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HYPOXIA-MEDIATED NITRO-HETEROCYCLIC DRUGS IN THE RADIO- AND CHEMOTHERAPY OF CANCER

AN OVERVIEW

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The likelihood that hypoxic cells probably present in most solid tumours may, in some cases, limit the successful local control of these tumours by radiotherapy, has been recognized by radiation oncologists for more than a quarter of a century. However, the possibility that such cells may be an important factor also in the treatment of cancer by chemotherapy has received relatively little attention.

Hypoxic cells develop in tumours as a result of growth essentially outstripping the tumour's vascular system hence reducing the supply of essential nutrients, particularly oxygen. Well-oxygenated tumour cells close to a microcapillary are the source of tumour growth. However, tissue oxygen tension decreases with distance from the capillary and gradually falls to a level insufficient for cell division. Eventually, the oxygen-deprived cells die and this causes the focal, or regional, necrosis usually observed in most solid tumours. Viable hypoxic cells, which occur in the interface regions between the well-oxygenated tissue and the necrotic regions, are radiation-resistant relative to oxic cells and it is now well established, in experimental murine tumour systems, that their radiation resistance is the largest factor influencing local tumour control by radiation. In an untreated tumour, hypoxic cells will eventually die, but in the event of tumour regression, i.e. during or after radiation treatment, some of these hypoxic cells may be reoxygenated, enter cycle and cause tumour regrowth.

Hypoxic cells may influence the overall response of tumours to cytotoxic drugs in several ways. Drug accessibility may be a problem since hypoxic cells are usually located some distance from the nearest microcapillary. Oxygen may be necessary for the energy requirements of a particular drug mechanism. Further, cells with a low oxygen status may progress only slowly through the cell cycle or be arrested altogether. Such cells would be less sensitive therefore to some cycle-specific drugs.

This paper comments on some of the advances made in the development of nitroheterocyclic drugs designed to overcome hypoxic cell radiation resistance, considers the implications of hypoxic cell resistance in cytotoxic chemotherapy and discusses the exploitation of tumour hypoxia by the use of bioreducible cytotoxic drugs.

MISONIDAZOLE AND ITS ANALOGUES

The 2-nitroimidazole, misonidazole was the first hypoxic cell radiosensitizer to be extensively investigated clinically. Overall, the data were generally disappointing although limited benefit was reported in some clinical trials. However, a large collaborative study, involving cancers of the head and neck region, has revealed substantial benefit in the radiotherapy of tumours of the pharyngeal region [1]. This has added impetus to the search for improved sensitizers.

The neurological toxicity of misonidazole is the major limitation in its application. Experimental studies in rodents have now established that the neurotoxic properties of misonidazole and some other nitroimidazole sensitizers are related directly to the *lipophilic* properties of the compounds [2]. Compounds with reduced lipophilicity show reduced uptake in neural tissue and appear to be less neurotoxic. Studies with a variety of appropriate analogues of misonidazole have led therefore to the clinical development of the nitroimidazole SR-2508 (Fig. 1). Experimentally, this compound has a comparable sensitizing efficiency to misonidazole, is substantially less neurotoxic and possibly may show improved tumour uptake. Phase I and II clinical studies have confirmed the much reduced neurotoxicity and total doses over 30 g/m^2 have been successfully administered to patients over a 6-week course of radiotherapy without undue neurological complications. Randomized trials with this drug are now in progress in the United States.

The Roche compound, Ro 03-8799 (Fig. 1), an analogue of misonidazole, appears to be superior to misonidazole. While the tumour penetration of misonidazole is fairly efficient, that of 8799 is considerably better. Experimental and clinical studies [3] show generally that the tumour levels substantially exceed those measured in plasma. This differential uptake has been attributed to the acid-base properties of the piperidine group in the side chain of the structure [4]. Clinical trials with this drug are in progress in the U.K.

During the development of the sensitizer field, numerous compounds have been identified which are substantially more efficient *in vitro* than would be predicted on the basis of the electron-affinity relationship. This is particularly true of some compounds that react efficiently with intracellular glutathione. Most of these compounds show little, if any, activity *in vivo*. More recently however, a class of abnormally efficient *mixed-function* sensitizers has been developed [5, 6]. These compounds are derivatives of misonidazole which contain, in the side chain,

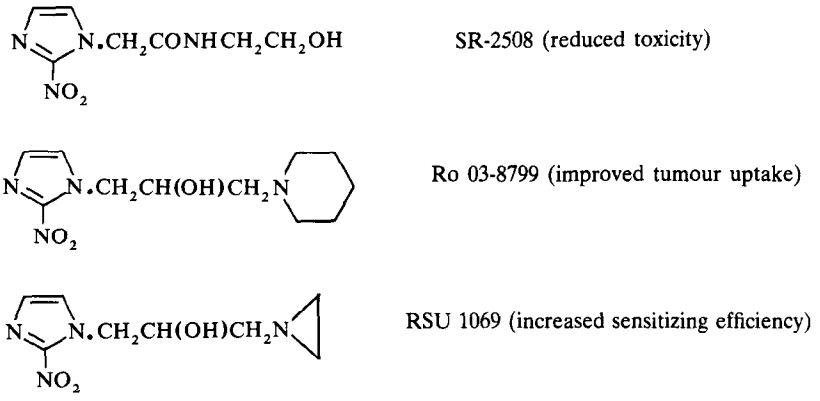

Fig. 1. Chemical structures of some current radiosensitizers.

monofunctional alkylating groups such as aziridine. The lead compound RSU 1069 (Fig. 1) can be up to 10-fold more efficient than misonidazole both as a radiation sensitizer and a chemosensitizer. It is, however, more toxic in humans than misonidazole. Various substituted aziridinyl analogues have been synthesized which retain the high sensitizing efficiency but are appreciably less toxic. A discussion of the various biological properties of RSU 1069 is contained in the paper by Stratford *et al.* [7].

BIOREDUCIBILITY OF NITROHETEROCYLICS

Differential cytotoxicity

Many nitroimidazoles and nitrofurans are considerably more toxic to hypoxic mammalian cells than they are to oxic cells. This was first observed with metronidazole using a cellular spheroid system [8] and has since been characterized in many mammalian systems *in vitro* and *in vivo* ([9–12] and references therein).

Figure 2 illustrates the differential cytotoxicity for these nitroheterocycles, metronidazole, misonidazole and nitrofurazone (compiled from data in refs. 13 and 14). Clearly, all these compounds are more toxic in nitrogen, and in some instances concentrations which are cytotoxic under these conditions will actually permit culture growth in air.

It is established that the efficiencies of nitroheterocyclic compounds as hypoxic cell radiation sensitizers correlate in the main (but not always) with the electron affinities of the compounds. Some data illustrating this are reproduced in Fig. 3a (data from ref. 15). The abscissa is the concentration of sensitizer required to give an enhancement ratio of 1.6 ($C_{1.6}$) and is a convenient measure of sensitizing efficiency. The ordinate is the one-electron reduction potential measured by a standard pulse radiolysis technique.

The data in Fig. 3b show that the cytotoxic efficiencies of these compounds also correlate with electron affinity (data from ref. 16). The abscissa in this

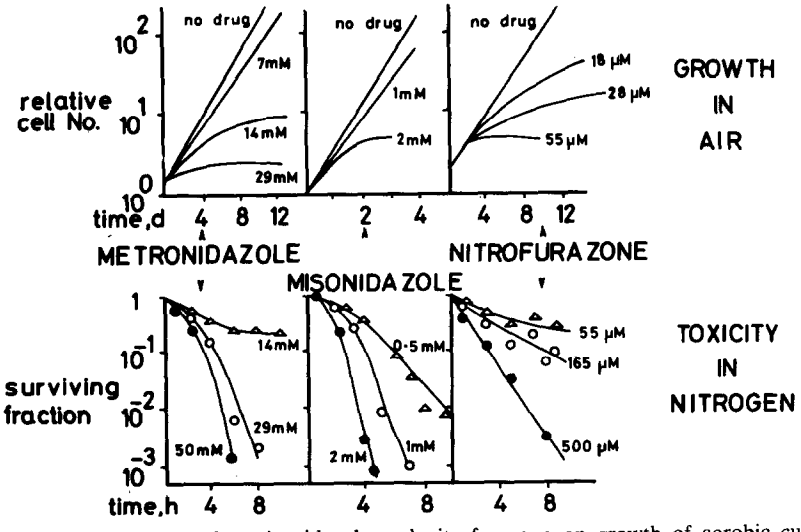

Fig. 2. Effect of metronidazole, misonidazole and nitrofurazone on growth of aerobic cultures of mammalian cells and cellular survival under hypoxic conditions. Metronidazole + nitrofurazone: suspension cultures of CHO cells (data from ref. 13); misonidazole: suspension cultures of V79 cells (data from ref. 14).

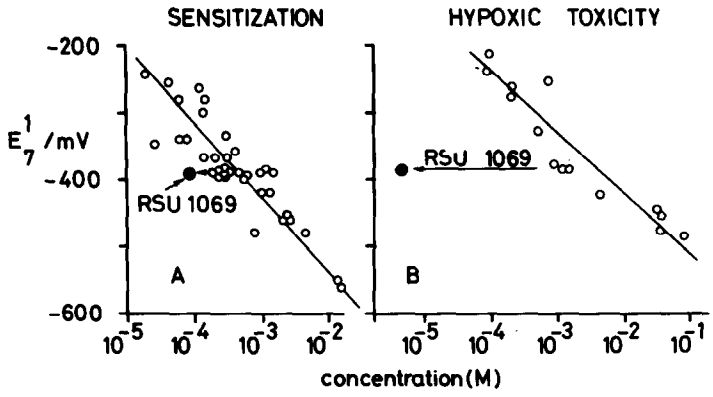

Fig. 3. Correlation of hypoxic radiation sensitization efficiency and hypoxic cytotoxicity efficiency of sone nitroimidazoles with one-electron reduction potential E_7^1. Radiation sensitization: abscissa, concentration of compound required to give an enhancement ratio of 1.6 ($C_{1.6}$) (data from ref. 15 except datum point for RSU 1069 (ref. 6)). Hypoxic cytotoxicity: abscissa, concentration of compound required to reduce surviving fraction by 10^{-2} for a contact time of 5 hr (data from ref. 16 except datum point for RSU 1069 (ref. 7)).

case is the concentration of compound required to reduce surviving fraction by 10^{-2} for a contact time of 5 hr at 37°.

The applicability of the redox relationship for both radiation sensitization and hypoxic cytotoxicity might suggest that the mechanisms of both phenomena are the same, and this has often been assumed. The evidence strongly indicates, however, that the mechanisms are quite different. Firstly, radiation sensitization occurs mainly via very fast free-radical processes initiated by radiation. Various fast-mixing studies show that sensitization can occur even when the pre-irradiation contact times are very short, i.e. less than one second [17]. In contrast, hypoxic cytotoxicity occurs only after substantial contact times. Secondly, the enhancement ratios for radiation sensitization generally show little dependence on the position of the cells in the mitotic cycle [18], whereas cytotoxicity shows a marked cell-cycle dependence with maximum efficiency in early S-phase. Thirdly, ascorbic acid greatly increases hypoxic cytotoxicity [19] but has little effect on radiation sensitization. Finally, the temperature dependencies of the two phenomena are quite different. The free radical nature of oxygen-mimetic radiation sensitization would predict that temperature would not have a large influence on the efficiency. This is generally true whereas temperature has a profound influence on hypoxic cytotoxicity [14, 20].

CHEMOSENSITIZATION

Information on the mechanism of hypoxic cytotoxicity has been obtained from studies of the phenomenon of chemosensitization. Evidence from experiments *in vitro* and *in vivo* had already indicated that various cytotoxic drugs used in cancer chemotherapy are often less effective against hypoxic cells compared with oxic cells. This led to speculation therefore that nitroimidazole drugs that are *more* effective against hypoxic cells could be added to existing chemotherapy schedules in order to increase therapeutic effectiveness.

Independently, Rose *et al.* [21] and Clement *et al.* [22] investigated the effect of misonidazole on the anti-tumour activity of various alkylating agents. Enhancement of efficacy was observed in various tumour systems although the effect was variable depending on the nature of the tumour and the type of alkylating agent used. In particular, both groups observed enhancement factors of up to about 2 which in terms of log kill represents a considerable increase in effectiveness. Since that time, numerous reports have been published confirming the phenomenon in a variety of different experimental tumour systems ([11, 12, 23] and references therein).

Chemosensitization of tumour response to nitrosoureas has been observed by several groups but, for this type of drug, chemosensitizing efficiency appears to correlate with carbamoylating activity rather than the alkylating activity of the nitrosoureas [24]. Slight enhancement of normal tissue morbidity has been observed in some studies but with few exceptions, this effect is substantially less than the enhancement of tumour response.

The relative efficiencies of nitroimidazoles as chemosensitizers differ according to chemical structure, redox properties and particularly lipophilicity [25]. Enhancement ratios greater than 2 are sometimes observed: an example is the sensitization of tumour response to melphalan by the aziridinyl nitroimidazole RSU 1069 [5, 6].

Figure 4 compares the efficiencies of misonidazole and RSU 1069 in sensitizing the response of the MT tumour in WHT/Cbi mice to melphalan [6, 26]. Misonidazole administered at a dose of 0.5 mg/g body weight gives an ER of 1.7 whereas 0.08 mg/g 1069 gives an ER of about 3.0. The latter compound is atypical in several respects, one of which is its very high toxicity to hypoxic cells (note the displacement of the datum point on the redox correlation plot for hypoxic toxicity shown in the figure). However, despite this, chemosensitization by RSU 1069 is observed at drug doses which by themselves, are too low to cause appreciable *direct* kill of hypoxic cells.

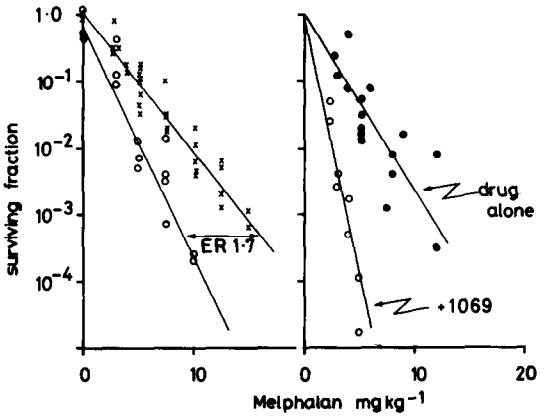

Fig. 4. Chemosensitization of the response of the MT tumour in WHT/Cbi mice to melphalan by misonidazole and RSU 1069. Left: 0.5 mg/g misonidazole administered i.p. to tumour bearing mice 30 min before various doses of melphalan (data from ref. 26). Right: 0.08 mg/g RSU 1069 administered 60 min before various doses of melphalan (data from ref. 6).

THE PRE-INCUBATION EFFECT

Pre-treatment of hypoxic mammalian cells with nitroheterocyclic compounds *in vitro* sensitizes the subsequent response of the cells to alkylating agents. The first study involved chemosensitization of melphalan by misonidazole [27] but it is now clear that chemosensitization by pre-incubation can be effected with various other nitroheterocyclic compounds. The effect is illustrated by the data in Fig. 5 (data from ref. 28). Exponential and plateau-phase cells were treated with 5 mM misonidazole for 2 hr in hypoxia at 37° followed by exposure for 1 hr to a range of concentrations of melphalan at 37°. Substantial sensitization occurs for both types of cell culture and is greater for plateau-phase cells.

A number of criteria for chemosensitization *in vitro* are now established. It is essential that the pre-incubation with the nitroimidazole (e.g. misonidazole) is carried out under hypoxic conditions. Interestingly chemosensitization is still observed even if the cells, following hypoxic pre-incubation, are washed free of the drug before exposure to the alkylating agent (Fig. 6). Further, sensitization also occurs even if the pre-incubated cells are exposed to oxygen during the treatment with melphalan.

There is evidence that the sub-lethal damage caused by the hypoxic pre-incubation with misonidazole is repairable [28]. If the cells, following pre-incubation with the sensitizer under hypoxic conditions, are then held for various times in oxygen *before* exposure to melphalan, the chemosensitization effect is gradually lost. For cells in exponential phase, a delay of 4 hr is sufficient to eliminate chemosensitization entirely. This effect is consistent with a repair phenomenon particularly in view of the fact that no recovery is observed if, during the delay period, the cells are held either in nitrogen at 37° or in oxygen at 0°.

MECHANISMS OF CHEMOSENSITIZATION *IN VITRO* AND *IN VIVO*

The common thread running through most of the chemosensitization studies both *in vitro* and *in vivo*

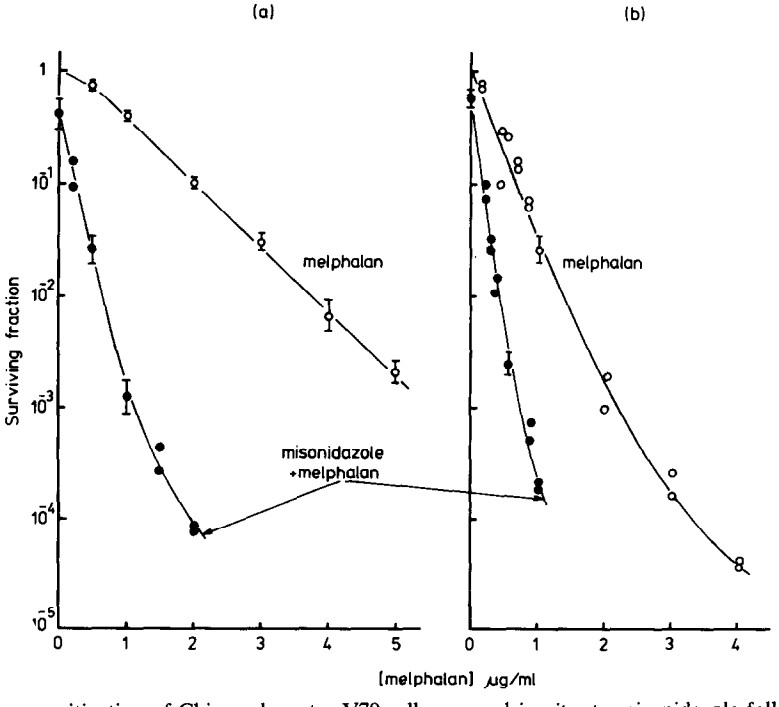

Fig. 5. Chemosensitization of Chinese hamster V79 cells exposed *in vitro* to misonidazole followed by melphalan: (a) exponential-phase cells; (b) plateau phase cells. The cells were exposed to 5 mM misonidazole for 2 hr in hypoxia at 37° before exposure to melphalan in air for 1 hr at 37°: open symbols, cells treated with melphalan alone; solid symbols, cells treated with misonidazole and melphalan.

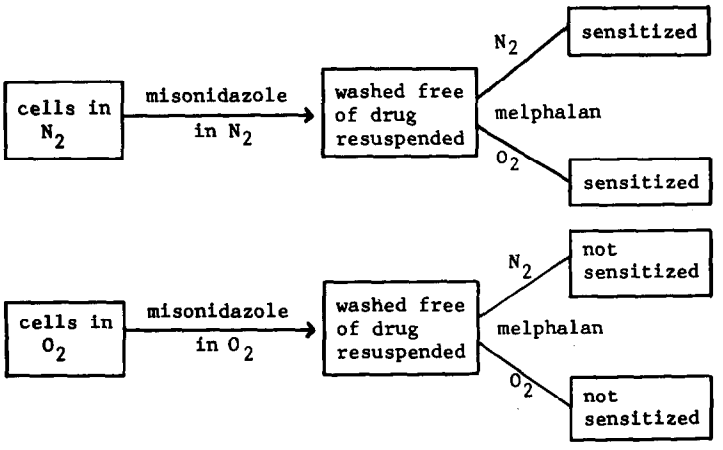

Fig. 6. The pre-incubation effect *in vitro* conditions for sensitization, by misonidazole, of cellular response to melphalan.

is bioreduction although it is clear that more than one mechanism is involved in the phenomenon. The *in vitro* pre-incubation effect is operative only under hypoxia although it is not reversible by oxygen. This has been explained on the basis that the sensitizer is metabolically reduced in hypoxic cells to give a product which, while cytotoxic if formed in sufficient amounts, can also damage the cells *sub-lethally*. This sub-lethal damage is then, according to the hypothesis, expressed, or fixed, by the alkylating agent.

Suppression of intra-cellular glutathione by reaction with the sensitizer appears to influence chemosensitization. However, this cannot account entirely for the phenomenon since under conditions of optimum GSH suppression, misonidazole still induces some chemosensitization [29]. The influence of intracellular GSH has led to much discussion of the role of repair inhibition in chemosensitization. There is evidence that nitroimidazoles can inhibit repair of "potentially lethal damage" (PLD) [29, 30]. Measurement of tumour response using cell-assay techniques shows that, in some tumours but not others, excision-delay inhibits but does not eliminate chemosensitization.

The evidence of a hypoxia-mediated mechanism for chemosensitization *in vitro* has some support from *in vivo* studies. The differential retention of ^{14}C labelled misonidazole in tumours [31] is believed to operate through the binding of a substance, or substances, formed by anaerobic reduction of the sensitizer in oxygen-deficient regions of the tumour.

Strong evidence for the involvement of hypoxia has been obtained through studies with the experimental 9L tumour [32]. This slowly-growing tumour appears not to contain hypoxic cells. Misonidazole does not enhance the cytotoxic effect of a nitrosourea in this tumour under normal conditions but does so if the tumour blood supply is occluded by clamping after misonidazole administration. In these experiments, the clamping rendered the tumour artificially hypoxic for a 2-hour period and treatment with the nitrosourea *after* the clamps were removed revealed the chemosensitization effect. Control experiments without misonidazole showed no effect of the clamping procedure on the response to the nitrosourea.

In addition to hypoxia-mediated processes, pharmaco-kinetic changes can also give rise to apparent chemosensitization [33]. Administration of some nitroimidazoles can affect the pharmaco-kinetics of some agents, particularly the nitrosoureas. Although the mechanisms are complex, this effect can differentially increase the antitumour response of the nitrosourea with respect to normal tissue.

Overall, the mechanism of chemosensitization in tumours is undoubtedly complex and can be influenced by various factors: the nature of the nitro-heterocyclic compound, the type of alkylating agent, tumour size and histological type, and drug sequence and timing. However, the evidence supporting the role of hypoxia-mediated bio-reductive processes in chemosensitization provides a sound rationale for the development of new strategies in cancer chemotherapy. There is evidence that hypoxia can develop in tumours of about 1 mm size, and therefore bioreductive drug activation and the associated phenomenon of chemosensitization could be a valuable new approach in the selective treatment of disseminated cancer by cytotoxic chemotherapy.

REFERENCES

1. J. Overgaard, S. H. Hansen, A. P. Anderson, M. Hjelm-Handsen, K. Jorgensen, K. Sandberg, J. Rygard, R. H. Jensen and M. Petersen, *Proc. 3rd Int. Meeting on Progress in Radio-Oncology, Vienna, Austria 1985*. Raven Press, New York, in press.
2. J. M. Brown and P. Workman, *Radiat. Res.* **82**, 171 (1980).
3. M. I. Saunders, P. J. Anderson, M. H. Bennett, S. Dische, A. Minchinton, M. R. L. Stratford and M. Tothill, *Int. J. Radiat. Oncol. Biol. Phys.* **10**, 1759 (1984).
4. M. F. Dennis, M. R. L. Stratford, P. Wardman and M. E. Watts, *Int. J. Radiat. Biol.* **47**, 629 (1985).
5. G. E. Adams, I. Ahmed, P. W. Sheldon and I. J. Stratford, *Br. J. Cancer* **49**, 571 (1984).

6. G. E. Adams, I. Ahmed, P. W. Sheldon and I. J. Stratford, *Int. J. Radiat. Oncol. Biol. Phys.* **10**, 1653 (1984).
7. I. J. Stratford, P. O'Neill, P. Sheldon, A. R. J. Silver, J. Walling and G. E. Adams, *Biochem. Pharmac.* **35**, 105 (1986).
8. R. M. Sutherland, *Cancer Res.* **34**, 3501 (1974).
9. Collected papers, in *Hypoxic Cell Sensitizers in Radiobiology and Radiotherapy. Br. J. Cancer* **37**, *Suppl* III (1978).
10. Collected papers, in *Radiation Sensitizers: Their Use in the Clinical Management of Cancer* (Ed. L. W. Brady). *Cancer Management*, Vol. 5. Masson, New York (1980).
11. Collected papers, in *Chemical Modification: Radiation and Cytotoxic Drugs* (Ed. R. M. Sutherland), *Int. J. Radiat. Oncol. Biol. Phys.* **8** (1982).
12. Collected papers, in *Chemical Modifiers of Cancer Treatment* (Eds. J. D. Chapman and G. F. Whitmore). *Int. J. Radiat. Oncol. Biol. Phys.* **10** (1984).
13. J. K. Mohindra and A. M. Rauth, *Cancer Res.* **36**, 930 (1976).
14. I. J. Stratford and G. E. Adams, *Br. J. Cancer* **35**, 307 (1977).
15. G. E. Adams, E. D. Clarke, I. R. Flockhart, R. S. Jacobs, D. S. Sehmi, I. J. Stratford, P. Wardman and M. E. Watts, *Int. J. Radiat. Biol.* **35**, 133 (1979).
16. G. E. Adams, I. J. Stratford, R. G. Wallace, P. Wardman and M. E. Watts, *J. natn. Cancer Inst.* **64**, 555 (1980).
17. G. E. Adams, B. D. Michael, J. C. Asquith, M. A. Shenoy, M. E. Watts and D. W. Whillans, in *Radiation Research: Biochemical, Chemical and Physical Perspectives* (Eds. O. F. Nygaard, H. I. Adler and W. K. Sinclair), pp. 478–492. Academic Press, New York (1975).
18. J. D. Chapman, A. P. Reuvers, J. Borsa, A. Petkan and D. R. McCalla, *Cancer Res.* **32**, 2630 (1972).
19. P. D. Josephy, B. Palcic and L. D. Skarsgard, *Nature, Lond.* **271**, 370 (1978).
20. E. J. Hall, M. Astor and C. Geard, *Br. J. Cancer* **35**, 809 (1977).
21. C. M. Rose, J. L. Millar, J. H. Peacock, T. A. Phelps and T. C. Stephens, in *Radiation Sensitizers: Their Use in the Clinical Management of Cancer* (Ed. L. W. Brady), *Cancer Management*, Vol. 5, pp. 250–257, Masson, New York (1980).
22. J. J. Clement, M. S. Gorman, I. Wodinsky, R. Catane and R. K. Johnson, *Cancer Res.* **40**, 4165 (1980).
23. D. W. Siemann and R. T. Mulcahy, *Biochem. Pharmac.* **35**, 111 (1986).
24. R. T. Mulcahy, N. L. Dembs and G. A. Ublacker, *Br. J. Cancer* **49**, 307 (1984).
25. D. G. Hirst, J. M. Brown and J. L. Hazelhurst, *Cancer Res.* **43**, 1961 (1983).
26. P. W. Sheldon, E. C. Batten and G. E. Adams, *Br. J. Cancer* **46**, 523 (1982).
27. I. J. Stratford, G. E. Adams, M. R. Horsman, S. Kandaiya, S. Rajaratnam, E. Smith and C. Williamson, *Radiation Sensitizers: Their Use in the Clinical Management of Cancer* (Ed. L. M. Brady), *Cancer Management*, Vol. 5, pp. 276–281. Masson, New York (1980).
28. E. Smith, I. J. Stratford and G. E. Adams, *Br. J. Cancer* **46**, 117 (1982).
29. J. M. Brown, *Int. J. Radiat. Oncol. Biol. Phys.* **8**, 675 (1982).
30. D. W. Siemann, *Int. J. Radiat. Oncol. Biol. Phys.* **10**, 1585 (1984).
31. J. D. Chapman, A. J. Franko and C. J. Koch, in *Biological Bases and Clinical Implications of Tumour Radioresistance* (Eds. G. H. Fletcher, C. Nervi and H. R. Withers), pp. 61–73, Masson, New York (1983).
32. K. T. Wheeler, C. A. Wallen, K. L. Wolf and D. W. Siemann, *Br. J. Cancer* **49**, 787 (1984).
33. F. Y. F. Lee and P. Workman, *Br. J. Cancer* **49**, 579 (1984).