Active versus Passive Absorption Kinetics as the Basis for Selective Protection of Normal Tissues by S-2-(3-Aminopropylamino)-ethylphosphorothioic Acid

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ABSTRACT

Through an in vivo and in vitro analysis of the absorption kinetics of S-2-(3-aminopropylamino)ethylphosphorothioic acid (WR-2721) in the normal tissues and solid tumors of mice, rats, and rabbits, it has been demonstrated that normal tissues actively concentrate WR-2721 against a concentration gradient, whereas solid tumors passively absorb it, or, if active concentration of WR-2721 is present in tumors, it operates at a far reduced rate relative to normal tissues. These observations can account for the ability of WR-2721 to selectively protect normal tissues against both radiation and alkylating agent injury.

INTRODUCTION

Ten years ago, we first reported the observation that injection of WR-2721 before radiation exposure significantly increased the radiation resistance of both the skin and bone marrow of mice, without altering the radiosensitivity of the solid tumors they bore (11). This observation has been confirmed and expanded by ourselves and others (see Refs. 8 and 10 for review), and this drug offers significant radioprotection to all normal tissues tested, with the exception of the central nervous system, but does not alter the radiosensitivity of the solid tumors tested. As a consequence, WR-2721 is now entering clinical trials both in the United States (3) and in Japan (4). Although they are preliminary, the results to date are encouraging. Kligerman et al. (3) have failed to detect any toxicity following single injections of WR-2721 as high as 250 mg/sq m, or approximately 500 mg/patient. Further, Sugahara and Tanaka (4) have observed a 1.6-fold increase in the resistance of head and neck cancer patients to radiation-induced stomatitis when they were given 50 mg or approximately 25 mg of WR-2721 per sq m 30 min prior to each daily radiation treatment; they have also observed as yet unquantified protection against radiation pneumonitis under the same conditions.

The mechanism whereby WR-2721 offers this selective normal tissue protection, however, is not entirely clear. The original proposal (11) which led to the testing of this drug was that deficient tumor vascularity would inhibit absorption of WR-2721, thereby allowing selective radioprotection of the well-vascularized normal tissues. Since deficient tumor absorption has been demonstrated (6) and since it had been reported that normal tissues and tumors alike absorb WR-2721 passively down a concentration gradient (2), it appeared that deficient tumor vascularity was, in fact, responsible for the poor tumor absorption of WR-2721.

The inconsistency of this conclusion occurred to us when we initiated studies with other presumably passively absorbed drugs, such as misonidazole (1). In contrast to WR-2721, these drugs reached roughly equal concentrations in the normal tissues and tumors (1), even when we used the same tumors which excluded WR-2721. In addition, injection of mice and rats with WR-2721 prior to such passively absorbed alkylating agents as nitrogen mustard (7) or cis-dichlorodiammine platinum (9) produced significant (1.7- to 2.0-fold) increases in resistance to hematopoietic injury and nephrotoxicity, respectively, but did not alter the response of any of the 5 tumors that these animals bore. If deficient vascularity were the only factor involved in selective WR-2721 absorption by normal tissues, these results would not be expected.

Since deficient tumor vascularity cannot be the only factor involved in producing inhibited absorption of WR-2721 by tumors, we have reexamined the WR-2721 absorption patterns by normal tissues and tumors of mice, rats, and rabbits both in vivo and in vitro. The data presented below have demonstrated that WR-2721 is actively concentrated by normal tissues but is passively absorbed by solid tumors.

MATERIALS AND METHODS

Experimental Animals. The female BALB/c mice used in these experiments were purchased at the age of 28 days (The Jackson Laboratory, Bar Harbor, Maine) and introduced into the experiments at 10 weeks of age. Female Fischer 344 rats (Charles River Breeding Laboratories, Wilmington, Mass.) were obtained and used at similar ages. Female NZW rabbits (King Rich Rabbit Ranch, Edgewood, N. M.) were obtained at the age of 10 weeks and used at the age of 10 months. Mice and rats were maintained as described elsewhere (7, 9), and rabbits were housed individually in conventional animal facilities.

Tumor Transplants. Transplants of 2 different mammmary carcinomas growing s.c. served as the source of tumor tissue for transplant. These were the 3M2N squamous cell carcinoma growing in the Fischer 344 rat and the MCA-11 adenocarcinoma growing in the BALB/c mouse. Tumors were harvested aseptically, minced, and prepared as a single-cell suspension as described elsewhere (7, 9). Each syngeneic recipient received a s.c. injection of 1 to 5 x 10⁶ viable tumor cells in 0.1 to 0.2 ml of PBS. When the tumors reached 7 mm (mice) or 10 mm (rats) in diameter, the animals were used for the experiments.

In Vivo Drug Distribution Studies. Unlabeled WR-2721 (NSC 296961) was obtained from Dr. Melvin Heiffer, Walter...
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1. **Chart 1.** Serum concentration (conc.) of WR-2721 in BALB/c mice as a function of time after they received WR-2721 (200 mg/kg) i.v. or i.p. Point, average of 3 to 6 determinations.

injected WR-2721 is protein bound, that the unbound drug is absorbed with a half-life of 5 to 8 min, and that the remaining protein-bound drug is absorbed far more slowly.

Following i.p. injection of WR-2721, the peak serum concentration is reached within 5 min in the mouse (Chart 1), 10 min in the rat, and 15 min in the rabbit (data not shown). In all 3 species, the serum concentration rises gradually to approach the serum levels seen in i.v.-injected animals during the second phase of clearance, and once the peak is reached 71 to 86% of the drug can be precipitated from the serum by trichloroacetic acid. It would appear therefore that unbound drug leaves the circulation at least as rapidly as it enters from the peritoneal cavity.

**Tissue Distribution of WR-2721.** The distribution of WR-2721 in the normal tissues and tumors of mice and rats, following both i.v. or i.p. injection, gave essentially identical results, in spite of gross differences in the serum kinetics for these 2 routes (Chart 1). For brevity and because the i.p. studies yielded less interanimal variation, we present below only the i.p. data for mice and rats, but the patterns observed and the conclusions reached are the same for the i.v. studies.

Chart 2 is a plot of the WR-2721 concentration in serum, 5 normal tissues, and the MCa-11 tumor as a function of the time after an i.p. injection of WR-2721 (200 mg/kg). Data for the brain and spinal cord are not included, since, as expected (6), they failed to absorb detectable quantities of WR-2721 over the time interval tested. With the exception of the kidney, the normal tissues initially contain less WR-2721 than did the serum, but by 15 min postinjection their concentrations exceed that of the serum by factors of 1.5 to 2.0. It is particularly noteworthy that, as the serum level is declining, the normal tissue levels continue to increase, indicating that these tissues are actively concentrating WR-2721 against a gradient (Chart 2). The concentration factors, relative to the serum, average 2.1 at 60 min postinjection and 2.5 at 90 min postinjection. Even at the earliest intervals tested, the concentration of WR-2721 in the kidney exceeds that of the serum, but it declines rapidly, with the kidney joining the other normal tissues by 30 min postinjection.

The kinetics of WR-2721 absorption by the MCa-11 tumor is
time after injection, the tumor concentration of WR-2721 rises quite unlike that of the normal tissues studied (Chart 2). With time after injection, the tumor concentration of WR-2721 rises gradually to meet the declining serum levels by the 90th min postinjection.

A similar study conducted in rats bearing the 3M2N mammary carcinoma is given in Chart 3. Essentially the same results were obtained in this model, with normal tissue to serum ratios all exceeding 1.9 by 30 min postinjection (Chart 3). The rate at which the 3M2N tumor absorbs WR-2721 is even slower than that of the MCA-11 tumor, however, since it does not reach equilibrium with the serum by the 90th min postinjection.

Similar studies have been performed with the line 1 lung carcinoma in BALB/c mice and with the R3230AC and DMBA-14 mammary carcinoma in Fischer rats. All of these systems gave results comparable to those presented in Charts 2 and 3. Furthermore, although no rabbit tumors were available to us, we were able to demonstrate active concentration of WR-2721 in all of the normal tissues studied in this species.

**Preliminary Characterization of the Active Concentration Mechanism.** The simplest proposal which might account for the low rate of absorption of WR-2721 by solid tumors is that the normal tissues have ready access to the drug and deficiently vascularized tumors do not; consequently, the normal tissues remove the drug from the circulation before the tumor can actively concentrate it. Secondly, the tumor may be composed of viable areas which actively concentrate the drug and necrotic areas which do not. To determine whether either or both of these variables were responsible for the results given above, we developed a simple in vitro assay in which 1-cu mm cubes of normal tissues and grossly viable tumor are incubated in 35S-labeled WR-2721 under varying conditions, and the tissue concentration is determined. Chart 4 is a plot of the WR-2721 concentration in cubes of liver and grossly viable MCA-11 tumor as a function of the amount of their incubation time in 100 μM WR-2721. If the liver cubes are incubated at 37° in either Hanks' balanced salt solution or PBS, the WR-2721 concentration increases linearly with time reaching a concentration which is 2.7 times greater than the medium by the fourth hr (Chart 4). However, incubation at 4° results in a slower rate of absorption by the liver, and the WR-2721 concentration does not reach equilibrium with the medium by the fourth hr. We conclude, therefore, that active concentration of WR-2721 by the liver against a concentration gradient is demonstrable in vitro and that it is temperature dependent. Data to be presented elsewhere have shown that similar temperature-dependent active concentration can be demonstrated for a variety of other organs including skin, lung, kidney, heart, salivary gland, and spleen. Furthermore, in each of these tissues, a temperature-dependent active concentration has been demonstrated in 3 to 14 separate experiments.

The rate of WR-2721 absorption by the cubes of MCA-11 tumor is also a linear function of time, but the rate is approximately 3-fold lower (p < 0.02) than in the liver so that equilibrium with the medium is not attained within 4 hr. Incubation of these tumor cubes at 37° for as long as 16 to 24 hr can result in tumor concentrations in excess of the surrounding medium (1.1- to 1.3-fold), indicating that tumor cubes can actively concentrate WR-2721 but that the rate of active concentration is far below that observed in normal tissues. Furthermore, similar kinetics of absorption have been observed for liver 1 lung carcinoma and for 3M2N, R3230AC, and DMBA-14 mammary carcinomas.

Although we had selected the tumor samples with care to avoid even the slightest amount of grossly necrotic tissue, we considered it necessary to test the possibility of necrotic area artifacts more directly. To do so, we compared the 4-hr absorption of WR-2721 by 3 different samples of MCA-11 tumor cubes: grossly viable, grossly necrotic, and samples with questionable necrosis. The results, which are summarized in Table 2, show clearly that not only are necrotic samples not deficient in their ability to absorb WR-2721 but they can actually actively concentrate it within 4 hr, while viable tumors show the same results as in Chart 4. The samples of questionable viability fall in between the other two. Coupled with the simple linear kinetics of absorption through 16 hr (data not shown) and the fact that killing of viable tumor samples with liquid nitrogen increases their rates of absorption (data not shown), these studies (Table 2) indicate that the slow rate of WR-2721 absorption by solid tumors (Chart 4) is not an artifact due to the presence of necrotic tissue within the tumor cubes.

**Comparative Tissue Distribution of CYC.** BALB/c mice bearing the MCA-11 tumor were given i.p. injections of 14C-labeled CYC (200 mg/kg), and the concentration of the drug was determined in the serum, normal tissues, and tumor as a function of time postinjection. Chart 5 is a plot of the concentrations of CYC in each sample, and equilibration of both normal tissues and tumors with the serum is apparent. Similar results were obtained with L-PAM, and in neither case did injection of WR-2721 (200 mg/kg) 30 min before the alkylating alter these patterns.

Chart 6 is a plot of the concentration of WR-2721 and CYC in the spleen and MCA-11 tumor, following i.p. injection of BALB/c mice with WR-2721 (200 mg/kg) followed 30 min later by CYC (200 mg/kg) i.e., the same protocol used in our differential protection studies (Table 1). The spleen was se-
Table 2
Absorption of 100 µM WR-2721 by viable and necrotic samples of the MCa-11 tumor in vitro

<table>
<thead>
<tr>
<th>Sample quality</th>
<th>Tumor WR-2721 concentration (µM)</th>
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<tbody>
<tr>
<td>Viable</td>
<td>82.4 ± 2.7³</td>
</tr>
<tr>
<td>Questionably necrotic</td>
<td>96.5 ± 5.5</td>
</tr>
<tr>
<td>Necrotic</td>
<td>150.5 ± 8.5</td>
</tr>
</tbody>
</table>

³ Mean ± S.E.

Differ in the rate at which WR-2721 diffuses across their respective membranes. We are presently studying solid tumors, voluntary muscle, the brain, and the spinal cord, tissues which fail to absorb WR-2721 readily, in the hope of elucidating the mechanisms of inhibited WR-2721 absorption.

At present, we cannot say whether solid tumors truly absorb
WR-2721 passively or whether their passive kinetics are more apparent than real. The low levels (1.1- to 1.3-fold) of active concentration observed with tumors following 16- to 24-hr incubations could be the result of small numbers of nontumorous cells within the cubes or cell death and the consequent loss of the proposed membrane restriction. Whatever the ultimate resolution, we will refer to the tumor absorption kinetics both in vivo and in vitro as being passive, while recognizing that this refers to the net effect.

We are not proposing that active concentration of WR-2721 by normal tissues versus passive absorption by tumors as seen in vitro is the only factor responsible for deficient tumor absorption in vivo. The far greater mass of normal tissues coupled with the poorer vascularity of the tumor would undoubtedly place the tumor at a selective disadvantage even if it could actively concentrate WR-2721. The primary value of the in vitro system is that it allows the study of one of the major components responsible for deficient tumor WR-2721 concentrations and provides a means of identifying those types of human tumors which can actively concentrate WR-2721. It should be noted that 1 of the 14 experimental animals tumors studied has absorbed significant amounts of WR-2721 following i.v. injection (10).

The fact that CYC (Chart 4), L-PAM (data not shown), and misonidazole (1) achieve essentially identical concentrations throughout the body while WR-2721 selectively concentrates in normal tissue explains why WR-2721 does not interfere with the radiosensitizing (12) or antitumor (7, 9) activity of these passively absorbed agents.

REFERENCES

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