

# **In Vivo Potentiation of 1-(2-Chloroethyl)-3-cyclohexyl-1-nitrosourea by the Radiation Sensitizer Benznidazole<sup>1</sup>**

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## **ABSTRACT**

Recent studies in mouse tumor systems have indicated a potential therapeutic advantage in combining the radiosensitizer misonidazole (MISO) with cancer chemotherapy drugs. One agent the antitumor activity of which has been enhanced to a greater extent than its hematological or gastrointestinal toxicities is the nitrosourea, 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU). Recently, sensitizers more lipophilic than MISO have been reported to give greater tumor response enhancement when combined with CCNU. The present studies compared the potential therapeutic benefit of combining MISO (partition coefficient, 0.43) or benznidazole (BENZO) (partition coefficient, 8.5) in KHT sarcoma or RIF-1 tumor-bearing C3H mice. Both sensitizers were administered i.p. and given either 30 min before (BENZO) or simultaneously with (MISO) the chemotherapeutic agent. Survival of clonogenic tumor cells assessed 22 to 24 hr after treatment or *in situ* tumor growth delay were used as assays of tumor response. Normal tissue toxicity was determined using the drug dose yielding 50% animal lethality in 30 days end point. When combined with CCNU, doses of MISO (5.0 mmol/kg) or BENZO (0.3 mmol/kg) were found to yield approximately equivalent increases in both the tumor effect (enhancement ratio, ~1.8 to 2.0) and normal tissue toxicity (enhancement ratio ~1.3 to 1.4). Both sensitizers therefore led to a therapeutic benefit. However, although a ~10-fold lower dose of the more lipophilic sensitizer BENZO proved to be as effective as MISO at enhancing the tumoricidal effects of CCNU, this dose reduction did not result in a greater therapeutic gain for BENZO.

## **INTRODUCTION**

Highly electron-affinic compounds such as the nitroimidazole MISO<sup>3</sup> have been shown to be effective radiation sensitizers of oxygen-deficient or hypoxic tumor cells (1). It also is known that combining MISO with conventional chemotherapeutic agents (primarily alkylating agents) can cause marked enhancements in the cytotoxic action of these agents both *in vitro* (14, 23-25, 28) and *in vivo* (4, 8, 12, 15, 17, 26). Of particular interest in these chemopotential studies, is the observation made in several animal tumor models that a therapeutic benefit, *i.e.*, a selective enhancement of the tumor response, may be achieved when MISO is added to the chemotherapeutic pro-

tolocol (for reviews, see Refs. 11 and 18).

Recently, it further has been suggested that more lipophilic sensitizers might be more effective chemopotentializers than MISO (32). One agent in particular, BENZO, was found to be as effective as MISO and at approximately 10-fold lower sensitizer doses (32). Since it has been shown previously that MISO can lead to a differential improvement in the response of the KHT sarcoma and RIF-1 tumor to the nitrosourea CCNU compared to the extent of the normal tissue toxicity enhancement (6, 11, 17-19, 30), these 2 tumor model systems were chosen to evaluate the CCNU:BENZO combination in the present series of experiments. The aim of these studies was to determine whether the lower doses of BENZO reported to yield chemopotentialization in tumors equivalent to or greater than that achieved with MISO would translate directly into a larger therapeutic gain for the CCNU:BENZO combination than for CCNU plus MISO.

## **MATERIALS AND METHODS**

### **Animals and Tumor Models**

KHT sarcoma cells (7) were maintained *in vivo* and prepared from solid tumors by a mechanical dissociation procedure (27). RIF-1 tumor cells were grown and passaged alternately *in vitro* and *in vivo* as described by Twentyman *et al.* (29). In experiments,  $2 \times 10^5$  KHT sarcoma or RIF-1 tumor cells were inoculated i.m. into the hind limbs of 8- to 14-week-old female C3H/HeJ mice (The Jackson Laboratory, Bar Harbor, Maine). For treatment evaluation, tumors weighing 0.2 to 0.3 g were selected.

### **Drug Treatments**

CCNU was received from Dr. Robert Engle, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute. MISO was obtained from Dr. Ven Narayanan, Drug Synthesis and Chemistry Branch, National Cancer Institute. BENZO was kindly provided by Dr. Carey Smithen, Roche Laboratories (Welwyn Garden City, England), and Dr. W. R. Scott, Roche Laboratories (Nutley, N. J.).

CCNU was dissolved in absolute ethanol (10 mg/ml) until just prior to injection when 9 ml of a 0.3% solution of hydroxypropyl cellulose in 0.9% NaCl solution was added to 1.0 ml of the stock solution. MISO was dissolved in 0.9% NaCl solution at 20 mg/ml. BENZO was prepared in a polyethylene glycol:0.9% NaCl solution (1:1, v/v). All drugs were administered i.p. according to animal body weight. On the basis of previous studies, MISO and CCNU were given simultaneously while BENZO preceded the CCNU treatment by 30 min (17, 32). Tumor response was not affected by giving animals injections of the CCNU or BENZO carriers alone, nor was the tumor response to CCNU altered by administering volumes of 0.9% NaCl solution equivalent to those given to mice receiving a sensitizer in combination with CCNU.

### **Measurement of Tumor Response**

**Clonogenic Cell Survival.** Cell survival was assessed 22 to 24 hr after treatment with the chemotherapeutic agent alone or in combina-

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<sup>3</sup> The abbreviations used are: MISO, misonidazole; BENZO, benznidazole; CCNU, 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea; ER, enhancement ratio.

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tion with the sensitizers. The tumors were aseptically excised, and single-cell suspensions were prepared using a combination of mechanical and enzymatic dissociation procedures (27, 29). The latter consisted of a 30-min incubation in trypsin (0.2%) plus DNase. The cells were counted using a hemocytometer, and various dilutions were prepared. KHT sarcoma cells were plated into 24-well multiwell plates with  $10^4$  lethally irradiated tumor cells in 0.2% agar containing  $\alpha$  minimum essential medium supplemented with 10% fetal calf serum. Two weeks later, the colonies from surviving cells were counted with the aid of a dissecting microscope. RIF-1 cells were plated into 60-mm plastic dishes containing  $\alpha$  minimum essential medium with 10% newborn calf serum. These dishes were incubated for 12 days at 37°, harvested, and stained with crystal violet, and colonies of over 50 cells were counted. In these experiments, cell recovery and plating efficiency were in the ranges of  $0.2$  to  $1.0 \times 10^8$  cells/g and 10 to 40%, respectively.

**Tumor Regrowth Delay.** Following treatment, the tumors were measured daily by passing the tumor-bearing legs through a plastic plate with holes of various diameters. The size of the hole the tumor-bearing leg would just pass through was determined and converted to a tumor weight using a calibration curve as previously described (21, 22). The median time for the tumors of each treatment group to grow to 4 times the starting size was determined, and confidence limits about the medium were calculated using nonparametric statistics (13).

**RESULTS**

The effect on clonogenic cell survival in the KHT sarcoma of combining a fixed dose of CCNU (5.0 mg/kg) with a range of doses of the sensitizer BENZO is illustrated in Chart 1. The data show that even a 0.1-mmol/kg dose led to a significant increase in tumor cell kill compared to CCNU alone. Increasing the sensitizer dose up to a dose of  $\sim 0.3$  mmol/kg further decreased survival rapidly. When larger BENZO doses were administered, little additional enhancement in the tumoricidal effect of CCNU was seen. On the basis of these results, a dose of 0.3 mmol/kg was selected for all subsequent investigations.

When KHT sarcoma-bearing C3H mice were treated with a combination of BENZO (0.3 mmol/kg) and a range of CCNU doses (Chart 2), the extent of tumor cell killing was increased significantly compared to that seen for CCNU treatment alone (● versus ○). From the data, an ER, defined as the ratio of the dose of CCNU alone divided by the dose of CCNU plus BENZO required to yield the same biological effect, *i.e.*, same level of cell kill, can be calculated. The results show that BENZO is dose modifying when combined with CCNU yielding an ER of  $\sim 2.0$  irrespective of the cell survival level used to calculate it. This enhancement in the efficacy of CCNU by a 0.3-mmol/kg dose of BENZO is similar to that reported previously for a MISO dose of 5.0 mmol/kg (17, 19).

Tumor response to CCNU plus BENZO also was assessed in the KHT sarcoma using a tumor growth delay assay (Chart 3). The data indicate that, just as was seen in the clonogenic cell survival studies, BENZO improved the tumor response to CCNU (■, ●). Also shown in this chart is the response of KHT sarcomas to CCNU plus 5.0 mmol/kg MISO (□, ○). Using tumor regrowth as the end point, the sensitizers were found to lead to ERs of  $\sim 1.8$  to 2.0 for CCNU combined with BENZO (0.3 mmol/kg) or MISO (5.0 mmol/kg).

For comparison with the KHT sarcoma, combinations of CCNU and BENZO or MISO also were evaluated in the RIF-1 tumor model (Chart 4). This tumor is considerably more resistant to nitrosourea treatments than the KHT sarcoma (9, 17, 19;

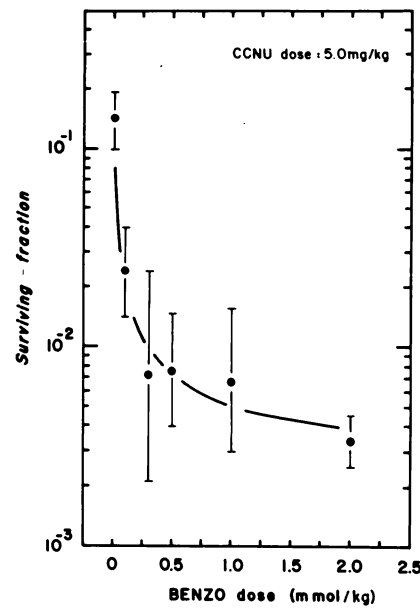


Chart 1. Clonogenic cell survival in the KHT sarcoma as a function of the dose of BENZO administered in combination with a 5.0-mg/kg dose of CCNU. In each experiment, 2 tumors were pooled at every dose. Points, mean of 3 to 6 experiments; bars, S.E.

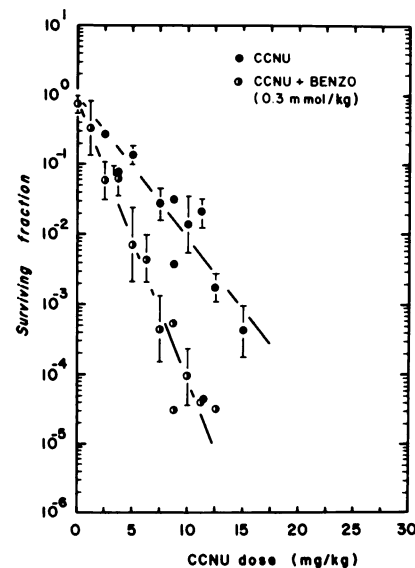


Chart 2. Cell survival assessed in KHT sarcomas treated 24 hr previously with single doses of CCNU either alone or in combination with a 0.3-mmol/kg dose of BENZO. Points, mean of 3 to 6 experiments; bars, S.E. Data points without errors are single determinations.

see also Charts 2 and 4). Nevertheless, as has been shown previously (19, 30), CCNU-induced tumor cell kill was enhanced significantly by the addition of MISO (○). BENZO at 0.3 mmol/kg (●) again appeared to be as effective in combination with CCNU as the larger dose of MISO. The ER from these data for both sensitizers was  $\sim 1.8$ .

To assess whether the addition of BENZO would lead to a therapeutic benefit, systemic toxicity due to CCNU alone or CCNU:BENZO was assessed (Table 1). Even though it has been shown previously in both our laboratories (12, 17, 19) as well as those of others (3) that most mice die within 2 weeks after single-dose nitrosourea exposure, the animals in the

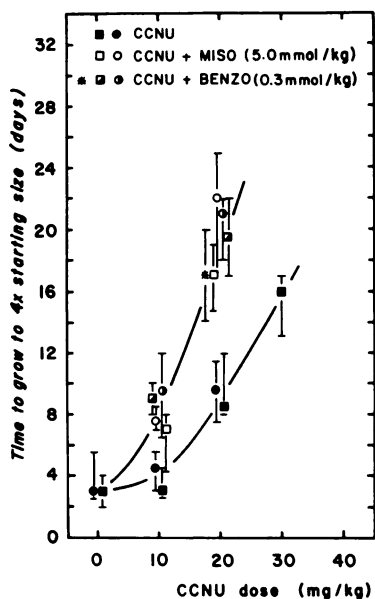


Chart 3. Days to grow to 4 times the starting size as a function of the CCNU dose for KHT sarcoma-bearing C3H mice treated with (a) CCNU alone, (b) CCNU plus MISO (5.0 mmol/kg) simultaneously, or (c) BENZO (0.3 mmol/kg) 30 min prior to CCNU. \*, group of mice treated simultaneously with a 0.3-mmol/kg dose of BENZO and a 20-mg/kg dose of CCNU. Points, median tumor responses; bars, 98% confidence limits of 7 to 10 mice. Confidence intervals about the median were calculated using nonparametric statistics (13).

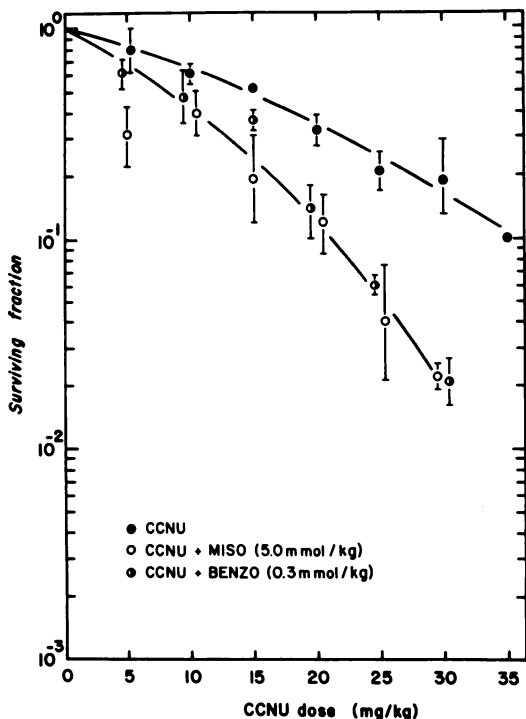


Chart 4. Cell killing assessed in RIF-1 tumors 22 to 24 hr after treatment with single doses of CCNU, CCNU plus a 5.0-mmol/kg dose of MISO, or CCNU plus a 0.3 mmol/kg dose of BENZO. The results shown are as described in Charts 1 and 2.

present experiments nevertheless were followed for 30 days after treatment. This was done to include both the gastrointestinal and bone marrow toxicity syndromes. The results of 3 experiments (Table 1) show that pretreating mice with a 0.3-

mmol/kg dose of BENZO significantly reduced the drug dose yielding 50% animal lethality in 30 days of CCNU. The combination of CCNU plus BENZO resulted in an enhancement in the systemic toxicity by a factor of ~1.3 to 1.4.

### DISCUSSION

The *in vivo* enhancement of the tumor response to alkylating chemotherapeutic agents, particularly melphalan, cyclophosphamide, and CCNU, by the radiation sensitizer MISO has been well established (for reviews, see Refs. 11 and 18). Many of these studies have suggested that a therapeutic benefit may be achieved through such a combination. Nevertheless, almost all investigations also have reported concomitant enhancements in normal tissue toxicity when alkylators and MISO were combined. This increased systemic toxicity probably was due to the large doses of MISO usually required to produce increased antitumor activity. Recent attempts to maintain the enhanced antitumor efficacy without increased normal tissue toxicity through the administration of repeated small doses of MISO has been successful in some (6, 11) but not all (31) investigations.

Alternatively, other nitroimidazoles have been compared to MISO in an attempt to select more effective chemopotentiators (18). In general, this approach has met with only limited success, although a few promising compounds have been found (10, 16, 32). In particular, Workman and Twentyman (32) have shown enhancement of the tumoricidal effects of CCNU, equivalent to that seen with MISO, by the administration of significantly lower doses of the more lipophilic radiation sensitizer BENZO.

To evaluate this compound further, chemopotential of CCNU by BENZO was assessed in the present study in 2 tumor models with intrinsically different responses to CCNU as well as in terms of systemic host toxicity. The results verified that BENZO is more effective than MISO on an administered dose basis and that lipophilicity is more important than electron affinity for CCNU enhancement as had been indicated previously by Workman and Twentyman (32). The findings show that a BENZO dose of 0.3 mmol/kg is essentially equivalent to a 5.0-mmol/kg dose of MISO at enhancing the response of both the KHT sarcoma and RIF-1 tumor to CCNU (Charts 2 to 4). For both tumor models, the inclusion of the sensitizer in the treatment protocol led to an ER of ~1.8 to 2.0. This increase in the antitumor efficacy of CCNU is similar to what has been reported previously for MISO (6, 17, 19, 32) and BENZO (32).

Table 1  
Systemic toxicity of CCNU or CCNU plus a 0.3-mmol/kg dose of BENZO in non-tumor-bearing female C3H/HeJ mice

| Experiment | LD <sub>50/30</sub> <sup>a</sup> |                  | ER <sup>b</sup> |
|------------|----------------------------------|------------------|-----------------|
|            | CCNU                             | CCNU + BENZO     |                 |
| A          | 48.5 (46.5-50.4) <sup>c</sup>    | 38.5 (36.6-40.7) | 1.26            |
| B          | 42.5 (40.5-44.5)                 | 30.0 (28.1-32.0) | 1.42            |
| C          | 47.2 (45.3-49.2)                 | 34.2 (32.1-36.2) | 1.38            |

<sup>a</sup> LD<sub>50/30</sub>, drug dose yielding 50% animal lethality in 30 days, calculated using a logit bioassay (2) and Scheffe's discrimination interval (5).

$$ER = \frac{LD_{50/30} \text{ CCNU}}{LD_{50/30} \text{ CCNU + BENZO}}$$

<sup>c</sup> Numbers in parentheses, 95% confidence intervals.

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Previous studies, evaluating primarily bone marrow and gut toxicity, have shown that the addition of MISO at 2.5 to 5.0 mmol/kg to CCNU treatments enhanced normal tissue damage by a factor of ~1.0 to 1.4 (for review, see Ref. 11). The present data (Table 1) illustrate that a similar increase in systemic toxicity was seen when 0.3-mmol/kg doses of BENZO and CCNU were combined.

Because the chemopotential effect was found to be greater in the tumor than in the normal tissue toxicity studies, CCNU:BENZO did lead to a therapeutic benefit. Nevertheless, the reduced doses of BENZO required for the potentiation of CCNU efficacy did not result in a therapeutic result which was significantly better than that previously achieved with a combination of CCNU and MISO. Consequently, on the basis of therapeutic gain, the present findings indicate no real advantage in using BENZO instead of MISO to potentiate the activity of CCNU. The lower doses of BENZO required to observe chemopotential may, however, possibly prove advantageous in the clinic since such doses will be more readily achievable.

## REFERENCES

- Adams, G. E. Hypoxic cell sensitizers for radio-therapy. In: F. F. Becker (ed.), *Cancer. A Comprehensive Treatise*, Vol. 6, pp. 181-224. New York: Plenum Publications, Inc., 1977.
- Berkson, J. Maximum likelihood and minimum  $\chi^2$  estimates of the logistic function. *J. Am. Statist. Assoc.*, 50: 130-162, 1955.
- Blackett, N. M., Courtenay, V. D., and Mayer, S. M. Differential sensitivity of colony-forming cells of hemopoietic tissue, Lewis lung carcinoma, and B16 melanoma to three nitrosoureas. *Cancer Chemother. Rep.*, 59: 929-933, 1975.
- Clement, J. J., Gorman, M. S., Wodinsky, I., Catane, R., and Johnson, R. K. Enhancement of anti-tumor activity of alkylating agents by the radiation sensitizer misonidazole. *Cancer Res.*, 40: 4165-4172, 1980.
- Finney, D. J. *Statistical Methods*. London: Griffin, 1971.
- Hirst, D. G., and Brown, J. M. The therapeutic potential of misonidazole enhancement of alkylating agent cytotoxicity. *Int. J. Radiat. Oncol. Biol. Phys.*, 8: 639-642, 1982.
- Kallman, R. F., Silini, G., and Van Putten, L. M. Factors influencing the quantitative estimation of the *in vivo* survival of cells from solid tumors. *J. Natl. Cancer Inst.*, 39: 539-549, 1967.
- Law, M. P., Hirst, D. G., and Brown, J. M. Enhancing effect of misonidazole on the response of the RIF-1 tumour to cyclophosphamide. *Br. J. Cancer*, 44: 208-218, 1981.
- Lelieveld, P., Twentyman, P. R., Kallman, R. F., and Brown, J. M. The effect of time between X-irradiation and chemotherapy on the growth of three solid tumors. VI. BCNU. *Int. J. Radiat. Oncol. Biol. Phys.*, 5: 1549-1552, 1979.
- Martin, W. M. C., McNally, N. J., and deRonde, J. The enhancement of effect of cytotoxic drugs by radiosensitizers. *Br. J. Cancer*, 43: 756-766, 1981.
- McNally, N. Enhancement of chemotherapy. *Int. J. Radiat. Oncol. Biol. Phys.*, 8: 593-599, 1982.
- Mulcahy, R. T., Siemann, D. W., and Sutherland, R. M. *In vivo* response of KHT sarcomas to combination chemotherapy with radiosensitizers and BCNU. *Br. J. Cancer*, 43: 93-99, 1981.
- Noether, G. *Introduction to Statistics—A Fresh Approach*. Boston: Houghton Mifflin, 1971.
- Roizin-Towle, L. A., and Hall, E. J. Enhanced cytotoxicity of antineoplastic agents following prolonged exposure to misonidazole. *Br. J. Cancer*, 44: 201-207, 1981.
- Rose, C. M., Miller, J. L., Peacock, J. H., Phelps, T. A., and Stephens, T. C. Differential enhancement of melphalan cytotoxicity in tumor and normal tissue by misonidazole. In: L. W. Brady (ed.), *Radiation Sensitizers: Their Use in the Clinical Management of Cancer*, p. 250. New York: Masson Publishing USA, Inc., 1980.
- Sheldon, P. W., and Batten, E. L. Potentiation *in vivo* of melphalan activity by nitroimidazole compounds. *Int. J. Radiat. Oncol. Biol. Phys.*, 8: 635-638, 1982.
- Siemann, D. W. *In vivo* combination of misonidazole and the chemotherapeutic agent CCNU. *Br. J. Cancer*, 43: 367-377, 1981.
- Siemann, D. W. Potentiation of chemotherapy by hypoxic cell radiation sensitizers—a review. *Int. J. Radiat. Oncol. Biol. Phys.*, 8: 1029-1034, 1982.
- Siemann, D. W. Response of murine tumours to combinations of CCNU with misonidazole and other radiation sensitizers. *Br. J. Cancer*, 45: 272-281, 1982.
- Siemann, D. W. Combinations of CCNU and radiosensitizers *in vivo*: misonidazole and benzimidazole. *Proc. Am. Assoc. Cancer Res.*, 23: 164, 1982.
- Siemann, D. W., Hill, R. P., and Bush, R. S. The importance of pre-irradiation breathing times of oxygen and carbogen (5% CO<sub>2</sub>:95% O<sub>2</sub>) on the *in vivo* radiation response of a murine sarcoma. *Int. J. Radiat. Oncol. Biol. Phys.*, 2: 903-911, 1977.
- Siemann, D. W., and Sutherland, R. M. *In vivo* tumor response to single and multiple exposures of Adriamycin. *Eur. J. Cancer*, 16: 1433-1440, 1980.
- Smith, E., Stratford, I. J., and Adams, G. E. The enhancing effect of pre-treatment of cells with misonidazole in hypoxia on their subsequent response to melphalan in air. *Br. J. Cancer*, 45: 117-126, 1982.
- Stratford, I. J., Adams, G. E., Horsman, M. R., Kandaiya, S., Rajaratnam, S., Smith, E., and Williamson, C. The interaction of misonidazole with radiation, chemotherapeutic agents or heat. *Cancer Clin. Trials*, 3: 231-236, 1980.
- Sutherland, R. M., Bareham, B. J., and Reich, K. A. Cytotoxicity of hypoxic cell sensitizers in multicell spheroids. *Cancer Clin. Trials*, 3: 73-83, 1980.
- Tannock, I. The *in vivo* interaction of anti-cancer drugs with misonidazole or metronidazole: cyclophosphamide and BCNU. *Br. J. Cancer*, 42: 871-880, 1980.
- Thomson, J. E., and Rauth, A. M. An *in vitro* assay to measure the viability of KHT tumor cells not previously exposed to culture conditions. *Radiat. Res.*, 58: 262-276, 1974.
- Twentyman, P. R. The response of EMT6 tumor spheroids to combined treatment with misonidazole and either nitrogen mustard, Adriamycin or BCNU. *Cancer Clin. Trials*, 3: 253-256, 1980.
- Twentyman, P. R., Brown, J. M., Gray, J. W., Franko, A. J., Scoles, M. A., and Kallman, R. F. A new mouse tumor model system (RIF-1) for comparison endpoint studies. *J. Natl. Cancer Inst.*, 64: 595-604, 1980.
- Twentyman, P. R., and Workman, P. The effect of radiosensitizer pretreatment of the response of the RIF-1 mouse sarcoma to cytotoxic drugs. *Int. J. Radiat. Oncol. Biol. Phys.*, 8: 611-614, 1982.
- Twentyman, P. R., and Workman, P. An investigation of the possibility of chemosensitization by clinically achievable concentrations of misonidazole. *Br. J. Cancer*, in press, 1982.
- Workman, P., and Twentyman, P. R. Enhancement by electron-affinic agents of the therapeutic effects of cytotoxic agents against the KHT tumour: structure-activity relationships. *Int. J. Radiat. Oncol. Biol. Phys.*, 8: 623-626, 1982.