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Distribution of nitroimidazoles and L-phenylalanine mustard in mammary adenocarcinoma 16/C tumors*

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Summary. Using the triphenylmethane dye, lissamine green, as an indicator of blood perfusion, we have demonstrated that L-phenylalanine mustard (L-PAM) is differentially distributed in mice bearing mammary adenocarcinoma 16/C tumors. Following i.p. administration, concentrations of L-PAM in various regions of the tumors vary by as much as 10-fold or more between regions of low and high perfusion. Since the nitroimidazoles, metronidazole and misonidazole, increase the cytotoxicity of certain antitumor agents, these compounds were investigated for their ability to increase the distribution of L-PAM into tumor regions of low perfusion. Administration of metronidazole (400 mg/kg) or misonidazole (800 mg/kg) 1 h prior to L-PAM and lissamine green resulted in elevated plasma levels of L-PAM and increased concentrations of L-PAM in tumor regions of high perfusion. A slight increase in the normally low levels of L-PAM in tumor regions of low perfusion was observed but the increase was not statistically significant. In contrast to the uneven distribution of L-PAM, metronidazole and misonidazole were evenly distributed throughout plasma and tumor regions of both high and low perfusion. Bioassay of tumors following in vivo exposure to metronidazole and L-PAM indicated decreased viability in fragments from tumor regions of high perfusion, but not from tumor regions of low perfusion. These studies demonstrate that the nitroimidazoles increased L-PAM levels in plasma and in tumor regions of both high and low perfusion but did not induce a uniform distribution of L-PAM throughout the tumors. The nitroimidazoles may enhance the effectiveness of L-PAM as an antitumor agent by increasing the concentration of drug that reaches a tumor.

Introduction

The nitroimidazole radiosensitizers, metronidazole (Metro) and misonidazole (Miso) enhance the cytotoxicity of certain chemotherapeutic agents in a variety of murine tumor systems [10, 13, 17, 19]. Miso displays greatest interaction with the nitrosoureas, especially 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU) [12], and the bifunctional alkylating agents, cyclophosphamide and L-phenylalanine mustard (L-PAM) [11, 14, 25]. At present, the mechanisms underlying the chemosensitization of tumors to alkylating agents remain unclear. One possible explanation is that the nitroimidazoles cause an alteration in the pharmacokinetics of the alkylating agent that leads to an increase in cytotoxic drug concentrations in tumors. Changes in L-PAM and CCNU elimination half-lives have been demonstrated following Miso administration [9, 11, 12].

By use of parenterally administered lissamine green, we previously demonstrated [15, 22] that the murine tumor, mammary adenocarcinoma 16/C (mam ad 16/C), which responds to chemotherapy with L-PAM [8], contains regions of both high perfusion (HP) and low perfusion (LP). Following i.p. administration of L-PAM and lissamine green to tumor-bearing mice, concentrations of L-PAM in different regions of these tumors varied as much as 10-fold at any given time after treatment and were directly correlated with the levels of lissamine green in the same tumor region, i.e., with the degree of tumor perfusion [22]. In view of these observations and the reported decrease in the clearance of L-PAM from plasma [9, 17] produced by nitroimidazole compounds, we investigated the effect of nitroimidazoles on the differential distribution of L-PAM in mam ad 16/C tumors. A preliminary report of this work has been presented [16].

Materials and methods

Chemicals. L-PAM, lissamine green B and metronidazole were obtained from Sigma Chemical Co. (St. Louis, Mo.). Misonidazole was the gift of Hoffmann-LaRoche, Inc. (Nutley, NJ).

Animals and tumor system. The transplantable mam ad 16/C tumor was propagated and maintained as previously described [21]. Tumor fragments were implanted subcutaneously, by trocar, in the axillary region of female B6C3F1 mice. Subsequent tumor growth was monitored by two-dimensional caliper measurements. Tumor weight was estimated from the formula for a prolate ellipsoid, assuming the specific density to be 1. Animals were used when tumors weighed 1200–1500 mg.

Drug administration. L-PAM and lissamine green were prepared as a single aqueous solution and administered i.p. (0.1 ml/10 g body wt.) at 20 and 50 mg/kg respective-

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ly. Metro and Miso were dissolved in water or saline and given i.p. at doses of 400 mg/kg (0.4 ml/10 g body wt.) and 800 mg/kg (0.32 ml/10 g body wt.), respectively. The nitroimidazoles were administered 0.5, 1, 2 or 4 h prior to L-PAM/lissamine green.

Sample collection and preparation. At 1 or 2 h after administration of L-PAM/lissamine green, mice were anesthesized with diethyl ether. A single, terminal blood sample was collected from the axillary region into a tube containing heparin. Plasma was obtained after centrifugation of cooled samples and immediately extracted with 4 volumes of methanol. Portions of each extract were assayed for L-PAM, lissamine green and nitroimidazole as described below.

Tumors were excised and immediately frozen on dry ice. After removal of grossly necrotic material, tumors were dissected into regions of HP and LP based on lissamine green content as assessed visually. Each region was homogenized in 4–9 volumes of methanol and then processed as described for plasma.

Variable patterns of lissamine green staining were observed among individual tumors. Regions of HP were not necessarily found in the tumor periphery, and regions of LP were not generally associated with the tumor center. In many instances "nodules" of HP and LP were present throughout.

The recoveries of L-PAM from plasma and the HP and LP regions of tumors were 90%, 107% and 102% respectively. For the nitroimidazoles, recoveries from the same sample types were 98%, 102% and 100%.

Sample analysis. HPLC analyses were accomplished with a Waters Associates (Milford, Mass.) apparatus equipped with a Model 6000A high pressure delivery pump, a Model 441 UV absorbance detector, a Model 710 automatic sample injector (WISP) and a Model 730 data module. The conditions for the analysis of L-PAM were as described previously [21]. Metro and Miso were analyzed by the procedure described by Workman et al. [27], with the exception that the mobile phase, consisting of methanol:water (20:80), was delivered at a flow rate of 1 ml/min. During the analysis of samples for Metro content, a metabolite peak was resolved. Based on its retention time, this component was tentatively identified as the hydroxylated derivative of Metro, 1-(2-hydroxyethyl)-2-hydroxymethyl-5-nitroimidazole [24]. Plasma and tumor levels of this metabolite were estimated using Metro standards.

Lissamine green was measured spectrophotometrically at 630 nm with a Beckman DU-2 spectrophotometer.

Bioassay experiment. Mice bearing mam ad 16/C tumors ranging in weight from 1500 to 2000 mg were randomly divided into four groups of 10 mice each. Animals in each group were dosed, i.p., with water or Metro (400 mg/kg), and 1 h later with either lissamine green (50 mg/kg) or lissamine green and L-PAM (10 mg/kg). One hour after treatment, the tumors were harvested and the HP and LP regions of each tumor were dissected and pooled with similar regions obtained from other tumors in the same group. Fragments from each pool were then implanted in 15–20 recipient mice and the growth of the tumors was monitored for several weeks.

Statistical analysis. *t*-Test evaluations were performed using a computerized program of SPSS/PC (SPSS/PC Information Analysis System Version 1.1, SPSS Inc., Chicago Ill., 1984).

Results

Administration of Metro 1 h prior to L-PAM resulted in significantly elevated plasma levels of L-PAM in mice bearing mam ad 16/C tumors (Table 1). This effect was observed in animals sacrificed either 1 or 2 h after L-PAM administration. In addition, L-PAM levels in the HP and

Table 1. Effect of Metro on L-PAM distribution in mice bearing mam ad 16/C tumors

Time of treatment with Metro or H_2O[a]	Time of killing	Treatment	μg L-PAM/g or ml			μg Lissamine green/g or ml			% Highly perfused tumor
			Plasma	Tumor regions		Plasma	Tumor regions		
				High perfusion	Low perfusion		High perfusion	Low perfusion	
−0.5 h	1 h	Metro	3.7±1.4	5.8±1.3	1.3±0.3	41.8±7.4	20.0±2.3	4.2±2.3	74± 5
		H_2O	2.7±0.5	3.8±0.9	0.8±0.4	38.1±8.6	18.8±3.5	2.4±2.1	85± 6
−1 h	1 h	Metro	3.7±0.5*	6.5±0.8*	2.0±0.4	34.9±2.1*	21.5±2.8	9.1±2.3	86± 9
		H_2O	1.6±0.1	3.6±0.2	1.2±0.7	25.2±3.6	17.7±2.5	6.3±2.1	72±10
−1 h	2 h	Metro[b,c]	0.6±0.1*	1.5±0.1*	1.3±0.1*	86.6±0.7	31.3±3.4	3.8±0.8	83±11
		H_2O[b,c]	0.3±0.2	0.8±0.2	0.7±0.1	87.1±7.8	26.0±4.3	2.8±1.0	79± 1
−2 h	1 h	Metro	2.4±0.2			25.8±7.6			
		H_2O	1.8±0.3			22.6±1.9			
−4 h	1 h	Metro[b]	2.0±0.7			23.8±4.5			
		H_2O[b]	1.9±0.6			27.3±6.8			

Results represent the mean ± SD for three separate experiments involving at least three mice per treatment group per experiment

[a] L-PAM and lissamine green were administered at time 0

[b] Results from one experiment are presented

[c] Lissamine green was administered separate from L-PAM at 30 min prior to sacrifice

* Value is significantly different ($P < 0.05$) from the corresponding value for mice administered water

LP tumor regions obtained from these mice were increased about twofold; however, the levels of L-PAM in the LP regions did not approach the levels in the HP tumor regions. Even though L-PAM levels were increased, the tumor to plasma ratio of L-PAM and the proportion of each tumor that was highly perfused were not altered by Metro. When Metro was administered 0.5 h prior to L-PAM, levels of L-PAM in plasma and the HP and LP tumor regions were increased about 1.5-fold, but the increases were not statistically significant. No change in L-PAM levels was observed when Metro was administered either 2 or 4 h before L-PAM.

Lissamine green levels in plasma were also increased about 1.4-fold in mice treated with Metro 1 h prior to L-PAM and sacrificed 1 h after L-PAM administration. This effect and the concurrent increase in L-PAM levels may have been related to a Metro-induced decrease in the volume of distribution or a decrease in the elimination rate of both the dye and L-PAM. In Metro-treated mice killed 2 h after L-PAM administration, the different schedule for the administration of lissamine green may have accounted for the absence of elevated lissamine green plasma levels in these animals.

At all times of analysis, Metro was evenly distributed throughout the HP and LP regions of mam ad 16/C tumors at concentrations equivalent to those in plasma (Table 2). Similarly, at any given time, levels of the OH metabolite of Metro were the same in plasma and the HP and LP tumor regions. Thus, in contrast to L-PAM, which was distributed in proportion to the tumor perfusion, Metro and its metabolite appeared to be distributed independent of the vascular supply.

In tumor-bearing mice administered Miso 1 h prior to L-PAM, the L-PAM levels in plasma and the HP tumor regions were about 3 times higher than those in saline-treated mice (Table 3). Levels of L-PAM in the LP tumor regions of the Miso-treated mice were increased only slightly compared to the levels in similar tumor regions obtained from saline-treated mice. As observed after Metro treatment, the proportion of each tumor that was highly perfused was not changed by Miso treatment. Also, similar to Metro, Miso was evenly distributed throughout plasma and the HP and LP tumor regions of each mouse (Table 3). In addition to increasing L-PAM levels, Miso also increased the levels of lissamine green in plasma (Table 3), suggesting that the mechanism of action of the compound was similar to that of Metro. At the doses administered, however, Miso was more effective in altering L-PAM and lissamine green levels than was Metro. Additional experiments with Miso were not conducted because of a limited supply of the compound.

A variety of chemopotentiation studies have established that Metro is preferentially cytotoxic to hypoxic cells [5, 23]. In view of this and the observed even distribution of Metro throughout the HP and LP regions of mam ad 16/C tumors, a study was conducted to determine whether Metro would enhance the cytotoxicity of L-PAM against cells from both of these tumor regions to the same

Table 2. Metro and metabolite levels in plasma and tumors (μg/g or ml)

Time of treatment with Metro	Time of killing	Compound	Plasma	Tumor regions of	
				High perfusion	Low perfusion
−0.5 h	1 h	Metro	221 ± 30	226 ± 23	199 ± 46
		Metab	8 ± 2	6 ± 1	2 ± 1
−1 h	1 h	Metro	227 ± 24	241 ± 36	245 ± 33
		Metab	13 ± 6	14 ± 7	8 ± 6
−1 h	2 h	Metro	143 ± 26	145 ± 13	161 ± 18
		Metab	12 ± 1	11 ± 3	12 ± 3
−2 h	1 h	Metro	138 ± 15		
−4 h	1 h	Metro	35 ± 14		

Data (mean ± SD) were obtained from the same mice presented in Table 1
Metab., 1-(2-hydroxyethyl)-2-hydroxymethyl-5-nitroimidazole

Table 3. Effect of Miso on L-PAM distribution in mice bearing mam ad 16/C tumors

Treatment[a]	μg L-PAM/g or ml			μg Miso/g or ml			μg Lissamine green/g or ml			% Highly perfused tumor
	Plasma	Tumor regions of		Plasma	Tumor regions of		Plasma	Tumor regions of		
		High perfusion	Low perfusion		High perfusion	Low perfusion		High perfusion	Low perfusion[b]	
Miso	6.3 ± 0.3*	9.0 ± 0.7*	2.1 ± 0.1	491 ± 13	509 ± 51	475 ± 2	51.1 ± 5.4*	22.3 ± 3.4	5.3	89 ± 12
Saline	1.8 ± 0.1	3.4 ± 0.5	1.4 ± 0.2				27.6 ± 2.5	16.6 ± 1.4	7.0	78 ± 31

[a] Miso or saline was administered 1 h prior to L-PAM and lissamine green. Samples were obtained 1 h after L-PAM and lissamine green
[b] Data from one animal are presented; analyses could not be conducted on samples from other mice in the treatment group because of insufficient sample
* Value is significantly different ($P < 0.05$) from corresponding value for mice administered saline

Table 4. Time for tumor fragments obtained from HP and LP regions of previously treated donor tumors to reach 1000 mg

Pretreatment of donor mice	Median days to reach 1000 mg	
	Regions of low perfusion	Regions of high perfusion
H_2O + lissamine green	20.7	14.6
Metro + lissamine green	> 15.5[a]	14.3
H_2O + L-PAM, lissamine green	21.7	19.0
Metro + L-PAM, lissamine green	21.8	23.6

[a] Tumors in several mice in this group did not reach 1000 mg during the period of observation

degree. Bioassay of fragments from LP and HP tumor regions obtained from mice treated with lissamine green only indicated that the LP regions contained fewer viable cells than the HP regions (Table 4). The time required for fragments of LP tumor regions to reach 1000 mg in recipient mice was not affected by treatment of donor mice with lissamine green and either Metro or L-PAM or all three agents in combination. Similarly, administration of lissamine green only or lissamine green and Metro to donor mice did not affect the time to appearance of tumors from fragments of HP regions. However, fragments from HP regions obtained from mice treated with L-PAM did not reach 1000 mg until day 19 and those treated with both L-PAM and Metro did not reach this size until almost day 24. Thus, Metro enhanced the cytotoxicity of L-PAM only against cells obtained from HP regions of tumors.

Discussion

These studies demonstrate that following parenteral administration to mice bearing mam ad 16/C tumors, Metro and Miso distributed evenly throughout plasma and the HP and LP regions of the tumors. The tumor distribution of the nitroimidazole radiosensitizers has been investigated in a number of murine and human tumor systems, but for the most part the studies have involved quantitation of biopsy specimens or whole tumors for levels of radiosensitizer, without regard for possible intratumor variations in concentrations [12, 17, 28]. Exceptions include the work of Ash and coworkers [1], who found that in patients administered Miso the radiosensitizer was essentially uniformly distributed in multiple samples of solid breast tumors at concentrations approximately 60%–70% of those in blood. Rich et al. [18] assayed serial biopsies of various carcinomas for Miso and conducted concurrent histological analyses on adjacent tumor sections. Their results demonstrated wide intratumor variations in Miso concentrations, the levels of which were inversely correlated with the degree of tissue necrosis. Utilizing ^{14}C-Miso, Blasberg et al. [2] assessed the regional distribution of Miso-derived radioactivity in RT-9 tumors in rats and found that, even during periods of relatively constant plasma levels of ^{14}C-Miso, the levels of Miso in the peripheral tumor regions were higher than in the central regions. These reported differences in the intratumor distribution of the nitroimidazoles may be a consequence of differences in the histology of the individual tumor types, the anatomical location of the tumors, the amount of non-tumor material (e.g., fat) associated with the sample analyzed, or the interval between nitroimidazole administration and sampling.

The uniform distribution that we observed for Metro and Miso throughout areas of both HP and LP in mam ad 16/C tumors was in contrast to the non-uniform distribution of L-PAM in the same tumors. These results indicated that the penetration of Metro and Miso into tissues was less dependent upon local blood flow than was L-PAM penetration. Although the delivery of Miso into some tumors has been correlated with blood flow [2], diffusion also appears to play a role in its distribution [1, 26]. This latter factor is probably related at least partially to the low degree of protein binding exhibited by the nitroimidazoles and their lipophilicity [20, 26]. The distribution of L-PAM should be less diffusion-dependent and more blood flow-dependent, since the compound, by virtue of its alkylating properties, binds extensively to cellular components [3].

The mechanism(s) underlying the observed increased L-PAM levels following nitroimidazole administration was not directly investigated but was unlikely to have been related to an inhibition of L-PAM metabolism, since this alkylating agent is not extensively metabolized in vivo [6, 7] or in vitro [4]. The elimination of L-PAM may have been affected by the nitroimidazoles, but in view of the fact that less than 2% of the dose was excreted in urine within 4 h of L-PAM administration to mice [7], interference with the renal elimination of L-PAM would probably not have influenced plasma levels of the drug during the time frame (1–2 h) of our experiments. Hinchliffe et al. [9] have proposed that Miso may alter plasma L-PAM concentrations by interfering either with the initial absorption of L-PAM from the peritoneum and/or with its subsequent distribution. That Miso, in particular, may have produced a decrease in the volume of distribution was suggested in our studies by the concurrent increase in plasma concentrations of lissamine green in the Miso-treated animals. No change in the percent of each tumor that was highly perfused was observed after nitroimidazole administration, indicating that the increased L-PAM levels in the HP regions were a direct result of the increased plasma levels. Presumably, then, the levels of L-PAM in the LP tumor regions remained low because the relative perfusion of the tumors was not changed by the nitroimidazoles, and, thus, the concentrations of L-PAM in these regions were predominantly diffusion-dependent.

The intratumor differences in the distribution of L-PAM and the nitroimidazoles in HP and LP tumor regions provided an insight into the results obtained from the bioassay study. Pretreatment with Metro or L-PAM, either alone or in combination, had no effect on the subsequent growth of tumor fragments obtained from LP regions following implantation into recipient mice. These results were consistent with the low cytotoxicity reported for Metro [11, 17] and the observed poor distribution of L-PAM into the LP regions. In separate studies [22], we determined that the thymidine labeling index and the grain count per cell in the LP regions were lower than in the HP regions, findings which were consistent with LP and reduced cell proliferation. These results indicated that the LP regions may function as sanctuaries for cells capable of producing tumor recurrence. For tumor fragments obtained from the HP tumor regions, the decreased viability produced by the combined administration of L-PAM and Metro, as compared with the cytotoxicity of either agent alone, was con-

sistent with results obtained in other tumor systems [11, 14, 17] and was most likely a consequence of the increased levels of L-PAM in the HP regions.

In summary, these studies demonstrate that Metro and Miso distributed evenly throughout the plasma and tumors of mice bearing mam ad 16/C tumors. In contrast, the intratumor distribution of L-PAM was uneven and was directly correlated with the degree of tumor perfusion. Although the nitroimidazoles produced an elevation in L-PAM levels in plasma and tumors, the levels in the LP tumor regions were not increased to the levels in the HP regions. These results would suggest that the clinical usefulness of the nitroimidazoles as chemopotentiators may be limited by the degree of vascularization of the tumor under treatment. Further investigations are planned to examine the distribution of nitroimidazoles and L-PAM in other tumor systems.

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