12. Hongo T, Fujii Y, Igarashi Y. An *in vitro* chemosensitivity test for the screening of anti-cancer drugs in childhood leukemia. *Cancer* 1990, **65**, 1263–1272.
13. Dietel M, Arps H, Gerding D, Trapp M, Niendorf A. Establishment of primary cell cultures: Experiences with 155 cell strains. *Klin Wochenschr* 1987, **65**, 507–512.
14. Simon WE, Albrecht M, Hänsel M, Dietel M, Hölzel F. Cell lines derived from human ovarian carcinomas: Growth stimulation by gonadotropic and steroid hormones. *J Natl Cancer Inst* 1983, **70**, 839–845.
15. Dietel M, Arps H, Lage H, Niendorf A. Membrane vesicle formation due to acquired mitoxantrone resistance in human gastric carcinoma cell line EPG85-257. *Cancer Res* 1990, **50**, 6100–6106.
16. Dietel M, Arps H, Bals U, *et al.* Individualisierung der chemotherapie durch prädiktive *In vitro*-bestimmung der zytostatikasensitivität maligner tumoren. *DMW* 1989, **114**, 1645–1652.
17. Dietel M, Arps H, Klapdor R, Müller-Hagen S, Sieck M, Hoffmann L. Antigen detection by the monoclonal antibodies CA 19-9 and CA 125 in normal and tumor tissue and patient's sera. *J Cancer Res Clin Oncol* 1986, **111**, 257–265.
18. Chabner B. The role of drugs in cancer treatment. In Chabner B, ed. *Pharmacologic Principles of Cancer Treatment*. Philadelphia, W.B. Saunders. 1982, 3–14.
19. WHO Handbook for Reporting Results of Cancer Treatment. WHO Offset publication No. 48. World Health Organization, Geneva, 1979.
20. Ozols RF, Garvin AJ, Costa J, Simon RM, Young RC. Advanced ovarian cancer. Correlation of histological grade with response to therapy and survival. *Cancer* 1980, **45**, 572–581.
21. Smith HS, Zoli W, Volpi A, *et al.* Preliminary correlations of clinical outcome with *in vitro* chemosensitivity of second passage human breast cancer cells. *Cancer Res* 1990, **50**, 2943–2948.
22. Campling BG, Pym J, Baker HM, Cole SPC, Lam YM. Chemosensitivity testing of small cell lung cancer using the MTT assay. *Br J Cancer* 1991, **63**, 75–83.
23. Wilbur DW, Camacho ES, Hilliard DA, Dill PL, Weisenthal LM. Chemotherapy of non-small cell lung carcinoma guided by an *in vitro* drug resistance assay measuring total tumour cell kill. *Br J Cancer* 1992, **65**, 27–32.
24. Metelmann HR, Schlesinger S. Therapieergebnisse mit Antionkogramm-orientierten Zytostatika-Kombinationen bei Patienten mit Mundhöhlen-Karzinomen. *Dtsch Zeitschr Mund- Kiefer- und Gesichtschir* 1988, **12**, 307–313.
25. Park CH, Wiernik PH, Morrison FS, *et al.* Clinical correlations of leukemic clonogenic cell sensitivity assessed by *in vitro* continuous exposure to drugs. *Cancer Res* 1983, **43**, 2346–2349.
26. Werner A, Diedrich K, Kurbacher C, *et al.* Chemosensitivitätstestung von Ovarial- und Mammakarzinomen im Kolonie-Test und seinen Modifikationen. *Tumor Diagnostik Therapie* 1988, **9**, 204–209.
27. Venesmaa P, Ylikorkala O. Subrenal capsule assay in selection of chemotherapy after operation for recurrent ovarian cancer. *Br J Cancer* 1991, **63**, 84–86.
28. Link KH, Aigner KR, Kuehn W, Schwemmle K, Kern DH. Prospective correlative chemosensitivity testing in high-dose intraarterial chemotherapy for liver metastases. *Cancer Res* 1986, **6**, 4837–4840.
29. Levy G. Relationship between pharmacological effects and plasma or tissue concentration of drugs in man. In Davies DS, Prickard BNC eds. *Biological Effects of Drugs in Relation to Their Plasma or Tissue Concentrations in Man*. Baltimore, University Park Press, 1973.
30. Dietel M. What's new in cytostatic drug resistance and pathology? *Pathol Res Pract* 1991, **187**, 892–905.

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Antitumour Activity of an Immunoconjugate Composed of Anti-human Astrocytoma Monoclonal Antibody and Neocarzinostatin

Seiji Kondo, Shouji Nakatsu, Harumi Sakahara, Hisataka Kobayashi, Junji Konishi and Yuziro Namba

Neocarzinostatin (NCS) linked to the thiol group on the hinge region of the Fab' fragment of GA-17, a murine monoclonal antibody reacting with tyrosine-specific phosphorylated antigens, which are exclusively expressed on the cell surface of human astrocytomas, was evaluated for *in vivo* activity. GA-17-NCS immunoconjugates significantly suppressed the growth of human malignant glioma cell line U87-MG subcutaneous xenografts in nude mice until day 50 when administered intravenously into the tail vein. Disulphide- and thioether- linked GA-17-NCS were nearly equipotent immunoconjugates, but thioether-linked GA-17-NCS was more effective than disulphide-linked conjugates with 250 U/kg NCS content on day 50 ($P < 0.05$). Thioether-linked GA-17-NCS was significantly more effective on day 50 than free NCS with 500 U/kg or 250 U/kg NCS content ($P < 0.05$, $P < 0.01$, respectively). These results suggest that GA-17-NCS may prove useful in the treatment of human malignant gliomas.
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INTRODUCTION

MALIGNANT GLIOMAS, the most common primary malignant tumours of the brain, are rapidly fatal. The best treatment currently available (surgery, radiation therapy and systemic chemotherapy) results in median survival times of less than 1 year [1].

In recent years, monoclonal antibodies (Mab) recognising tumour-associated cell surface antigens have been widely utilised as tumour-specific carriers for cytotoxic agents such as toxins, radioisotopes, and anticancer drugs [2–5]. The efficacy of immunoconjugates depends upon both the Mab and drug components, and an immunoconjugate may potentially have advantages over

conventional forms of malignant glioma treatment if it possesses higher tumour specificity.

In this paper, we describe immunoconjugates formed by linkage of a proteinaceous anticancer antibiotic neocarzinostatin (NCS) to the thiol group on the hinge region of the Fab' fragment of Mab GA-17 via a disulphide or thioether bond [6–8]. We show that NCS linked to GA-17 (GA-17-NCS) can suppress the growth of solid-malignant glioma growing subcutaneously in nude mice when administered intravenously into the tail vein.

MATERIALS AND METHODS

Cells

The human malignant glioma cell line U87-MG was maintained in Dulbecco's modified essential medium (DMEM, Nissui, Tokyo, Japan) supplemented with 10% fetal calf serum (FCS, GIBCO, Grand Island, NY). All assays were conducted using antigen-positive U87-MG cells established in nude mice.

Monoclonal antibodies and fragmentations

Mab GA-17 is a murine IgG_1 which reacts with tyrosine-specific phosphorylated proteins expressed exclusively on the cell surface of human astrocytomas [9, 10]. $F(ab')_2$ fragments of GA-17 were prepared from whole antibody by digestion with immobilised pepsin. The $F(ab')_2$ of GA-17 was reduced to monovalent fragments (Fab') prior to conjugation.

Immunoconjugate GA-17-NCS

NCS (Kayaku, Tokyo, Japan) was conjugated with Fab' of GA-17 via a disulphide or thioether bond using a method described previously [11–13]. Briefly, NCS was treated with a heterobifunctional reagent, *N*-succinimidyl 3-(2-pyridyldithio) propionate (SPDP) or *N*-succinimidyl 4-(*N*-maleimidomethyl) cyclohexane-1-carboxylate (SMCC) (Pharmacia AB, Uppsala, Sweden) in order to introduce 3-(2-pyridyldithio) propionyl groups or maleimide groups, respectively, which were then permitted to react with the thiol group on the hinge region of the Fab'. The two-type conjugation rate was about 1 mol of NCS per mol of GA-17 (Fab').

In vivo *localisation of GA-17*

Mab GA-17 $F(ab')_2$ fragments were labelled with ^{131}I using the chloramine-T method. BALB/c (*nu/nu*) female athymic mice with U87-MG cells transplanted into the right flank were fed Lugol's solution for 2 days prior to antibody treatment. Radiolabelled Mab (3.7 MBq) was injected intravenously into the tail vein. At 48 h after injection of [^{131}I]Mab, imaging of mice was undertaken with a gamma camera.

In vivo *activity of GA-17-NCS*

Nude mice received subcutaneous injections in the right flank of 5×10^6 exponentially growing U87-MG cells on day 1. The tumours were permitted to establish on day 14, and free NCS, free GA-17, mixtures of NCS with GA-17, or GA-17-NCS was administered intravenously into the tail vein using a 100 μl volume of sample in PBS on days 21 and 23. The growth of tumour was monitored with the use of calipers at 2- or 3-day intervals. Tumour volume (V) was calculated from $(L \times W^2)/2$, where L = length (mm) and W = width (mm). All control groups had five mice each, as did the treatment groups. Data were statistically analysed using the Student's t-test.

RESULTS AND DISCUSSION

In vivo *localisation of GA-17*

The ability of Mab GA-17 to localise in malignant glioma xenografts was determined. Gamma camera imaging clearly demonstrated localisation of ^{131}I-GA-17 $F(ab')_2$ in tumour xenografts (Fig. 1).

In vivo *activity of GA-17-NCS*

The *in vivo* antitumour activity of GA-17-NCS against well-established U87-MG xenografts was as shown in Fig. 2. Panels (a) and (b) show a comparison of the effect of 500 U/kg or 250 U/kg NCS content of GA-17-NCS with that of free NCS. As is shown in Fig. 2(a), significant regression of solid tumours was obtained with two treatments of GA-17-NCS (GA-17, 2.5 mg/kg; NCS, 500 U/kg). Use of disulphide and thioether bond GA-17-NCS resulted in almost equipotent immunoconjugates. The mean tumour volume of thioether-linked GA-17-NCS-treated mice was 30 ± 8 mm^3 on day 50, significantly smaller than that of control mice receiving PBS (3080 ± 320 mm^3) ($P < 0.01$). The average V_T/V_C ratio determined from tumour volumes of immunoconjugate-treated and control mice was 0.01 on day 50. Free NCS, on the other hand, was not able to induce tumour regression when tested at the same dose. The mean tumour volume of immunoconjugate-treated mice was also significantly smaller than that of free NCS-treated mice (1045 ± 205 mm^3) ($P < 0.05$). As is shown in Fig. 2(b), treatment of the tumour xenografts with GA-17-NCS (GA-17, 1.25 mg/kg; NCS, 250 U/kg) did not induce tumour regression, but did result in suppression of tumour growth until day 30; moreover, a rebound in tumour growth was observed on day 40. In contrast, free NCS (250 U/kg) did not suppress tumour growth. On day 50, the mean tumour volume of thioether-linked GA-17-NCS-treated mice was 540 ± 55 mm^3, slightly smaller than that of disulphide-linked conjugate treated mice (865 ± 150 mm^3) ($P < 0.05$). It is also apparent that, on day 50, the mean tumour volume of thioether-linked GA-17-

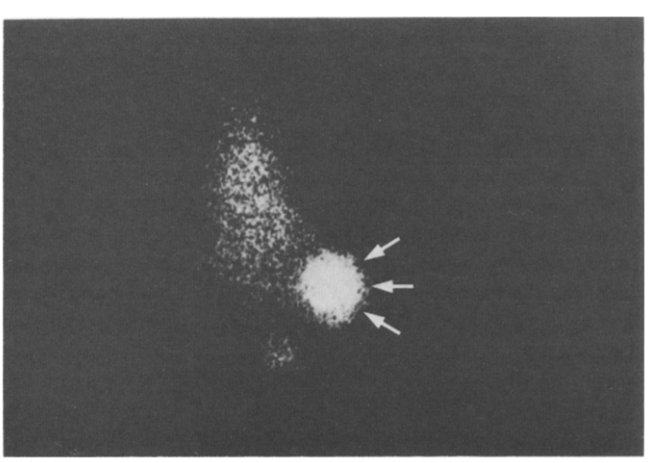

Fig. 1. *In vivo* localisation of [^{131}I]Mab GA-17. Nude mice with subcutaneously transplanted U87-MG cells were given intravenous injections of 3.7 MBq radiolabelled GA-17 $F(ab')_2$. Tumour xenografts were located in the right flank of mice. Arrows indicate the specific accumulation of ^{131}I-GA-17 in the tumour.

Correspondence to S. Kondo.
S. Kondo is at the Department of Neurosurgery, Utano National Hospital, 8-Ondoyama-cho, Narutaki, Ukyo-ku, Kyoto 616, Japan; S. Nakatsu is at the Department of Neurosurgery; H. Sakahara, H. Kobayashi and J. Konishi are at the Department of Nuclear Medicine, Faculty of Medicine; and Y. Namba is at the Institute for Virus Research, Kyoto University, Kyoto, Japan.
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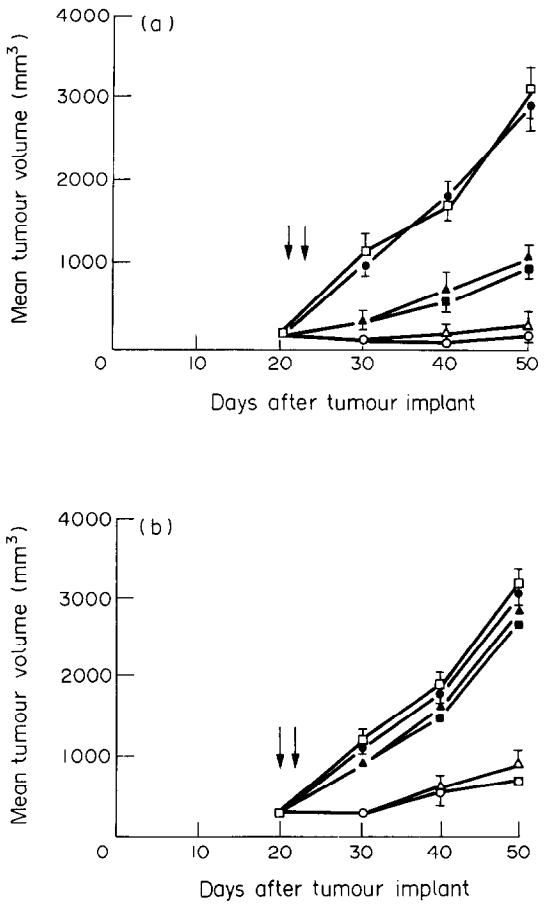

Fig. 2. Effectiviness of GA-17-NCS against well-established U87-MG xenografts. Tumour cells (5×10^6) were implanted subcutaneously in the right flank of nude mice. Control PBS (□), free GA-17 (Fab') (●), free NCS (▲), mixtures of GA-17 with NCS (■), disulphide- (△) or thioether-linked (○) GA-17-NCS was given intravenously on days 21 and 23 after tumour implant. (a) Dose with 500 U/kg NCS content, (b) Dose with 250 U/kg NCS content. Treatments are indicated by the arrows. Mean tumour volumes and standard error bars are shown for each group.

NCS-treated mice was significantly smaller than that of control (3105 ± 290 mm^3) or free NCS-treated mice (3020 ± 215 mm^3) ($P < 0.01$ and $P < 0.01$, respectively). The average V_T/V_C ratios determined from tumour volumes of thioether-linked GA-17-NCS- or free NCS-treated mice and control mice were 0.17 and 0.97, respectively. Treatment with GA-17 (Fab') alone had no significant effect on tumour growth, and mixtures of free NCS with free GA-17 were no more effective than free NCS alone. The above data indicate that GA-17-NCS is significantly more efficacious than free NCS as a therapeutic agent directed against the human malignant glioma xenograft.

We chose to use the thiol group on the hinge region of Fab' fragment for coupling to NCS rather than the amino groups of IgG, F(ab')$_2$ or Fab' based on the following considerations: (1) F(ab')$_2$ and Fab' penetrate more extensively into the tumour mass than IgG [5]; (2) malignant human brain tumours are more permeable to Fab' than to IgG [14, 15]. Of course, it is difficult to estimate the efficacy of this high molecular weight immunoconjugate GA-17-NCS in clinical therapy of malignant glioma. However, GA-17-NCS administered directly into the cerebrospinal fluid or tumour to avoid delivery problems caused by the blood brain barrier may provide effective response [4]; (3) the blood-clearance of F(ab')$_2$ or of Fab' is faster than that of IgG; so clinical application with high drug dosages may therefore be undertaken without serious adverse effects; (4) the linking enzyme via a disulphide or thioether bond to the thiol group on the hinge region of F(ab')$_2$ or Fab' is consistently more specific than that to the amino group of F(ab')$_2$ or Fab' [11]; the linkage using this thiol group may have no effect on the antigen binding activity of antibodies because this site of the thiol group is far from the antigen binding site; (5) thioether linkage with Fab' is highly efficient in yielding 1:1 molar ratio conjugates; this efficiency is very important for the large scale manufacturing necessary for clinical trials [13].

It has also been reported that human anti-mouse antibody (HAMA) was detectable in sera from patients receiving immunotherapy with mouse Mab [16]. This HAMA might reduce not only the level of both spontaneous cytotoxicity and antibody-dependent cell-mediated cytotoxicity (ADCC) in most patients, but also the anticancer effect of immunoconjugates [17, 18]. Takahashi *et al.* reported that there were no serious side effects in any patient receiving the A7-NCS conjugate (NCS; 1000–6000 U) for treatment of colorectal cancer [3], but recommended repeated administration of A7-NCS over a short period of time for clinical use [19]. Our current efforts are focused upon evaluating and reducing the toxicity of GA-17-NCS while maintaining its *in vivo* potency. Notably, the production of murine/human chimeric antibodies may also be considered for the purpose of avoiding HAMA [20].

1. Walker MD, Green SB, Byar DP. Randomized comparisons of radiotherapy and nitrosoureas for the treatment of malignant glioma after surgery. *N Engl J Med* 1980, **303**, 1323–1329.
2. Bjorn MJ, Groetsema G, Scalapino L. Antibody-Pseudomonas exotoxin A conjugates cytotoxic to human breast cancer cells *in vitro*. *Cancer Res* 1986, **46**, 3262–3267.
3. Takahashi T, Yamaguchi T, Kitamura K, *et al.* Clinical application of monoclonal antibody-drug conjugates for immunotargeting chemotherapy of colorectal carcinoma. *Cancer* 1988, **61**, 881–888.
4. Johnson VG, Wrobel C, Wilson D, *et al.* Improved tumor-specific immunotoxins in the treatment of CNS and leptomeningeal neoplasias. *J. Neurosurg* 1989, **70**, 240–248.
5. Roffler SR, Yu M-H, Chen BM, Tung E, Yeh M-Y. Therapy of human cervical carcinoma with monoclonal antibody-Pseudomonas exotoxin conjugates. *Cancer Res* 1991, **51**, 4001–4007.
6. Maeda H, Takeshita J, Yamashita A. Lymphotropic accumulation of an antitumor antibiotic protein, Neocarzinostatin. *Eur J Cancer Clin Oncol* 1979, **16**, 723–731.
7. Uemura S, Matsukado Y, Sonoda H, *et al.* Treatment of malignant glioma with Neocarzinostatin. A combined chemoradiotherapy with Intra-arterial Neocarzinostatin perfusion and irradiation. *Neurol Med Chir* 1986, **26**, 304–310.
8. Jung G, Kohnlein W, Luders G. Biological activity of the antitumor protein neocarzinostatin coupled to a monoclonal antibody by *N*-succinidyl 3-(2-pyridylthio)-propionate. *Biochem Biophys Res Commun* 1981, **101**, 599–606.
9. Kondo S, Miyatake S, Iwasaki K, *et al.* Human glioma-specific antigens detected by monoclonal antibodies. *Neurosurgery* 1992, **30**, 506–511.
10. Kondo S, Miyatake S, Matsumoto M, *et al.* Analysis of the close relationship between human astrocytoma-specific antigens detected by murine monoclonal antibodies and c-kit proto-oncogene product. *Biochem Biophys Res Commun* 1992, **182**, 474–480.
11. Yoshitake S, Yamada Y, Ishikawa E, Masseyeff R. Conjugation of glucose oxidase from *Aspergillus niger* and rabbit antibodies using *N*-hydroxysuccinimide ester of *N*-(4-carboxycyclo-hexylmetyl)-maleimide. *Eur J Biochem* 1979, **101**, 395–399.
12. Yoshitake S, Imagawa M, Ishikawa E, *et al.* Mild and efficient conjugation of rabbit Fab' and horseradish peroxidase using a maleimide compound and its use for enzyme immunoassay. *J Biochem* 1982, **92**, 1413–1424.
13. Morgan AC, Sivam G, Beaumier P, Mcintyre R, Bjorn M, Abrams PG. Immunotoxins of Pseudomonas exotoxin A (PE): effect of

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.

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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.
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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.
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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.