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Evidence for a therapeutic gain when AQ4N or tirapazamine is combined with radiation

SR McKeown[1], OP Friery[1], IA McIntyre[1], MV Hejmadi[1], LH Patterson[2] and DG Hirst[1]

[1]*School of Biomedical Sciences, University of Ulster at Jordanstown, N Ireland BT37 0QB;* [2]*Department of Pharmaceutical Sciences, De Montfort University, Leicester LE1 9BH, UK.*

Summary The use of bioreductive drugs as an adjunct to radiotherapy in the treatment of cancer is presently being tested in several clinical trials worldwide. We have developed a novel bioreductive compound AQ4N (1,4-bis-{[2-(dimethylamino-*N*-oxide)ethyl]amino}5,8-dihydroxy-anthracene-9,10-dione) which can be reduced to a stable cytotoxic agent AQ4. The anti-tumour efficacy of AQ4N has been studied using male BDF mice bearing the T50/80 tumour. AQ4N in combination with single dose radiation (12 Gy) and also with two fractionated radiation regimens was examined (5 × 3 Gy, one fraction per day; or 10 × 2 Gy fractions, 2 fractions per day with an 8 h interval). Results show that in all combinations tested there was a marked increase in anti-tumour efficacy. This was also found in the single dose regimen for the bioreductive drug tirapazamine (SR 4233; 3-amino-1,2,4-benzotriazine-1,4-dioxide). Normal tissue toxicity of drug-radiation combinations was measured by assessing function in the eccrine sweat gland of the mouse hind foot. When combined with 10 Gy radiation neither AQ4N nor tirapazamine showed any enhancement of functional loss as compared with radiation alone. This was in contrast to mitomycin C which had a marked effect on the radiation induced functional deficit. In conclusion, in our model, an increase in the therapeutic index was obtained for radiation treatment when either AQ4N or tirapazamine was administered concurrently.

Keywords: AQ4N; tirapazamine; radiation; therapeutic gain

Bioreductive drugs are designed to target treatment-resistant hypoxic tumour cells (Workman and Stratford, 1993). This subpopulation of cells does not normally make a major contribution to tumour growth unless the well-oxygenated tumour cells have been damaged. If bioreductive drugs are to have a clinical role, it is necessary to devise protocols which combine a second treatment modality that is toxic to aerobic cells. One of the major uses of bioreductive drugs will therefore be as an adjunct to radiotherapy.

Several chemical classes of bioreductive drugs have been described, including the nitroimidazoles, benzotriazene di-*N*-oxides and mitosenes (Workman, 1992). A new class of compounds has been developed which are analogues of the chemotherapeutic agent mitoxantrone (Patterson, 1993). The most promising is the alkylaminoanthraquinone *N*-oxide, AQ4N (1,4-bis-{[2-(dimethylamino-*N*-oxide)ethyl]amino}5,8-dihydroxy-anthracene-9,10-dione). Under hypoxic conditions AQ4N can be reduced to a positively charged, stable compound AQ4 that possesses marked affinity for DNA and the ability to inhibit topoisomerase II. This is in marked contrast to AQ4N which is electrically neutral and shows weak DNA binding and limited ability to inhibit topoisomerase II (Patterson, 1993). In order to assess the clinical potential of AQ4N we have examined the combination of AQ4N and radiation in single and in fractionated regimens. To evaluate the possible therapeutic gain we have also assessed whether AQ4N can exacerbate radiation-induced damage in a normal tissue using the murine eccrine sweat gland assay (Johns *et al.*, 1995). For comparison we have included data on the effect of the bioreductive drugs tirapazamine (SR 4233; 3-amino-1,2,4-benzotriazine-1,4-dioxide) and mitomycin C in these models.

Correspondence: S McKeown, School of Biomedical Sciences, University of Ulster, Jordonstown, Newtownabbey, N Ireland BT37 0QB

Materials and methods

In vivo *tumour model*

Male B6D2F$_1$ mice aged 8-12 weeks were used for all studies which were carried out in accordance with the UK Animals (Scientific Procedures) Act 1986. Tumours, induced by intradermal injection of 0.05 ml of tumour brei on the rear dorsum, were treated when they reached a diameter of 6.5-7.5 mm (geometric mean of three orthogonal diameters). Tumour doubling time (TDT) was used to assess anti-tumour efficacy; for untreated tumours TDT was 4.43 ± 0.4 days. Tumour growth delay was calculated by subtracting the mean TDT for untreated tumours from that obtained in the test situations.

Anti-tumour studies: combination of AQ4N or tirapazamine with radiation

AQ4N was prepared in sterile water and SR 4233 was prepared in phosphate buffered saline as described previously (McAleer *et al.*, 1992). The drugs were administered i.p. at a range of single or multiple doses. X-radiation was administered by a 300 kVp Siemens Stabilipan (dose rate 2.56 Gy min^{-1}). Unanaesthetised mice were immobilised in lead jigs with the dorsal tumour exposed to the radiation beam using lateral parallel opposed fields to ensure homogeneous dose distribution. In experiments using single fraction irradiation a dose of 12 Gy was administered (this dose was chosen from previous dose-response experiments). A range of AQ4N doses (50-200 mg kg^{-1}) was given as a single i.p. injection 30 min before irradiation. Tirapazamine was also given in this regimen over a range of doses (25-50 mg kg^{-1}).

AQ4N was also administered in combination with two approximately isoeffective fractionated radiation regimens. The first (5 × 3 Gy) was administered over five consecutive days. AQ4N (200 mg kg^{-1}) was administered 30 min before irradiation using three different drug schedules: 200 mg kg^{-1} on day one, 100 mg kg^{-1} on days 1 and 3, or 40 mg kg^{-1} on each of the five consecutive radiation days. The second fractionation schedule (10 × 2 Gy) was administered twice per

day with 8 h and 16 h intervals between radiation doses; when required AQ4N was administered daily 30 min before the first of the two fractions. In this schedule a range of total AQ4N doses was given in 5 equal fractions of 12.5, 25 or 50 mg kg^{-1} daily.

Murine eccrine sweat gland assay

Male BDF mice were used to establish a time dependent response over 0–16 weeks following single dose irradiation to the plantar surface of the left hind foot (8.0–17.0 Gy). This was carried out on unanaesthetised mice restrained in lead jigs. The left hind leg was extended into the radiation beam and exposed to predetermined single doses of radiation using a 300 kVp Siemens Stabilipan (dose rate 2.56 Gy min^{-1}). When drug–radiation combinations were being assessed mice were pretreated 20 min before irradiation by i.p. administration of AQ4N (200 mg kg^{-1}), SR 4233 (50 mg kg^{-1}) or mitomycin C (5 mg kg^{-1}).

The murine sweat gland assay (Johns *et al.*, 1995) was performed 1 day before the irradiation procedure to allow clearance of the pilocarpine before irradiation, and at a series of intervals post-irradiation (2–26 weeks). To assess eccrine gland function the mice were first injected with 0.05% pilocarpine (i.p.) to stimulate sweating. They were then restrained unanaesthetised with both hind legs extended. A hydrophobic silicone elastomer base was mixed thoroughly with an organic solvent and a hardening catalyst added. This was applied immediately to the plantar surface of both hind feet approximately 15 min after injection. After approximately 2 min the set cast was carefully peeled off. This provides a permanent record of sweat gland function. Using a low-power microscope (×3 magnification) indentations could be scored on the cast as evidence of the presence of a functioning sweat gland. For each group of animals, the α-pad count was averaged for the irradiated (left) and unirradiated (right) foot. To obtain a measure of functional loss the mean number of sweat glands in the irradiated foot was divided by the mean value for the unirradiated foot in each treatment group.

Results

Figure 1 shows the dose–response curves for AQ4N and tirapazamine when combined with a single dose of radiation (12 Gy). The drugs have only a limited effect on the tumour growth when used alone whereas they both show a marked enhancement of the anti-tumour effect when combined with radiation.

When AQ4N was combined with fractionated radiation a significant anti-tumour effect was obtained with both regimens studied. In Figure 2a it can be seen that with radiation (5 × 3 Gy) and AQ4N (200 mg kg^{-1}) administered in three different drug schedules, there was an enhanced anti-tumour effect with all schedules. Small doses of drug given on a daily basis had a slight advantage over a single dose given at the beginning of treatment. With a fractionated regimen of 10 × 2 Gy (two fractions per day) we gave a daily dose of AQ4N of 12.5, 25 or 50 mg kg^{-1} (Figure 2b). All regimens gave a significant enhancement of anti-tumour effect compared with radiation alone. Fractionated drug alone also produced a significant anti-tumour effect at the highest dose (5 × 50 mg kg^{-1}).

In order to assess normal tissue toxicity of drug–radiation combinations we used the murine eccrine sweat gland as a model. This assay is based on the detection of sweat droplets by impressions made in the silicone cast. Since the silicone elastomer is hydrophobic it retracts from actively sweating glands forming an indentation in the elastomer. There are six pads in the mouse hind foot and each contains a number of functional glands. The model assumes that the functional unit of the system is a single sweat pore. In our experiments the largest pad (α-pad) was scored: the mean number of α-pad sweat glands upon maximum stimulation is 17.5 ± 5. Any functional loss is shown by a reduction in the number of indentations formed.

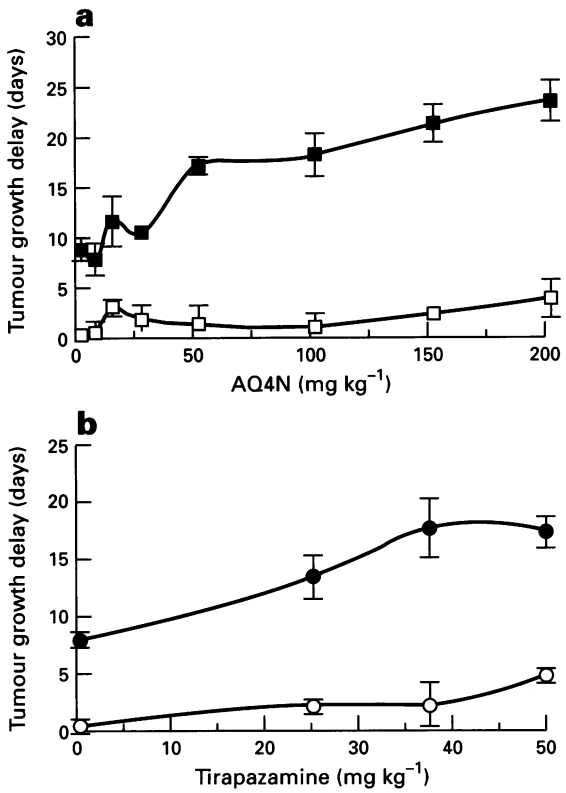

Figure 1 Tumour growth delay following treatment with AQ4N or tirapazamine and single dose radiation. Tumour growth delay was used to measure anti-tumour efficacy in male BDF mice bearing the T50/80 tumour following treatment with drug ± single fraction radiation (12 Gy). Drugs were administered i.p. 30 min before irradiation. (**a**) AQ4N alone (□), AQ4N + radiation (■); (**b**) tirapazamine alone (○), tirapazamine + radiation (●). Error bars show ±1 s.e.m.

Initially a range of single doses (8–17 Gy) was administered and functional loss assessed at predetermined time intervals (Figure 3a). A reproducible dose-dependent loss of function was seen with a nadir at 8 weeks and a dose-dependent partial recovery by 16 weeks. Groups of mice were also treated with AQ4N (200 mg kg^{-1}), tirapazamine (50 mg kg^{-1}) or mitomycin C (5 mg kg^{-1}) in combination with 10 Gy radiation and the percentage functional loss was followed as before. The nadir of functional deficit for all drugs was also found at 8 weeks (Figure 3b). Combination of AQ4N or tirapazamine with 10 Gy radiation did not show any appreciable difference from radiation alone at all time points studied. However mitomycin C showed a marked enhancement of the radiation-induced functional deficit with only 15% of normal function remaining at 8 weeks: this was more than that observed with 17 Gy of radiation as a single dose. Functional recovery was also related to the extent of deficit at 8 weeks with only 40% recovery by 16 weeks when mitomycin C was combined with radiation.

Discussion

This study investigated the potential of the novel bioreductive drug AQ4N as an agent which can provide a therapeutic gain when combined with radiation. Using the T50/80 tumour grown in BDF mice we have shown that AQ4N gives a significant increase in anti-tumour efficacy in combination with both single and fractionated radiation regimens. When AQ4N was combined with a single dose of radiation (12 Gy) a marked dose–response was shown with little effect at

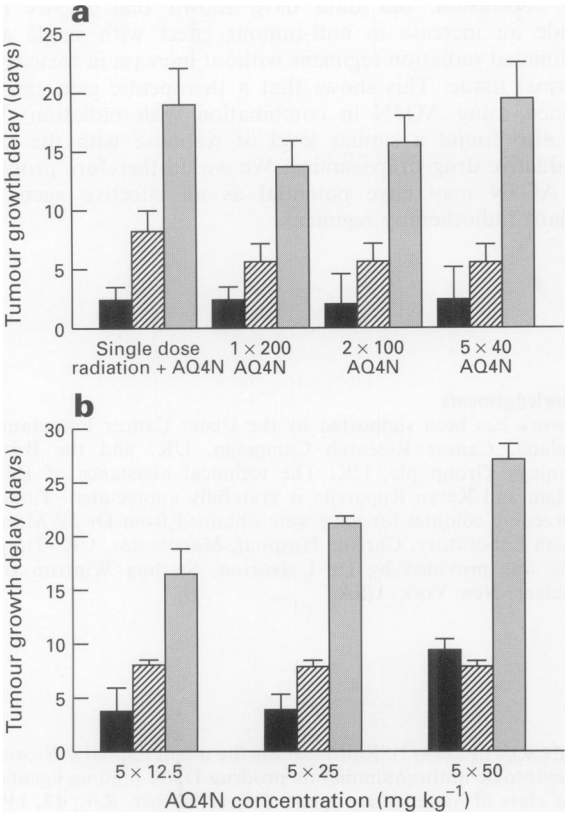

Figure 2 Tumour growth delay following treatment with fractionated radiation in combination with AQ4N. Two schedules were examined (**a**) 5×3 Gy in combination with AQ4N (200 mg kg^{-1}) given as a single dose on the first day of treatment, or in multiple doses as indicated. AQ4N was given 30 min before radiation. For comparison single dose radiation (12 Gy) + single dose AQ4N (200 mg kg^{-1}) is also included. Single dose of AQ4N (200 mg kg^{-1}) combined with fractionated radiation was significantly different to fractionated radiation alone ($P<0.01$). Multiple dose drug (5×40 mg kg^{-1}) with fractionated radiation was significantly more effective than single dose of drug when combined with fractionated radiation ($P<0.01$). (**b**) Dose of 10×2 Gy given as two daily fractions with an 8 h interval. Drug was given once daily in the doses indicated 30 min before the first daily fraction of radiation. ■, drug alone; ▨, radiation alone; ▩, AQ4N–radiation combinations. Error bars show ±1 s.e.m.

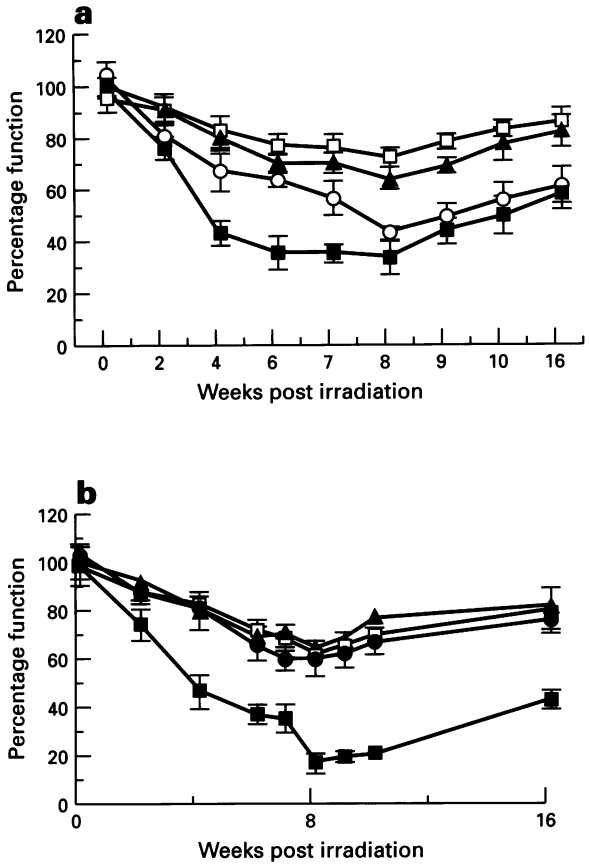

Figure 3 The effect of radiation and radiation–drug combinations on murine eccrine sweat gland function. (**a**) Functional deficit with time following a range of radiation doses given as a single fraction: 8 Gy (□), 10 Gy (▲), 13 Gy (○), 17 Gy (■). (**b**) Functional deficit with time when mice were treated with 10 Gy radiation (▲) or 10 Gy radiation in combination with 200 mg kg^{-1} AQ4N (□), 50 mg kg^{-1} tirapazamine (●), or 5 mg kg^{-1} mitomycin C (■). Error bars show ±1 s.e.m.

25 mg kg^{-1} and about half maximal effect by 50 mg kg^{-1}. Although the drug can be given at a dose of at least 320 mg kg^{-1} (data not shown) we have used 200 mg kg^{-1} in the majority of experiments as this gives an almost maximal effect, suggesting that all hypoxic cells available to the drug given as a single injection are being targeted at this dose. When AQ4N was administered alone a small anti-tumour effect was observed at higher doses, with both single and fractionated drug dosing schedules. This suggests that there may be killing of acutely hypoxic cells, which are known to be present in tumours (Trotter *et al.*, 1990). Similar dose–response curves were obtained for tirapazamine with a maximal effect being observed above 37.5 mg kg^{-1}.

The clinical use of bioreductive drugs will necessitate the concurrent use of fractionated radiation. It has already been shown that tirapazamine is effective when given in this manner (Brown and Lemmon, 1991). Two different fractionated radiation schedules have been studied in combination with AQ4N and both have shown considerable anti-tumour efficacy similar to, or greater than, that found in the single dose experiments. In Figure 2a it can be seen that giving the drug as five small daily doses is marginally more effective than as a large single dose. We have previously shown that AQ4N can be given in combination with a single dose of radiation with maximal efficacy over a long time interval. A maximal anti-tumour effect was shown when AQ4N (200 mg kg^{-1}) was administered up to 4 days before to 6 h after 12 Gy radiation (McKeown *et al.*, 1995). This long interval of interaction is attributed to the properties of the reduction product AQ4 which is highly DNA-affinic, in contrast to AQ4N which shows no measurable affinity for DNA (Patterson, 1993). It is suggested that the slight advantage in giving the drug in a daily schedule is due to the effect of the drug on acutely hypoxic cells. Each day an additional cohort of acutely hypoxic cells would be compromised by the drug administered.

Results from the second fractionation schedule (10×2 Gy) would also support this proposition. Although all regimens gave a significant effect the most beneficial regimen was obtained with the highest dose (5×50 mg kg^{-1}). This was also significantly different from the two lower doses (5×12.5 and 5×25 mg kg^{-1}). When given as a single agent in multiple doses there was also a significant effect with the AQ4N alone. This suggests that acutely hypoxic cells were being targeted by the daily administration of less than maximal doses of drug (50 mg kg^{-1}). When this schedule of 50 mg kg^{-1} per day for 5 days was combined with 10×2 Gy radiation we obtained the most effective growth delay (27 days) of any regimen studied.

If AQ4N is to be accepted into clinical trial as an adjunct to radiotherapy it is of critical importance to show that there is no exacerbation of radiation induced damage by the drug within the radiation field. The murine eccrine sweat gland assay was used for quantification of radiation-induced damage in a normal tissue (Judas *et al.*, 1995; Johns *et al.*, 1995). We found a reproducible time course of response with radiation which was dose-dependent within the range 10–17 Gy. A nadir of functional deficit was observed at 8 weeks

(Figure 3a), which is consistent with that of the studies cited above. Drug was administered 20 min before irradiation so that the dose in the foot would be high during exposure to the radiation: the elimination half-life of AQ4N is 30 min (MA Graham and LH Patterson, personal communication) and this is similar to the value for tirapazamine. Neither AQ4N nor tirapazamine had any effect on the radiation response with 10 Gy. For comparison we have included data on mitomycin C, which has toxic effects on both oxygenated and hypoxic cells (Rauth *et al.*, 1983). Mitomycin C in combination with radiation (10 Gy) caused a large increase in the normal tissue toxicity at all time points. The unirradiated feet, i.e. those which were only exposed to mitomycin C, did not show any decrease in functional sweat pores as compared with the untreated feet of animals in the radiation alone experiment (data not shown). This shows that mitomycin C produced a major enhancement of the radiation induced normal tissue damage with an effect which was greater at all time points than 17 Gy single fraction radiation. Functional recovery at 16 weeks was also poor (about 40% of original function) compared to the other drugs combined with radiation all of which had recovered to about 85% function by 16 weeks (Figure 3a).

In conclusion, our data have shown that AQ4N can provide an increase in anti-tumour effect with single and fractionated radiation regimens without increase in toxicity to a normal tissue. This shows that a therapeutic gain can be obtained using AQ4N in combination with radiation. We have also found a similar level of response with the lead bioreductive drug tirapazamine. We would therefore propose that AQ4N may have potential as an effective agent in standard radiotherapy regimens.

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