Effect of the Chemoprotective Agent WR-2721 on Disposition and Biotransformations of Ormaplatin in the Fischer 344 Rat Bearing a Fibrosarcoma

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INTRODUCTION

WR-2721 is a phosphorothioate compound currently in Phase III clinical trials investigating its efficacy at reducing the adverse side effects of both radiotherapy and alkylating agent chemotherapy of neoplastic disease (1–3). This chemoprotective effect is purported to be selective for nontumor tissues (4, 5). The proposed mechanism for this selectivity is a relative lack of alkaline phosphatase activity in tumor tissues as compared to normal tissues and the lesser conversion of the inactive parent compound, WR-2721, to its postulated active thiol metabolite, WR-1065, within tumor tissues. Because WR-1065 is postulated to bind to reactive platinum species within the cell, this would provide a mechanistic basis for selective protection of normal tissues as compared to tumor tissues (3, 6, 7). This hypothesis has never been tested directly. Ormaplatin (formerly tetraplatin) is a platinum(IV) analogue of cisplatin recently in Phase I clinical trials (8–10). This laboratory has previously reported investigations of the biotransformations of ormaplatin under a variety of conditions in vitro (11), in cell culture (12, 13), and in vivo (14–16). These studies have shown that ormaplatin is very rapidly (τ1/2 ~ 3 s) reduced to the corresponding platinum(II) complex, Pt(dach)Cl2, in the presence of plasma protein sulfhydryl (11), and that subsequent biotransformations of this complex appear to be similar to those reported for cisplatin (17, 18). The present studies have also shown that the intracellular biotransformations of ormaplatin in vivo are very similar in all tissues examined to date (19). The present report discusses investigations into the effects of WR-2721 on ormaplatin biotransformations in tumor-bearing male rats as a direct test of the proposed mechanism of action of WR-2721.

MATERIALS AND METHODS

Materials. [4,5-3H2(n)] Ormaplatin (formerly tetraplatin) was prepared in the Radiosynthesis Laboratory, Division of Medicinal Chemistry and Natural Products, School of Pharmacy, at the University of North Carolina at Chapel Hill with a specific activity of 0.520 Ci/mmol. The synthesis and purity of this compound have been described elsewhere (20). Reductive titration of the cyclohexene ring yields a radiolabel that is not chemically exchangeable (20). Preparation of a solution of the drug for administration was as described previously (14). WR-2721 and its corresponding free thiol, WR-1065, were obtained from US Bioscience (West Conshohocken, PA). This is different from the clinical formulation available through the National Cancer Institute, which contains a 1:1 mix by weight of WR-2721 and mannitol. The drug was dissolved either in H2O (20 mg/ml) for i.p. injection or in PBS (pH 7.5; 100 mg/ml) for i.v. injection and filtered immediately before use. YMT ultralipidation membranes (Mf cutoff, 30,000) were purchased from Amicon (Danvers, MA). HPLC grade reagents were obtained from commercial sources, and solutions were filtered and degassed before use. A Barnstead Nanopure water purification system with an organics filter provided the water used with solvents and for dissolution. All other general laboratory reagents were commercial reagent grade or better and were used without additional purification.

Animals. Male Fischer 344 adult rats (150–200 g) were obtained from Charles River Breeding Laboratories (Raleigh, NC) and housed in clear plastic cages on a 12-h light/12-h dark cycle with access to water and Purina rodent chow ad libitum. Room temperature was maintained at approximately 22°C. Animals were housed in one to five cages per cage. Animals were allowed at least a 1-week acclimatization period before use in experiments. A transplantable fibrosarcoma (originally induced by methylcholanthrene) was obtained from Dr. M. W. Dewhirst (Duke University Medical Center, Durham, NC) with permission from Dr. J. M. C. Bull (University of Texas Health Science Center, Houston, TX), who originally developed the tumor (21). The tumor was maintained via regular passage involving s.c. hind...
flank implantation of solid donor tumor (100 mm³) under ketamine-xylazine anesthesia. Experiments were routinely performed at 10 days posttransplantation, at which time tumor volume was on average 2.5–3.0 cm³.

Methods. Animals were administered WR-2721 or corresponding vehicle by either of two protocols. For most of the experiments, a dual i.p. paradigm was used, in which case the WR-2721 (20 mg/ml and 200 mg/kg, 34-fold molar excess to platinum in a per kg body weight basis) or H₂O vehicle was administered i.p. under light ether anesthesia at time zero (4). At t = 30 min, ormaplatin was administered (12.5 mg/kg, i.p.), also under light ether anesthesia. These animals were all then sacrificed at t = 60 min. The second protocol used an i.v./i.p. paradigm in which the WR-2721 (100 mg/ml in PBS, pH 7.5, vehicle, 200 mg/kg) was administered i.v. via the lateral vein under light ether anesthesia at time zero. For this protocol, ormaplatin (12.5 mg/kg, i.p.) was administered at either t = 5 min or t = 30 min. These animals were then sacrificed at either 15, 30, or 60 min after ormaplatin administration. Sacrifice was by exsanguination via the inferior vena cava under light ether anesthesia; blood was collected immediately into heparinized tubes on ice. Peritoneal fluid (in the dual i.p. paradigm) was aspirated from the open abdominal cavity and placed on ice immediately before catherization of the vein. After exsanguination, tumor and normal tissues of interest were harvested into ice-cold 0.15 M NaCl. Plasma was prepared by centrifugation of whole blood at approximately 1000 × g for 15 min at 4°C (22). Plasma and peritoneal fluid were then diluted 1:5 in ice-cold PBS (pH 7.5) and filtered over Amicon YMT membranes by centrifugation at approximately 2000 × g for 45 min at 4°C (Sorvall RC2-B with SS-34 rotor; Norwalk, CT; Refs. 15 and 23). Aliquots from the diluted fluids and from the ultrafiltrates were removed and allowed to stand overnight in scintillation cocktail before LSC.

Slices from several lobes of the liver, the cortex, and outer medulla of each kidney, the entire spleen, and portions of viable, noncancerous tumor tissue were isolated on ice, weighed, and homogenized according to Mistry et al. (18). Cytosol and cytosolic ultrafiltrate were separated essentially as described (18). Aliquots from homogenate, cytosol, and ultrafiltrate were removed and prepared for LSC as described for plasma. The remainder of the ultrafiltrates from peritoneal fluid, plasma, and each of the tissues was frozen at −20°C until analysis by HPLC.

Radioactivity was assessed with an LKB model 1215 scintillation counter. The difference between counts in the cytosol and in the ultrafiltrate was considered to represent macromolecular-bound platinum (15, 22). Counts in the ultrafiltrate, although frequently referred to as “free platinum,” are more aptly termed “filterable,” because it is considered likely that they represent platinum bound to low molecular weight substances from the plasma and/or cytosol (24). For these experiments quantitation of platinum biodistribution and biotransformations was based on conversion of radioactivity to amounts of platinum by the use of specific activity of the ormaplatin (0.520 Ci/mmol). This conversion is based on the assumption that the platinum-nitrogen bond of the parent drug was still intact. Previous findings from this laboratory (19) and others (25) using LSC in parallel with AAS analysis appear to validate this assumption for the early time point used in this study (see “Results”).

The biotransformation products from the ultrafiltrates of peritoneal fluid, plasma, and tissues were separated by a two-column HPLC system developed by Mauldin et al. (26). Briefly, an initial separation on a Partisil octadsyl silica-3 RP column with heptane sulfonate as the ion-pairing reagent provided resolution of species as neutral paired ions on the basis of polarity. Peak fractions of interest from this separation were pooled and then injected onto a Partisil 10 SCX cation exchange column for additional resolution of the positively charged species. Fractions from both separations were collected and analyzed for radioactivity by LSC. The total radioactivity of any peak of interest was expressed as a percentage of the total radioactivity recovered from the column. Individual biotransformation products were identified by comparison of their retention times on RP and SCX HPLC with those of standards prepared in the laboratory (13, 26). Initially, standards for the reaction products of both WR-2721 and WR-1065 with ormaplatin were prepared, as were other standards, by displacement of the malonate ligand from Pt(dach)(malonate) at 37°C in the dark overnight. Subsequently, it was determined that the reaction of platinum complexes with WR-2721 resulted in displacement of the phospho moeity. Thus, whether WR-2721 or WR-1065 was used as the starting material, the reaction products formed were identical. Henceforth, only the Pt(dach)(WR-1065) was used in column standardization, along with the other standards routinely tested: Pt(dach)(cysteine), Pt(dach)(GSH), Pt(dach)(methionine), Pt(dach)(serine), and the free dach ligand. These were the standards the chromatographic characteristics of which most closely approximated those of the RP HPLC peaks that appeared routinely in greatest abundance in the biological samples.

Statistics. Independent (unpaired) t tests on control (ormaplatin only) and treated (WR-2721 plus ormaplatin) variables were performed with the SYSTAT statistical package (SYSTAT, Inc., Evanston, IL). Assessment of the significance of any effect of WR-2721 treatment on ormaplatin disposition or biotransformation was at the P = 0.05 level.

RESULTS

Many preclinical studies of WR-2721 prevention of platinum-induced toxicity have used a dual i.p. paradigm in which both the WR-2721 and the platinum drug were injected into the peritoneal cavity in a variety of temporal relationships (4, 27–32). Some of the earliest studies were those of Yuhas et al. (4, 29), and one animal model they used was the F344 rat. Thus, for these initial studies, a similar paradigm (4, 29) was used in which WR-2721 (200 mg/kg, i.p.) was administered 30 min before the ormaplatin (12.5 mg/kg, i.p.). We chose to examine biotransformation product profiles at 30 min after ormaplatin administration based on our previous biotransformation studies at this same time point in nontumor-bearing animals (19). The biotransformation product profiles of ultrafiltrates of plasma, liver, and tumor from control and treated animals shown in Fig. 1 give dramatic evidence from a qualitative standpoint that WR-2721 does affect the biotransformations of ormaplatin in the whole animal. The peaks are labeled according to nomenclature established previously (13, 15, 26). In Fig. 1, peak b contains the reduced Pt(dach)Cl₂; the peak/shoulder eluting immediately before the peak corresponds to the unchanged parent drug, ormaplatin. In Fig. 1, peak e is predominantly the cysteine complex; peak f could contain the GSH, serine, threonine, glutamine, or asparagine complexes; peak g (unlabeled on Fig. 1) is predominantly the methionine complex and generally appeared as a shoulder to peak h in these experiments; and peak h is primarily the free dach ligand. In Fig. 1, peak i in treated animals behaves chromatographically like a standard of the Pt(dach)(WR-1065) complexes. The shoulder or peak most closely corresponding to peak i in control animals is most likely the Pt(dach)(citrato) complex (11).

Because the identification of peaks g, h, and i are crucial for interpretation of these studies, the complete HPLC profiles for the relevant standards are shown in Fig. 2. The retention times of Pt(dach)(methionine) (Fig. 2D), free dach carrier ligand (Fig. 2, ∇), Pt(dach)(citrato) (Fig. 2G), and Pt(dach)(WR-1065) (Fig. 2A) were similar by RP HPLC, although clearly distinguishable. Moreover, SCX HPLC produced a distinctive profile that was different for each of the standards. For example, RP HPLC (Fig. 2A) resolved the Pt(dach)(WR-1065) complexes into at least two distinct peaks (the third peak as seen in Fig. 2A was not reproducibly formed). SCX HPLC of the major RP HPLC peak gave two peaks at pH 4 (Fig. 2B). The major peak at pH 4 was clearly resolved from the free dach carrier ligand (∇), Pt(dach)(methionine) (Fig. 2E) and Pt(dach)(citrato) (Fig. 2F). There was a minor peak on SCX HPLC that had the same retention time as the free dach carrier ligand at both pH 4 (Fig. 2B) and pH 2.3 (Fig. 2C). This most likely represents free dach carrier ligand released by trans-labilization between the RP and SCX HPLC steps. The major peak at pH 4 (Fig. 2B) is resolved into two distinct

Pt(dach)(WR-1065) complexes at pH 2.3 (Fig. 2C), which also have retention times that are distinct from those of the free dach carrier ligand (V) and Pt(dach)(methionine) (Fig. 2F). Pt(dach)(citrate) was not run at pH 2.3 because its SCX HPLC profile at pH 4 was sufficiently distinct from all known standards. Thus, RP and SCX HPLC allow resolution of at least three distinct Pt(dach)(WR-1065) complexes that form in vitro (two peaks by RP HPLC, with one of the RP peaks being resolved into two peaks that are distinct from free dach carrier ligand by SCX HPLC at pH 2.3). Possible structures for those complexes have been suggested elsewhere. All three Pt(dach)(WR-1065) complexes are clearly distinguishable by RP and SCX HPLC from all other known dach-platinum standards, of which over 40 have been prepared and characterized to date. It should also be noted that the dach-platinum complexes formed with WR-2721 are indistinguishable from those formed with WR-1065, most likely because the phosphate group is lost from WR-2721 in the reaction with platinum(II) complexes.4

Fig. 3 shows SCX HPLC analyses of the prominent, late-eluting RP HPLC peaks shown in Fig. 1. The profiles derived from additional SCX HPLC resolution of peak i (Fig. 3D) from the treated kidney at pH 4.0 (Fig. 3E) and at pH 2.3 (Fig. 3F) are remarkably similar to profiles derived from a similar resolution of the Pt(dach)(WR-1065) standard (Fig. 2, B and C) and very different from the SCX HPLC profiles shown of peak h (Fig. 3A) from control kidney at pH 4.0 (Fig. 3B) and at pH 2.3 (Fig. 3C) and from the SCX HPLC profile of the Pt(dach)(citrate) standard at pH 4 (Fig. 2H). In the SCX HPLC profiles from the control animal (Figs. 3, B and C), the peak with a retention time of 34—35 min at pH 4.0 (Fig. 3B), which is resolved into two peaks at 37—38 min and 44—45 min at pH 2.3 (Fig. 3C), has been shown previously to contain primarily free dach carrier ligand (Fig. 3C; 37—38 min) and Pt(dach)(methionine) (Fig. 3C; 44—45 min; Ref. 19). The biotransformation products resolved from peak i of the treated animals by SCX HPLC (Fig. 3, E and F) appear to represent at least two distinct Pt(dach)(WR-1065) complexes that form both in vivo and in vitro. The Pt(dach)(WR-1065) complex formed in vitro with a retention time of 42—44 min by SCX HPLC at pH 2.3 (Fig. 2C) does not appear to form to a significant extent in vivo (Fig. 3F). These complexes are characterized in a related paper and will simply be referred to as the Pt(dach)(WR-1065) complexes throughout the rest of this paper.

After having observed the qualitative effects of WR-2721 on ormaplatin biotransformations evident in Figs. 1 and 3, these effects were then quantified by comparing individual peak quantitations among treated and control animals (for each n = 4). Table 1 summarizes the effects of WR-2721 treatment on total platinum distribution at 30 min after ormaplatin administration. The platinum was quantitated by conversion of radioactivity into an amount of platinum from the specific activity of the labeled ormaplatin. Previous work from this laboratory in the absence of WR-2721 (19) has shown that biodistribution and HPLC profiles are essentially identical whether platinum is measured directly by AAS or by conversion from LSC. Parallel analyses with AAS and LSC were also done on one control and one treated animal in the group of experiments reported here (data not shown). No major differences were observed in the quantitation of platinum by the two methods; thus, treatment with WR-2721 did not

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Fig. 1. Qualitative comparison of RP HPLC profiles from control and treated rats. Ultrafiltrates of plasma (A and D), liver (B and E), and tumor (C and F) were prepared as described in "Materials and Methods" from control rats (A-C) given vehicle i.p. and from treated rats (D-F) given WR-2721 (200 mg/kg, i.p.), 30 min before ormaplatin (12.5 mg/kg, i.p.). Separation of ultrafiltrates on RP HPLC was as described in "Materials and Methods" from control rats (A-C) given vehicle i.p. and from treated rats (D-F) given WR-2721 (200 mg/kg, i.p.), 30 min before ormaplatin (12.5 mg/kg, i.p.). Note that Y-axes for the plasma profiles are on a different scale than are those for the liver and tumor profiles. The nomenclature used for peak identification has been described previously (13, 15, 26). These peaks are discussed in the text and elsewhere (13, 15, 26) in terms of the most likely biotransformation products of ormaplatin that they represent.

Fig. 2. Standards for the in vivo studies. Pt(dach)(malonate) (0.05 mM) and either Pt(dach)(methionine) (Fig. 2F). Pt(dach)(citrate) was not run at pH 2.3 because its SCX HPLC profile at pH 4 was sufficiently distinct from all known standards. Thus, RP and SCX HPLC allow resolution of at least three distinct Pt(dach)(WR-1065) complexes that form in vitro (two peaks by RP HPLC, with one of the RP peaks being resolved into two peaks that are distinct from free dach carrier ligand by SCX HPLC at pH 2.3). Possible structures for those complexes have been suggested elsewhere. All three Pt(dach)(WR-1065) complexes are clearly distinguishable by RP and SCX HPLC from all other known dach-platinum standards, of which over 40 have been prepared and characterized to date. It should also be noted that the dach-platinum complexes formed with WR-2721 are indistinguishable from those formed with WR-1065, most likely because the phosphate group is lost from WR-2721 in the reaction with platinum(II) complexes.
WR-2721 treatment had a similar effect (i.e., of decreasing the percentage of platinum bound to protein in the peritoneal compartment), but this could not be confirmed due to an unexplained variability of this parameter among our control animals.

WR-2721 treatment was also found to have measurable but variable effects on total platinum distribution and disposition among the tissues examined in this study (Table 1). These tissues, as noted in previous work from this laboratory (19), represent a range of target and nontarget sites for ormaplatin toxicity, with kidney and spleen exhibiting significant sensitivity to platinum toxicity and liver exhibiting relatively no sensitivity. Our tumor model has been shown previously to have limited but detectable sensitivity to platinum-induced cytotoxicity (21). As demonstrated in Table 1, effects of WR-2721 treatment were most pronounced in the spleen. Distribution of total platinum to the spleens of treated rats was decreased by 67%, and all other parameters were decreased as well. Total platinum distributed to liver was decreased by 53%, whereas in the kidney and tumor, total platinum was relatively unaffected by WR-2721 treatment. The amount of total platinum that remained associated with the cytosolic fraction of the whole tissue homogenates was decreased in all normal tissue of treated rats but not in tumor tissue. The percentage of this cytosolic platinum that, in turn, was bound to cytosolic proteins and/or macromolecules was decreased in the spleen and tumor tissue of treated animals but not in the kidney and liver.

As described, HPLC was used to resolve the various biotransformation products of ormaplatin found in the filterable fraction (or ultrafiltrates) of Table 1. The quantitation of these separations, examples of which are shown in Figs. 1 and 3, are summarized in Table 2. Several effects of WR-2721 treatment were detectable: (a) the amount of unchanged parent drug, ormaplatin, was decreased as a percentage of the filterable platinum in the peritoneal fluid and plasma. The amount of the reduced product, Pt(dach)Cl₂ (i.e., Fig. 1, peak b), was apparently unaffected by the presence of WR-2721, except that it, too, was decreased on a percentage basis in the peritoneal fluid. In Fig. 1, peaks e/f and g, both in terms of absolute amounts and as a percentage of the filterable platinum, were decreased almost uniformly across all tissues and fluids. In Fig. 1, peak i, as a percentage of filterable platinum, was increased in the peritoneal fluid, plasma, kidney, and spleen of treated animals. It was also increased in terms of absolute amount in the peritoneal fluid, plasma, and kidney. Apparent differences in Fig. 1, peak i, between treated and control livers and tumors appear to have affected the stability of the platinum-nitrogen bond of ormaplatin at 30 min.

It is evident from Table 1 that 50% of the total plasma platinum in the control animals was bound to plasma proteins at 30 min. This is in contrast to previous findings from this laboratory (15, 19) of approximately 70% of plasma platinum bound at 30 min after i.v. administration in animals without tumors. WR-2721 treatment had no effect on total plasma platinum levels but decreased by 50% the amount of plasma platinum that was bound to protein in both percentage and absolute terms. The data suggest (Table 1) that WR-2721 treatment had a similar effect (i.e., of decreasing the percentage of platinum bound to protein in the peritoneal compartment), but this could not be confirmed due to an unexplained variability of this parameter among our control animals.
A-C). In Fig. 1, peak i from our control animals appears to be...route (see "Discussion"). Clinical regimens, in contrast, have generally used an i.v. infusion of WR-2721 (1, 2, 33). We retained the i.p. route of ormaplatin administration because our previous biotransformation data with ormaplatin in tumor-bearing animals (Figs. 1 and 3; Table 2) had been obtained via that route, and previous studies have shown rapid influx of platinum compounds into the systemic circulation when administered by the i.p. route (34, 35). There are also clinical situations where the malignancy being treated has a peritoneal exposure, such as ovarian carcinoma, and in these situations, i.p. administration of a platinum agent has demonstrated some benefit clinically (36). Furthermore, systemic protection from platinum-induced toxicity has been reported after concurrent i.p. administration of cisplatin and i.v. administration of another sulfhydryl-containing compound, sodium thiosulfate (37, 38). Thus, we also investigated the effects of i.v. administration of WR-2721 on biotransformations of i.p.-administered ormaplatin. For this paradigm, we administered WR-2721 i.v., either 5 or 30 min before ormaplatin, and characterized the ormaplatin biotransformation products at 15, 30, and 60 min after ormaplatin administration. It was of particular interest to know, given the rapid tissue uptake and clearance of WR-1065 after i.v. administration, rather than to provide a quantitative comparison of...
Quantitation of the major peaks in plasma and all tissues of animals treated with WR-2721 and ormaplatin biotransformations after i.v. pretreatment with WR-2721 (200 mg/kg iv) + ormaplatin (12.5 mg/kg, i.p.) via four different paradigms

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* Each peak is represented in terms of its percentage of the total radioactivity recovered from the RP HPLC column.

* The first time of the paradigm refers to the interval between WR-2721 administration and ormaplatin administration; the second time refers to the interval between ormaplatin administration and sacrifice of the animal (n = 1 for each paradigm).

Profiles of ormaplatin biotransformations after i.v. pretreatment with WR-2721 compared to those after pretreatment by the i.p. route. In none of the paradigms tested in Fig. 4 was peak i as prominent as it was after i.p. administration of WR-2721 (Figs. 1, D-F, and 3D). However, a small peak or shoulder in the position of peak i was observed in all tissues of the treated animals (Fig. 4 and data not shown).
plexes appear to represent only a very small percentage of filterable platinum at any time point and are found in both normal and tumor tissue.

DISCUSSION

Previous investigations from this laboratory have been concerned with the relationship between biotransformations of platinum(dach) compounds and the toxicity/cytotoxicity of these agents (9, 11, 13–16, 19, 26, 40). Most recently (19) we have examined the organ-specific biotransformations of ormaplatin in nontumor-bearing Fischer 344 rats. The present studies extend this line of investigation to determine if another drug, WR-2721, which has purported chemoprotective activity that is selective for nontumor tissue, would have selective effects on in vivo biotransformations of ormaplatin. We have examined a select group of target and nontarget tissues of platinum-induced toxicity similar to our previous studies (19) but have included a transplanted fibrosarcoma in the present investigation. Unfortunately, given the recent reports of neurotoxicity in clinical trials with ormaplatin (9, 10), it was not possible to study the effects of WR-2721 on ormaplatin biotransformations in neural tissue. As was discussed in the previous report (19), the target site of both ormaplatin- and cisplatin-induced neurotoxicity appears to be peripheral nerve fibers (41–43), which offer too little material for these types of biotransformation studies. We have chosen WR-2721 for this investigation because it has shown promise in both preclinical (4, 29, 44) and clinical (1, 2) studies. Carfagna et al. (16) have previously shown that WR-2721 pretreatment protected kidney and intestinal tissue from ormaplatin-induced toxicity but has little effect on toxicity to lymphoid tissue. The present investigations examine the relationship between this chemoprotective effect of WR-2721 and its effects on ormaplatin biotransformations.

The effects of WR-2721 on ormaplatin biodistribution were inconsistent and not readily interpretable. For example, i.p. administration of WR-2721 caused a significant decrease in total platinum in the spleen and liver but not in the kidney and tumor (Table 1). In contrast, the percentage of cytosolic platinum bound to macromolecules was decreased in the spleen and tumor but not in the kidney and liver (Table 1). Moreover, because the Pt(dach)(WR-1065) complex appears to form by direct reaction of Pt(dach)Cl2 and/or ormaplatin with WR-2721 in the peritoneal cavity (see below), the altered biodistribution pattern most likely reflects differences between the biodistribution of Pt(dach)Cl2, which is the major plasma biotransformation product in control animals, and Pt(dach)(WR-1065), which is the major plasma biotransformation product in WR-2721-treated animals. Thus, we consider the biodistribution data as providing a better assessment of the effects of WR-2721.

The biotransformation studies rely primarily on HPLC separation of platinum complexes to assess the effects of WR-2721 on ormaplatin biotransformation. In data reported elsewhere, we have characterized the RP and SCX HPLC profiles of over 40 platinum(II) standards (11, 12, 13, 15, 26). More importantly, we have characterized ormaplatin biotransformations in vitro (11), in cell culture (12, 13), and in the Fischer 344 rat (14–16, 19). The only complexes that have somewhat similar retention times to Pt(dach)(WR-1065) on RP HPLC are the free dach carrier ligand and the Pt(dach)citrate) complex, and both of these are clearly resolved from the Pt(dach)(WR-1065) complexes by SCX HPLC. Because of the difficulties inherent in quantitating Pt(dach)(WR-1065) complexes by SCX HPLC, we have relied on RP HPLC for quantitative comparisons (Tables 2 and 3). However, the combination of our RP (Figs. 1 and 4) and SCX (Figs. 3 and 5) HPLC data clearly demonstrate that the Pt(dach)(WR-1065) complexes are a major biotransformation product in all body fluids and tissues tested from animals treated with WR-2721 and ormaplatin by the i.p./i.p. route. In contrast, the same data demonstrate that Pt(dach)(WR-1065) complexes are only a minor, but detectable, biotransformation product in the plasma and all tissues of animals receiving WR-2721 by the i.v. route. We think that these differences between i.p. and i.v. administration of WR-2721 are best explained by models in which most of the ormaplatin and Pt(dach)Cl2 are converted to Pt(dach)(WR-1065) complexes in the peritoneal cavity with subsequent distribution of the Pt(dach)(WR-1065) complexes to plasma and all tissues when WR-2721 is administered by the i.p. route, whereas little of the Pt(dach)(WR-1065) complexes form in either plasma or tissues when WR-2721 is administered i.v. because both WR-2721 and Pt(dach)Cl2 are cleared from those compartments before ormaplatin and/or Pt(dach)Cl2 can reach them. These models are discussed in more detail below.

It was, of course, difficult to predict the optimal time for detection of Pt(dach)(WR-1065) complexes in vivo. The highest cytosolic WR-1065 concentrations are detected immediately after WR-2721 administration and then rapidly decrease to much lower steady-state levels (39, 45, 46). This would seem to suggest that the optimal time for detection of Pt(dach)(WR-1065) complexes would be shortly after administration of WR-2721. However, Treskes et al. (47) have predicted, based on in vitro reaction rates and likely tissue levels in vivo, that the reaction between platinum complexes and WR-1065 would be relatively slow. Thus, we examined tissue cytosols for the presence of Pt(dach)(WR-1065) complexes when WR-2721 was administered i.v., either 30 or 5 min before ormaplatin, and at times ranging from 15–60 min after i.p. ormaplatin administration. This makes it very unlikely that we missed significant formation of Pt(dach)(WR-1065) complexes in vivo because of an incorrect choice of sampling times. For the i.p./i.p. paradigm, we used only a single time point for analysis because our data indicated the likely inactivation of ormaplatin and/or Pt(dach)Cl2 in the peritoneal cavity by that route (see below).

The most frequently described mechanism (2, 3) by which WR-2721 is proposed to selectively protect normal tissue from platinum-induced toxicity has to do with the relative amounts and activities of alkaline phosphatase between tumor and nontumor tissue. WR-2721 is a prodrug that is converted to the more active WR-1065 by in vivo dephosphorylation. It has been postulated that the undeveloped vascularity frequently encountered in tumor tissue results in a lowered pH in the tumor microenvironment, which yields a net deficit of alkaline phosphatase activity in tumor tissue. This results in a relative lack of conversion of WR-2721 to and uptake of WR-1065 in tumor tissue as compared to normal tissue. Because previous pharmacokinetic studies had indicated that WR-2721 is rapidly cleared from the plasma (39, 48), the prediction has been that only the WR-1065 that accumulates preferentially in normal tissue would be present in sufficient concentration to react with and inactivate any reactive platinum species encountered. These predictions are summarized in schematic form in Model A of Fig. 6. The essential points of Model A are: (a) there is predicted to be little or no direct reactivity between the parent drug, WR-2721, and ormaplatin or its biotransformation products in the peritoneal cavity or plasma; and (b) little or no WR-1065 should be encountered by any platinum species except in the cytosols of normal tissues.

As stated previously, the primary reason for our use of an i.p./i.p. paradigm in the first group of experiments was the inhibition reported previously of cisplatin-induced nephrotoxicity with this same animal model and paradigm (4). Past decisions to use this i.p./i.p. paradigm have also been influenced by pharmacokinetic analyses showing rapid absorption and distribution of WR-2721-derived radioactivity into (primarily normal) tissues after i.p. administration in this same rat model (45). However, that study did not analyze the pharmacokinetics...
of WR-2721 and/or WR-1065 in the peritoneal cavity. Moreover, in comparing the plasma pharmacokinetics of WR-2721-derived radioactivity after administration of WR-2721 by the i.p. and i.v. routes in that study, it was clear that a significant amount of WR-2721 and/or WR-1065 never reached the plasma compartment when WR-2721 was administered i.p. (45). Our data suggest that this missing pool of WR-2721 remains in the peritoneal cavity at 30 min and is present at sufficiently high concentrations to rapidly convert the majority of ormaplatin and/or Pt(dach)Cl₂ to Pt(dach)(WR-1065) complexes before the parent compounds are able to react with other biological nucleophiles or to reach other compartments. For example, Model A (Fig. 6) predicts that WR-2721 would not react with ormaplatin or Pt(dach)Cl₂ and, therefore, would have little effect on the levels of platinum bound to protein and low molecular weight nucleophiles in either the peritoneal cavity or the plasma. However, our data show a statistically significant decrease in protein-bound platinum in the plasma and platinum bound to low molecular weight nucleophiles (peaks e, f, and g) in both the peritoneal cavity and plasma. Because data reported elsewhere show that platinum complexes can react directly with WR-2721 (47) and that this reaction results in the displacement of inorganic phosphate and formation of the Pt(dach)(WR-1065) complexes, our data are more consistent with an alternative pathway of biotransformation for ormaplatin in the presence of WR-2721, which we have delineated schematically in Model B of Fig. 6. In this model, Pt(dach)(WR-1065) complexes are postulated to form directly in the peritoneal cavity (and possibly in the plasma as well) and to subsequently distribute nonspecifically to all tissues and/or be excreted. The direct reaction between WR-2721 and ormaplatin and/or Pt(dach)Cl₂ in the peritoneal cavity would readily explain the decreased level of protein-bound platinum and platinum bound to low molecular weight nucleophiles in the peritoneal cavity and plasma. It would also account for our second major finding with this paradigm, which was a lack of any apparent specificity between tumor and nontumor tissue in the effects of WR-2721 on ormaplatin biodistribution and biotransformations. These findings, taken together, would seem to indicate clearly that a dual i.p./i.p. paradigm is not a useful model by which to investigate the ability of WR-2721 to selectively protect normal tissue from platinum-induced toxicity. Moreover, our findings would particularly call into question previous reports from other investigators in which WR-2721 chemoprotection against platinum-induced toxicity was tested in a dual i.p./i.p. paradigm, but the effect of WR-2721 on antitumor efficacy of the platinum agent was tested with a WR-2721 i.p./platinum agent i.v. paradigm (27, 32).

The change to i.v. administration of WR-2721 in the second group of experiments, as described above, was in part an attempt to more closely mimic clinical protocols in which the WR-2721 is usually given as a 15-min infusion before infusion of the platinum agent (33). We, however, chose to continue administering the ormaplatin by the i.p. route because we already had the data from the first group of experiments that were also based on i.p. dosing of ormaplatin. Furthermore, as mentioned above, there does exist a precedent for such a paradigm of i.p. administration of platinum agents in conjunction with i.v. chemoprotection (37, 38). Such an i.v./i.p. paradigm obviously would not be subject to the same sort of difficulties as the dual i.p./i.p. paradigm, mainly because the WR-2721 has been shown to be very rapidly cleared from the circulation when administered i.v. (τ₁/₂ = 5 min; Refs. 3 and 39). On the basis of the in vitro reactivity of WR-2721 and WR-1065 with cisplatin and achievable in vivo concentrations of these compounds, Treskes et al. (47) have predicted that not only is WR-2721 unlikely to directly inactivate platinum complexes in plasma, but that WR-1065 is also unlikely to cause significant inactivation of cytosolic platinum complexes. Our findings appear to confirm the predictions of Treskes et al. (47) because we detected at most 10–20% of recovered radioactivity by HPLC in the form of Pt(dach)(WR-1065) complexes after i.v. administration of WR-2721 (Table 3). This should be considered a maximum estimate because only a portion of peak i (Fig. 4, A and D) can be identified unambiguously as a Pt(dach)(WR-1065) complex (Fig. 5). This amount of inactivation of filterable platinum species would hardly be sufficient for protection from toxicity, nor would it be predicted to add much to the protective effects of the methionine, cysteine, and GSH complexes detected in peaks e, f, and g (Fig. 4).

Thus, these data suggest that the classic model for WR-2721 action (Fig. 6, Model A) is also inadequate to explain the protection of normal tissues when WR-2721 is administered i.v. That is, our data strongly suggest that mechanisms other than a simple, nonspecific inactivation of reactive platinum species in the cytosol of normal tissues must be considered to explain the protective effects of WR-2721. There have been several alternative mechanisms proposed, e.g., reduction in the formation of reactive oxygen species by depletion of oxygen (49), the inactivation of radicals by a direct interaction (49), proton donation to DNA damaged by radicals (49), and modification of enzymes involved in DNA metabolism and repair (46, 50). The effects of WR-2721 on the formation and/or inactivation of free radicals could be significant in light of several reports that platinum complexes cause an increase in lipid peroxidation by reducing the concentration of protective sulfhydryl compounds (51, 52). One recent report has suggested that platinum complexes can generate free radicals when they react with DNA (53). Several investigators (3, 46, 54) have also proposed that WR-1065, which is positively charged at cellular pH, has a tendency to concentrate near the DNA by counterion condensation. Thus, this would act to create pockets of high WR-1065 concentration amid an apparently low total cytosolic concentration and increase the rate of reaction with any platinum species or free radicals appearing in the vicinity of the DNA. Such a model could, in theory, be used to explain the protective effects of WR-2721 on DNA in the nucleus, although inactivation of reactive platinum species in the cytosol was minimal. Whether this concentration around the DNA actually occurs in the in vivo situation becomes a moot question, of course, if DNA is not the target for platinum-induced toxicity.

WR-2721 AND ORMAPLATIN BIOTRANSFORMATIONS IN F344 RATS

Fig. 6. Schematic of possible reaction pathways of WR-2721 and ormaplatin in the F344 rat. Model A, predicted reaction pathways based on postulated mechanism of action of WR-2721 found in the literature (2, 3). Model B, reaction pathways, which would appear to be operative based on the findings of the investigations reported here.
toxicity. Particularly with respect to platinum-induced nephrotoxicity, this very much remains a subject for debate (55).

In terms of a mechanistic basis for the tissue specificity of the protective effects of WR-2721, our data demonstrated an apparent lack of any specificity between tumor and nontumor tissue when the WR-2721 is given by the i.v. route because we detected P(dach)WR-1065 complexes in tumor and nontumor tissue alike. There are two possible explanations for these data. Either the fibrosarcoma used in these experiments did not exclude WR-1065, or the selective protection of normal tissues by WR-2721 involves mechanisms other than the selective inactivation of platinum complexes in the cytosols of normal tissues. Unfortunately, ormaplatin at nontoxic doses did not cause a statistically significant increase in tumor growth delay with the selective inactivation of platinum complexes in the cytosols of normal tissues. This confirms the predictions of Treskes et al. (47) and suggests that new models for the mechanism of WR-2721 protection of normal tissues from platinum-induced toxicity need to be explored. Finally, our data showed no selective effect of WR-2721 on normal versus tumor tissue when WR-2721 was administered by either the i.p. or the i.v. route. These data suggest that the selective protection of normal versus tumor tissue by WR-2721 is unlikely to be a universal phenomenon, or the mechanism for the selective protection of normal tissue is unknown at present.

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Effect of the Chemoprotective Agent WR-2721 on Disposition and Biotransformations of Ormaplatin in the Fischer 344 Rat Bearing a Fibrosarcoma

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