Biochemical Pharmacology, Vol. 46, No. 7, pp. 1251–1256, 1993.
Printed in Great Britain.

0006-2952/93 $6.00 + 0.00

SR2508 (ETANIDAZOLE) PHARMACOKINETICS AND BIOCHEMICAL EFFECTS IN TUMOR AND NORMAL TISSUES OF *SCID* MICE BEARING HT-29 HUMAN COLON ADENOCARCINOMA

PETER J. O'DWYER,* LISA PANTING, FRANK P. LACRETA and MARGIE L. CLAPPER
Fox Chase Cancer Center, Philadelphia, PA 19111, U.S.A.

(*Received* 24 *July* 1992; *accepted* 13 *May* 1993)

Abstract—Several lines of evidence implicate glutathione (GSH) depletion and/or GSH transferase inhibition in the sensitizing action of nitroimidazoles to alkylating agents. To characterize this interaction, *scid* mice bearing subcutaneously implanted HT-29 colon tumor (0.75 to 1.25 cm diameter) were treated with SR2508 (2 g/kg, i.p.). At intervals following treatment, samples of blood, liver, spleen, kidney and central non-necrotic tumor core and tumor periphery were obtained and analyzed for SR2508 content by high-pressure liquid chromatography. Tissues were assayed spectrophotometrically for GSH and GSH transferase. SR2508 plasma pharmacokinetics in this model were similar to those described previously ($t_{1/2\beta}$ = 5.83 hr). The volume of distribution of 0.32 L/kg suggests minimal tissue binding. In tumor periphery and core samples SR2508 levels peaked at 1 hr, and declined exponentially in parallel with plasma. During the terminal phase core SR2508 levels were 10-fold and tumor periphery levels 4.3-fold those of concurrent plasma concentrations. Consistent with these data, tumor GSH levels in both periphery and core fell below 30% of control at 4 hr, and remained depressed >12 hr. Delayed recovery of GSH content of tumor tissue may explain in part the selectivity of SR2508 for tumor (oxic or hypoxic). GSH transferase activity in tumor was inhibited both at the center and periphery to 75 and 71% of control, respectively, and it appeared that recovery occurred more slowly in the hypoxic core. The mild degree of inhibition observed does not support an important role for inhibition of GSH transferase in sensitization by SR2508 in this tumor. The pronounced selective depletion of GSH in tumor supports the further development of SR2508 in the reversal of alkylating agent resistance.

SR2508 (etanidazole, NSC 301467) is a less lipophilic 2-nitroimidazole analogue of misonidazole which is in clinical trials as a hypoxic cell radiosensitizer and chemosensitizer. It is probable that the mechanistic bases for radiosensitizing and chemosensitizing effects are different: the former is dependent on high drug levels at the time of irradiation, and is fully expressed immediately upon adding the drug to the system. Chemosensitization, on the other hand, is enhanced by preincubation of cells with the sensitizing drug, and the phenomenon persists following drug removal [1].

A biochemical action of SR2508 consistent with such a preincubation phenomenon may occur at the level of glutathione (GSH) and associated enzymes. Depletion of GSH is known to enhance the cytotoxicity of several alkylating agents and platinum compounds [2]. Nitroimidazoles have been shown previously to deplete cells of GSH, and to inhibit certain GSH transferases [3, 4]. We have demonstrated depletion of GSH and inhibition of GSH transferases in the peripheral mononuclear cells of patients treated with SR2508 in a clinical trial [5].

The present study was performed in human tumor-bearing *scid* mice to: (a) characterize the plasma and tissue pharmacokinetics in this model; (b) investigate the effects of SR2508 upon tumor and normal tissue GSH concentrations and GSH transferase activity; and (c) observe differences in biochemical and pharmacologic effects between the well-oxygenated tumor periphery and the hypoxic tumor core.

* Corresponding author: Peter J. O'Dwyer, M.D., Fox Chase Cancer Center, 7701 Burholme Ave., Philadelphia, PA 19111. Tel. (215) 728-3513; FAX (215) 728-2741.

MATERIALS AND METHODS

Drugs. SR2508 was obtained from the Pharmaceutical Resources Branch, National Cancer Institute, Bethesda, MD, and stored at room temperature in the dark. All other reagents were of analytic grade and purchased from Sigma.

Tumor xenografts. HT-29 human colon adenocarcinoma carried *in vitro* was injected i.p. (10^5 cells in Hanks' buffered saline) in athymic mice (Balb/c, age 7 months). The palpable solid tumors were excised, and fragments (about 1 mm^3) were implanted in *scid* mice, from a colony derived at Fox Chase Cancer Center [6]. These tumors have been carried *in vivo* in this laboratory for several years. Recipient mice of mean weight 20 g were anesthetized with ether, and a fragment of tumor tissue was implanted into a subcutaneous pocket cut in each flank. The incision was closed with clips that were removed 2 days later. The tumor take rate was in excess of 90%. When tumors had reached dimensions of 0.75 to 1.25 cm in diameter (which required 6–8 weeks), animals were randomly assigned to various drug dose levels.

Drug treatment and sample acquisition. SR2508 was dissolved in phosphate-buffered saline to a

concentration of 50 mg/mL; fresh solutions were made on the day of treatment. Mice were weighed immediately before dosing, and injected i.p. with a 2 g/kg dose. At intervals following treatment, blood samples were obtained in heparinized capillary tubes by orbital puncture, and centrifuged; plasma was separated and stored at −70°. At further intervals, groups of 4–6 animals were killed by cervical dislocation. Normal organs (liver, kidney, spleen) were excised immediately, washed in phosphate-buffered saline, and frozen. Two specimens were obtained from each tumor: one from the surface, from which a 2–3 mm thick arc was dissected with a scalpel, and one from the central area, avoiding areas of necrosis if present. Plasma and tumor samples were analyzed for SR2508 concentrations. Normal organs and tumors were assayed for GSH concentrations and GSH transferase activity.

Pharmacokinetic assay. SR2508 concentrations in plasma were measured by the method of Workman *et al.* [7, 8]. Briefly, samples were thawed and deproteinized with methanol, and the supernatant was evaporated to dryness under a stream of nitrogen at room temperature. The residue was resuspended in mobile phase and injected (by way of a Hewlett–Packard automated injector) onto a C_{18}-Versapack column (30 × 4.1 mm i.d.) in a Hewlett–Packard HP1090 high pressure liquid chromatography system. The mobile phase was 5% methanol in water; SR2508 had a retention time of 4 min. Detection was by UV absorbance at 323 nm; peak heights were calculated and stored by an HP integrator, and concentrations were determined by reference to a standard curve that was run with each batch of samples. No other nitro-containing species have been detected in plasma, urine, or tissue samples using this analytic procedure. The day-to-day coefficient of variation (CV) was 5–10% from 0.1 to 50 μg/mL, and the within-day CV was <4%. The lower limit of quantitation was 50 ng/mL, and the detector response was linear up to 50 μg/mL. At each time interval, 3–6 samples were obtained.

Pharmacokinetic analysis was conducted using the arithmetic mean value of concentrations obtained at each time interval. These data were analyzed with NONLIN84 (Statistical Associates, Lexington, KY). The plasma levels best fit a two-compartment open model, represented by the equation:

$$C_t = Ae^{-\alpha t} + Be^{-\beta t}$$

where C_t represents the concentration at time t, and A, α, B and β are constants. Sample weighting of $1/y^2$ (the square of the measured value) was used throughout. The area under the plasma concentration–time curve was obtained by integration. Clearance and volume parameters were calculated by standard methods [9].

Assays for glutathione and GSH transferase. GSH and GSH transferase were measured in samples of tumor, liver, spleen, and kidney. Tissues were processed by suspending them in an equal volume (w/v) of phosphate-buffered saline, followed by homogenization using a Polytron (Brinkmann Instruments, Westbury, NY). Homogenates were spun at 10,000 *g* for 20 min and the supernatant was assayed for GSH and GSH transferase activity. Total GSH (oxidized and reduced) was measured by a modification of the method of Griffith [10] in which the rate of formation of a GSH conjugate of 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) is determined spectrophotometrically. GSH transferase was assayed spectrophotometrically by measuring the rate of formation of a conjugate of glutathione and 1-chloro-2,4-dinitrobenzene (CDNB) as described by Habig and Jakoby [11]. The GSH and GSH transferase activities were normalized to protein content using the Bradford protein assay (Bio–Rad, Hercules, CA).

Table 1. Pharmacokinetics of SR2508 (2 g/kg, i.p.) in *scid* mice

Kinetic parameters	Value
A	7200 μg/mL
B	15.50 μg/mL
α	1.196 hr^{-1}
β	0.1189 hr^{-1}
AUC	6151 $\mu g \cdot min \cdot mL^{-1}$
Half-lives	
k_{10}	0.591 hr
α	0.580 hr
β	5.831 hr
Clearance	
Cl_{tot}	5.42 $mL \cdot min^{-1} \cdot kg^{-1}$
Volumes	
V_c	0.277 L/kg
V_{ss}	0.324 L/kg
V_β	2.73 L/kg

Abbreviations: AUC, area under the concentration–time curve; Cl_{tot}, total body clearance; V_c, volume of central compartment; V_{ss}, volume of distribution at steady state; V_β, β phase apparent volume of distribution.

RESULTS

A minimum of three samples was obtained at each time interval for plasma pharmacokinetics. The plasma elimination curve fit a two-compartment open model, with an elimination half-life of 5.8 hr. At this dose (2 g/kg), SR2508 was still detectable at 24 hr. The major pharmacokinetic parameters are shown in Table 1.

The plasma elimination curve fit from all the samples is shown in Fig. 1 (solid line). The interrupted lines in this figure show simultaneous tumor concentrations both in the well-oxygenated, well-perfused periphery and in the hypoxic core. It is evident that peak tissue concentrations were achieved with an hour of dosing, and that drug disappearance, especially from the tumor core, was considerably slower than from plasma. Indeed, from the 8-hr point, the concentrations of SR2508 in the tumor were in excess of 10-fold those in plasma. This suggests that sensitizing concentrations of the drug may be maintained at its hypoxic target for extended periods, and supports the further investigation of SR2508 as a chemosensitizer.

The effects of SR2508 administration on GSH levels in various tissues are shown in Figs. 2 and 3.

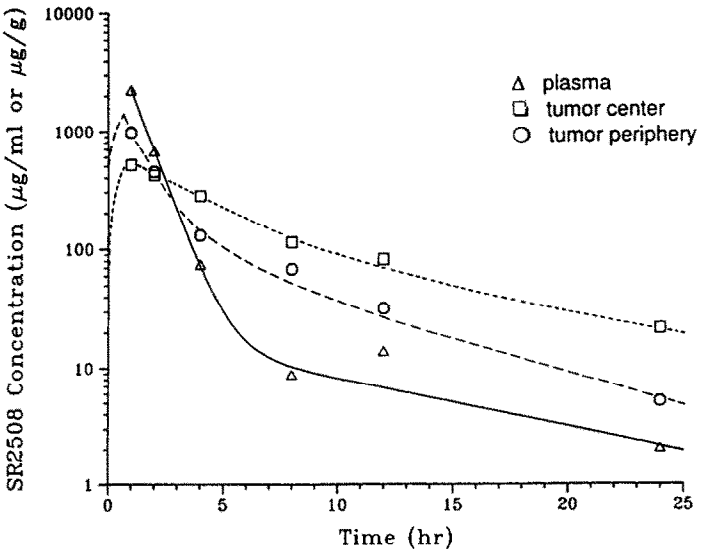

Fig. 1. Concentration–time curves of SR2508 disappearance from mouse plasma (solid line), tumor center (small dashes), and tumor periphery (long dashes). Plasma concentrations are expressed as μg/mL, and tumor drug content as μg/g wet weight.

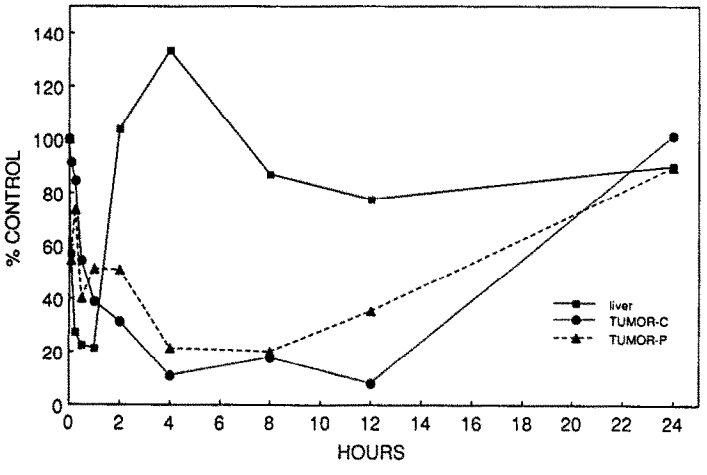

Fig. 2. Effect of SR2508 on glutathione content (normalized to a pretreatment value of 100%) in mouse liver, HT-29 tumor center, and tumor periphery. Each point represents the mean of 4–6 animals. Mean pretreatment values were: liver 29.4, tumor center 0.256, and tumor periphery 2.21 nmol/mg protein.

The most profound effects were found in tumor tissue, in which depletion of GSH to below 20% of control was found in both central and peripheral tumor specimens. Maximal depletion was not reached until 4 hr, when tissue SR2508 concentrations had already declined (Fig. 1). An indication at the 12-hr time point that recovery was delayed in the tumor core is consistent with the higher concentrations of SR2508 remaining in this region of the tumor. GSH content had returned to control levels by 24 hr.

A very different pattern of GSH depletion was found in liver and spleen: in both organs depletion of GSH occurred early (to 20 and 40% of control, respectively) with recovery by 2 hr, following which levels remained unchanged. The GSH content of kidney appeared to be minimally affected by this dose of SR2508.

The effects of SR2508 administration on GSH transferase activity are shown in Figs. 4 and 5. In no case was the enzyme inhibited to less than 50% of control activity. In the tumor core, activity was less than that in tumor periphery and liver at 12 and 24 hr, but it seems unlikely that this level of inhibition would have important consequences for sensitization. Curiously, GSH transferase inhibition was observed

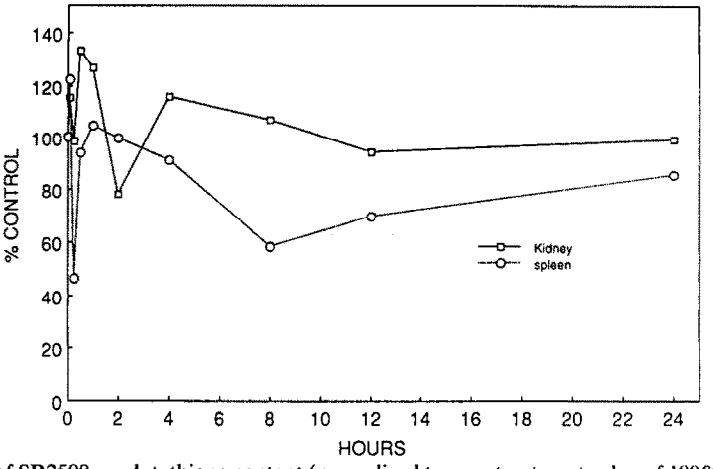

Fig. 3. Effect of SR2508 on glutathione content (normalized to a pretreatment value of 100%) of mouse kidney and spleen. Mean pretreatment values were: kidney 0.036 and spleen 6.28 nmol/mg protein.

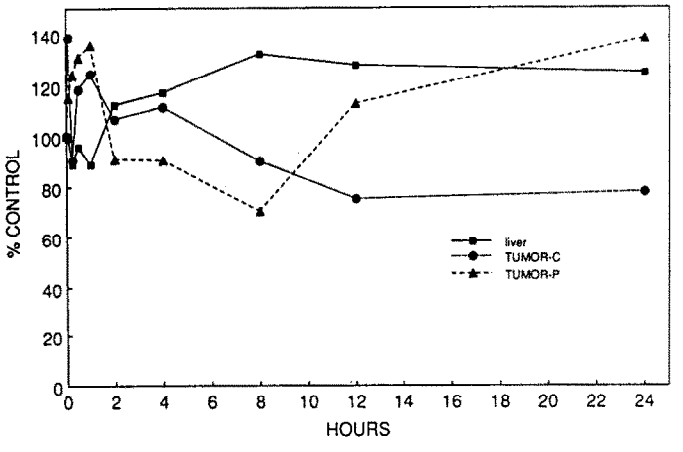

Fig. 4. Effect of SR2508 on glutathione transferase activity (normalized to a pretreatment value of 100%) in mouse liver, HT-29 tumor center and tumor periphery. Mean pretreatment values were: liver 247.3, tumor center 21.6, and tumor periphery 40.9 nmol/min/mg protein.

to a greater extent in the kidney than in other organs, despite the lack of GSH depletion in this organ.

DISCUSSION

The pharmacokinetic results show that in the *scid* mouse the terminal half-life is longer, and the total body clearance less than in the Balb/c model studied by Workman and Brown [12]. In addition to kinetic effects resulting from differing routes of administration (i.p. vs i.v.), such differences between strains are not unusual. Comparison of our murine data with the human pharmacokinetics is, however, of some interest. The dose received by the mice was 2 g/kg or 6 g/m^2. We have determined previously the pharmacokinetics of SR2508 at 5.5 g/m^2 in humans [13]. The terminal half-lives in these two species are identical. However, total body clearance (on a per m^2 basis) is 3-fold higher in humans (16.3 vs 46.6 mL/min/m^2). The characteristics of drug distribution are also markedly different: the volumes of distribution in mice are an order of magnitude less than those in humans. As a result, the total area under the concentration–time curve at this dose is 20-fold higher in humans. These characteristics may favor achieving the tissue concentrations needed for chemosensitization in humans.

The differences in drug content in tumor core versus both plasma and tumor periphery undoubtedly reflect tissue perfusion and clearance. Since SR2508 is not metabolized irreversibly in oxic tissues, parent drug concentrations should reflect total drug content. However, in solid tumors capable of nitroreduction, conversion of the 2-nitro function to various reactive intermediates may occur. Since the detection of SR2508 by UV absorbance depends on an intact 2-

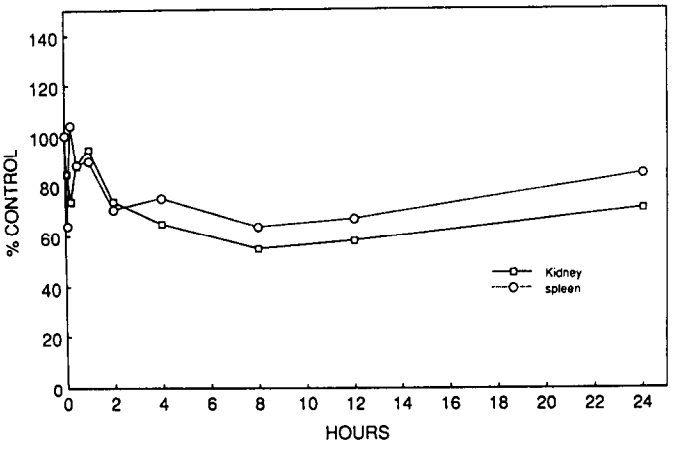

Fig. 5. Effect of SR2508 content on glutathione transferase activity (normalized to a pretreatment value of 100%) in mouse kidney and spleen. Mean pretreatment values were: kidney 74.3 and spleen 77.4 nmol/min/mg protein.

nitro function, the hypoxic core of the tumor may contain additional metabolites of SR2508 (e.g. the hydroxylamine and the amine) not detected by this methodology. Therefore, the concentrations described represent a minimum estimate of total tissue drug content.

These results also confirm *in vivo* that SR2508 causes marked depletion of GSH with evidence of selectivity for tumor over normal tissue in the recovery period from 2 to 12 hr after treatment. Depletion of GSH by inhibition of its synthesis is known to sensitize tumors *in vitro* and *in vivo* to several classes of active cytotoxic drugs. Further, differences in recovery of GSH content in tumors and normal tissue have been observed with buthionine sulfoximine (an inhibitor of γ-glutamylcysteine synthetase) [14]; these differences may account for the apparent selective action of both drugs in sensitizing tumors to alkylating agents. GSH depletion may therefore contribute to the chemosensitization obtained by SR2508. The time-course observed supports the scheduling of SR2508 *before* the cytotoxic drug, to allow maximal depletion of GSH in tumor cells to occur.

However, it is likely that depletion of GSH is not the sole mechanism of chemosensitization [15]. Early studies demonstrated that even in cells maximally depleted of GSH by diethyl maleate, addition of a 2-nitroimidazole resulted in further sensitization [16]. In addition, a regimen of buthionine sulfoximine administered together with 2-nitroimidazoles has been shown to have supraadditive effects in sensitization studies [17, 18]. Further studies to investigate the metabolism of SR2508 in the hypoxic core of the tumor are warranted.

Finally, the very modest effects of SR2508 upon GSH transferase activity were unexpected. In normal human peripheral mononuclear cells we previously found GSH transferase inhibition to result from administration of SR2508 at doses as low as 1 g/m^2 [5]. Indeed, the time-courses of GSH depletion and of transferase inhibition were generally parallel. However, it should be noted that GSH transferase isoenzyme expression varies from one tissue to another [19, 20]. In peripheral mononuclear cells the μ isoenzyme predominated, while in HT-29 cells, the π isoenzyme alone was expressed (Clapper ML, unpublished results). Characterization of the inhibitory action of SR2508 on the various GSH transferase isoenzymes will assist in reconciling these results.

Acknowledgements—The authors gratefully acknowledge the expert secretarial assistance of Catherine Thompson. This work was supported in part by Grant CA 49820 from the NCI.

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