Stimulation by Localized Tumor Hyperthermia of Reductive Bioactivation of 2-Nitroimidazole Benznidazole in Mice

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ABSTRACT

We have investigated the effects of localized tumor hyperthermia (LTH; 43.5°C × 30 min) on the reductive bioactivation of the 2-nitroimidazole benznidazole in C3H mouse normal tissues and KHT tumors. Mice were allocated to one of three treatment groups: (a) unrestrained controls, (b) sham tumor treatment, and (c) LTH. Concentrations of benznidazole and its amine metabolite were determined by high-performance liquid chromatography. Conscious mice were given LTH or sham treatment 2.5 h after 2.5 mmol/kg benznidazole i.p. This gave steady-state plasma benznidazole concentrations of 120–170 µg/ml at 2–5 h in all three groups. Plasma amine concentrations were very low at 0.1–1 µg/ml in all cases. Liver benznidazole concentrations were similar to plasma but amine concentrations were 30–40-fold greater at 20–40 µg/g in all three groups, implicating the liver as a major site of reductive metabolism. Benznidazole concentrations in tumors from unrestrained mice were comparable to those in plasma and liver, with tumor/plasma ratios of 85–113%. Tumor amine concentrations were intermediate at about 2–3 µg/g, indicating reductive bioactivation had occurred. Sham treatment decreased tumor benznidazole concentrations by 25–50%, particularly at later times, and amine concentrations were correspondingly increased. This may be a result of sham tumor treatment at 37°C, a temperature 3–4°C higher than in unrestrained controls. More importantly, LTH further decreased tumor benznidazole concentrations over sham treatment, e.g., by 59% from 114 to 47 µg/g (P < 0.01) immediately after heating. Amine concentrations were correspondingly elevated, e.g., by 40% from 5.1 to 8.4 µg/g (P < 0.01). These results clearly show that LTH can selectively enhance the reductive bioactivation of benznidazole in KHT tumors in mice, and support a particular role for the use of bioreductive agents with heat.

INTRODUCTION

Hypoxic tumor cells are resistant to radiation and certain common anticancer drugs (1). Strategies to overcome this problem include the use of 2-nitroimidazole radiosensitizers such as misonidazole which are also preferentially cytotoxic towards hypoxic cells, as well as local hyperthermia which can likewise be used to sensitize tumor cells to radiotherapy (2–4).

Previous studies combining misonidazole and LTH2 in mice have demonstrated enhanced tumor cytotoxicity (5, 6). On the basis of previous in vitro studies (7–9) this is probably due, at least in part, to heat-enhanced reductive bioactivation of the drug to cytotoxic species (10, 11). A number of enzymes have been implicated in this metabolic activation process (12, 13) which is highly sensitive to inhibition by oxygen (14). To date, however, there appears to have been no reported attempts to quantify the effects of hyperthermia on reductive bioactivation in vivo.

Benznidazole is a lipophilic 2-nitroimidazole used in the treatment of Chagas' disease (15). It is as potent a radiosensitizer as misonidazole and also showed promise as a chemosensitizer in combination with the nitroreductase [1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea] in humans (16, 17). We have previously shown that 44°C hyperthermia preferentially enhanced benznidazole reductive bioactivation in murine KHT tumor homogenates compared to liver microsomes under anoxia in vitro (9). This might result via several mechanisms, including the expression of different nitroreductase enzymes in the two tissues.

Temperatures in the range 43–45°C can be achieved using LTH in vivo (18). The therapeutic advantage of nitroimidazole-hyperthermia-radiation combinations will of course be improved by the selective heating of tumor versus normal tissue by LTH. Furthermore, LTH has also been shown to preferentially decrease blood flow and pO2 in heated tumors compared to normal tissues (19, 20), thus creating a relatively more hypoxic tumor environment which should further facilitate nitroreduction (21, 22). Consequently there is a clear rationale for selective enhancement of the activation of benznidazole and other bioreductive agents by tumors in vivo through the application of LTH.

We now report that LTH (43.5°C × 30 min) does stimulate the reductive metabolism of benznidazole by KHT tumors in C3H mice. The results support a particular role for bioreductive agents in combination with localized tumor hyperthermia and radiotherapy (4).

MATERIALS AND METHODS

Mice and Tumors. Adult male C3H/He mice were obtained from our own breeding colony or Olac Ltd. (Bicester, UK). They were allowed food (PRD nuts; Labourse, Poole, Dorset, UK) and water ad libitum, and were used at 24–33 g body weight.

The KHT sarcoma was grown in the gastrocnemius muscle of the hind right leg as previously described (23). Mice were treated when orthodiagonal tumor diameters reached 10–13 mm.

Drug Supply and Administration. Benznidazole [Ro 07-1051; Radanil; N-benzyl(2-nitroimidazoyl)acetamide] and the analytical internal standard Ro 07-0602 [1-(2-nitroimidazol-1-yl)-3-4-butoxyxpropan-2-ol] were supplied by Roche Laboratories (Welwyn Garden City, Herts, UK). Benznidazole amine [Ro 11-1721; N-benzyl(1-imida
dazole)acetamide] was supplied as the hydrochloride salt from Hoffmann La Roche (Basle, Switzerland). Benznidazole was prepared as a suspension in 50% polyethylene glycol (molecular weight, 400) in Hank's balanced salt solution, and injected i.p. in a volume of 0.01 ml/g at a dose of 2.5 mmol/kg (650 mg/kg).

Localized Tumor Hyperthermia (LTH). Groups of three to four unaesthetized mice received a standard LTH treatment of 43.5°C × 30 min. This is equivalent to a heat dose (Eq 43 of 42.2 min (24). LTH was initiated 2.5 h after benznidazole administration using a microcomputer-controlled, combined radiofrequency-waterbath heating system described in detail elsewhere (25). Mice were given either drug alone, drug with LTH or drug with sham treatment. For the latter, mice were restricted in the perspex LTH jigs with PTFE-coated wires simulating rectal and tumor thermocouples in place. Jigs were then placed in a circulating waterbath (Grant Instruments, Shepreth, Cambridge, UK) set at 37°C. Mice were held in this way for 30 min, starting 2.5 h after benznidazole administration. All mice were loaded into the jigs 5–15 min before LTH or sham treatments. After LTH and sham treatment mice were rubbed dry with tissue paper and briefly warmed under a 40-W lamp to prevent hypothermia.
Core (rectal) temperatures were measured using a BAT-12 digital thermometer (± 0.1°C) fitted with a RET-3 murine rectal probe (Baileys’ Instruments, Saddle Brook, NJ).

Sample Preparation and Analysis. Samples were prepared essentially as previously described (26). Briefly, whole blood was obtained under diethyl-ether anaesthesia by cardiac puncture into heparinized syringes. Plasma was obtained by centrifugation at 3,000 x g for 15 min at 4°C, and stored at −20°C for up to 6 weeks prior to analysis. Plasma (200 μl) was treated with 2 volumes of methanol containing internal standard Ro 07 6062 (3 mg/l) thoroughly mixed and then centrifuged at 4,000 x g for 20 min at −15°C. Supernatants were evaporated to dryness in vacuo and residues resuspended in running buffer (100 μl) before injection (20–45 μl) into the HPLC apparatus for analysis.

Whole tumor and livers were excised rapidly. Livers were washed three times in cold distilled water (4°C) after gallbladder removal. Both tissues were quickly snap-frozen in dry ice to minimize ex vivo metabolism and stored as for plasma. Liver and tumor homogenates (33% w/v in distilled water) were prepared in all-glass homogenisers and treated as for plasma. All samples were handled on ice.

Benznidazole and benznidazole amine concentrations in plasma and tissue samples were determined by reversed-phase HPLC as previously described (26). Briefly, chromatography was carried out on equipment and columns supplied by Waters Ass (Milford, MA). Separations were carried out on reversed-phase C18 Bondpak Rad-Pak cartridge columns, which were eluted isocratically with 25% acetonitrile in 0.2 M glycine/hydrochloric acid buffer, pH 2.45, and containing 5 mM octane sulphonic acid at a flow rate of 3.5 ml/min. Benznidazole amine was detected at 229 nm and benznidazole at 313 and 229 nm. The lower limits of detection were 80 and 120 ng/ml, respectively, for an injection volume of 30 μl. Coefficients of variation were typically 3–5%.

Statistics. Levels of significance were determined using the Student’s t test.

RESULTS

Effects of LTH and Benznidazole on Tumor and Core Temperatures. In common with other 2-nitroimidazoles (27, 28), benznidazole (2.5 mmol/kg i.p.) decreased mouse rectal temperature (± 0.1 °C) fitted with a RET-3 murine rectal probe (Baileys’ Instruments, Saddle Brook, NJ). Localized tumor hyperthermia had no effect on either liver parent drug or amine metabolite concentrations compared to sham-treated mice (P > 0.05). The mean benznidazole liver/plasma ratios (± 2 SE) from 1–5 h were 95.8 ±16.5% after sham treatment (N = 25; P > 0.4) for unrestrained controls and sham-treated animals respectively. This is the most sensitive index of the extent of nitroreduction and it can be concluded that sham treatment had a relatively modest effect.

Local tumor hyperthermia had no effect on either liver parent drug or amine metabolite concentrations compared to sham-treated mice (P > 0.05). The mean benznidazole liver/plasma ratios (± 2 SE) from 1–5 h were 95.8 ± 16.5% after LTH and 94.9 ± 15.5% after sham treatment (N = 25; P > 0.5). The corresponding ratios for liver amine metabolite/plasma ratios were 34.1 ± 7.8 and 31.2 ± 5.5%, respectively (N = 25; P > 0.5). Thus LTH had no effect on nitroreduction in the host liver.

Effects of LTH on Tumor Benznidazole and Amine Concentrations. Fig. 3 shows benznidazole and its amine metabolite in C3H mice given benznidazole (2.5 mmol/kg i.p.) either alone or together with LTH or sham treatment. A, plasma benznidazole; O, plasma benznidazole/amine concentrations in mice bearing sham-treated tumors. O, plasma benznidazole; O, amine concentrations in mice bearing locally heated tumors. Results are mean ± 2 SE. Pooled data from two to three independent experiments with five to nine mice per point. **, values significantly different from sham-treated mice (P < 0.01).
HYPERTHERMIA-ENHANCED REDUCTIVE BIOACTIVATION IN VIVO

Fig. 2. Liver concentrations of benznidazole and amine in C3H mice administered benznidazole (2.5 mmol/kg i.p.) either alone or in combination with LTH or sham treatment. △, benznidazole; ∗, amine concentrations in livers from unrestrained control mice. ◊, benznidazole; •, amine concentrations in livers from mice bearing sham-treated tumours. ○, benznidazole; ●, amine concentrations in livers from mice bearing locally heated tumours. Results are mean ± 2 SE. Pooled data from two to three independent experiments with five to nine mice per point. **, values significantly different from sham-treated mice (P < 0.01).

2.5 h, similar to that seen in plasma. Over the subsequent 4 h, tumor benznidazole concentrations were remarkably stable at around 140–180 µg/g. Corresponding amine metabolite concentrations over this period were 2–3 µg/g, intermediate between plasma and liver values.

Sham-treated tumors showed consistently lower benznidazole concentrations compared to those for unrestrained control mice (P < 0.05 at 2.66 and 5 h). Benznidazole amine concentrations were concomitantly elevated by between 0.6–5 µg/g in sham-treated tumors relative to unrestrained controls, and this difference was particularly clear at 3 and 5 h (P < 0.01).

Most importantly, Fig. 3 shows that LTH clearly reduced benznidazole concentrations by 25–59% in heated compared to sham-treated tumors. This difference was particularly marked during the heating period (P < 0.01). Localized tumor hyperthermia further decreased benznidazole tumor/plasma ratios by up to 59% compared to sham-treated values, with the more dramatic decreases occurring specifically during the heating period (P < 0.01).

Table 1 summarizes the benznidazole tumor/plasma ratios for these experiments. Steady-state ratios were achieved by 2.5 h in unrestrained control mice. The ratios of 85–113% between 2.5 and 5 h indicated excellent drug penetration. By comparison, benznidazole tumor/plasma ratios in sham-treated tumors were 47–80% between 2.66 and 5 h. Ratios were consistently less than in tumors from unrestrained control mice, with significantly lower values at 3 and 5 h (P < 0.01 and P < 0.05, respectively). Localized tumor hyperthermia further decreased benznidazole tumor/plasma ratios by up to 59% compared to sham-treated values, with the more dramatic decreases occurring particularly during hyperthermia. For example LTH reduced average benznidazole tumor/plasma ratios over the interval 2.66–5 h by 47%, from 68.5 ± 11.5 to 36.5 ± 18.6% (mean ± 2 SE, N = 28 and 26, respectively; P < 0.01).

Table 2 shows the ratio for amine metabolite/total drug-
related material (metabolite conversion ratio). After equilibration at about 1 h, this ratio was very low in tumors from unrestrained control mice (1.4–2.7%) reflecting a modest level of benznidazole reduction. Sham tumor treatment consistently increased these ratios compared to unrestrained controls, a trend which became significant particularly at later times (3–5 h, P < 0.05). LTH produced an impressive 2- to 4-fold elevation in this conversion ratio as compared to sham-treated tumors, with significant increases occurring throughout the heating period (2.67–3 h, P < 0.05). The average conversion ratio over this period was increased from 7.1 ± 3.4% in sham-treated tumors to 22.5 ± 8.9% with LTH (P < 0.01).

**DISCUSSION**

Using a sensitive HPLC technique we have shown that mouse liver and KHT tumor tissue readily catalyze the nitroreduction of benznidazole to its amine metabolite in vivo. Furthermore, localized tumor hyperthermia greatly enhanced this bioreductive metabolism in KHT tumors with minimal effects on plasma and liver concentrations.

Plasma benznidazole concentrations in unrestrained control mice were in good agreement with previous observations (29, 30). The low plasma concentrations of benznidazole amine, representing <1% total detected drug-related material, were similar to those for misonidazole amine (31). Sham tumor and LTH treatment had minimal effects on plasma drug concentrations and LTH has also been reported to have little influence on either plasma misonidazole (32) or pimonidazole (Ro 03-30). The low plasma concentrations of benznidazole amine were similar for sham and LTH treatments. However, there was a clear indication that both LTH and sham treatment (i.e., both involving restraint) gave a modest elevation in liver amine concentrations relative to unrestrained control animals, possibly as a result of stress or slightly elevated core temperatures on hepatic metabolism.

Liver benznidazole concentrations equilibrated completely with plasma in unrestrained control mice after 1 h. The high amine concentrations in this tissue are consistent with a role for liver as a major site of nitroreduction in vivo (30). Misonidazole also undergoes extensive metabolism to its amine in perfused hypoxic rat liver in vitro (34) and mouse livers in vivo (31). Liver concentrations of benznidazole were similar for sham and LTH treatments. However, there was a clear indication that both LTH and sham treatment (i.e., both involving restraint) gave a modest elevation in liver amine concentrations relative to unrestrained control animals, possibly as a result of stress or slightly elevated core temperatures on hepatic metabolism.

Tumor benznidazole concentrations equilibrated with plasma after 1 h in unrestrained control mice. The corresponding tumor benznidazole amine concentrations were some 10-fold lower than in liver tissue at 2–3 μg/g. This is in close agreement with our previous results for benznidazole amine in murine EMT6 and RIF-1 tumor (30), but in marked contrast to the reported biodistribution of misonidazole amine which was comparable in both liver and KHT tumor tissue (31).

Sham-treated tumors exhibited markedly decreased benznidazole concentrations compared to unrestrained controls with up to 50% lower tumor/plasma ratios, particularly at later times. This decrease was accompanied by elevations in tumor amine concentrations, suggesting that sham-treatment enhanced reductive drug metabolism in the tumor. Similarly, sham treatment reduced pimonidazole tumor concentrations by up to 83% relative to unrestrained controls (33). We believe that this apparent increase in reductive metabolism is most likely to be a consequence of treating sham tumors in a water-bath set at 37°C, resulting in a temperature 3–4°C higher than those occurring in unrestrained mice treated with this dose of benznidazole. Temperature increases of this order can enhance benznidazole amine formation rates by up to 35% in vitro (9). Alternatively, leg restriction and manipulation, though kept to an absolute minimum, may alter tumor physiology, possibly reducing tumor blood perfusion and Po2 in such a way as to facilitate increased reductive metabolism.

More importantly, localized tumor hyperthermia further decreased tumor benznidazole concentrations by up to 59% especially during the heating period. Benznidazole tumor/plasma ratios were significantly decreased and tumor amine concentrations were correspondingly elevated 2- to 4-fold, particularly over the 30-min heating phase. This is in good accord with previous results where mISONIDAZOLE concentrations were reduced by up to 70% in locally heated EMT6 mouse tumors compared to unheated controls (32). Similarly, tumor/plasma ratios for pimonidazole were also shown to be significantly reduced when LTH was applied 5 min after drug administration (33).

The present study differs in at least three important respects from previous work. First, LTH was given when tumor drug concentrations were uniformly high and stable ruling out the possibility that decreased tumor benznidazole concentrations with LTH were a result of impaired tumor drug delivery or uptake. Second, concentrations of amine metabolite were measured alongside the parent drug to give a direct indication of bioreductive metabolism. Third, benznidazole reductive metabolism was monitored in locally heated tumors as well as unheated liver tissue. In view of the elevated amine metabolite levels in tumor after LTH and the in vitro results reported elsewhere (9), it is difficult to avoid the conclusion that LTH results in a marked potentiation of nitroreductive bioactivation.

The precise mechanisms involved in this hyperthermia-enhanced reductive bioactivation of benznidazole in heated tumors are likely to be complex. In vivo concentrations of benznidazole amine will depend on several factors including tissue temperature, the nature and quantity of the tissue nitroreductases and the ambient concentrations of reducing equivalents and inhibitory oxygen. Modest increases in temperature over the range 33–41°C can markedly increase benznidazole amine formation rates for liver microsomal nitroreductases, by elevating $V_{max}$ without altering the apparent $K_m$ (9). Furthermore, KHT tumor but not liver nitroreduction can be potentiated further between 41 to 44°C, possibly due to the presence of different nitroreductases with dissimilar temperature-dependencies (9, 35, 12). More work is required on the enzymology of
bioreductive activation, in order to establish which nitroreductases are most suitable for thermal potentiation.

Additional mechanisms may also be important. LTH can decrease blood flow in certain rodent tumors (21), leading to decreased pO2 values (20, 36). Since benznidazole reduction is decrease blood flow in certain rodent tumors (21), leading to oxic cells located close by. This may apply not only to nitro


demonstration of a tumor-specific increase in nitroreductive bioactivation with LTH has important therapeutic implications. If LTH increases the amount of drug metabolized to cytotoxic species and, perhaps in addition, enhances the reactivity of the intermediates, then tumor cytotoxicity should be potentiated. Depending on the reactivity of these intermediates, damage may be confined to the hypoxic cells or extend to oxic cells located close by. This may apply not only to nitro compounds but also to other bioreductively activated drugs. The present results suggest that combination of LTH with such agents is a promising area for further study.

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