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DEGRADATION OF 2-(3-AMINOPROPYLAMINO)-ETHANETHIOL (WR-1065) BY Cu-DEPENDENT AMINE OXIDASES AND INFLUENCE ON GLUTATHIONE STATUS OF CHINESE HAMSTER OVARY CELLS*

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Abstract—The radioprotective drug 2-(3-aminopropylamino)ethanethiol (WR-1065) can be degraded when incubated in cell culture medium *in vitro*. The degradation reaction consumes oxygen and results from the action of Cu-dependent amine oxidases present in the serum content of the medium. Analysis of the degradation products of WR-1065 demonstrates the formation of cysteamine, acrolein and H_2O_2. WR-2721, the inactive prodrug of WR-1065, is not a substrate for these enzymes. Extracellular degradation of WR-1065 by Cu-dependent amine oxidases leads to an intracellular depletion of glutathione (GSH) in Chinese hamster ovary (CHO) cells and to reduction of clonogenic cell survival. Addition of aminoguanidine, an inhibitor of Cu-dependent amine oxidases, protects CHO cells from the toxic effects of WR-1065 and under these conditions an increase of intracellular GSH levels occurs. These data demonstrate that WR-1065 can be degraded to toxic compounds by the presence of Cu-dependent amine oxidases which might have further implications for the clinical use of WR-2721.

Key words: WR-1065; WR-2721; amine oxidase; acrolein; cysteamine; glutathione

WR-2721‡ is a well-known radioprotective drug often used to protect tissues against the damaging effects of radiation. These effects of WR-2721 were demonstrated to be selective for normal but not for tumour cells [1]. Several studies have demonstrated that WR-2721 can also protect against cytotoxic effects of chemotherapeutic agents [2–4]. Therefore, WR-2721 is used in combination with chemotherapeutic agents, because it is able to suppress side-effects without reducing antitumour activity [5–7]. WR-2721 is considered to be a prodrug which needs dephosphorylation to the active form WR-1065. The dephosphorylation reaction is mediated by alkaline phosphatases [8]. Pharmacokinetical studies demonstrate that WR-2721 is rapidly converted to WR-1065 *in vivo* [9, 10]. WR-1065 can be taken up by cells and accumulates intracellularly [11]. WR-2721 and WR-1065 have a polyamine-like structure similar to spermidine as shown in Fig. 1. Polyamines are naturally occurring polycations found in millimolar concentrations within mammalian cells and have been implicated in normal patterns of cell growth and differentiation [12, 13]. Degradation of polyamines is mediated by amine oxidases, an ubiquitous class of enzymes with a broad spectrum of substrate specificities [14]. Previous data from our group suggest that WR-1065 might be a substrate for amine oxidases [15]. The purpose of this study was to characterize the degradation reaction of WR-1065 and to investigate the consequences of an extracellular degradation reaction on survival and GSH status of CHO cells *in vitro*.

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‡ Abbreviations: AG, aminoguanidine; AP, alkaline phosphatase; CHO, Chinese hamster ovary; GSH, glutathione; HPLC, high-performance liquid chromatography; WR-1065, 2-(3-aminopropylamino)ethanethiol; WR-2721, *S*-2-(3-aminopropylamino)ethyl phosphorothioic acid; DTT, dithiothreitol

MATERIAL AND METHODS

Trypsin (0.25%), newborn and foetal calf serum, McCoy's 5A medium, glutamine and antibiotics were purchased from Gibco (Eggenstein, Germany). WR-2721 and WR-1065 were a gift from Dr Leo Gerweck of the Department of Radiation Medicine (Massachusetts General Hospital, Boston, MA, U.S.A.). The purified bovine plasma amine oxidase was a gift from Dr Bruno Mondovi (Dipartimento di Scienze Biochimiche, Rome, Italy). The inhibitor of FAD-dependent polyamine oxidase, MDL 72.521,

Spermidine NH_2-CH_2-CH_2-CH_2-NH-CH_2-CH_2-CH_2-CH_2-NH_2

NH_2-CH_2-CH_2-CH_2-NH-CH_2-CH_2-S-R

WR-2721: R = PO_3H_2; WR-1065: R = H

Fig. 1. Molecular structures of spermidine, WR-2721 and WR-1065.

was a gift from Merrell Dow Research Institute (Strasbourg, France). Alkaline phosphatase (type VII-NL from bovine intestine), catalase (from bovine liver, 17,600 U/mL), acrolein, aminoguanidine, cysteamine, DTT, sodium hydrosulfite and spermidine were obtained from Sigma Chemical Co. (Deisenhofen, Germany).

Determination of WR-1065 and cysteamine. WR-1065 was analysed using a method developed for the determination of polyamines [16]. Perchloric acid extracts were incubated with 1 mM DTT to reduce the disulphide forms of WR-1065. The extracts were injected onto a C18 reversed phase column and eluted at pH 4.5 as ion pairs with octane sulfonate. The eluted amines were derivatized on-line with *o*-phthaldialdehyde and quantified with a fluorescent detector. For quantification diaminoheptane was used as internal standard. Detection of cysteamine was not possible under these HPLC conditions. Cysteamine was separated with a C18 reversed-phase column using 0.1 M monochloracetic acid/methanol (82/18) with 6 mM octane sulphonate at a flow rate of 1 mL/min. The eluted cysteamine was electrochemically detected using a BAS detector at +150 V auxiliary voltage. The retention time of cysteamine under these conditions was 15 min.

Determination of intracellular GSH. Total soluble intracellular GSH (reduced and oxidized form) was quantified using previously described methods [17, 18]. Briefly, the cell pellet was treated with 1 N perchloric acid and γ-glutamylglutamate was added as an internal standard. After derivatization of the free thiol groups with iodoacetic acid and of the aminogroups with 1-fluoro-2,4-dinitrobenzene the reaction mixtures were separated by HPLC. Aliquots were injected onto a μBondapak amine column (4 × 250 mm, Waters, Eschborn) and eluted with a sodium acetate gradient (flow rate 2 mL/min) in a water/methanol/acetic acid solvent at pH 4.5. The dinitrophenyl derivatives were detected at 360 nm. GSH was quantified in relation to the internal standard. Protein was determined according to the method of Lowry [19].

Measurement of PAO-activity. Oxygen uptake and peroxide production were measured as described using the Yellow Springs Oxygen Apparatus with a Clark electrode [20]. All measurements were done at 37°. The test chamber was equipped with a magnetic stirrer. The cell culture medium was buffered with 20 mM HEPES and allowed to equilibrate to the desired temperature. The drug was added using a syringe introduced into the test chamber through an access slot. Sodium hydrosulfite was used to calibrate recorder output. PAO-activity was expressed as nmol O_2 uptake per min per mL beginning 1–2 min after the addition of the drugs.

Determination of acrolein. A fluorimetric determination of acrolein was used as described [21]. Two millilitres of the samples were mixed with 0.5 mL of mixed reagent (250 mg *m*-aminophenol plus 300 mg hydroxylamine hydrochloride, dissolved in 25 ml 1 N HCl) and 0.5 ml of 5 N HCl. Blanks and standards were prepared in a similar manner. The resulting mixtures were heated in a boiling water bath for 10 min and subsequently cooled in tap water. In samples containing serum the precipitated protein was removed with a short centrifugation step. Fluorescence was determined at room temperature immediately after derivatization with a fluorimeter (Kontron Spectrofluorimeter SFM 23). A calibration curve of acrolein standard solutions was used to quantify the acrolein content of the samples.

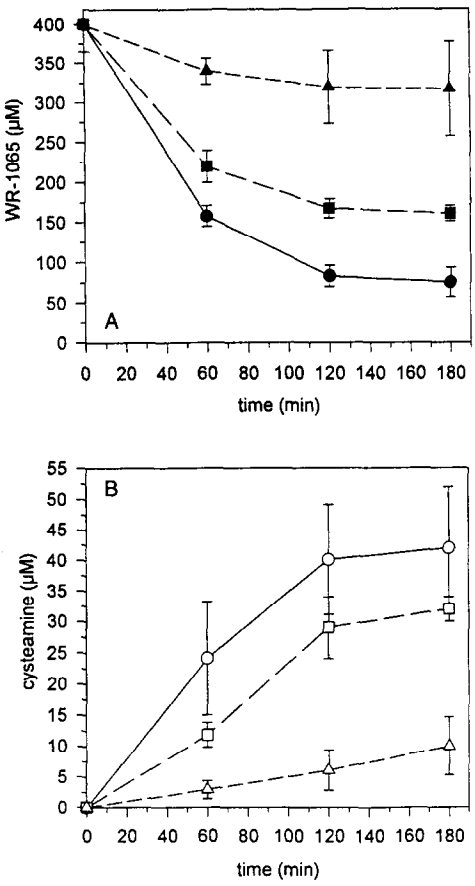

Fig. 2. Determination of (A) WR-1065 and (B) cysteamine in CHO medium. CHO medium was incubated for 1 hr at 37° with 0.4 mM WR-1065 (circles), 1 mM AG + 0.4 mM WR-1065 (squares), 10 mM AG + 0.4 mM WR-1065 (triangles). Means ± SD from three independent experiments are shown.

Cell culture. Chinese hamster ovary (CHO) cells were routinely grown and subcultured in McCoy's 5A medium supplemented with 10% newborn and 5% foetal calf serum with glutamine and antibiotics. Cell viability was determined by the Trypan blue exclusion method. The assay for the clonogenic survival of CHO cells was performed as described [18].

RESULTS

Measurement of WR-1065 in CHO-medium and detection of cysteamine

In a previous study we described differential effects of WR-1065 on cystine uptake and GSH

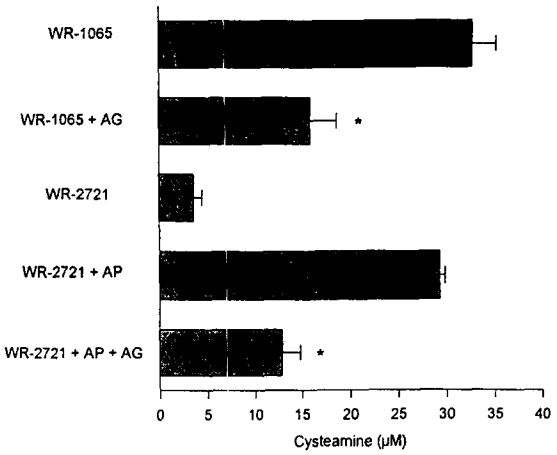

Fig. 3. Determination of cysteamine in CHO medium under different conditions. WR-1065 (0.4 mM), AG (1 mM), WR-2721 (0.4 mM) and AP (20 U/mL) were added to CHO medium as indicated and incubated for 1 hr at 37°. Means ± SD from three different independent experiments. Asterisks indicate a significant difference from control values without AG ($P < 0.05$).

content in two different types of CHO cells, but the mechanisms of this action remained elusive [15]. Here we show that the effects of WR-1065 *in vitro* mainly depend upon the formation of breakdown products. When WR-1065 was incubated in CHO medium in the absence of cells we found a decrease in the concentration of WR-1065 as shown in Fig. 2(A). After 180 min less than half of the initial concentration of WR-1065 was detectable in the medium. This decrease was not the result of a disulphide formation of WR-1065 with other thiols in the medium. As mentioned above (see the Materials and Methods Section) all samples were treated with DTT before HPLC analysis to convert disulfides to the reduced thiol molecules. Because of the polyamine-like structure of WR-1065 we proposed that polyamine oxidases are involved in the degradation of WR-1065. We tested AG, a competitive inhibitor of Cu-dep. amine oxidases, on its ability to reduce degradation of WR-1065. One millimolar AG reduced the degradation of WR-1065 which was even more pronounced when an AG concentration of 10 mM was used. Simultaneously with the decrease of WR-1065 concentration in CHO medium, the aminothiol cysteamine accumulated as shown in Fig. 2(B). Addition of 1 and 10 mM AG reduced the levels of cysteamine. A quantitative analysis of Figs 2(A) and (B) showed that the detected concentration of cysteamine is about 10–30% of the degraded concentration of WR-1065. This indicates that cysteamine is a major degradation product of WR-1065 in CHO medium.

High concentrations of cysteamine are detectable after incubation of CHO medium with WR-1065 but not with WR-2721, as shown in Fig. 3. Coincubation of AP with WR-2721, which dephosphorylates WR-2721 to WR-1065, also leads to high concentrations of cysteamine. Addition of AG to WR-1065 or WR-2721 + AP significantly reduces cysteamine concentrations. The formation of cysteamine shows that WR-1065 but not WR-2721 is a substrate for Cu-dependent amine oxidases. The production of cysteamine by WR-2721 alone can be explained by a low dephosphorylating activity of CHO medium which leads to the formation of WR-1065 (HPLC data not shown).

Measurement of serum amine oxidases by oxygen uptake and detection of H_2O_2

Cu-dependent amine oxidases can catabolize a broad spectrum of polyamines [14]. To determine the activity of polyamine oxidases in CHO medium we used the polyamine spermidine as a substrate. Enzyme activity was determined by measurement of the initial oxygen uptake with Clark's oxygen electrode as described. In Fig. 4(A) the addition of spermidine to CHO medium led to rapid initial oxygen uptake. The initial oxygen uptake of 0.4 mM spermidine at 37° was 16.7 ± 2.4 nmol O_2/min/mL. Addition of different concentrations of AG to the medium before the addition of spermidine reduced oxygen uptake. At 1 mM AG nearly no uptake of oxygen (1.2 ± 0.8 nmol O_2/min/mL) could be detected.

Incubation of CHO medium with WR-1065 also leads to rapid oxygen uptake as shown in Fig. 4(B). Addition of 1 mM AG, which nearly completely inhibited polyamine oxidation activity as shown in Fig. 4(A), strongly reduced oxygen uptake. The remaining oxygen uptake results from the non-enzymatic autoxidation reaction of the free thiol group of WR-1065 [22] and the low enzyme activity which remains after addition of 1 mM AG. The autoxidation reaction can also be observed in aqueous solutions containing no serum, while AG (1 mM) did not influence this reaction of WR-1065 (data not shown). Therefore, oxygen uptake of WR-1065 in CHO medium is caused by two different reactions: (a) a non-enzymatic autoxidation reaction, and (b) an enzymatic reaction which can be inhibited with AG. Calculation of the oxygen uptake without and with AG from the curves in Fig. 4(B) results in values of 26.4 ± 3.4 nmol O_2/min/mL and 12.1 ± 2.9 nmol O_2/min/mL, respectively. Subtraction of the autoxidation rate from the total oxygen uptake gives the oxygen uptake for the enzymatic reaction which is inhibited by 1 mM AG. The resulting value of 14.3 ± 3.4 nmol O_2/min/mL is not significantly different ($P > 0.05$) from the oxygen uptake of 0.4 mM spermidine under the same conditions.

The accumulation of hydrogen peroxide was measured also in Fig. 4(B). Addition of catalase (50 μg/mL) to the medium after 5 min leads to the rapid generation of oxygen. This was demonstrated in the presence and absence of AG. The results indicate that approximately half of the oxygen uptake of WR-1065 under either condition was utilized for the generation of H_2O_2.

WR-2721 led only to a small uptake of oxygen in CHO medium as shown in Fig. 4(C), which can be explained by the dephosphorylating activity of CHO medium as mentioned above. Adding AP to the medium before WR-2721 increased oxygen uptake.

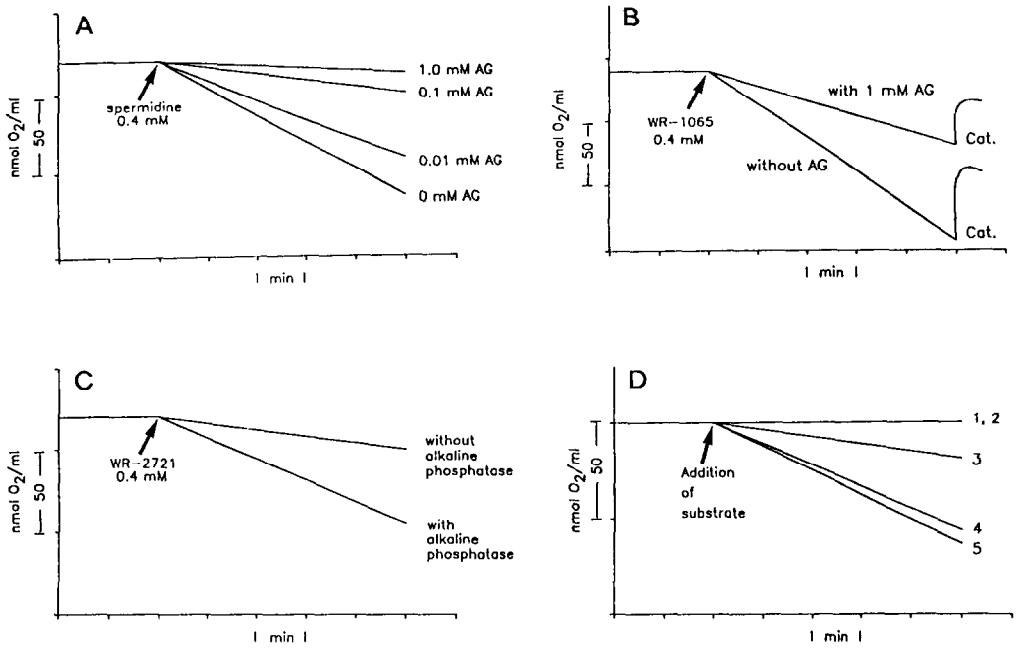

Fig. 4. Determination of oxygen consumption induced by WR-2721, WR-1065 and spermidine. (A) Spermidine (0.4 mM) ± AG in CHO medium; (B) WR-1065 (0.4 mM) ± AG (1 mM) in CHO medium; (C) WR-2721 (0.4 mM) ± AP (20 U/mL) in CHO medium; (D) summarizes data from a purified bovine plasma amine oxidase: (1) spermidine (0.4 mM) + AG (1 mM); (2) WR-2721 (0.4 mM); (3) WR-1065 (0.4 mM) + AG (1 mM); (4) spermidine (0.4 mM); (5) WR-1065 (0.4 mM). Each of the figures represents data from the average of at least five independent experiments.

Table 1. Determination of acrolein concentrations in CHO-medium

	Acrolein (μM)
Control	<0.05
WR-1065 (400 μM)	20.4 ± 5.9
WR-1065 (400 μM) + AG (1 mM)	11.6 ± 2.4
Acrolein (500 μM)	46.4 ± 2.0

After addition of the compounds the CHO medium was incubated at 37° for 1 hr. Means ± SD from at least three independent measurements.

Table 2. Determination of acrolein concentrations in PBS

	Acrolein (μM)
Control	<0.05
WR-1065 (400 μM)	<0.05
Acrolein (100 μM)	87.9 ± 8.3
WR-1065 (400 μM) + acrolein (100 μM)	3.1 ± 1.3
Acrolein (100 μM) + AG (1 mM)	84.9 ± 8.6

After addition of the compounds the PBS was incubated at 37° for 1 hr. Means ± SD from at least three independent measurements.

These data also confirmed that WR-1065 (but not WR-2721) can be a substrate for Cu-dependent amine oxidases.

To support the finding that Cu-dependent amine oxidases were responsible for the degradation of WR-1065 we tested a purified bovine plasma amine oxidase. The results (summarized in Fig. 4D) demonstrate that addition of WR-1065 or spermidine led to a strong uptake of oxygen. No oxygen uptake was observed when WR-2721 or spermidine + AG were tested, whereas WR-1065 + AG again showed a reduced uptake compared to WR-1065 alone. AG completely blocked the oxygen uptake of spermidine, but in the case of WR-1065 oxygen uptake due to the autoxidation reaction of the free thiol group remained. Therefore, bovine plasma amine oxidase exhibits similar substrate and inhibitor properties as the enzymes responsible for the oxidation of WR-1065 and spermidine in the CHO medium.

Detection of acrolein as breakdown product of WR-1065

Incubation of CHO medium with WR-1065 led to the formation of the aldehyde acrolein as summarized in Table 1. Coincubation of WR-1065 with AG reduced the detectable concentration of acrolein. A quantitative analysis of the data showed that the concentration of acrolein resulting from the degradation of WR-1065 was less than expected. After 1 hr incubation time the concentration of WR-1065 was reduced from 400 μM to approximately 200 μM (see Fig. 2A), while an acrolein concentration

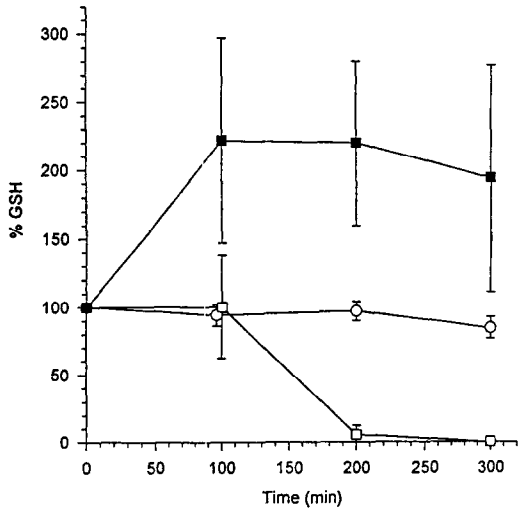

Fig. 5. Modulation of glutathione levels in CHO cells by WR-2721 under different conditions. (□) WR-2721 (0.4 mM) + AP (20 U/mL); (■) WR-2721 (0.4 mM) + AP (20 U/mL) + AG (1 mM); (○) control. Means ± SD from three different independent experiments.

of only 20.4 μM was detectable. To investigate side reactions we added acrolein alone to CHO medium. Approximately 10% of the added acrolein could be recovered after an incubation time of 1 hr. This demonstrates that acrolein reacts with molecules of the CHO medium and only low concentrations of acrolein can be detected. Experiments designed without the use of CHO medium did not circumvent the problem of acrolein side reactions. The data in Table 2 summarize the recovery of acrolein concentrations in PBS under different incubation conditions. Approximately 90% of acrolein could be detected after an incubation time of 1 hr at 37°. The addition of WR-1065 thus strongly reduces the detectable concentration of acrolein. These data are in agreement with data from the literature stating that the favourite reaction molecules of acrolein in aqueous solutions are those with free thiol groups [23]. It is to be noted that acrolein did not significantly react with AG, as shown in Table 2.

Table 3. Toxic effects of WR-2721 on CHO cells

	Trypan blue negative cells	Clonogenic surviving fraction	Number of independent experiments
Control	>95%	0.98 ± 0.05	7
WR-2721 + AP	>95%	0.14 ± 0.11*	4
WR-2721 + AP + AG	>95%	0.86 ± 0.20	4

CHO cells were incubated with WR-2721 (0.4 mM), AP (20 U/mL), AG (1 mM) for 5 hr at 37° as indicated. The asterisk indicates a significant difference from control ($P < 0.05$).

Influence of degradation of WR-1065 on GSH levels and survival of CHO cells

Earlier data from our group demonstrate that WR-2721 + AP is able to promote the cystine uptake of CHO cells *in vitro* [15]. A promotion of cystine uptake leads to an increase in GSH biosynthesis and GSH content of CHO cells as demonstrated for several thiols, because cysteine is in most cases the limiting amino acid for GSH biosynthesis [24]. Addition of WR-2721 + AP leads to a rapid depletion of GSH in CHO cells as demonstrated in Fig. 5. In contrast inhibition of amine oxidase by addition of 1 mM AG to the medium before WR-2721 + AP increased the GSH levels of CHO cells.

WR-2721 + AP did not influence the viability of CHO cells as measured by Trypan blue exclusion assay (Table 3). In contrast, the clonogenic survival of the cells was significantly reduced after incubation with WR-2721 + AP, but addition of 1 mM AG completely prevented this cellular toxicity. One millimolar AG alone had no influence on glutathione levels and clonogenic survival of CHO cells, but higher concentrations of AG (e.g. 10 mM) lead to a reduced clonogenic survival (data not shown). The effects of WR-2721 + AP was also tested using a carcinoma cell line. Incubation of CHO carcinoma cells with WR-2721 + AP showed a similar influence on GSH levels and clonogenic survival when incubated under the same medium conditions like CHO cells (data not shown).

DISCUSSION

The data demonstrate a degradation pathway of WR-1065 by Cu-dependent amine oxidases present in the culture medium of CHO cells. The results confirm our previous observations where we found evidence for a degradation of WR-1065 in cell culture medium [15]. Many data are available on the pharmacokinetics of WR-2721 in animals and humans. These studies show that only low amounts of the administered dose of WR-2721 are excreted as WR-2721, WR-1065 or WR-33278 [25]. WR-33278 is the disulphide form of WR-1065 and can be formed by a non-enzymatic autoxidation reaction from WR-1065 in aqueous solutions [22]. Studies with radioactively-labelled WR-2721 demonstrate the excretion of unidentified breakdown products of WR-2721 [26]. These studies implicate that degradation reactions of WR-2721/WR-1065 also occur *in vivo*, but the mechanism of degradation and the resulting products remained elusive. In our *in vitro* experiments we found that the aminothiol cysteamine is one major breakdown product of WR-1065. In a previous study, cysteamine was also proposed as a breakdown product of WR-1065 from data of cystine depletion by WR-1065 in cystinotic cells [27]. The observed degradation of WR-1065 in CHO medium was most likely mediated by amine oxidases. To investigate the source of amine oxidase activity in our system we tested the major components of the CHO medium. Amine oxidase activity was only detected in the newborn calf serum portion of the medium, whereas in foetal calf serum no amine oxidase activity could be found (data not shown).

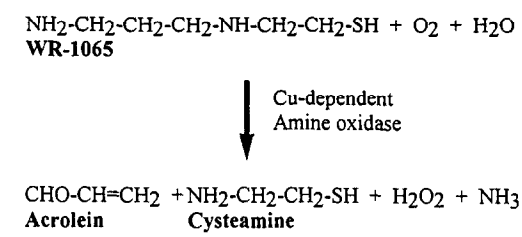

Fig. 6. Proposed degradation pathway of WR-1065.

Amine oxidase activity is present in the sera of a number of mammalian species [28] and these enzymes are quite heterogeneous in regard to structure, catalytic mechanism and mode of substrate oxidation [29]. One simple classification divides amine oxidases into FAD- and Cu-dependent enzymes in reference to the nature of the prosthetic group [14]. Natural substrates of amine oxidases are polyamines and related compounds. The use of amine oxidase inhibitors—particularly the nontoxic carbonyl reagent aminoguanidine, a specific inhibitor of Cu-dependent amine oxidases—has often been demonstrated to reduce the growth-inhibitory effects of polyamines dose-dependent [30, 31]. From our results the degradation and toxic effects of WR-1065 *in vitro* could be inhibited by AG, which demonstrates that Cu-dependent amine oxidases are responsible for the degradation reaction. We were able to confirm this observation by the use of a purified and characterized amine oxidase from bovine plasma [32]. The observed breakdown product acrolein is an α,β-unsaturated aldehyde which in general can be formed by oxidation from organic compounds. In summary we propose a degradation pathway of WR-1065 by Cu-dependent amine oxidases as shown in Fig. 6.

Formation of acrolein as a breakdown product of polyamine (e.g. spermidine and spermine) oxidation has been described [33]. Acrolein is also a metabolite of the cystostatic drugs cyclophosphamide and ifosfamide [34]. As a direct-acting alkylating agent acrolein is capable of covalent binding to cellular electrophilic groups [23]. The unsaturated double bond in acrolein conjugates nonenzymatically with sulphydryl groups as demonstrated for the thiols cysteine [35] and GSH [36]. Reactions with other thiols have also been described; e.g. 2-mercaptoethanesulphonate (mesna) prevents toxic side-effects of cyclophosphamide chemotherapy by protecting cells from the toxic effects of acrolein [37, 38]. Exposure of cells to acrolein leads to an intracellular depletion of GSH [38, 39]. This high reactivity of acrolein with sulphydryl groups might explain the low concentrations of acrolein measured in our experiments as described in Tables 1 and 2. The data indicate that acrolein reacts non-enzymatically with the thiols WR-1065 and cysteamine present in the medium.

CHO cells have intracellularly high levels of FAD-dependent polyamine oxidase activity [40]. We also tested whether WR-1065 is a substrate for this kind of enzyme. An assay was used which is based on the specific inhibitor MDL 72.521 of FAD-dependent polyamine oxidase [31, 41] as described [40]. We found no indication that WR-1065 is degradated by this kind of enzyme (data not shown).

GSH is the major intracellular non-protein thiol that is responsible for a variety of cellular functions [42, 43]. The data available in the literature concerning the influence of WR-1065 on intracellular GSH levels are not consistent. Some authors find no influence on GSH levels [10], whereas others describe a depletion [44] or increase [15] of intracellular GSH induced by WR-1065. In our *in vitro* system using CHO cells we observed a depletion of GSH under conditions where degradation of WR-1065 occurs in the medium. The breakdown product responsible for this GSH depletion is most likely acrolein, because acrolein binds to GSH. In contrast, exposure of CHO cells to cysteamine increased GSH levels [24]. An increase in GSH levels is observed after exposure of CHO cells to WR-1065 when the acrolein-producing oxidation reaction is reduced with AG. GSH synthesis is regulated in most cases by the availability of the amino acid cysteine [42]. As demonstrated with other thiols (e.g. cysteamine, *N*-acetylcysteine) WR-1065 promotes cystine uptake from the medium and increases intracellular GSH level [15, 24].

In vitro data using different cell systems demonstrate that molecules with a free thiol group can exhibit a strong toxicity *in vitro* [18, 45]. This toxicity may be accounted for the generation of reactive oxygen species during the autoxidation reaction of the free thiol group. However, the extent of thiol toxicity depends on the cell system, the environmental conditions and the structure of the thiol compound [46]. Addition of catalase markedly reduced the toxicity of thiol compounds, which indicates that generation of H_2O_2 is the most important factor for thiol toxicity [45]. Data from the literature demonstrate that the toxicity of WR-1065 due to autoxidation of the free thiol group is low [46]. Therefore the observed toxicity of WR-1065 in our CHO cell system is not caused by autoxidation of the free thiol group but by production of breakdown products, because the inhibition of enzymatic degradation by AG blocks the cytotoxic effects.

High WR-2721 toxicity has been reported and suggested to be based on the action of polyamine oxidases [47]. Cytotoxic effects of the polyamines spermidine or spermine are mediated by degradation reactions in the culture medium of cells [48, 49]; e.g. exogenous spermidine is oxidized by Cu-dependent amine oxidases to putrescine, acrolein and H_2O_2 [50]. Our data strongly suggest that the cytotoxic effects of WR-1065 on CHO cells are caused by similar mechanisms as described for the toxicity of polyamines. The enzymatic degradation of WR-1065 leads to the formation of toxic breakdown products (e.g. acrolein and H_2O_2), which are able to damage cellular structures. For clinical situations it is very difficult to make meaningful predictions in regard to the levels of toxic degradation products which may be reached *in vivo*. The amount of amine oxidases present in the serum of humans seems to be low in

general, but under certain conditions, e.g. during pregnancy, in cancer patients or after administration of heparin, an increase in serum amine oxidase activity was observed [51]. However, it is not clear whether the enzyme characteristics of human and bovine serum amine oxidases are comparable. The detection of cysteamine after administration of WR-2721 *in vivo* (52) indicates that the described degradation mechanism might be of importance for the clinical use of WR-2721.

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REFERENCES

1. Yuhas JM, Active versus passive absorption kinetics as the basis for selective protection of normal tissues by *S*-2-(3-aminopropylamino)-ethylphosphorothioic acid. *Cancer Res* **40**: 1519–1524, 1980.
2. Kligerman MM, Blumberg AL, Glick JH, Nelson DF, Glover D, Yuhas JM, Amols HI and Goodman RL, Phase I trials of WR2721 in combination with radiation therapy and with the alkylating agents cyclophosphamide and *cis*-platinum. *Cancer Clin Trials* **4**: 469–474, 1981.
3. Glover D, Glick JH, Weiler C, Yuhas J and Kligerman MM, Phase I trials of WR-2721 and cis-platinum. *Int J Radiat Oncol Biol Phys* **10**: 1781–1784, 1984.
4. Glover D, Glick JH, Weiler C, Fox K and Guerry D, WR-2721 and high-dose cisplatin: an active combination in the treatment of metastatic melanoma. *J Clin Oncol* **5**: 574–578, 1987.
5. Glick JH, Glover D, Weiler C, Norfleet L, Yuhas J and Kligerman MM, Phase I controlled trials of WR-2721 and cyclophosphamide. *Int J Radiat Oncol Biol Phys* **10**: 1777–1780, 1984.
6. Glover D, Glick JH, Weiler C, Hurowitz S and Kligerman MM, WR-2721 protects against the hematologic toxicity of cyclophosphamide: a controlled phase II trial. *J Clin Oncol* **4**: 584–588, 1986.
7. Treskes M and van-der-Vijgh WJ, WR2721 as a modulator of cisplatin- and carboplatin-induced side effects in comparison with other chemoprotective agents: a molecular approach. *Cancer Chemother Pharmacol* **33**: 93–106, 1993.
8. Calabro-Jones PM, Fahey RC, Smoluk GD and Ward JF, Alkaline phosphatase promotes radioprotection and accumulation of WR-1065 in V79–171 cells incubated in medium containing WR-2721. *Int J Radiat Biol Relat Stud Phys Chem Med* **47**: 23–27, 1985.
9. McGovern EP, Swynnerton NF, Steele PD and Mangold DJ, HPLC assay for 2-(3-aminopropylamino)ethanethiol (WR-1065) in plasma. *Int J Radiat Oncol Biol Phys* **10**: 1517–1520, 1984.
10. Utley JF, Seaver N, Newton GL and Fahey RC, Pharmacokinetics of WR-1065 in mouse tissue following treatment with WR-2721. *Int J Radiat Oncol Biol Phys* **10**: 1525–1528, 1984.
11. Calabro-Jones PM, Aguilera JA, Ward JF, Smoluk GD and Fahey RC, Uptake of WR-2721 derivatives by cells in culture: identification of the transported form of the drug. *Cancer Res* **48**: 3634–3640, 1988.
12. Tabor CW and Tabor H, Polyamines in microorganisms. *Microbiol Rev* **49**: 81–99, 1985.
13. Pegg AE, Recent advances in the biochemistry of polyamines in eukaryotes. *Biochem J* **234**: 249–262, 1986.
14. Mondovi B, Riccio P and Agostinelli E, The biological functions of amine oxidases and their reaction products: an overview. *Adv Exper Med Biol* **250**: 147–161, 1988.
15. Issels RD and Nagele A, Promotion of cystine uptake, increase of glutathione biosynthesis, and modulation of glutathione status by *S*-2-(3-aminopropylamino)ethyl phosphorothioic acid (WR-2721) in Chinese hamster cells. *Cancer Res* **49**: 2082–2086, 1989.
16. Seiler N and Knodgen B, Determination of amino acids by separation of their ion pairs with dodecyl sulphate. *J Chromatogr* **341**: 11–21, 1985.
17. Reed DJ, Babson JR, Beatty PW, Brodie AE, Ellis WW and Potter DW, High-performance liquid chromatography analysis of nanomole levels of glutathione, glutathione disulfide, and related thiols and disulfides. *Anal Biochem* **106**: 55–62, 1980.
18. Issels RD and Nagele A, Influence of thiols on thermosensitivity of mammalian cells *in vitro*. *Meth Enzymol* **186**: 696–708, 1990.
19. Lowry OH, Rosebrough NJ, Farr AL and Randall RJ, Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**: 265–275, 1951.
20. Biaglow JE, Issels RW, Gerweck LE, Varnes ME, Jacobson B, Mitchell JB and Russo A, Factors influencing the oxidation of cysteamine and other thiols: implications for hyperthermic sensitization and radiation protection. *Radiat Res* **100**: 298–312, 1984.
21. Alarcon RA, Fluorometric determination of acrolein and related compounds with *m*-aminophenol. *Anal Chem* **40**: 1704–1708, 1968.
22. Tahsildar HI, Biaglow JE, Kligerman MM and Varnes ME, Factors influencing the oxidation of the radioprotector WR-1065. *Radiat Res* **113**: 243–251, 1988.
23. Witz G, Biological interactions of alpha,beta-unsaturated aldehydes. *Free Radic Biol Med* **7**: 333–349, 1989.
24. Issels RD, Nagele A, Eckert KG and Wilmanns W, Promotion of cystine uptake and its utilization for glutathione biosynthesis induced by cysteamine and *N*-acetylcysteine. *Biochem Pharmacol* **37**: 881–888, 1988.
25. Shaw LM, Turrisi AT, Glover DJ, Bonner HS, Norfleet AL, Weiler C and Kligerman MM, Human pharmacokinetics of WR-2721. *Int J Radiat Oncol Biol Phys* **12**: 1501–1504, 1986.
26. Anderson KW, Krohn KA, Grunbaum Z, Phillips RB, Mahler PA, Menard TW, Spence AM and Rasey JS, Analysis of S-35 labeled WR-2721 and its metabolites in biological fluids. *Int J Radiat Oncol Biol Phys* **10**: 1511–1515, 1984.
27. Butler JD, Gahl WA and Tietze F, Cystine depletion by WR-1065 in cystinotic cells. Mechanism of action. *Biochem Pharmacol* **34**: 2179–2185, 1985.
28. Hegre OD, Marshall S and Hickey GE, Spermidine cytotoxicity *in vitro*: effect of serum and oxygen tension. *In Vitro* **20**: 198–204, 1984.
29. Yasunobu KT, Ishizaki H and Minamiura N, The molecular mechanistic and immunological properties of amine oxidases. *Molec Cell Biochem* **13**: 3–29, 1976.
30. Gahl W and Pitot HC, Reversal by aminoguanidine of the inhibition of proliferation of human fibroblasts by spermidine and spermine. *Chem Biol Interact* **22**: 91–98, 1978.
31. Seiler N, Inhibition of Enzymes Oxidizing Polyamines. In: *Inhibition of Polyamine Metabolism* (Eds. McCann PP, Pegg AE and Sjoerdsma A), pp. 49–77. Academic Press, San Diego, 1987.
32. Mondovi B, Turini P, Befani O and Sabatini S, Purification of Bovine Plasma Amine Oxidase. *Meth Enzymol* **94**: 314–318, 1983.
33. Alarcon RA, Acrolein. IV. Evidence for the formation of the cytotoxic aldehyde acrolein from enzymatically oxidized spermine or spermidine. *Arch Biochem Biophys* **137**: 365–372, 1970.
34. Brock N, Stekar J, Pohl J, Niemeyer U and Scheffler G, Acrolein, the causative factor of urotoxic side-effects of cyclophosphamide, ifosfamide, trofosfamide

and sufosfamide. *Arzneimittelforschung* **29**: 659–661, 1979.
35. Esterbauer H, Ertl A and Scholz N, The reaction of cysteine with α,β-unsaturated aldehydes. *Tetrahedron* **32**: 285–289, 1976.
36. Grafstrom RC, Dypbukt JM, Willey JC, Sundqvist K, Edman C, Atzori L and Harris CC, Pathobiological effects of acrolein in cultured human bronchial epithelial cells. *Cancer Res* **48**: 1717–1721, 1988.
37. Brock N and Pohl J, The development of mesna for regional detoxification. *Cancer Treat Rev* **10** (Suppl A): 33–43, 1983.
38. Meier T, Allenbacher A, Mueller E, Multhoff G, Botzler C, Wiesnet W and Issels R, Ifosfamide induced depletion of glutathione in human peripheral blood lymphocytes and protection by mesna. *Anti-Cancer Drugs* **5**: 403–409, 1994.
39. Patel JM and Block ER, Acrolein-induced injury of cultured pulmonary artery endothelial cells. *Toxicol Appl Pharmacol* **122**: 46–53, 1993.
40. Carper SW, Tome ME, Fuller DJ, Chen JR, Harari PM and Gerner EW, Polyamine catabolism in rodent and human cells in culture. *Biochem J* **280**: 289–294, 1991.
41. Bey P, Bolkenius NF, Seiler N and Casara P, *N*-2,3-butadienyl-1,4-butanediamine derivatives: potent irreversible inactivators of mammalian polyamine oxidase. *J Med Chem* **28**: 1–2, 1985.
42. Meister A and Anderson ME, Glutathione. *Ann Rev Biochem* **52**: 711–760, 1983.
43. Multhoff G, Meier T, Botzler C, Wiesnet M, Allenbacher A, Wilmanns W and Issels RD, Differential effects of ifosfamide on the capacity of cytotoxic T lymphocytes and natural killer cells to lyse their target cells correlate with intracellular glutathione levels. *Blood* **85**: 2124–2131, 1995.
44. Schor NF, Depletion of glutathione by the radioprotective agent S-2-(3-aminopropylamino)ethyl phosphorothioic acid (WR2721). *Biochem Pharmacol* **37**: 562–563, 1988.
45. Issels RD, Biaglow JE, Epstein L and Gerweck LE, Enhancement of cysteamine cytotoxicity by hyperthermia and its modification by catalase and superoxide dismutase in Chinese hamster ovary cells. *Cancer Res* **44**: 3911–3915, 1984.
46. Held KD and Biaglow JE, Mechanisms for the oxygen radical-mediated toxicity of various thiol-containing compounds in cultured mammalian cells. *Radiat Res* **139**: 15–23, 1994.
47. Gaugas JM, Mechanisms of *in vitro* cytotoxicity of *S*-2-(3-aminopropylamino)ethylphosphorothioic acid. In: *Advances in Polyamine Research* (Ed. Caldarera CM), pp. 441–450. Raven Press, New York, 1981.
48. Russo A, Mitchell JB, DeGraff W, Friedman N and Gamson J, Depletion of cellular glutathione by exogenous spermine in V79 cells: implications for spermine-induced hyperthermic sensitization. *Cancer Res* **45**: 4910–4914, 1985.
49. Henle KJ, Moss AJ and Nagle WA, Mechanism of spermidine cytotoxicity at 37° and 43° in Chinese hamster ovary cells. *Cancer Res* **46**: 175–182, 1986.
50. Harari PM, Fuller DJ and Gerner EW, Heat shock stimulates polyamine oxidation by two distinct mechanisms in mammalian cell cultures. *Int J Radiat Oncol Biol Phys* **16**: 451–457, 1989.
51. Baylin SB and Luk GD, Diamine oxidase activity in human tumors: clinical and biological significance. In: *Structure and Functions of Amine Oxidase* (Ed. Mondovi B), pp. 187–194. CRC Press, Boca Raton, 1985.
52. Shaw LM, Bonner HS and Brown DQ, Metabolic pathways of WR-2721 (ethyol, amifostine) in the Balb/c mouse. *Drug Metab Dispos* **22**: 895–902, 1994.