM, respectively) (Fig. 2).

The inhibitory effects of TMX, OHTMX, and DMTMX toward purified recombinant iNOS were also investigated. TMX weakly inhibited the oxidation of L-Arg to L-Cit catalyzed by purified iNOS (*ca.* 10% inhibition at 10 μM, with an IC_{50} value of approximately 300 μM), whereas 25 μM NO_2Arg strongly inhibited this iNOS activity (Table 1). OHTMX and DMTMX were also poor inhibitors of iNOS (Table 1). These results indicated a clear selectivity of TMX and its two metabolites for nNOS (an IC_{50} more than 100 times lower for nNOS than for iNOS). Moreover, neither the expression nor the activity of iNOS from lipopolysaccharide-induced murine macrophages (detected

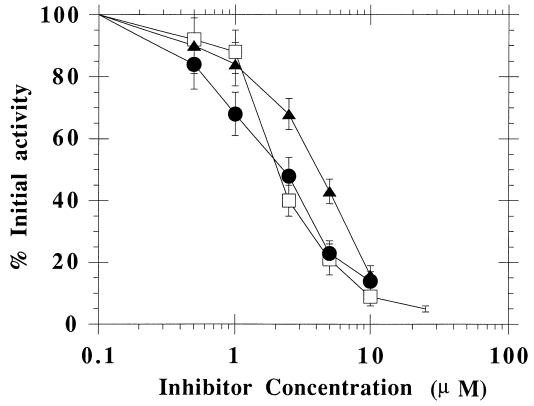

FIG. 2. Effects of increasing concentrations of TMX, OHTMX, and DMTMX on the oxidation of L-Arg to L-Cit catalyzed by purified nNOS. Rates of oxidation of [^{3}H]L-Arg to [^{3}H]L-Cit were measured as described under "Materials and Methods" in the presence of increasing concentrations of TMX (□), OHTMX (●), or DMTMX (▲). Activity in the absence of TMX (100%) was 350 ± 50 nmol L-Cit min^{-1} mg protein^{-1}. Results are means ± SEM from four experiments.

by the release of nitrite ions) was inhibited by 10 μM TMX (data not shown).

Double-reciprocal plots of the nNOS activity ([^{3}H]L-Cit formation from [^{3}H]L-Arg) as a function of L-Arg concentration showed that increasing concentrations of TMX (5 and 10 μM) caused a decrease in the V_m value (310 ± 40, 250 ± 30, and 220 ± 30 nmol min^{-1} mg^{-1} at 0, 5, and 10 μM TMX, respectively) without changing the K_m value of L-Arg for nNOS (10 ± 2 μM, Fig. 3). These results suggested that TMX was not a competitive inhibitor of the nNOS-dependent oxidation of L-Arg. The lack of interaction of TMX at the L-Arg binding site was further confirmed by spectral studies showing that addition of increasing amounts (up to 50 μM) of TMX to nNOS failed to lead

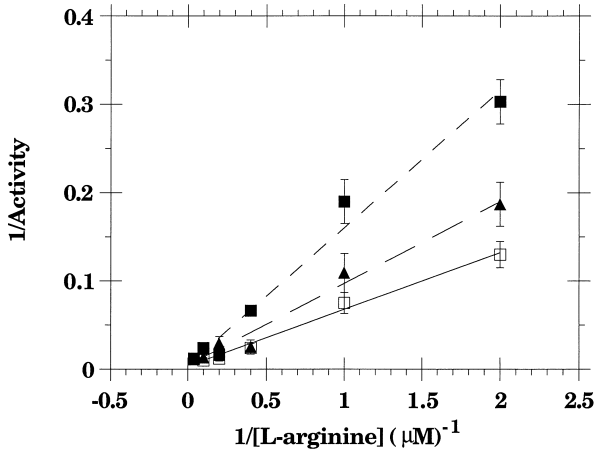

FIG. 3. Double-reciprocal plots of the nNOS activity (L-Cit formation) as a function of L-Arg concentration in the presence of various concentrations of TMX. Oxidation of [^{3}H]L-Arg to [^{3}H]L-Cit was measured as described under "Materials and Methods" in the absence (□) or in the presence of 5 (▲) or 10 μM (■) TMX. Activity expressed in nmol L-Cit min^{-1} mg protein^{-1}. Results are means ± SEM from three determinations.

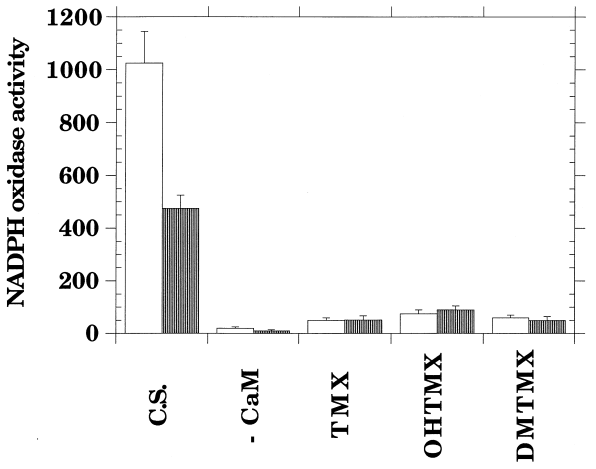

FIG. 4. Effect of CaM, TMX, OHTMX, and DMTMX on NADPH oxidation catalyzed by purified nNOS. NADPH oxidation was assayed as described under "Materials and Methods." C.S., complete system. Maximal activities were 1020 ± 110 and 475 ± 70 nmol of NADPH consumed min^{-1} mg protein^{-1} in the absence (open bars) and in the presence of 1 mM L-Arg (hatched bars), respectively. Experiments were performed in the absence of CaM (−CaM) or in the presence of 10 μM TMX, OHTMX, or DMTMX. Results are means ± SEM from three determinations.

to any change in the visible spectrum of the heme. Furthermore, addition of 10 μM TMX did not modify the K_s value of L-Arg for nNOS (data not shown).

As previously reported, nNOS catalyzes the oxidation of NADPH both in the presence and absence of L-Arg. The reaction did not occur in the absence of CaM (Fig. 4), and its rate was twofold higher in the absence of L-Arg [21]. TMX (10 μM) also strongly inhibited both the L-Arg-independent (Fig. 4, open bars) and the L-Arg-dependent (Fig. 4, hatched bars) oxidation of NADPH. Similar behaviour was also observed with OHTMX and DMTMX (Fig. 4). These results further confirmed that TMX and its metabolites did not simply interact with nNOS at the L-Arg binding site.

The hypothesis of an interaction of TMX with the CaM-dependent activation of nNOS was then tested by studying the effects of TMX on the nNOS-catalyzed reduction of Cyt *c* by NADPH. This flavin-dependent reduction is much faster in the presence of CaM [23] as shown by the 15-fold stimulation of the activity (2.90 ± 0.3 μmol instead of 0.18 ± 0.03 μmol of reduced Cyt *c* min^{-1} mg protein^{-1}) (Fig. 5). TMX and its analogues had no significant effect on the CaM-independent activity, whereas they clearly inhibited the CaM-dependent nNOS-catalyzed reduction of Cyt *c* (*ca.* 75% inhibition at 10 μM TMX, Fig. 5). The aforementioned hypothesis was also tested by measuring the effects of TMX on the oxidation of [^{3}H]L-Arg to [^{3}H]L-Cit in the presence of NADPH, O_2, and various CaM concentrations. An increase in the IC_{50} values of TMX toward nNOS was observed (IC_{50} = 2.0 ± 0.5, 5.0 ± 0.5, and 30 ± 2 μM) when concentrations of CaM used in the incubate were 10, 25, and 50 μg/mL, respectively. These results

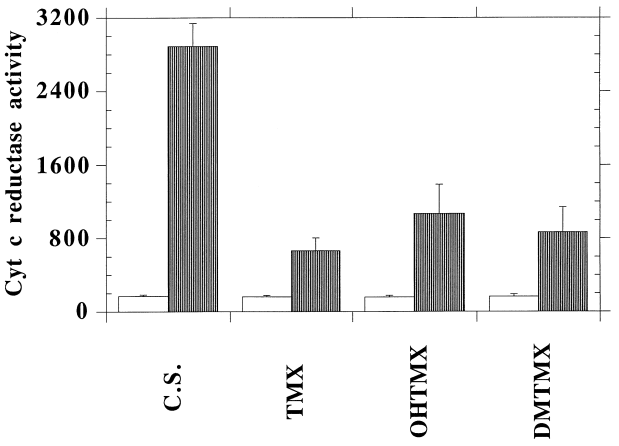

FIG. 5. Effects of TMX and TMX metabolites on the reduction of Cyt *c* by NADPH catalyzed by purified nNOS. Cyt *c* reduction was assayed as described under "Materials and Methods" without CaM (open bars) or in the presence of 10 μg/mL CaM (hatched bars). C.S., complete system without TMX. TMX and analogues (10 μM) were added to the complete system. Activities in nmol Cyt *c* reduced min^{-1} mg $protein^{-1}$. Results are means ± SEM from three determinations.

strongly suggest that TMX, and two of its metabolites, interfered with the CaM-dependent electron transfer from NADPH via the nNOS reductase domain.

DISCUSSION

The aforementioned results show that TMX and its two metabolites, OHTMX and DMTMX, are strong inhibitors of the nNOS-dependent oxidation of L-Arg (an IC_{50} value of approximately 2.0 μM), a concentration of pharmacological relevance. This inhibition could be partly overcome by an increase in calmodulin concentration. TMX does not interact with the L-Arg binding site (Fig. 3 and lack of appearance of any difference spectrum in UV-visible spectroscopy). However, it strongly inhibits the oxidase activity of nNOS in the absence of L-Arg as well as the calmodulin-dependent reduction of cytochrome *c* catalyzed by nNOS (Figs. 4 and 5). Moreover, TMX is a very poor inhibitor of inducible NOS from murine macrophages (IC_{50} > 100 μM). All these results suggest that TMX inhibits nNOS by interfering with the necessary activation of this enzyme by CaM. Its poor inhibitory effects on iNOS (either purified or in LPS-stimulated mouse macrophages) are probably due to the much higher affinity of CaM for iNOS than for nNOS [18]. TMX could thus be compared with calmidazolium, trifluoperazine, or chlorpromazine, which are nNOS inhibitors (IC_{50}: 2, 7, and 15 μM, respectively) [34] and potent CaM antagonists. Our results are in good agreement with previous data concerning the interactions of TMX with CaM-dependent proteins. For instance, the IC_{50} value of TMX for inhibition of bovine brain cAMP-phosphodiesterase is 2 μM [9].

The presence of NOSs in various tumor cell lines has been reported, with some of them being Ca^{2+}/CaM-dependent [18, 35]. Moreover, a correlation between the estrogen receptor status, NOS expression, and tumor grade has been suggested [35], and it has been proposed that estradiol is a strong enhancer of NO release in breast tissue and endothelial cells [35, 36]. Since NO and peroxynitrite (resulting from the reaction of NO with superoxide ion [37]) have been implicated in mutagenic and carcinogenic mechanisms [38, 39], this effect of estradiol could participate in the induction of mutations and malignant diseases. One thus may speculate that the inhibitory effect of TMX toward CaM-dependent constitutive NOS could play a role in the antiproliferative effects of this drug.

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