

# Non-Nuclear Localized Human NOSII Enhances the Bioactivation and Toxicity of Tirapazamine (SR4233) in Vitro

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## ABSTRACT

Tirapazamine (TPZ) is the lead member of a class of bioreductive drugs currently in phase II and III clinical trials. TPZ requires metabolic activation to give a cytotoxic free radical species, and this hypoxia-mediated process is carried out by a variety of cellular reductases, including NADPH cytochrome c (P450) reductase (P540R). Nitric-oxide synthase (NOS) is widely expressed in human tumors, and this enzyme consists of an oxidase and a reductase domain, the latter showing striking homology to P450R. Thus, in this article, we have investigated the role of one of the cytosolic isoforms of NOS [inducible NOS (NOSII)] in the bioactivation of this DNA-damaging antitumor agent. To achieve this, we have constitutively overexpressed NOSII in human breast tumor MDA231 cells by employing an optimized expression vector in which the strong human polypeptide chain elongation factor 1 $\alpha$  promoter drives a bicis-

tronic message encoding the genes for human NOSII and the puromycin-resistant gene (*pac*). Subcellular localization of NOSII in the stably transfected clones was determined after differential centrifugation and showed that NOSII catalytic activity was exclusively cytosolic as determined by conventional activity assay. This was confirmed by immunostaining followed by fluorescent microscopy studies. The increase in NOSII activity in a series of transfected clones was associated with an increase in TPZ metabolism and toxicity under hypoxic conditions. There was no similar increase in aerobic toxicity. These findings are of significance for two reasons. First, cellular NOSII activity, similar to that seen in human breast cancer, could contribute to TPZ toxicity; second, this will be a result of NOS-derived/cytosol-associated TPZ radicals.

Tirapazamine [TPZ (3-amino-1,2,4-benzotriazine-1,4-dioxide, SR 4233, Tirazone)] is a promising antitumor agent that has a unique spectrum of activity in that it selectively causes DNA damage in hypoxic tumor cells after one-electron bioreductive activation. Cytotoxicity results from activation by a reductive enzyme(s) that adds an electron to the parent drug to produce radical species that cause DNA single-strand breaks, double-strand breaks (DSBs), and chromosome aberrations (Wang et al., 1992; Brown, 1993; Daniels et al., 1998; Kotandeniya et al., 2002). It is currently undergoing phases II and III clinical trials for the treatment of various cancers (Lee et al., 1998; Del Rowe et al., 2000; von Pawel et al., 2000; Gandara et al., 2002).

Considerable advantage would be gained by exploiting the inherent metabolic differences that distinguish malignant from normal tissues. Many cellular enzymes are able to me-

tabolize TPZ, and their location within the cell could be significant in governing the cellular response to the drug. In addition, knowledge gained of the enzyme(s) involved in drug reduction and toxicity would provide an ideal means of identifying tumors that are likely to be responsive to the drug. The high incidences of tumor nitric oxide synthase (NOS) expression reported in many clinical studies (Thomsen et al., 1994, 1995; Cobbs et al., 1995; Rosbe et al., 1995; Gallo et al., 1999; Swana et al., 1999), strongly suggest that NOS is widely expressed and often up-regulated in multiple tumor tissues. Hence, it presents an attractive target to improve cancer therapy. First, it generates nitric oxide (NO), which is important in tumor angiogenesis and maintaining vascular homeostasis. Second, NOS-dependent pro-drug activation offers a number of fundamental advantages over other endogenous enzyme-prodrug paradigms. Its dimeric nature provides two distinct catalytic domains that will bioactivate a broad repertoire of established and novel chemotherapeutic

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**ABBREVIATIONS:** TPZ, tirapazamine; P450R, NADPH cytochrome c (P450) reductase; NO, nitric oxide; NOS, nitric-oxide synthase; iNOS, inducible nitric-oxide synthase (NOSII); MTT, 3-(4,5-dimethylthiazal-2-yl) 2,5-diphenyltetrazolium bromide; DHR, dihydrorhodamine; DAPI, 4'-6-diamidino-2-phenylindole; DSB, double-strand break; SR4233, 3-amino-1,2,4-benzotriazine-1,4-dioxide; SR4317, 3-amino-1,2,4-benzotriazine-1-N-oxide; FCS, fetal calf serum; PBS, phosphate-buffered saline; wt, wild type.

prodrugs. The reductase domain shares a high degree of sequence homology with NADPH cytochrome *c* (P450) reductase (P450R), known to be important for the bioactivation of many bioreductive drugs (Bredt et al., 1991). We have recently exploited the structural similarities between NOS and P450R and demonstrated that the former could metabolize a number of anticancer cytotoxins, including TPZ (Garner et al., 1999). However, nontumor NOS expression is of little consequence to bioreductive drug activation because cytotoxicity is restricted by the presence of oxygen. This requirement for a reductive environment refines the specificity of the pro-drug target, because low oxygen tension (hypoxia) is a unique characteristic of solid tumors. In addition, unlike P450R, NOSII is essentially cytosolic (remote from the nuclear compartment), thereby suggesting that cytosolic activation of antitumor agents such as TPZ away from the vicinity of DNA can still contribute significantly to its toxicity. The aim of this study is to employ a specifically designed plasmid vector to highly express cytosolic NOSII and to characterize its role in the metabolic activation and toxicity of TPZ.

## Materials and Methods

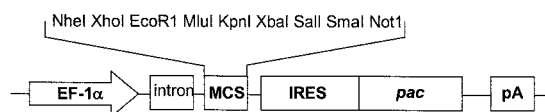
### Drugs and Chemicals

TPZ (3-amino-1,2,4-benzotriazine-1,4-dioxide, SR4233) and its 2-electron reduced product SR4317 were generously provided by Dr. M. Jaffar (Drug Action and Design, School of Pharmacy, University of Manchester, UK.). All other reagents (unless otherwise stated) were of high analytical grade and were purchased from Sigma Chemical Co. (Poole, Dorset, UK).

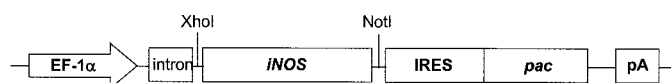
### Construction of the Eukaryotic pEF-iNOS Expression Unit

*EcoRI*-linked NOSII cDNA (GenBank accession no. X73029) cloned as a *NotI* fragment into pBluescript SKII (+), was kindly provided by Prof. Ian Charles (The Rayne Institute, University of London, UK). The NOSII cDNA was then isolated by partial *EcoRI* digestion followed by restriction with *XhoI/NotI* and then cloned into a mammalian expression vector, F373 (kindly provided by Dr. Stephen Hobbs, CRUK Centre for Cancer Therapeutics, ICR, London, UK) (Fig. 1) cut with the same enzymes. The resultant plasmid (pEF-iNOS-puro; Fig. 1) encoded a bicistronic message driven by the human elongation factor 1 $\alpha$  promoter. This allowed the translation of both NOSII and *pac* (the gene responsible for puromycin resistance) from a single transcript by virtue of an internal ribosomal entry site upstream of the *pac* gene. By linking the expression of the cDNA of interest with the selectable marker gene (*pac*) at the level of the mRNA, antibiotic selection directly enforces expression of NOSII. We have observed that this arrangement will efficiently produce stable clones.

#### F373



#### pEFiNOS-puro



**Fig. 1.** Schematic illustration of the mammalian expression vector, F373, and the vector construct, pEFiNOS-puro, containing the NOSII gene that encodes NO synthase and the puromycin resistance gene.

### Cell Culture

Tissue culture medium was obtained from Invitrogen (Carlsbad, CA) and fetal calf serum (FCS) from Sigma. The human tumor cell line MDA231 (breast adenocarcinoma), was obtained from the European Collection of Animal Cell Cultures (ECACC, Salisbury, UK). Cells were grown in Dulbecco's modified Eagle's medium supplemented with 2 mM glutamine, 10% FCS at 37°C, and 5% carbon dioxide. Puromycin-resistant clones were grown under identical conditions in media containing 2.5  $\mu$ g/ml puromycin.

### Transfection of the NOSII cDNA Expression Unit into MDA231 Cells

Before transfection into MDA231 cells, the pEF-iNOS-puro expression vector was linearized by restriction at the unique *NdeI* site and 10  $\mu$ g was used to transfect  $5 \times 10^6$  MDA231 cells in exponential growth, using standard electroporation techniques (van den Hoff et al., 1992). Forty-eight hours later, cells were plated at low density into Dulbecco's modified Eagle's medium supplemented with 2 mM glutamine and 10% FCS and containing 2.5  $\mu$ g/ml puromycin. Individual colonies were isolated 10 to 14 days later. The F373 empty vector served as the control for the NOSII transfection in all experiments. To avoid loss of viability through the cytotoxic consequences of excessive NO production, clones were grown in the presence of a nontoxic dose (100  $\mu$ M) of the NOS inhibitor *N*<sup>ω</sup>-nitro-L-arginine. Clonal lineages were assessed for uniformity by immunohistochemical techniques combined with flow cytometric single-cell analysis and those of single cell parentage were expanded for detailed characterization. Stability of established clonal populations were monitored by carrying out multiple passages ( $n = 10$ ) in both the presence and the absence of puromycin.

### Clonal Characterization

**Preparation of Cell Lysates.** Cells in exponential growth phase were washed twice with phosphate-buffered saline (PBS) and then gently scraped in ice-cold PBS. Subsequent steps in lysate preparation were as described previously (Chinje et al., 1999). The resultant extract was stored in aliquots at  $-80^\circ\text{C}$  until used.

**Preparation of Cytosolic Fractions.** Cytosolic fractions from the various cell pellets were prepared from cells in the exponential growth phase and lysed as above. The cellular lysate was centrifuged at 100,000g at 4°C for 45 min. The cytosolic supernatant was stored at  $-70^\circ\text{C}$  until use.

**Isolation of Intact Nuclei.** Isolation of intact nuclei was achieved by employing a Sigma Nuclei Pure Prep Isolation Kit. Essentially, cells in exponential growth phase in T-75-cm<sup>2</sup> flasks were washed twice with ice-cold PBS. After addition of 10 ml of ice-cold lysis solution (provided in kit) containing 1  $\mu$ M DL-dithiothreitol and 10% Triton X-100 to each flask, cells were harvested and lysed by thoroughly scraping each flask with a small-bladed cell scraper. The entire cell lysate from each flask was then transferred to a separate 15-ml centrifuge tube, vortexed briefly, and incubated on ice for 5 min. Nuclei were isolated and purified by centrifugation through a 1.8 M sucrose cushion at 30,000g at 4°C in a swinging bucket rotor with transparent centrifuge tubes. The nuclei were visible as a small, thin pellet at the bottom of each tube. The supernatant (cytosol and cell debris) and the clear sucrose cushion layers were aspirated without disturbing the pellet of purified nuclei. The nuclear pellet was washed twice to remove any contamination and finally resuspended in a small volume of nuclei storage buffer (provided in kit) and set on ice. After triturating 5 to 10 times with a micropipette to help break up clumps of nuclei, aliquots were then stored at  $-80^\circ\text{C}$  until analyzed.

### NOSII Oxygenase Activity by Citrulline Assay

NOS oxygenase catalytic activity was measured by monitoring the conversion of L-[U-<sup>14</sup>C]arginine to L-[U-<sup>14</sup>C]citrulline as described previously (Chinje et al., 2002).

## NOSII Reductase Activity

NOS reductase activity was determined spectrophotometrically as the NADPH-dependent reduction of cytochrome *c*. Each incubation comprised 500  $\mu$ l of cytochrome *c* (final concentration, 200  $\mu$ M), 100  $\mu$ l of 10 mM potassium cyanide (final concentration, 1 mM), 5  $\mu$ l of 1.5 mM tetrahydrobiopterin (final concentration, 7.5  $\mu$ M), calmodulin (250 units/ml), and 10 to 100  $\mu$ g of lysate protein made up to 0.98 ml with 100 mM potassium phosphate buffer, pH 7.6, containing 3  $\mu$ M calcium chloride. The reaction was equilibrated to 37°C and was initiated by addition of 20  $\mu$ l of 10 mM NADPH (final concentration, 200  $\mu$ M), and the rate of reduction of cytochrome *c* was monitored at 550 nm for 2 min against a blank without NADPH. Initial rates were determined and activity was calculated using a molar extinction coefficient of 21 mM<sup>-1</sup> cm<sup>-1</sup> at 550 nm and expressed as nmol cytochrome *c* reduced per minute per milligram of cell lysate protein.

## DT-Diaphorase Assay

DT-diaphorase activity was determined spectrophotometrically as the dicoumarol-inhibitable component of the NADPH-dependent reduction of cytochrome *c* (Robertson et al., 1994).

## Western Blotting

For Western immunodetection, cytosol and nuclei proteins were separated by SDS-polyacrylamide gel electrophoresis and electrophoretically transferred to a protein binding membrane. A highly purified rabbit NOSII polyclonal antibody raised from a synthetic peptide derived from the extreme C terminus of the human NOSII protein (Zymed Laboratories, Inc., South San Francisco, CA) was used at a final concentration of 2  $\mu$ g/ml. Samples were loaded based on equal amounts of protein.

## Immunocytochemical Analysis

For immunocytochemical staining of NOSII protein expression within the clones, 10<sup>4</sup> cells/well were seeded into six-well plates into which sterile glass cover slips had been placed. Cells were allowed to attach for 24 h at 37°C, washed with PBS, and fixed with 1 ml of 10% formalin (in PBS) for 20 min at room temperature. After each incubation step, the cells were washed three times with PBS. To stain for intracellular NOSII, the cells were permeabilized with PBS/0.1% Triton X-100 for 15 min on ice. The cells were blocked with PBS/10% horse serum for 30 min followed by incubation with PBS/1% horse serum for a further 10 min before incubating with the primary antibody. The cells were incubated with the primary antibody rabbit polyclonal  $\alpha$  NOSII (Zymed Laboratories), diluted 1:1000 in PBS/1% horse serum, for 2 h in a damp atmosphere. The secondary antibody fluorescein isothiocyanate-labeled goat  $\alpha$  rabbit IgG (DAKO, Bucks, United Kingdom) diluted 1:50 in PBS/1% horse serum, was used to coat the cells for a further 2 h in a damp and dark atmosphere. After completion of two PBS washes, the cell-coated coverslips were coated with 1 $\times$  DAPI solution (Sigma) for 10 min in the dark, and then mounted onto glass slides using Mowiol (12% Mowiol, 34.5% glycerol, and 0.12 M Tris-HCl, pH 8.5).

## Protein Determination

The amount of protein in the cell lysate and nuclei prep was determined by the Pierce bicinchoninic acid (BCA) assay (Smith et al., 1985) using bovine serum albumin as a protein standard.

## Drug Sensitivity by MTT Proliferation Assay

Drug evaluation was carried out using clonal selected lines with incremental levels of NOSII expression relative to a negative control clonal population.

Dose-response curves were determined using the 3-(4,5-dimethylthiazal-2-yl)-2,5-diphenyltetrazolium bromide (MTT) proliferation assay, which is based on the ability of viable cells to convert a soluble tetrazolium salt, MTT, into purple formazan crystals. The optical density of the dissolved crystals is proportional to the number of

viable cells. Details of this assay have been described previously (Patterson et al., 1995) except that hypoxic exposure was achieved in an anoxic cabinet (Sheldon Manufacturing, Inc., Cornelius, OR). The IC<sub>50</sub> values quoted are the means of at least three independent experiments conducted on different days.

## Drug Sensitivity by Clonogenic Survival Assay

The method employed using TPZ has been described previously (Saunders et al., 2000). After hypoxic exposure, cells were incubated at 37°C for between 2 and 4 weeks. For every individual clonogenic survival determination, three replicate wells were exposed to each TPZ concentration. When colonies were seen in the control wells (no drug exposure), the media was discarded and the cells washed with PBS and fixed as described previously. Individual colonies were judged by more than 50 mauve-stained cells in each well.

## Metabolism of TPZ by Cell Lysates and Subcellular Fractions

Incubations were carried out in air or under nitrogen at 37°C in 4-ml amber glass vials (Chromacol, Welwyn Garden City, UK) sealed with subaseal (Aldrich, Gillingham, UK). Hypoxic conditions were generated using zero-grade nitrogen gas passed through an oxy-trap (Alltech, Camforth, UK). Experimental details have been described elsewhere (Chinje et al., 1999). Detection of metabolites was at 267 nm. Approximate retention times under these conditions were 2.7, 5.6, and 9.4 min for TPZ, SR4317, and 4-nitroquinoline *N*-oxide, respectively. Concentrations of metabolites were calculated from peak height ratios and comparison with calibration curves (0–500  $\mu$ M) prepared by spiking inactivated lysate preparations with known amounts of metabolite.

## Detection of TPZ Radical Formation with Dihydrorhodamine 123

A fluorescence-based assay was employed to detect TPZ free radicals by using DHR 123 oxidation as an indicator of free radical formation (Henderson and Chappell, 1993). DHR 123 is a nonfluorescent compound that is oxidized by highly reactive species, including the TPZ radical, to form the highly fluorescent rhodamine 123 product (Delahoussaye et al., 2001). DHR 123 (final concentration, 25  $\mu$ M) was added to the TPZ metabolism system described in the previous section in a final volume of 500  $\mu$ l. The incubation mixture was then exposed to hypoxic conditions at 37°C as described above by passing over a stream of zero-grade nitrogen gas for 1 h with continuous shaking. At the end of incubation, the incubation mixture was transferred to a cuvette, and the fluorescence was measured using a luminescence spectrophotometer (LS-50B; PerkinElmer, Boston, MA), set up with an excitation wavelength at 509 nm and an emission wavelength at 529 nm, with a slit width of 10 nm. The amount of rhodamine 123 generated was determined by reading the values off a calibration curve that was set up using incubation buffer.

## Statistical Analysis

Statistical analysis was performed using Student's unpaired *t* test between two experimental groups. Data are expressed as means  $\pm$  S.E.M. for the indicated numbers of experiments. A *p* value of less than 0.05 was considered of statistical significance in all experiments.

## Results

**Enzyme Profiling of NOS-Transfected Clones.** NOS is a dimeric protein containing two catalytic domains (a reductase and an oxygenase domain). Table 1 shows both activities determined in MDA231 parental (wt), F373 vector control, and four NOSII-transfected lines (iNOS-8, iNOS-9, iNOS-10, and iNOS-12). Both the parental MDA231 and its vector control showed very little detectable NOS oxygenase activity.



In contrast, the NOSII-transfected cell lines exhibited at least a 13- to 85-fold increase in activity over both parental MDA231 and vector control. Similarly, NOSII reductase activity exhibited a 2- to 6-fold increase in activity over the parental line, in which residual reductase activity may be attributed to the low constitutive expression of P450R. Also included in Table 2 is DT-diaphorase activity determined across the panel of clones, and this remained unchanged after transfection.

**Subcellular Localization of NOSII by Immunocytochemical Analysis.** The clones were grown on glass coverslips to which they adhered. The subcellular distribution of the NOSII protein was determined by immunocytochemical staining of permeabilized cells using a rabbit polyclonal antibody specific for NOSII followed by a secondary antibody against rabbit immunoglobulin G conjugated to the fluorochrome fluorescein isothiocyanate. The cytoplasmic distribution of NOSII protein can be clearly seen in Fig. 2 (green staining pattern) with no localization within the nucleus (stained blue by DAPI). Western blotting experiment was also carried out to confirm the subcellular localization of NOSII. Fig. 3 shows the expression of NOSII in cytosolic fractions obtained from parental (wt) MDA231, F373 vector control, and two NOSII transfected lines with varying levels of expression, iNOS-10 (high) and iNOS-12 (intermediate). NOSII was readily detected in the cytosol of both clones except for the parental and vector control. In contrast, we could not detect NOSII protein expression in the corresponding isolated nuclei (data not shown).

**Sensitivity to TPZ.** The toxic effect of TPZ on the transfected clones was determined by exposing cells to the drug for 3 h under aerobic and hypoxic conditions. Cytotoxicity was measured by the MTT proliferation assay, and the data obtained are summarized in Table 2. Also included in the table are values of differential toxicity. After stable transfection, each clonal line shows increased sensitivity to TPZ under hypoxia, with values of differential toxicity ranging from 12 to 33. This represents at least between 1.2- to 3.3-fold over either the parental line or the vector control. Our results suggest that a significant contribution to this change in sensitivity may be a consequence of the increase in NOS reductase activity. We exploited the potential role of intracellular NOS reductase activity in the toxicity of TPZ across the panel of clones by examining the interrelationship between NOSII reductase activity and  $IC_{50}$  under short-term (3 h) aerobic and hypoxic exposures. A plot showing the dependence of  $IC_{50}$  on NOS reductase activity after 3-h hypoxic and aerobic exposures to TPZ is presented in Fig. 4. There was a clear relationship between NOSII reductase activity of the cell lysates and TPZ cytotoxicity under hypoxic condition. Clones

with increased reductase activity were more sensitive (lower  $IC_{50}$  value) than the parental untransfected cells. Hence, intracellular activity of NOSII seemed to predict cytotoxicity under hypoxic but not aerobic exposure condition.

To further demonstrate the significance of NOSII protein overexpression in TPZ bioactivation and toxicity, we employed a long term cytotoxicity assay. The results for the clonogenic survival assay after exposure to tirapazamine under hypoxic conditions are given in Fig. 5. From the data presented, it can clearly be seen that the transfection of NOSII into the parental MDA231 cell line significantly enhances TPZ cytotoxicity.

**Metabolism of TPZ by HPLC.** The rate at which TPZ was converted into the two-electron reduction product SR4317 is included in Table 2. The increase in product formation corresponded with an increase in NOSII reductase activity.

**Detection of the TPZ Radical.** Analysis of TPZ metabolism by HPLC determines the amount of drug that is converted into the two-electron reduction product SR4317. However, by such analysis, it is not always possible to demonstrate that the enzyme formed the toxic one-electron intermediate. Other cellular reductases, such as DT-diaphorase, have been shown to metabolize TPZ in a single two-electron step, thereby bypassing the free radical formation (Wang et al., 1993). By employing a fluorescence-based assay, TPZ radical formation was detected by measuring the rate of DHR123 oxidation to a highly fluorescent rhodamine 123 compound (Rh-123). Fig. 6 shows Rh-123 formation rate correlates well with the rate of SR4317 formation ( $r = 0.91$ , slope = 0.038). This provides further evidence of the generation of free radical intermediates that mediate the cytotoxicity caused by TPZ.

## Discussion

We have successfully engineered stable clones that constitutively express human NOSII in an MDA231 human breast cell line background. The expressed protein was confirmed by Western blotting and assessed to be catalytically functional. Thus, its role as a bioreductive enzyme implicated in TPZ bioactivation could be investigated.

The cytotoxicity of TPZ is selective for hypoxic cells in solid tumors and is thought to arise as a consequence of activation by reductive enzymes that donate single electrons to the parent drug to produce radical species that cause DNA single-strand breaks, DSBs, and ultimately chromosome aberrations (Brown, 1993). The present investigation examined the role of soluble non-nuclear human NOSII reductase in the hypoxic metabolism of TPZ after stable overexpression of

TABLE 1

Activity of functional domains of NOS (reductase and oxygenase) and DT-diaphorase in MDA231 parental and NOSII-transfected clones

All values are mean  $\pm$  S.D. of at least triplicate experiments.

Clone	NOS Reductase <i>nmol of cyt c/min/mg</i>	NOS Oxygenase <i>pmol of citrulline/min/mg</i>	DT-Diaphorase <i>nmol of cyt c/min/mg</i>
Parental	3.53 $\pm$ 0.37	0.87 $\pm$ 0.11	8.86 $\pm$ 0.94
Vector control	4.86 $\pm$ 0.70	0.78 $\pm$ 0.17	7.72 $\pm$ 0.67
iNOS-9	6.71 $\pm$ 0.20	11.62 $\pm$ 0.93	8.45 $\pm$ 1.11
iNOS-12	14.96 $\pm$ 0.63	32.30 $\pm$ 1.58	9.86 $\pm$ 1.03
iNOS-8	21.15 $\pm$ 1.41	64.51 $\pm$ 2.41	10.12 $\pm$ 2.4
iNOS-10	22.11 $\pm$ 1.63	66.20 $\pm$ 3.11	6.86 $\pm$ 1.41

the enzyme in the MDA231 cells. The data clearly demonstrated that the level of cytosolic NOSII reductase activity in the transfected clones correlates strongly with the rate of formation of TPZ free radical formation and hence DNA damage. The assay gave a linear concentration response for TPZ radical formation with increased NOSII enzyme activity. The activity of two other representative reductase enzymes that may metabolize TPZ, cytochrome P450 reductase and DT-diaphorase, remained unchanged after transfection. In addition to catalytic activity, by stably overexpressing NOSII activity in MDA231 cell line, the sensitivity of TPZ as determined by the MTT proliferation assay increased significantly under hypoxic conditions; unlike P450R overexpression, however, aerobic toxicity was not significantly altered. Further evidence in support of the importance of NOSII in the cytotoxicity of TPZ was obtained from clonogenic survival data. Both sets of data are compatible with the hypothesis that NOSII activity is involved with the hypoxic activation of TPZ. It has been suggested that the mechanisms implicated in aerobic and hypoxic cytotoxicity are distinct, and a wealth of experimental data indicate that DNA is an important cellular target for TPZ (Biedermann et al., 1991; Siim et al., 1996; Peters et al., 2001). However, the exact nature of the chemical species responsible for TPZ-mediated strand cleavage remains unresolved. Under hypoxic conditions, a free radical intermediate is generated by a one-electron reduction of the parent compound (Lloyd et al., 1991). This TPZ radical apparently directly abstracts hydrogen atoms from the DNA backbone, thereby generating deoxyribose radicals. It has also been shown that, in addition to generating deoxyribose radicals, TPZ (and its mono-*N*-oxide metabolites) can further react with these DNA radicals, thus converting them into strand breaks and chromosome aberrations (Jones and Weinfield, 1996; Hwang et al., 1999). Further evidence that TPZ does, in fact, cause significant amounts of oxidative DNA damage has been provided by studies employing base excision repair enzymes (Kotandeniya et al., 2002). These studies revealed that reductive activation of TPZ yields a pattern of DNA damage that is typical of a small diffusible oxidizing radical (e.g., hydroxyl radical), in which damage to both DNA backbone and the heterocyclic bases occurs (Daniels and Gates, 1996). The results lend further support to the work previously carried out by Laderoute et al. (1988) that revealed that the "natural" lifetime of the radical generated was sufficiently long that it could diffuse over significant distances within hypoxic cells and thus inflict further oxidative damage on cellular targets. Therefore, it seems reasonable to predict that cytotoxic radicals generated by the re-

duction of TPZ by non-nuclear NOSII reductase may be able to diffuse into the nucleus. However, the exact chemical nature of the base damage inflicted by this drug remains to be fully characterized and also to determine whether such damage occurs *in vivo*.

Under aerobic conditions, the unstable TPZ radical is rapidly back-oxidized to the parent compound, generating superoxide radicals, which can undergo the Fenton reaction to generate hydroxyl radicals. These are highly reactive and have a limited diffusion distance (Halliwell and Gutteridge, 1999); thus, when generated in the cytosol, they will be unlikely to contribute to the aerobic toxicity of TPZ. We have recently demonstrated that purified recombinant NOS isoforms, albeit from nonhuman sources (rat, mouse, bovine), were capable of reducing TPZ under both aerobic and hypoxic conditions and that, overall, NOS isoforms may have a greater affinity for TPZ than P450R (Garner et al., 1999). In addition, we were able to demonstrate that NOSII can catalyze the formation of DNA-damaging products from TPZ, which were mainly single-strand breaks. Our present study involving human NOSII therefore widens the accepted role of NOS to include a function as an important enzyme implicated in the chemotherapeutic effects of TPZ. Therefore, the close relationship between TPZ activation and cytotoxicity in human cytosolic NOSII MDA231 transfected clones suggests that a major determinant of cytotoxicity is most probably attributable to the accumulation of TPZ-mediated DNA damage arising from the overexpressed cytosolic NOSII reductase. We could not detect NOS activity in the prepared nuclear fractions from either parental or transfected clones, consistent with its soluble cytoplasmic localization.

It is well documented that NOS is overexpressed in a wide variety of human tumors (Thomsen and Miles, 1998). In many cases, it is clear from immunohistochemical analyses of tumor sections that this overexpression is not evenly distributed. One reason for this may be that the NOSII gene contains a hypoxia-responsive element in its promoter region (Melillo et al., 1995). This will drive expression of the gene under hypoxic conditions and thereby lead to focal overexpression of the protein in the hypoxic regions of tumors. We have shown the overall level of NOS to be elevated in human breast carcinomas; however, these measurements will underestimate the actual level of focal activity of NOS in the tumors (E. Chinje, unpublished observations). Therefore, it is likely that the level of activity of NOS created in the transfected clones will be similar to that focally expressed in human breast tumors. To support this contention, we have shown previously through immunostaining that NOS expres-

TABLE 2

TPZ metabolism and cytotoxicity (3-h exposure) against MDA231 parental and NOSII-transfected clones

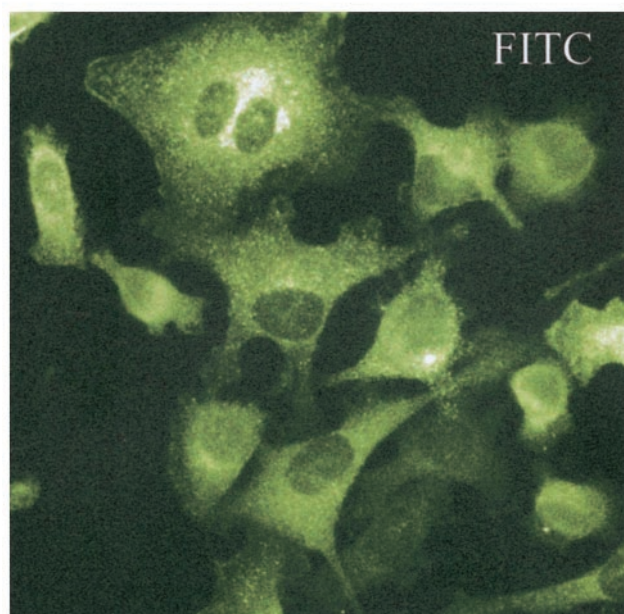
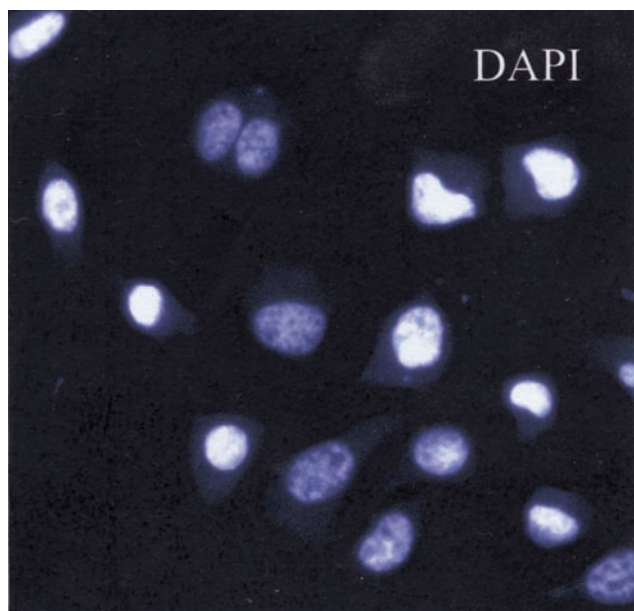
Values are mean  $\pm$  S.D. of at least triplicate experiments. HCR is defined by the aerobic-to-hypoxic IC<sub>50</sub> ratio. Metabolism of TPZ to SR4317 was determined under hypoxic conditions as outlined in the text

Clone	Tirapazamine (IC <sub>50</sub> )			
	3-h Aerobic Exposure	3-h Hypoxic Exposure	Differential Tox. (HCR)	SR4317 Rate
		$\mu\text{M}$		$\text{nmol/min/mg}$
Parental	352.5 $\pm$ 27.2	44.7 $\pm$ 4.6	7.9	3.53 $\pm$ 1.0
Vector	389.5 $\pm$ 15.3	39.0 $\pm$ 8.4	10.0	5.98 $\pm$ 1.4
iNOS-9	321.0 $\pm$ 26.0	26.6 $\pm$ 2.3	12.1	12.07 $\pm$ 1.1
iNOS-12	454.6 $\pm$ 76.0	15.4 $\pm$ 3.2	29.5	18.11 $\pm$ 3.6
iNOS-8	335.9 $\pm$ 19.2	14.8 $\pm$ 3.6	22.7	20.89 $\pm$ 2.4
iNOS-10	544.2 $\pm$ 86	16.5 $\pm$ 5.1	33.0	25.58 $\pm$ 3.5

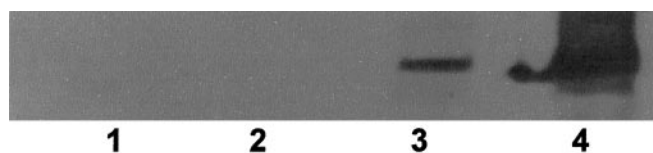
sion can be highly localized in tumors, particularly in areas interfacing with necrotic tissue, that are a region of hypoxia (Chinje et al., 2002). Therefore, the actual NOS activity

within this tumor subpopulation could be much higher than our estimation of NOS activity using whole tumor homogenate. Thus, it is likely that the focally expressed high levels of NOS could make significant contributions to TPZ metabolism and toxicity.

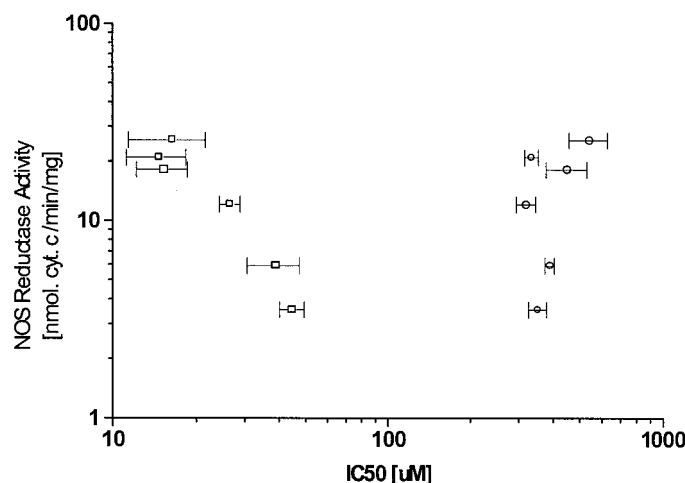
Interestingly, the role of P450R in the bioactivation of TPZ has been brought into question (Elwell et al., 1997; Evans et al., 1998), thus casting doubt about a similar role by NOSII reductase activity. Elwell et al. (1997) evaluated the biochemical consequences of long-term aerobic exposure of the A549 human lung adenocarcinoma cell line to TPZ to elucidate the mechanism(s) of toxicity. The results showed that, as a consequence, the adapted cell lines expressed extremely low levels of P450R activity (1–3% of the parental activity), but apparently only aerobic toxicity was significantly modified (9.6-fold). Hypoxic toxicity was only marginally modified (1.5-fold), which did not reflect the reduced enzyme expression. In questioning this relationship, we restored P450R activity in one of the TPZ-resistant clones by stable transfection (Saunders et al., 2000). This significantly increased both hypoxic and aerobic sensitivities and restored metabolism so



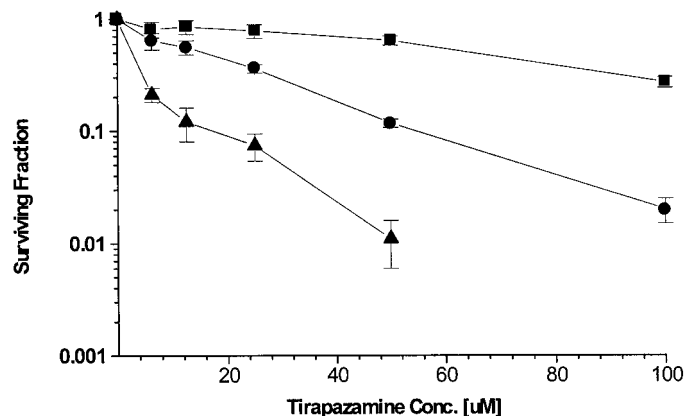
**Fig. 2.** Immunocytochemical staining of NOSII protein expression within adherent MDA231 NOSII transfected clones. Experimental procedure was as detailed in the text. The cytoplasmic distribution of NOSII protein is visible (FITC green staining pattern) with no localization within the nucleus (stained blue by DAPI).



**Fig. 3.** Western blot for NOSII protein in cytosolic fractions of parental (wt) MDA231 (lane 1), F373 vector control (lane 2), and two NOSII transfected clones, iNOS-12 (intermediate, lane 3) and iNOS-10 (high, lane 4)

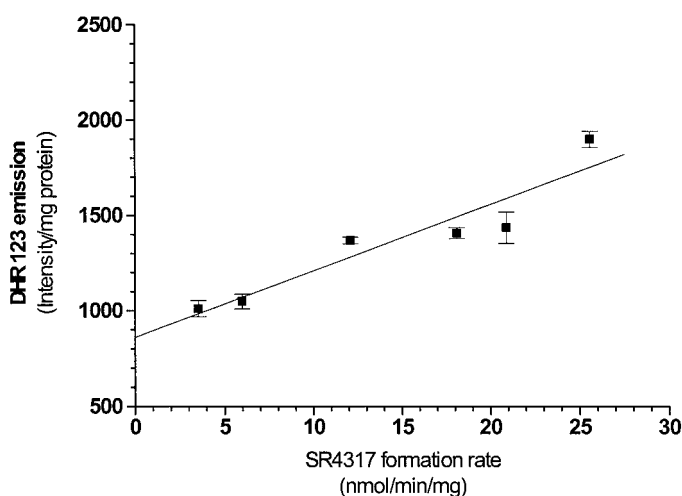


**Fig. 4.** MTT proliferation assay shows dependence of  $IC_{50}$  values of the transfected MDA231 clones exposed to tirapazamine for 3 h under aerobic (○) or hypoxic (□) conditions, on NOSII reductase activity. Bars indicate S.D. for at least triplicate experiments.



**Fig. 5.** Dependence of survival fraction, as determined using clonogenic survival assay, on TPZ concentration after 3-h hypoxic exposure. Parental MDA231 (■), iNOS-12 clone (●), and iNOS-10 (▲). The control cloning efficiency for each cell line was: parental (37.3%), iNOS-12 (29.6%), and iNOS-10 (21.8%). Results are from an experiment representative of three determinations.





**Fig. 6.** Graph shows correlation between TPZ radical formation detected by the DHR123 assay and SR4317 formation rate measured by HPLC after hypoxic exposure. The means ( $\pm$  S.D.) for four independent experiments are shown.

that it resembled that of the original parental line. Thus, P450R clearly contributes to both oxic and hypoxic TPZ-mediated toxicity as well as other yet-to-be-identified flavoenzymes. We have presented evidence that demonstrates the reductase domain of NOSII will carry out similar metabolic and cytotoxic activities.

Our findings on the importance of non-nuclear metabolism of TPZ on its cytotoxicity are in direct contrast with studies carried out by other workers (Evans et al., 1998, Delahoussaye et al., 2001), which suggests that TPZ radicals formed outside the nuclei cannot account for and do not cause damage to intranuclear DNA. The studies carried out by Delahoussaye et al. (2001) were based on cofactor dependence of the reductases that are implicated in TPZ metabolism and the ensuing DNA damage. They concluded that such damages are probably caused by the most abundant source of unidentified reductase(s) in the nucleus. In agreement with our recent findings (Patterson et al., 1997), these workers similarly could not detect the presence of P450R in the nucleus but have used this as argument against its involvement in TPZ toxicity via DNA damage. However, these same workers concede that tumors expressing high levels of the reductases involved in producing DNA DSBs would be expected to be highly sensitive to TPZ under hypoxic conditions. We have overwhelming support for such an important role for P450R (Patterson et al., 1995, 1997; Saunders et al., 2000).

In conclusion, we have demonstrated that cytosolic human derived NOSII reductase has an important contribution in the activation and toxicity of TPZ and the data argue against the dogma that TPZ radicals formed outside the nucleus do not cause damage to intracellular DNA.

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