linkage on conjugate yield, potency, selectivity and toxicity. *Mol Immunol* 1990, 27, 273–282.
14. Neuwelt EA, Specht HD, Hill SA. Permeability of human brain tumor to ^{99m}Tc-glucoheptonate and ^{99m}Tc-albumin. Implications for monoclonal antibody therapy. *J Neurosurg* 1986, 65, 194–198.
15. Nazzaro JM, Rosenbaum LC, Pagel MA, Neuwelt EA. A new model of systemic drug rescue based on permeability characteristics of the blood–brain-barrier in intracerebral abscess-bearing rats. *J Neurosurg* 1991, 74, 467–474.
16. Curtet C, Maurel C, Douillard JY, *et al.* Enzyme-linked immunosorbent assay to monitor colorectal carcinoma patients treated with a monoclonal antibody (17-1A). *J Immunol Methods* 1985, 83, 193–199.
17. Blottiere HM, Maurel C, Douilard J. Immune function of patients with gastrointestinal carcinoma after treatment with multiple infusions of monoclonal antibody 17.1A. *Cancer Res* 1987, 47, 5238–5241.
18. Goodman GE, Beaumier P. Pilot trial of murine monoclonal antibodies in patients with advanced melanoma. *J Clin Oncol* 1985, 3, 340–352.
19. Honda M, Takahashi T, Yamaguchi T. Immunoresponses in clinical use of immunoconjugate A7-NCS with special reference to changes in human anti-mouse antibody level. *Biotherapy* 1990, 4, 844–847.
20. Goldenberg DM. Targeted cancer treatment. *Immunology Today* 1989, 10, 286–288.

Eur J Cancer, Vol. 29A, No. 3, pp. 423–425, 1993.
Printed in Great Britain

0964–1947/93 $6.00 + 0.00

In vitro Activity of the Benzotriazene Dioxide SR 4233 Against Human Tumour Colony-forming Units

Axel-R. Hanauske, Michael Ross, Donna Degen, Susan G. Hilsenbeck and Daniel D. Von Hoff

SR 4233 (3-amino-1,2,4-benzotriazine 1,4-dioxide) is a novel bioreductive agent selectively toxic to hypoxic cells. It is active as a radiation sensitiser *in vitro*. Using a human tumour cloning system we have studied the effects of SR 4233 against freshly explanted human tumour specimens under hypoxic and non-hypoxic culture conditions. For hypoxic conditions, final concentrations of SR 4233 of 10.0–500 μmol/l were used in short-term (1 h) exposure experiments. Final concentrations in non-hypoxic experiments ranged from 10 to 1350 μmol/l. 25 tumour specimens were tested under each culture condition. Of those, 14 (56%) were evaluable. The most common tumour types recruited included ovarian, non-small cell lung, and breast cancer. A moderate concentration-dependent increase in the frequency of inhibited tumour specimens under non-hypoxic conditions was observed with zero out of 10 sensitive specimens at 10 μmol/l as compared with five out of 14 (36%) sensitive specimens at 500 μmol/l ($P < 0.02$). However, when hypoxic conditions were used SR 4233 had a profound antitumour activity, (two out of 14 specimens sensitive at 10 μmol/l compared with 10 out of 10 specimens sensitive at 500 μmol/l, $P < 0.00005$). We conclude that SR 4233 is active against tumour colony-forming units *in vitro* and that its antitumour activity is greatly increased against hypoxic tumour cells.
Eur J Cancer, Vol. 29A, No. 3, pp. 423–425, 1993.

INTRODUCTION

THE BENZOTRIAZINE dioxide SR 4233 (3-amino-1,2,4-benzotriazine 1,4-dioxide) is a novel bioreductive compound that has been developed as a radiation sensitiser [1–3]. Unlike earlier generations of radiation sensitisers, this agent had an intrinsic antitumour activity particularly against hypoxic cells *in vitro* [1, 4–7]. Under hypoxic conditions, a free radical 1-electron reduction product is generated and appears to be responsible for DNA single and double strand breaks [4, 8–10]. *In vitro* experiments suggest that the extent of cytotoxicity of SR 4233 may be influenced by metabolic inactivation and the ability to repair DNA breaks [11, 12]. SR 4233 has been shown to effectively augment the radiation-induced damage to tumour cells *in vitro* [4, 10, 13]. It has also been reported to be beneficial as part of a triple modality tumour therapy including radiation, hyperthermia, and radiation sensitisation [14].

In the present study we have utilised a human tumour cloning system to determine and compare the direct antitumour effects of SR 4233 under hypoxic and non-hypoxic culture conditions against a variety of freshly explanted human tumour specimens *in vitro*.

Correspondence to D.D. Von Hoff.
A.-R. Hanauske is at the Technische Universität München, Abteilung Hämatologie und Onkologie, Klinikum rechts der Isar, F.R.G.; M. Ross is at the Sterling Research, Great Valley, Pennsylvania; and D. Degen, S.G. Hilsenbeck and D.D. Von Hoff are at the Section of Drug Development, Division of Oncology, Department of Medicine, University of Texas Health Science Center at San Antonio and Cancer Therapy and Research Center, 7703 Floyd Curl Drive, San Antonio, Texas 78240, U.S.A.
Revised 11 June. 1992; accepted 17 Aug. 1992.

MATERIALS AND METHODS

Compounds

SR 4233 was kindly provided by Sterling Research (Great Valley, Pennsylvania). Stock solutions were prepared in distilled water and stored at −20°C until used. Final concentrations ranged from 10 to 1350 μmol/l for non-hypoxic culture conditions and from 10 to 500 μmol/l for hypoxic culture conditions.

Human tumour cloning system

After obtaining informed consent in accordance with federal and institutional guidelines, tumour specimens were collected by sterile standard procedures as part of routine clinical measures. Biopsies of solid tumours were stored in McCoy's 5A medium containing 10% v:v newborn calf serum, 10 mmol/l Hepes, 90 U/ml penicillin and 90 μg/ml streptomycin (all from Gibco, Grand Island, NY) for transport to the laboratory. Preservative-free heparin (10 U/ml, O'Neill, Johns and Feldman, St. Louis, Missouri) was added immediately after collection of fluids to prevent coagulation. Solid specimens were minced and repeatedly passed through metal meshes with mesh widths of 40 μm (E.C. Apparatus, St. Petersburg, Florida) to obtain a single cell suspension. Effusions were centrifuged at 150 *g* for 5–7 min and passed through 25 gauge needles when necessary to obtain single cell suspensions. All specimens were suspended in McCoy's 5A medium containing 5% v:v horse serum (HS), 10% v:v fetal calf serum (FCS) (both Hyclone, Logan, Utah), 2 mmol/l sodium pyruvate, 2 mmol/l glutamine, 90 U/ml penicillin, 90 μg/ml streptomycin and 35 μg/ml L-serine (all from Gibco).

The single cell suspension was mixed with the specified concentrations of SR 4233 in 15-ml polystyrene conicals (Corning) and transferred to air-tight rubber-sealed 20-ml glass serum bottles (Wheaton). For hypoxic conditions, the serum bottles were flushed with N_2 five times using a Bactec 460 machine (Johnston Laboratories, Becton Dickinson). Each flush consisted of 180 ml 100% nitrogen giving a total of 30 volume exchanges. For non-hypoxic conditions, the bottles were flushed with 5% v:v CO_2 in air five times. The bottles were incubated at 37°C in a shaking water bath for 1 h. The cell–drug mixture was then transferred to 15-ml conical tubes. The drug was removed by repeated washings with medium and centrifugation at 150 *g*.

The human tumour cloning assay (HTCA) was performed using the two-layer system described by Hamburger and Salmon with several modifications [15]. Base layers contained 0.5% w:v agar (DIFCO, Detroit, Michigan) in a mixture of McCoy's 5A medium as described above, 0.6% w:v soy broth (Difco), and 100 μg/ml asparagine (Gibco). Cells were plated at a density of 5×10^5/dish in 35-mm Petri dishes (Corning) in a mixture of 0.3% w:v agar in CMRL medium 1066 (Irvine Scientific) containing 15% v:v HS, 2% v:v FCS, 50 mg/l vitamin C (Gibco), 90 U/ml penicillin, 90 μg/ml streptomycin, 0.1 mmol/l non-essential amino acids, 2 mmol/l glutamine (all from Gibco), 2 U/ml insulin (Iletin I^R, Eli Lilly). Immediately prior to plating, Hepes (Gibco, 10 mmol/l final concentration), asparagine (100 μg/ml final concentration), and glutamine (2 mmol/l final concentration) were added. All determinations were done in triplicate. Each experiment included a set of solvent controls (water) and a set of plates containing the highly cytotoxic compound orthosodium vanadate (10^{-3} mol/l, Sigma) to assure the presence of a good single cell suspension [16]. Plates were incubated at 37°C, 5% v:v CO_2, 100% humidity. After 14 days, colonies were counted with an inverted microscope. An experiment was considered evaluable when the water control had ≥ 20 colonies/plate and the positive control showed ≤ 30% colony formation compared with the solvent control. A decrease in tumour colony formation was considered significant if survival of colonies was ≤ 0.5-fold compared to the control.

Statistical analysis

Data were expressed as means and standard deviations of triplicate determinations. The average number of tumour colony forming units from SR 4233-treated cells was expressed in relation to the average number of tumour colony forming units from untreated controls. Statistical analyses were performed using the χ^2-test for association and the χ^2-test for linear trend.

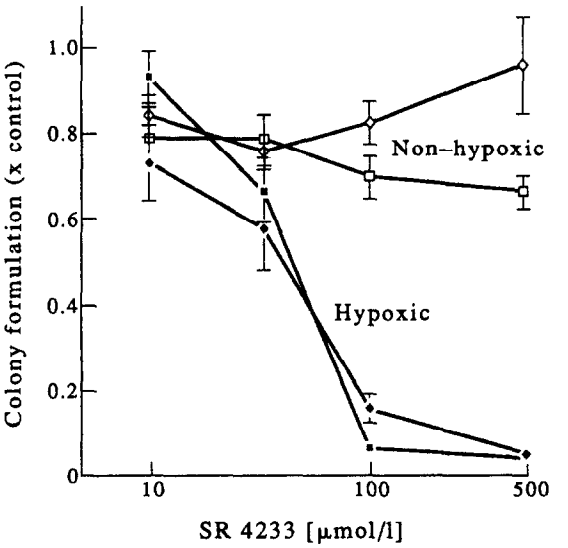

Fig. 1. Representative concentration–response effects of SR 4233 on clonogenic growth from freshly explanted human tumours. Two ovarian cancer specimens are shown. SR 4233 strongly inhibited clonal proliferation of cells incubated with the agent using hypoxic conditions, but had little effect on cells exposed to the agent using non-hypoxic conditions. Solid symbols: hypoxic culture conditions; open symbols: non-hypoxic culture conditions.

RESULTS

The effects of SR 4233 on *in vitro* tumour colony formation were studied using hypoxic and non-hypoxic culture conditions in a total of 25 tumour specimens each. For both experimental settings cells were exposed to the agent for 1 h. The types of tumours studied are summarised in Table 1. For both settings, 14 (56%) tumour specimens were evaluable. Final concentrations of SR 4233 during hypoxic exposure were 10, 33, 100 and 500 μmol/l. For non-hypoxic exposure, final concentrations were the same and also included 1350 μmol/l. As shown in Table 2, SR 4233 was moderately active against tumour colony forming units under non-hypoxic conditions. At 500 μmol/l, five out of 14 (36%) evaluable tumour specimens were significantly inhibited (colony survival ≤ 0.5 × control, $P = 0.02$). However, a more profound concentration-dependent increase in the frequency of significant growth inhibition was notable when tumour cells were exposed to SR 4233 under hypoxic conditions (Table

Table 1. Tumour types studied with SR 4233. For hypoxic and non-hypoxic experiments, the number of evaluable and attempted specimens were identical

Tumour type	No. evaluable/ no. attempted (%)
Ovary	8/9 (89)
Lung, non-small cell	3/6 (50)
Breast	2/5 (40)
Other tumour types	1/5 (20)
Total	14/25 (56)

Table 2. Concentration-dependent inhibition of colony formation by SR 4233 (1-h exposure) using non-hypoxic culture conditions

Tumour type	No. specimens with inhibition*/no. specimens evaluable (μmol/l) 10	33	100	500	1350
Ovary	0/7	0/7	1/8	2/8	0/1
Lung, non-small cell	—	—	0/3	1/3	2/3
Breast	0/2	0/2	1/2	1/2	—
Other	0/1	1/1	1/1	1/1	—
Total	0/10 (0%)	1/10 (10%)	3/14 (21%)	5/14 (36%)	2/4 (50%)

*Colony survival ≤ 0.5 × control.

Table 3. Concentration-dependent inhibition of colony formation by SR 4233 (1-h exposure) using hypoxic culture conditions

Tumour type	No. specimens with inhibition*/no. specimens evaluable (μmol/l) 10	33	100	500
Ovary	2/8	4/8	7/8	7/7
Lung, non-small cell	0/3	0/3	1/3	—
Breast	0/2	2/2	2/2	2/2
Other	0/1	0/1	1/1	1/1
Total	2/14 (14%)	6/14 (43%)	11/14 (79%)	10/10 (100%)

*Colony survival ≤ 0.5 × control.

3). While two out of 14 specimens were sensitive at 10 μmol/l, colony formation of 10 out of 10 specimens was significantly decreased at 500 μmol/l ($P < 0.00005$). In head-to-head comparisons of each concentration, no significant difference in antitumour activity between hypoxic and non-hypoxic exposure conditions was detectable for 10 and 33 μmol/l ($P = 0.6$ and 0.2, respectively). However, at 100 and 500 μmol/l, hypoxic tumour cells were significantly more sensitive to SR 4233 than non-hypoxic cells ($P = 0.008$ and 0.005, respectively). Chart 1 summarises representative effects of SR 4233 on two ovarian cancer specimens. A steep concentration–response relationship was noted for hypoxic cells. The agent had only minor activity under non-hypoxic culture conditions. The difference between hypoxic and non-hypoxic cells was most pronounced at concentrations ≥ 100 μmol/l.

DISCUSSION

SR 4233 is a novel radiation sensitiser with intrinsic antitumour activity *in vitro*. It has been reported to have significantly higher activity against hypoxic tumour cells [1]. Its mechanism of action is believed to be due to the generation of a free radical 1-electron product that damages pyrimidine residues and leads to DNA strand scission [4, 10]. We have studied the effects of SR 4233 against tumour colony forming units from freshly explanted human tumours *in vitro* and have compared exposure to this compound under hypoxic and non-hypoxic conditions. Our data indicate that SR 4233 does have cytotoxic activity against human tumour colony forming units even under non-hypoxic conditions although this effect is rather moderate. As observed by other investigators using *in vitro* experiments with tumour cell lines, we noted the cytotoxic activity of SR 4233 was significantly increased when tumour cells were kept hypoxic [1, 4, 5]. This effect, however, was only statistically significant at concentrations of ≥ 100 μmol/l. The reasons for this concentration dependency (e.g. increased activity at only the higher concentration) are not clear but may involve the small sample size studied. While the increased activity of SR 4233 was clearly seen in ovarian cancer specimens, too few specimens of lung cancer or breast cancer were studied to permit further conclusions.

In summary, we have extended the earlier reports on animal and human tumour cell lines that SR 4233 is intrinsically active against *in vitro* tumour colony-forming units. This activity is significantly increased in hypoxic tumour cells. SR 4233 may thus be a promising agent for further clinical studies either alone or as a radiation sensitiser.

1. Zeman EM, Brown JM, Lemmon MJ, *et al.* SR-4233: a new bioreductive agent with high selective toxicity for hypoxic mammalian cells. *Int J Radiat Oncol Biol Phys* 1986, **12**, 1239–1242.
2. Brown JM, Lemmon MJ. Tumor hypoxia can be exploited to preferentially sensitize tumours to fractionated irradiation. *Int J Radiat Oncol Biol Phys* 1991, **20**, 457–461.
3. Zeman EM, Baker MA, Lemmon MJ, *et al.* Structure-activity relationships for benzotriazine di-N-oxides. *Int J Radiat Oncol Biol Phys* 1989, **16**, 977–981.
4. Zeman EM, Hirst VK, Lemmon MJ, *et al.* Enhancement of radiation-induced tumor cell killing by the hypoxic cell toxin SR 4233. *Radiother Oncol* 1988, **12**, 209–218.
5. Baker MA, Zeman EM, Hirst VK, *et al.* Metabolism of SR 4233 by Chinese hamster ovary cells: basis of selective hypoxic cytotoxicity. *Cancer Res* 1988, **48**, 5947–5952.
6. Cahill A, White IN. Reductive metabolism of 3-amino-1,2,4-benzotriazine-1,4-dioxide (SR 4233) and the induction of unscheduled DNA synthesis in rat and human derived cell lines. *Carcinogenesis* 1990, **11**, 1407–1411.
7. Zeman EM, Lemmon MJ, Brown JM. Aerobic radiosensitization by SR 4233 *in vitro* and *in vivo*. *Int J Radiat Oncol Biol Phys* 1990, **18**, 125–132.
8. Laderoute K, Wardman P, Rauth AM. Molecular mechanisms for the hypoxia-dependent activation of 3-amino-1,2,4-benzotriazine-1,4-dioxide (SR 4233). *Biochem Pharmacol* 1988, **37**, 1487–1495.
9. Zeman EM, Brown JM. Pre- and post-irradiation radiosensitization by SR 4233. *Int J Radiat Oncol Biol Phys* 1989, **16**, 967–971.
10. Brown JM, Lemmon MJ. Potentiation by the hypoxic cytotoxin SR 4233 of cell killing produced by fractionated irradiation of mouse tumors. *Cancer Res* 1990, **50**, 7745–7749.
11. Biedermann KA, Wang J, Graham RP, *et al.* SR 4233 cytotoxicity and metabolism in DNA repair-competent and repair-deficient cell cultures. *Br J Cancer* 1991, **63**, 358–362.
12. Walton MI, Workman P. Enzymology of the reductive bioactivation of SR 4233. A novel benzotriazine di-N-oxide hypoxic cell cytotoxin. *Biochem Pharmacol* 1990, **39**, 1735–1742.
13. Brown JM, Lemmon MJ. SR 4233: a tumor specific radiosensitizer active in fractionated radiation regimes. *Radiother Oncol* 1991, **20** (suppl. 1), 151–156.
14. Herman TS, Teicher BA, Coleman CN. Interaction of SR-4233 with hyperthermia and radiation in the FSaIIC murine fibrosarcoma tumor system *in vitro* and *in vivo*. *Cancer Res* 1990, **50**, 5055–5059.
15. Hamburger AW, Salmon SE. Primary bioassay of human tumor stem cells. *Science* 1977, **197**, 461–463.
16. Hanauske U, Hanauske A-R, Marshall MH, *et al.* Biphasic effects of vanadium salts on *in vitro* tumor colony growth. *Int J Cell Cloning* 1987, **5**, 170–178.

Acknowledgements—This work was supported in part by grants from Sterling Research and the Cancer Therapy and Research Foundation of South Texas.