ABSTRACT:

AQ4N (1,4-bis-[2-(dimethylamino-N-oxide)ethyl]amino)5,8-dihydroxyanthracene-9,10-dione) is in a class of bioreductive agents incorporating the aliphatic N-oxide functionality and is well documented as a very effective enhancer of radiotherapy and chemotherapy. The compound is shortly to enter Phase I clinical trials in the United Kingdom, and this study describes the preclinical pharmacokinetics and metabolism of AQ4N in mice. AQ4N was administered by i.v. injection at doses of 200, 100, and 20 mg/kg and was quantified by high-performance liquid chromatography and liquid chromatography/mass spectroscopy. There was a linear increase in the maximum plasma concentration (C_max) proportional to dose with a C_max of 1171 μg/ml at the maximum tolerated dose of 200 mg/kg. The area under plasma concentration versus time curve (AUC) increased disproportionally with dose from 14.1 μg/h/ml at 20 mg/kg to 247 μg/h/ml at 200 mg/kg with a subsequent decrease in clearance. Terminal elimination half-lives ranged from 0.64 to 0.83 h. The spectra of the two major metabolites matched those from authentic standards with the molecular ions [M + H]^+ being detected at m/z 445.4 (AQ4N), m/z 429.5 (AQ4N mono-N-oxide) and m/z 413.5 (AQ4). Only low concentrations of the toxic metabolite (AQ4) were detected in plasma at all three doses, with the AUC and C_max at 200 mg/kg being 3.54 μg/h/ml and 3.7 μg/ml, respectively, representing <2% of AQ4N. Concentrations of the intermediate AQ4 M represented 8, 10, and 18% of those for AQ4N at the doses of 20,100, and 200 mg/kg. The concentrations necessary for a therapeutic response in vivo have been described in this pharmacokinetic study.

Oxygen starvation of tissues (hypoxia) is a pathophysiological condition associated with major disease including cardiac arrhythmias, brain stroke, arthritis, and cancers. Paradoxically harnessing the reductive conditions that persist during the hypoxic episode may facilitate treatment of these diseases. The treatment of solid cancers by chemotherapeutic agents designed to kill hypoxic tumor regions is currently of intense interest because hypoxic tumor cells are resistant to radiation and conventional chemotherapy (Stratford and Workman, 1998). The occurrence of hypoxic cancer cells is a consequence of the metabolic demand of dividing cells in proximity to the blood supply depleting the available oxygen for more distant cells. The condition is exacerbated by the often chaotic and incomplete nature of tumor vasculature, which lacks tonicity and is subject to collapse as a result of the high interstitial pressure and blockage due to blood and cell debris (Vaupel et al., 1989). To facilitate hypoxic tumor cell kill, a large number of potential anticancer agents, which predominantly possess quinone or nitro functionalities, have been progressed that are enzymically capable of generating cytotoxic intermediates (Stratford and Workman, 1998; Patterson and Raleigh, 1998). We have now developed a class of bioreductive agent incorporating the aliphatic N-oxide functionality and typified by AQ4N (1,4-bis-[2-(dimethyl amino-N-oxide)ethyl]amino)5,8-dihydroxyanthracene-9,10-dione) (Patterson, 1993; Patterson et al., 1994). AQ4N is unique because it is the only agent currently described that is converted to a persistent anticancer agent unaffected by tumor reoxygenation (Hejmadi et al., 1996). In other words, AQ4N will continue to act even after an acute hypoxic episode. It is also the only bioreductive prodruk topoisomerase II inhibitor in advanced preclinical development (Smith et al., 1997). Targeting this enzyme is especially important in hypoxic cells, i.e., radiation-resistant tumor cells, because its inhibition (by AQ4N) synchronizes cell growth. Therefore, AQ4N will sensitize tumors to the repeated (fractionated) courses of radiation commonly used in radiotherapy (McKeown et al., 1995). AQ4N is well documented as a very effective enhancer of radiotherapy (McKeown et al., 1995, 1996; Patterson et al., 2000) as well as cisplatin and cyclophosphamide chemotherapy (Friery et al., 2000) in mice. Studies of the metabolism of AQ4N show that cytochrome P450 hemoprotein acts as a reductase for the hypoxia-dependent reduction to the active cytotoxin (Fig. 1). Human and mouse CYP 3A (Raleigh et al., 1998) and rat CYP 2B and CYP 2E (Raleigh et al., 1999) are among the isozymes that bioreduce AQ4N. This is in contrast to other bioreductive agents currently under investigation, notably tirapazamine, in which flavoprotein reductases are demonstrably involved in their activation (Patterson et al., 1998).

AQ4N is to enter Phase I clinical trials shortly in the UK under the auspices of the Cancer Research Campaign. It is therefore important to have a thorough understanding of the pharmacokinetic properties of AQ4N before the clinical trials because the trials themselves are likely to be pharmacokinetically guided (Collins et al., 1986).

Therefore, the intention of this study was to monitor the pharmacokinetics and metabolism of AQ4N in the mouse to complement the anti-tumor data already available in this species. The doses chosen for...
study were 200 mg/kg, which is the MTD, a likely starting dose for the clinical trial, and an intermediate 100 mg/kg. This information should also give an indication as to whether the pharmacokinetics of AQ4N and its toxic metabolites are likely to be linear as the dose is increased.

Materials and Methods

Animals. Female NMR1 mice aged 6 to 8 weeks were obtained from B and K Universal Ltd (Hull, UK). They were fed a CRM pellet diet (Special Diets Service, Witham, Essex, UK) and water ad libitum. All animal experiments were carried out under a project license approved by the Home Office, London, UK, and UK Co-ordinating Committee on Cancer Research (Workman et al., 1998) guidelines were followed throughout.

Chemicals and Reagents. AQ4N, AQ4M, and AQ4 were synthesized as described previously (Patterson, 1992). AQ4N was dissolved in physiological saline at an appropriate concentration for the desired dose to be administered in 0.1 ml/10 g of body weight by i.v. injection.

High purity HPLC grade solvents (Fisher Scientific, Loughborough, UK), analytical grade chemicals (Sigma Chemical Co. Ltd., Poole, UK), and triple-distilled water were used throughout. Mitoxantrone, the internal standard, was purchased from Sigma.

Drug Analysis. Chromatographic analysis of AQ4N was as described by Swaine et al. (2000). A Hichrom RPB (25-cm × 4.6-mm i.d.) column (Hichrom Ltd., Reading, UK) was used for the separation. A Waters 996 Photodiode Array Detector (λ1 = 240 nm, λ2 = 612 nm) with Millenium Software (Waters Ltd., Watford, UK) was used for spectral analysis of the peaks of interest. The flow rate was set at 1.2 ml/min with Waters model 510 HPLC pump and samples introduced via Waters 717 plus Autosampler. The mobile phase used was 78% ammonium formate buffer (0.05 M)/22% acetonitrile with final pH adjusted to 3.6 with formic acid.

Mass Spectroscopy. LC/MS analysis was carried out using a Finnigan MassLab Navigator (Manchester, UK) quadrupole mass spectrometer and Waters Alliance 2690 (Milford, MA) quaternary pump chromatography system. The mass spectrometer was operated in positive ion electrospray mode with a voltage of +3.00 kV applied to the capillary. A solvent flow of 1 ml/min with a nitrogen gas flow of 400 liters/h and a source temperature of 180°C were employed to produce stable spray conditions. The cone voltage was set at 25 V, and this gave clear mass spectra from these samples. The mass spectra were continuously scanned from m/z 1000 to m/z 100 every 3.5 s throughout the entire HPLC separation. MassLab software was used to process the mass spectral data and produce total ion chromatograms for the separation. The mass spectra shown were produced by averaging a number of scans across the top of the peaks recorded in the total ion chromatogram. All HPLC conditions, solvents, column, and flow rates were as previously detailed by Swaine et al. (2000).

Drug Extraction. Blood samples from three mice per time point (2, 5, 15, 30, 60, 120, 240, and 360 min) were taken by cardiac puncture under ether anesthesia and placed into heparinized tubes on ice; plasma was separated by centrifugation at 3000g for 5 min and 4°C. To 50 μl of plasma, 150 μl of methanol was added followed by centrifugation at 7000g for 8 min, and 60 μl of the supernatant was used for HPLC analysis. All plasma samples were analyzed individually. Linear calibration over a range of 0.1 to 10 μg/ml was achieved using HPLC by spiking 45 μl of plasma with 5 μl of AQ4N/AQ4 at a range of doses and addition of 150 μl of methanol followed by centrifugation at 7000g × 8 min. This extraction method produced extraction efficiencies of >80% (Swaine et al., 2000). Urine was collected from three mice 1 h following administration of AQ4N (200 mg/kg) i.p., using a metabowl apparatus (Harvard Apparatus Ltd., Kent, UK). Drug and metabolites were extracted from the pooled urine as for plasma.

Abbreviations used are: MTD, maximum tolerated dose; AQ4N, 1,4-bis-[[2-(dimethylamino)-N-oxide]ethyl]amino]-5,8-dihydroxyanthracene-9,10-dione; AQ4M, 1-[[2-(dimethylamino)-N-oxide]ethyl]amino]-4-[2-(dimethylamino)ethyl]amino]-5,8-dihydroxyanthracene-9,10-dione; AQ4, 1,4-bis-[[2-(dimethylamino)ethyl]amino]-5,8-dihydroxyanthracene-9,10-dione; AUC, area under the plasma concentration versus time curve; HPLC, high-performance liquid chromatography; MS, mass spectroscopy.

Pharmacokinetic Parameters. Pharmacokinetic calculations use standard formulas (Bibby et al., 1992). Cmax is the maximum plasma concentration at 2 min. Area under the plasma concentration versus time curve (AUC) was calculated from 0 to 6 h using the trapezoidal rule and when extrapolated to infinity used the equation Cz/Kel where Cz is the concentration at the last time point tz. The elimination rate constant (Kel) was calculated using log linear regression analysis and the half-life (t1/2) calculated from the equation t1/2 = 0.693/Kel.

Results

Drug Analysis and Chromatography. Authentic standards of AQ4N and the metabolites AQ4 and mono-N-oxide (AQ4M) gave good separation with limits of quantification for AQ4N and the two major metabolites in mouse plasma of 50 ng/ml. The analytical method was sensitive enough to be able to measure plasma levels of AQ4N at 1/10 of the MTD (20 mg/kg). Limits of quantification for the analysis of AQ4N in human plasma are expected to be below 10 ng/ml due to the larger sample size.

Pharmacokinetics. A summary of the pharmacokinetic parameters calculated for AQ4N following i.v. injection at 200, 100, and 20 mg/kg is given in Table 1, and the data are shown graphically in Fig. 2. A triphasic curve best described all three sets of data. There was a linear increase in the maximum plasma concentration ([Cmax] proportional to dose with a Cmax of 1171 μg/ml at the MTD of 200 mg/kg. The AUC increased disproportionately with dose from 14.1 μg/h/ml at 20 mg/kg to 247 μg/h/ml at 200 mg/kg (an increase of 1.75 over which that was expected, assuming linearity) with a subsequent decrease in clearance. Terminal elimination half-lives ranged from 0.64 to 0.83 h.

Metabolites were also detected in plasma and example traces are given in Fig. 3. Two of these metabolites co-eluted with authentic standards for AQ4 and AQ4M, and their identification was confirmed by HPLC/MS. Spectra obtained for AQ4N and the metabolites from the HPLC/MS analysis are shown in Fig. 4. The spectra of the metabolites matched those from authentic standards with the molecular ion [M + H]+ being detected at m/z 445.4 (AQ4N), m/z 429.5 (AQ4M), and m/z 413.5 (AQ4). A summary of the pharmacokinetic data for the two metabolites is presented in Table 2, and the plasma concentrations of all metabolites following a 200 mg/kg injection (i.v.) of AQ4N are shown graphically in Fig. 5. Only low concentrations of the toxic metabolite (AQ4) were detected in plasma. The calculated AUC for AQ4 following a dose of 200 mg/kg AQ4N was 3.54 μg/h/ml with a maximum plasma concentration of 3.7 μg/ml, which represents less than 2% of the parent compound AQ4N. Similarly, low concentrations of AQ4 were seen at all three doses.

Concentrations of the intermediate AQ4M were higher than those of AQ4. The AUC for the mono-N-oxide represented 8, 10, and 18% of those for AQ4N at the doses of 20, 100, and 200 mg/kg, respectively, with peak concentrations occurring after 2 min. Two other metabolites could be detected in plasma samples (Fig. 3), but the concentrations of both of these metabolites was too low to be accu-
rately quantified or for identification by MS. It is likely that these two metabolites are an N-demethylated AQ4N and AQ4 as they co-eluted with authentic standards. The urine from mice monitored 1 h after administration of a dose of 200 mg/kg AQ4N contained 8% of the administered dose. It contained predominantly AQ4N (99%) and trace levels (≤1%) of the mono-N-oxide and one of the unidentified metabolites. We were unable to detect any AQ4 in the urine after 1 h.

Discussion

One of the key requirements of prodrug cancer therapy is the selective activation of the drug within the tumor environment. There is ample evidence in the literature that AQ4N acts synergistically with other chemotherapies and radiation (McKeown et al., 1995, 1996; Hejmadi et al., 1996; Patterson et al., 2000). AQ4N is relatively nontoxic due to its poor affinity for DNA, but following reduction to AQ4, this affinity increases severalfold due to the cationic charge of the metabolite allowing interaction with the sugar phosphate backbone of the DNA (Smith et al., 1997). It is therefore important to understand the pharmacokinetic characteristics of both the parent AQ4N and this toxic metabolite before the compound enters clinical trial. This study has therefore monitored the pharmacokinetics of AQ4N in mice with a particular emphasis on the release of the potentially toxic metabolite AQ4 in plasma.

Peak levels of AQ4N are high (1 mg/ml) at the MTD of 200 mg/kg, and the drug is eliminated from the plasma in a triphasic manner at all three doses. The C<sub>max</sub> increases in a linear fashion with respect to dose. There was a slight decrease in terminal plasma half-life accompanied by a slight increase in AUC as the dose increased, and clearance was 1.75 times slower at the MTD than at 20 mg/kg.

Mass spectral analysis has confirmed the presence of AQ4 in plasma with a molecular ion (m/z 413.5) identical to that of synthetic standards although pharmacokinetic studies show that the release of AQ4 systematically is minimal, representing <2% of the AQ4N at all doses administered. There is a C<sub>max</sub> for AQ4 of 3.7 μg/ml and an AUC of 3.5 μg·h/ml. These low plasma levels of AQ4 are supported by both in vivo data, showing the MTD of AQ4N (250 mg/kg) is 5 times that of AQ4 (50 mg/kg; unpublished data), and in vitro with human liver microsomes, showing that the metabolic conversion of AQ4N to AQ4 is very dependent on decreased oxygen tension (Raleigh et al., 1998, 1999). Due to the high binding affinity of the metabolite AQ4 it is unlikely to diffuse out of the hypoxic regions of tumors in which it was reduced, therefore these low plasma concentrations would be expected. Previous studies have demonstrated AQ4 to be more than 100-fold more potent in vitro than the parent AQ4N, and a reflection on this tumor-specific activation of the drug would be lack of AQ4 in plasma.

Peak concentrations of the intermediate AQ4M (81 μg/ml and
Fig. 4. A, a total ion chromatogram (300–500 m/z) from a plasma sample following administration of AQ4N; B, mass spectra of the major peaks seen in plasma (see Figs. 3 and 4A) following administration of AQ4N.

The boxed numbers represent the following: 1 = AQ4, m/z 413.5; 2 = AQ4M, m/z 429.5; and 3 = the parent compound AQ4N, m/z 445.4.
TABLE 2
Pharmacokinetic data summary for AQ4N and the metabolites AQ4M and AQ4 following i.v. administration of AQ4N at 20, 100, and 200 mg/kg

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>C_{max} (µg/ml)</th>
<th>AUC_{0-\infty} (µg h/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>AQ4 0.3</td>
<td>AQ4M 6.49 ± 2.49</td>
</tr>
<tr>
<td></td>
<td>AQ4N 99.5 ± 22.5</td>
<td>AQ4M 6.49 ± 2.49</td>
</tr>
<tr>
<td>100</td>
<td>1.0 ± 1.08</td>
<td>85.14 ± 78.66</td>
</tr>
<tr>
<td></td>
<td>80.77 ± 58.1</td>
<td>505.5 ± 168.5</td>
</tr>
<tr>
<td>200</td>
<td>3.71 ± 4.81</td>
<td>1172 ± 84</td>
</tr>
</tbody>
</table>

Each data point represents the mean value from three mice (±1 S.D.). The pharmacokinetic parameters for these data are given in Table 2.

<10% of the AQ4N are seen immediately at 2 min, although the calculated AUC at 44.1 µg h/ml represents 18% of that of the parent compound AQ4N. Preliminary stability studies suggest that AQ4N is reduced by reductases in murine blood to the 2e reduced product AQ4M, but only trace levels of AQ4 could be detected in murine blood during these stability studies (data not shown).

The majority of Phase I clinical trials use 1/10 of the mouse LD_{50} as a starting dose, therefore allowing a large safety margin (Collins et al., 1986). Systemic concentrations that need to be achieved in patients must reflect those achieved in mice at therapeutic doses where AQ4N has been shown to enhance other chemotherapies and radiation therapy. The target AUC for such trials is generally taken as the AUC in mice at the MTD. The majority of combination studies in mice used doses of 200 mg/kg although increases in anti-tumor efficacy were seen with doses between 50 mg/kg and 200 mg/kg.

With prodrug therapy, it is clearly not only the AUC of the parent compound that is important but also the AUC of the active metabolite. Therefore, the AUC of AQ4 or AQ4M must be monitored carefully in patients as it approaches the profile seen in mice at 200 mg/kg, and subsequent dose escalation must be cautious. It may be that it is the AUC of AQ4 in plasma that determines the limiting dose of AQ4N.

This investigation has used a newly developed analytical method to study the pharmacokinetics of AQ4N in mice. The concentrations necessary for a therapeutic response in vivo have been described, and the study has shown that the two major metabolites detected systematically were the mono-N-oxide (AQ4M) and AQ4. The presence of these two metabolites has been confirmed by mass spectral analysis.

References


Hejmadi MV, McKeown SR, Ferri OP, McIntyre IA, Patterson LH and Hirst DG (1996) DNA damage following combination of radiation with the bioreductive drug AQ4N. Possible selective toxicity to oxic and hypoxic tumour cells. Br J Cancer 73:499–505.


