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 mammary 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 5×10⁶ 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...
Reed Army Institute of Research, Washington, D. C. (Sample AN). The contamination of this sample by free sulfhydryl and symmetrical disulfide (5) was less than 1%. 

For each drug, labeled and unlabeled samples were mixed just prior to dissolution so that mice and rats received 20 µCi of the labeled drug per kg of body weight in the desired total dose per kg. Rabbits received 30 µCi/kg. The drugs were dissolved just prior to use and injected i.p. or i.v. (lateral tail vein in mice and rats; ear vein in rabbits). At intervals of 3 to 120 min after injection, the animals were bled (superpapribital plexus for mice and rats; opposite ear vein for rabbits) into capillary tubes, which were capped and centrifuged to allow harvesting of the serum. Each animal was bled at all intervals up to the time of sacrifice. In the charts, each value represents the mean of 3 to 8 separate determinations. To avoid confusion, standard errors have been deleted from the charts; these ranged from 3 to 11% (mean, 4.9).

To determine the amount of radioactivity in each serum sample, we added 25 µl of serum to 0.5 ml of Soluene (Amer- sham/Searle, Arlington Heights, Ill.). Following a 4-hr period for digestion, the sample was diluted in 3 ml of PCS® (Amer- sham/Searle). Standards for the serum assays were 25 µl of appropriately diluted drug from the injection vial. For these samples and the tissue samples described below, selected samples were spiked with known quantities of the labeled drug so that we could determine the relationship between the external standard ratio and the counting efficiency. Each sample, including the standards, was then corrected for the counting efficiency. From a comparison of the cpm in the standard which contained a known amount of total drug, we then estimated the serum concentration in terms of micromolarity.

Fifty- and 100-mg samples of each tissue were harvested within 5 min after sacrifice, washed 3 times, weighed, and placed in 1.0 ml of Soluene. Following a 24-hr digestion, the samples were diluted with 10 ml of PCS. Standards contained 25 µl of the injected drug in 0.1 ml of water. Procedures followed were similar to those for the serum samples. To convert tissue concentration to µM, we assumed unit density for tissue.

In Vitro Absorption of WR-2721. Samples of liver and grossly viable tumors from mice were removed aseptically and diced into 1-cm³ mm cubes. In certain studies, we intentionally selected grossly necrotic tumor samples for comparison. These were incubated under varying conditions with 35S-labeled WR-2721 at a concentration of 100 µM in Hanks’ balanced salt solution or PBS (Grand Island Biological Co., Grand Island, N. Y.). The WR-2721 was first equilibrated with the appropriate incubation conditions, and then 250 mg of the respective tissues were added as 1-cm³ mm cubes. At intervals of up to 4 hr later, 5 samples of ~50 mg of the tissue cubes were harvested, washed 3 times in PBS, weighed, and handled as described above for determination of the concentration of WR-2721 in the tissue. This washing procedure was not responsible for the differences noted below, since essentially the same results were obtained when the labeled medium was removed by dilution instead of washing, when the tissues were washed in 500 µM WR-2721, or when the tissues were incubated for 4 hr in PBS prior to assay. This last observation reflects the fact that the majority of the drug is bound by the tissues. These data are being expanded and will be reported elsewhere. 3

RESULTS

Selective Protection of Normal Tissues. WR-2721 has been shown to effectively protect virtually all normal tissues studied against radiation injury, with the exception of the central nervous system, yet offer little protection to the 15 solid tumors studied (see Ref. 10 for review). A similar selective protection has been observed against nitrogen mustard-induced lethality (2.0-fold) (7) and against cis-dichlorodiammine platinum-in- duced nephrotoxicity (1.7-fold) (9), yet none of the 5 tumors studied were protected against these alkylating agents. Table 1 summarizes the results of similar studies with CYC, and again selective normal tissue protection is observed. We present these preliminary data with CYC because we compare the absorption patterns of this drug with WR-2721 below. A more complete description of the effects of WR-2721 on the responses of normal tissues and tumors to CYC will be presented elsewhere, including daily to weekly treatments. 3

Table 1

<table>
<thead>
<tr>
<th>CYC LD₅₀ (mg/kg)</th>
<th>MCA-11 growth delay (days) at following CYC doses</th>
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<tbody>
<tr>
<td>100 mg/kg</td>
<td>200 mg/kg</td>
</tr>
<tr>
<td>Controls</td>
<td>372 ± 21</td>
</tr>
<tr>
<td>pretreated</td>
<td>540 ± 19</td>
</tr>
<tr>
<td>DRF</td>
<td>1.45 ± .07</td>
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<tr>
<th>Givens</th>
<th>200 mg/kg</th>
<th>300 mg/kg</th>
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<tbody>
<tr>
<td>a Based on 3 of the 5 animals which survived.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>b Given an i.p. injection of 0.9% NaCl solution 30 min before CYC.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>c Mean ± S.E.</td>
<td></td>
<td></td>
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<tr>
<td>d Based on 3 of the 5 animals which survived.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>e Given an i.p. injection of WR-2721 (200 mg/kg) 30 min before CYC.</td>
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Serum Clearance of WR-2721. Chart 1 is a plot of the serum concentration of WR-2721 following i.v. or i.p. injection of 200 mg/kg. Mice given an i.v. injection cleared the WR-2721 from their serum biphasically, with an initial serum half-time of 5.5 min, followed by a second phase with a half-time in excess of 1 hr. Similar biphasic patterns were observed in i.v.-injected rats and rabbits (data not shown), with the initial half-times being 7.4 and 6.8 min, respectively, and the second-phase half-life being in excess of 1 hr for both species.

The relatively slower second-phase clearance in these i.v. studies would appear to result from binding of the WR-2721 to protein. Precipitation of the serum which had been harvested 30 to 60 min postinjection with trichloroacetic acid before counting removes 68 to 92% of the WR-2721, while similar treatment of serum harvested at 5 min after injection produces no alteration in the estimated WR-2721 concentration. We interpret these data as indicating that a small amount of the

3 J. M. Yuhas, unpublished observations.
Active versus Passive Absorption

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
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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 3S-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 has been observed for line 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>
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<tr>
<th>Sample quality</th>
<th>Tumor WR-2721 concentration (µM)</th>
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<tr>
<td>Viable</td>
<td>82.4 ± 2.7</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>

* Mean ± S.E.

lected as a typical organ, but data for other organs (Chart 2) would lead to similar conclusions. Within 30 min after injection, the concentration of WR-2721 in the spleen is 790 µM, while in the tumor it is 185 µM (Chart 6). In our therapy studies, we normally inject CYC at this time, and Chart 6 shows quite clearly that CYC is absorbed equally by the spleen and normal tissues. Because of differential absorption of WR-2721 by the spleen and tumor but similar absorption of CYC by these same tissues, the reason for selective protection of normal tissues (Table 1) is apparent. Similar patterns were observed with WR-2721 versus L-PAM (data not shown).

DISCUSSION

The data presented above make it quite clear that WR-2721 is actively concentrated by normal tissues and not passively absorbed as was originally proposed (2). The exact nature of this mechanism remains to be determined, but preliminary studies indicate that facilitated diffusion as opposed to true active transport is involved. As an example, classical inhibitors of active transport (e.g., KCN) fail to inhibit the active concentration. Furthermore, the vast majority of the absorbed drug is in the bound state, as would be the case in facilitated diffusion. If indeed facilitated diffusion is the mechanism underlying the active concentration of WR-2721, this would explain why Harris and Phillips (2) failed to detect active concentration. Their wet chemical methods would not detect the bound form of the drug. Studies on the nature of this temperature-dependent active concentration mechanism are continuing.

Solid tumors, on the other hand, have failed to express active concentration of WR-2721 in vivo (Charts 2 and 3) or in vitro (Chart 6) unless the incubations are extended to 16 hr, and even then the concentration factors observed are small (1.1 to 1.3). This deficiency would not appear to be the result of tumor necrosis, since necrotic tumors can actively concentrate WR-2721, while viable tumors cannot (Table 2). This observation provides an interesting possible explanation for the difference between most normal tissues and all of the solid tumors tested. Cell death would result in loss of any membrane-related control of absorption and expose intracellular contents to unrestricted concentrations of WR-2721. Once within the limits of the cell membrane, the drug could be bound, fail to participate in equilibrium with the medium, and thereby allow entrance of more drug. The fact that exposure of viable MCa-11 tumor cubes to liquid nitrogen or to hypotonic shock allows them to actively concentrate WR-2721 would be consistent with our working hypothesis that normal and tumor cells alike can bind the WR-2721 once it is inside the cell, but these 2 cell types 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 (6), 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

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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