Mammalian cell polyamine homeostasis is altered by the radioprotector WR1065

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Mammalian cells become more susceptible to radiation-induced death and mutagenesis when restricted in their production of the natural polyamines putrescine, spermidine and spermine. The effects of polyamine deprivation are reversed by *N*-(2-mercaptoethyl)-1,3-diaminopropane (WR1065), a simple aminothiol that has been extensively studied for its radioprotectant properties. Because this compound and its oxidized derivative WR33278 bear some resemblance to the polyamines, it was hypothesized that radioprotection by WR1065 or its metabolites is derived, at least in part, from their ability to supplement the natural polyamines. To evaluate the ability of these aminothiol compounds to emulate polyamine function in intact cells, rat liver hepatoma (HTC) cells were treated with radioprotective doses of WR1065; the ability of this compound to affect various aspects of normal polyamine metabolism was monitored. Although cellular WR1065 was maintained at levels exceeding those of the polyamines, this aminothiol did not have any polyamine-like effect on the initial polyamine biosynthetic enzyme, ornithine decarboxylase, or on polyamine degradative reactions. On the contrary, treatment with relatively low levels of WR1065 resulted in an unexpected increase in putrescine and spermidine synthesis. WR1065 treatment enhanced the stability, and consequently the activity, of ornithine decarboxylase. This stabilization seems to result from a WR1065-induced delay in the synthesis of antizyme, a critical regulatory protein required in the feedback modulation of polyamine synthesis and transport. The increase in cellular spermidine induced by WR1065 might explain its antimutagenic properties, but is probably not a factor in protection against cell killing by radiation. This is the first evidence that compounds can be designed to control polyamine levels by targeting the activity of the regulatory protein antizyme.

INTRODUCTION

The polyamines putrescine, spermidine and spermine are ubiquitous organic cations essential for normal cell physiology. In accord with their polycationic nature, the polyamines, and in particular spermidine and spermine, are thought to be integral components of cell chromatin structure [1,2]. Analogues that affect polyamine levels and their normal interaction with nucleic acids have been found to alter cellular responses to agents reacting with DNA [3,4]. It is particularly noteworthy that inhibitors that decrease cellular polyamine levels have been reported to increase the sensitivity of cells to radiation, resulting in enhanced mutagenesis and cell death [5,6]. Further, spermine has been shown to protect cells from radiation-induced mutagenesis even when administered after exposure to radiation [7,8].

The mechanism of radioprotection by the natural polyamines is not clear. Løvaas [9] and the Tabors [10] have reported an antioxidant activity of the polyamines, whereas Held and Awad [11] have suggested that in aerobic conditions the radioprotection by polyamines involves scavenging of hydroxyl radicals. In contrast, polyamine-deprivation studies show the involvement of polyamines in DNA repair mechanisms [5,6,12].

Over the past 50 years many compounds have been examined in the hope of uncovering potential drugs that would protect human tissues from the harmful effects of radiation. One of the most effective of these compounds is *N*-(2-mercaptoethyl)-1,3-diaminopropane (WR1065), which has been studied extensively for its ability to protect cells from ionizing radiation [13–15]. The structural resemblance of WR1065 to the polyamines has long been noted [16–19]. At cellular pH, WR1065 is a divalent cation similar to putrescine, and its 1,3-diaminopropane moiety is common to spermidine and spermine. Further, the oxidized (disulphide) derivative of this aminothiol (WR33278) has approximately the same charge distribution as the tetravalent cation, spermine. Functionally, WR1065 and WR33278 seem to bind DNA equivalently to polyamines of equal charge [20,21] and they can reverse much of the sensitivity induced by polyamine deprivation [5,6]. It seems likely that radioprotection by the aminothiol and its metabolites is associated with their ability to enhance natural polyamine actions in affecting DNA structure, replication and function.

The polyamine-like nature of these compounds has been examined in a physiological system [19]. WR1065 and WR33278 were each tested as a potential substrate for the polyamine transporter of the mammalian cell cytoplasmic membrane. In this study, WR33278 was determined as being a close analogue of spermidine in that it not only inhibited spermidine incorporation but also was transported at the same velocity as the natural substrate. By these same criteria, WR1065 was not found to be nearly as good an analogue.

If intracellular WR1065, or the disulphide WR33278, really does mimic polyamines in function, one might expect that the very high levels of these compounds entering cells during radioprotective treatments would have profound effects on natural polyamine metabolism. Normally, cellular polyamine levels are precisely maintained by a complex of feedback regulatory mechanisms controlling both biosynthetic and biodegradative enzymes, and cytoplasmic membrane transporters. Putrescine is produced by the activity of ornithine decarboxylase (ODC; EC 4.1.1.17), a very labile and highly regulated protein. Elevations in putrescine levels stimulate the activity of *S*-adenosylmethionine decarboxylase necessary for the synthesis of spermidine and spermine. Increases in the levels of either spermidine or spermine induce the synthesis of ODC-antizyme,

Abbreviations used: ODC, ornithine decarboxylase; WR1065, *N*-(2-mercaptoethyl)-1,3-diaminopropane; WR33278, the disulphide dimer of WR1065.

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a small regulatory protein that both stimulates the rapid degradation of ODC [22,23] and reversibly inhibits the activity of the polyamine uptake mechanism in the cytoplasmic membrane [24,25]. Elevations in polyamine levels also stimulate the polyamine biodegradative enzyme spermidine/spermine N^1-acetyltransferase. In view of these multiple regulatory mechanisms it is likely that a compound behaving like a polyamine in some respects would elicit a corresponding response on the feedback mechanisms controlling polyamine homeostasis. In the present study we have tested this hypothesis by evaluating changes in polyamine synthesis and homeostasis resulting from the treatment of mammalian cells with WR1065. Contrary to initial expectations, the results indicate that this analogue actually enhances natural polyamine levels by interfering with normal feedback mechanisms.

EXPERIMENTAL

Chemicals

Polyamines, aminoguanidine, dithiothreitol, *o*-phthalaldehyde, cyclohexylammonium sulphate, cycloheximide and general biochemicals were from Sigma Chemical Co. WR1065 and WR33278 were generously provided by the Drug Synthesis and Chemistry Branch (Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD 20892, U.S.A.).

Cell culture and sampling

Rat hepatoma (HTC) cells were grown as monolayers and stirred flasks in Swim's −77 medium and 10% (v/v) heat-inactivated calf serum as described previously [25]. For most studies cells in stirred flasks were resuspended at 7×10^5 cells/ml in fresh medium containing 3 mM aminoguanidine 1 h before treatments with either aminothiols or polyamines. The aminoguanidine protects the aminothiols [26] and polyamines [27] from amine oxidases present in calf serum. In the experiments described in Figure 4 the cells were resuspended in medium containing horse serum instead of calf serum. Although the induction of ODC is generally not as robust as with calf serum, the horse serum does not contribute amine oxidases and therefore the addition of aminoguanidine can be avoided. Additions of WR1065 were made from a frozen stock of 100 mM in 2 mM dithiothreitol. When WR1065 was used at 2 mM the material was freshly weighed from stocks kept in a desiccator at −20 °C and the powder was added directly to the culture medium to initiate the treatment. Samples were subsequently withdrawn for enzyme assays (5 ml) and polyamine/aminothiol analysis (3 ml). The latter were placed directly into 10 ml of ice-cold phosphate-buffered (pH 7.2) isotonic saline and quickly pelleted by centrifugation, washed twice more and frozen before analysis.

Polyamine and aminothiol analysis

Polyamines, acetylpolyamines, WR33278, WR1065 and mixed disulphides of WR1065 with cysteine, glutathione and 2-mercaptoethanol were analysed simultaneously with the polyamine assay procedure that we have described previously [19,28]. In evaluations of the different states of oxidized and reduced WR1065, cell samples were extracted directly with 0.2 M perchloric acid. This acidification precipitated proteins and, by minimizing any thiol–disulphide exchanges, served to fix the proportions of reduced and variously oxidized states of the aminothiol present in the cell at the moment of extraction.

Portions (100 μl) of the perchloric acid extract were applied to a 5 cm Mono-S cation-exchange column (LKB/Pharmacia) equilibrated with 50 mM sodium acetate buffer, pH 4.5. A Waters 600E HPLC system was used to elute the sample with a linear 0–1.0 M NaCl gradient over 36 min at 1 ml/min. The column effluent was mixed in equal volume with *o*-phthalaldehyde/mercaptoethanol reagent as described previously [28], then detected with a Waters 470 Scanning Fluorescent Detector. Baseline separation was obtained for the polyamines and the oxidized and reduced aminothiols indicated above. Peak quantification was by comparison with external standards run every 7–10 analyses with the Waters Max-825 peak integration software.

Assay of ODC

ODC activity was assayed by measuring the release of $^{14}CO_2$ from L-[^{14}C]ornithine as described previously [25,29]. One unit of activity is defined as the release of 1 nmol of CO_2 per h.

RESULTS

WR1065 uptake affects cellular polyamine levels

High concentrations (2–4 mM) of WR1065 are reported to exert radioprotective effects within the first hour of treatment of cells in culture [30], whereas the antimutagenic effects of this aminothiol are associated with much lower doses and longer exposures [8,18]. To test whether one or both of these protective effects are a consequence of the emulation of polyamine functions by either WR1065 or its derivative WR33278, experiments were conducted to assess potential physiological interactions between these compounds and the polyamines. In the initial studies, HTC cells were exposed to a level of WR1065 (2 mM) known to produce radioprotection. As shown in the experiment described in Figure 1, this level induced a rapid incorporation of the compound, consistent with a passive uptake system as suggested by previous studies [19,31]. In this experiment less than 1 h was required before the cellular WR1065 concentration exceeded the concentration of all the natural polyamines combined, and this high level was maintained for the duration of the study (Figure 1A).

If radioprotection by WR1065 is due to this compound's ability to augment cellular polyamine actions, the very high levels of WR1065 obtained would be expected to affect other polyamine-induced responses as well. In particular, the first enzyme in the polyamine biosynthetic pathway, ODC, is very sensitive to slight changes in spermidine or spermine levels yet is relatively insensitive to its immediate product, putrescine [23,32]. Normally, the process of refreshing cell culture medium results in an increase in ODC activity and polyamine synthesis, peaking between 3 and 7 h later. This induction of ODC is completely inhibited by exposure to polyamines, and as little as 50 μM spermidine in culture medium can block the appearance of any ODC activity for at least 10 h. Surprisingly, the very high level of cellular WR1065 maintained in the experiment shown in Figure 1 did not result in a similar inhibition in the activity of the polyamine biosynthetic enzyme. On the contrary, the normal induction of ODC activity seen in cells on renewing culture medium was actually enhanced in the cells treated with WR1065 (Figure 1B).

A second projection of the working hypothesis is that if the aminothiols emulate polyamine functions, elevation of their cellular levels should induce a feedback decrease in the levels of the natural polyamines. As shown in Figure 1(A), prolonged exposure to WR1065 induced an increase, rather than a decrease, in the cellular level of spermidine. This significant aminothiol-

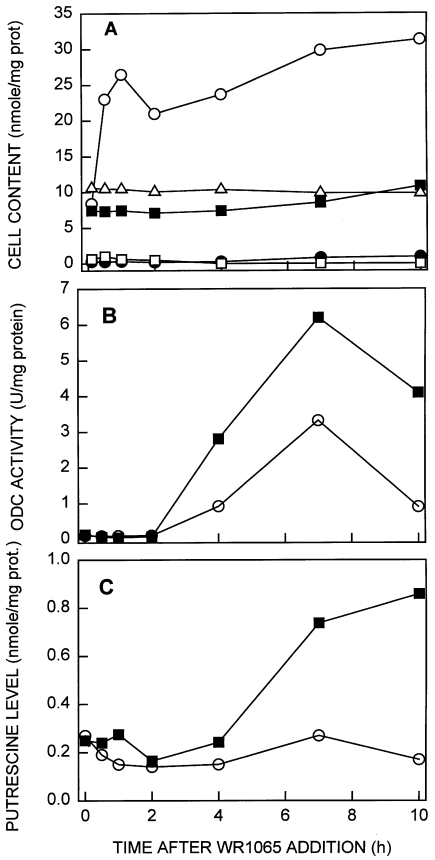

Figure 1 Effect of WR1065 on cellular polyamines

HTC cells were suspended in fresh medium in spinner flasks 24 h before the start of the experiment. Cells were then resuspended in fresh medium either with or without 2 mM WR1065, and samples were subsequently removed for enzyme or polyamine analysis. (**A**) Cellular content of WR1065 (○) and its derivative, WR33278 (□), were determined in comparison with the normal cell polyamines putrescine (●), spermidine (■) and spermine (△). (**B**) Changes in the levels of ODC activity in cells exposed to WR1065 (■) in comparison with control cultures (○). (**C**) Changes in putrescine levels with time after treatment with WR1065 (■) in comparison with control cells (○).

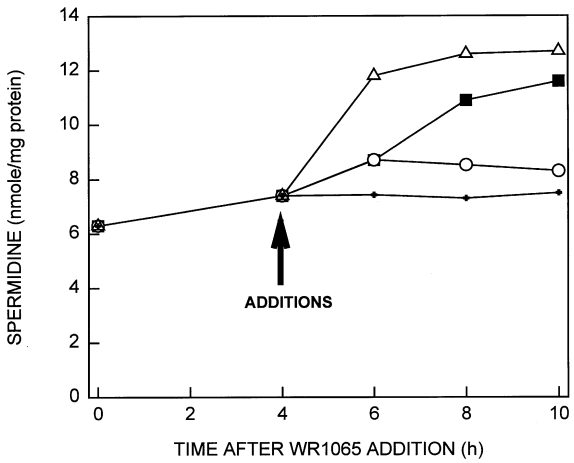

Figure 2 Conditions that alter the WR1065-induced effect on cellular polyamine levels

HTC cells were prepared and exposed to 2 mM WR1065 as described in the legend to Figure 1. After 4 h the culture was divided into five parts and the following additions were made: 0.1 mM spermidine (△), 0.2 mM cycloheximide (○), 0.125 mM cyclohexylammonium sulphate (●) or none (■). Samples were subsequently extracted and analysed for cellular polyamine levels. The results plotted are averages of duplicate determinations and represent the changes in the cellular levels of spermidine. Spermine concentrations remained constant during the course of this experiment.

induced increase in spermidine probably resulted from the increase in putrescine synthesis (Figure 1C) associated with the enhancement in ODC activity (Figure 1B). It should be noted that cellular levels of spermidine and spermine are generally very tightly regulated; increases in concentration, especially that of spermidine, are generally associated with cell and tissue growth [33,34].

Previous studies on the use of the polyamine transporter by the aminothiols had indicated that WR33278 is a better polyamine analogue than the reduced WR1065 form [19]. In contrast with the very high cellular levels of WR1065 achieved in the experimental conditions described in Figure 1, the levels of the oxidized disulphide derivative, WR33278, remained very low (less than 10%) compared with those of either spermidine or spermine. Attempts were made to induce significant cellular levels of WR33278 to assess the effects of this form of the radioprotectant *in vivo*. Exposing cells to high concentrations of this disulphide resulted in even lower cellular levels of WR33278 than those induced by WR1065 treatments (results not shown). This is consistent with the previous suggestion that there is no mechanism of passive uptake of the disulphide and its transport is strictly dependent on the limited activity of the polyamine transporter [19,31]. Additionally, of the WR33278 that is incorporated, most is converted rapidly to WR1065 owing to the reducing environment of cell cytoplasm.

In cells treated with WR1065, the predominant form of the compound, the reduced state, easily exceeds the concentrations of the natural polyamines, yet there is no evidence that this drug has any polyamine-like effect on either ODC activity or polyamine level homeostasis. On the contrary, these studies suggest an alternative model. Instead of mimicking the polyamines, it seems that either WR1065 or its disulphide derivative might act by enhancing the cellular levels of one of the natural polyamines, and spermidine in particular.

WR1065 stimulates an increase in spermidine synthesis

To confirm that the elevation in spermidine-to-spermine ratio is the result of spermidine synthesis and not a decrease in spermine concentration, the conversion of putrescine to spermidine was blocked by the use of an inhibitor of spermidine synthase, cyclohexylammonium sulphate [32,35]. As shown in Figure 2, the inhibitor completely prevented any WR1065-induced changes in spermidine levels. Further, the inhibition of spermidine synthesis was accompanied by an accumulation of putrescine equivalent to the extra spermidine that was produced in the uninhibited controls (results not shown). It therefore appears that the additional spermidine in WR1065-treated cells is a direct result of an enhancement in putrescine synthesis, and its further modification to form spermidine. The cell concentrations of the intermediate putrescine are quite low in comparison with the net increase in spermidine, suggesting that the synthesis of putrescine is the rate-limiting step in this biosynthetic pathway under these growth conditions. Further evidence that the increase in spermidine was due to an increase in the activity of ODC was found on examination of the effect of cycloheximide on the polyamine ratio change. This inhibitor of protein synthesis blocked any elevation in ODC protein and thereby prevented the expected increase in spermidine concentration (Figure 2).

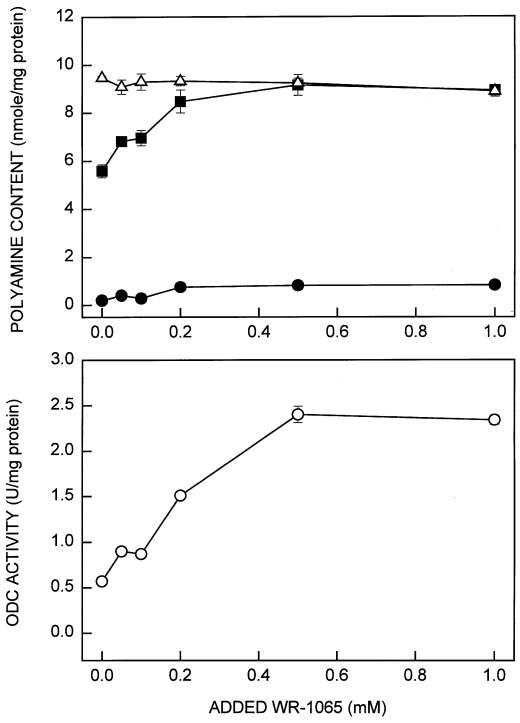

Figure 3 Comparison of the induction of ODC activity and the alteration in cellular polyamine levels with exposure to varying WR1065 levels

HTC cells were pretreated as described in the legend to Figure 1 and then resuspended in fresh medium containing different levels of WR1065. After an additional 8 h of culture, samples were extracted for the quantification of putrescine (●), spermidine (■) and spermine (△). Lower panel: the cultures were compared for the relative amount of peak ODC activity achieved. Values are means ± S.D. for triplicate assays. U, units.

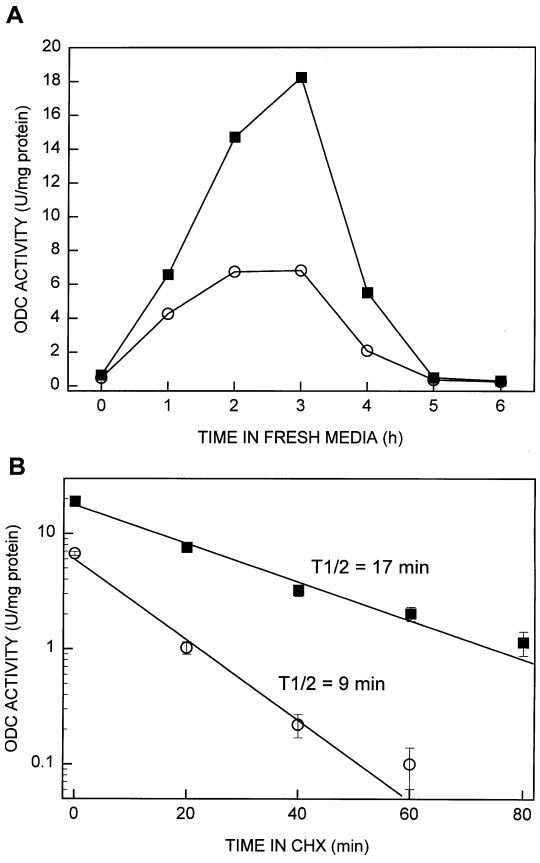

Figure 4 Effect of WR1065 on ODC activity and stability

HTC cells were suspended in fresh medium containing 10% (v/v) horse serum. After 24 h the culture was divided into two parts: one was treated with 0.5 mM WR1065 (■); the other served as an untreated control (○). (**A**) Samples were subsequently removed and analysed for ODC activity. (**B**) After 3 h each culture was divided and 0.2 mM cycloheximide (CHX) was added to part. Samples were withdrawn at 20 min intervals and assayed for ODC activity to show the rate of loss of existing enzyme.

The significance of the increase in spermidine concentration associated with exposure to WR1065 was evaluated by the comparison with cells exposed to exogenous spermidine. As shown in Figure 2, the addition of 0.1 mM spermidine to the culture medium induced a rapid elevation in cellular spermidine concentration, which was only slightly greater than that induced by exposure to WR1065. Such exposure of cells to exogenous polyamines, and the resultant increase in intracellular spermidine concentration, has been associated with a large variety of physiological responses [36–39].

Effects of WR1065 on ODC

Although radioprotection is exerted by treating cells with millimolar levels of the aminothiols, some antimutagenic effects are noted at much lower concentrations. As shown in Figure 3, an increase in ODC activity and enhanced spermidine synthesis can both be observed in HTC cells treated with as little as 50 μM WR1065; the effect seems to be maximally induced with approx. 0.5 mM WR1065.

It is counterintuitive that a putative polyamine analogue such as WR1065 would stimulate ODC activity. Because this low-abundance enzyme is noted for its short half-life, such an increased induction of ODC protein levels would be expected to entail either enhanced synthesis of ODC or diminished degradation. The latter mechanism was examined by inhibiting protein synthesis with cycloheximide at the peak of the ODC response curve. In the experiments summarized in Figure 4, the stability of ODC activity in cells treated with WR1065 was found to be about twice that of controls, a difference consistent with the elevation in ODC activity achieved in the treated cells. ODC degradation is known to be facilitated by its specific interactions with a small labile protein, antizyme [40,41]. Synthesis of this regulatory protein, in turn, is controlled by an unusual translational frame-shift that is dependent on the cellular levels of free spermidine [42]. In five repeats of this experiment, the half-life of ODC at the peak of induction in treated cells was uniformly doubled relative to controls. The level of free antizyme activity appearing after the ODC peak was the same in the presence and the absence of the aminothiol. Thus the presence of WR1065 seems only to retard the appearance of antizyme activity. Attempts were made to use immunodetection techniques [43] to evaluate the effect of WR1065 on the increase in antizyme protein. Unfortunately the levels of antizyme protein present during this peak in ODC activity were too low to yield meaningful data.

DISCUSSION

These studies were designed to test whether a major factor in the radioprotective and antimutagenic activities of WR1065 is the ability of this aminothiol, or its disulphide derivative WR33278, to enhance endogenous polyamine pools. This hypothesis was

based on previous studies showing that (1) tissue radiosensitivity induced by the lowering of cellular polyamine levels can be readily reversed by treatment with WR1065 [5,12,18] and (2) WR33278 is a good substrate for the polyamine-specific cytoplasmic membrane transporter [19]. In the present study, WR1065-treated cells were shown to rapidly accumulate levels of this compound exceeding those of the natural polyamines, yet even at these cytoplasmic concentrations there was no evidence that WR1065 mimicked polyamines in either the down-regulation of ODC or the stimulation of feedback mechanisms responsible for polyamine homeostasis. This evidence suggests that WR1065 does not behave like a general polyamine analogue *in vivo*, a result consistent with our previous observation that WR1065 was not an effective substrate for the cytoplasmic membrane polyamine transport system [19].

Because previous studies [19] indicated that WR33278 was a much better polyamine analogue than WR1065, it would be helpful to know whether the radioprotectant's oxidized form acts as a polyamine *in vivo*. Unfortunately this hypothesis was not testable because, as others have noted [19,30,31], WR33278 was taken up only slowly into cells, and most of what was incorporated was rapidly converted to WR1065 by the reducing potential of normal cell cytoplasm. The low levels of WR33278 that were achieved in these studies seemed to make a negligible contribution to the total polyamine pool, as there were no measurable responses in the polyamine feedback systems.

These results are not consistent with our initial hypothesis that the aminothiol WR1065 exerts radioprotection or antimutagenesis by emulating normal polyamine functions. Instead, the studies revealed a surprising alternative mode of action. Rather than supplementing the endogenous polyamine pools, WR1065 or its derivative WR33278 actually stimulate the synthesis of the natural polyamine spermidine. Cells exposed to relatively low doses of WR1065 responded with increases in spermidine concentration that were of approximately the same magnitude as those in cells treated with spermidine directly. The increase in spermidine concentration is significant; however, it is unlikely that this change is responsible for the radioprotective activity of WR1065. Peak radioprotection is generally noticed approx. 30 min after exposure to WR1065 [44], yet the increase in spermidine concentration is not evident until several hours after treatment. Further, the radioprotective effect of WR1065 increases up to treatment concentrations of 2–4 mM, yet full stimulation of spermidine synthesis is achieved by concentrations of this compound one-tenth of that. Thus although spermidine might have a role in a cell's primary response to radiation [6], the increase in spermidine concentration induced by WR1065 appears to be too late, and perhaps too little, to alter radiosensitivity.

In contrast, the delayed timing and lower effective dose levels for enhanced spermidine synthesis observed in this study are quite consistent with those reported by Grdina et al. [18] for protection by WR1065 against mutagenesis. These investigators suggested that the mechanism by which WR1065 protects against mutagenesis is different from that for protection against cell killing. Because spermidine and spermine have repeatedly been shown to be important in DNA replication and repair processes [5,6,12], it is likely that at least part of WR1065's antimutagenic activity is associated with its interesting ability to increase cellular levels of spermidine.

Spermidine and spermine concentrations are normally regulated closely by a complex of product feedback systems. It was therefore surprising that WR1065, a putative polyamine analogue, would enhance rather than decrease the level of cytoplasmic spermidine. The mechanism of this unexpected response was clearly of concern. Because the WR1065-induced increase in spermidine concentration was blocked by the spermidine synthase inhibitor cyclohexylammonium sulphate, the effect must involve spermidine synthesized from putrescine, not retroconverted from spermine. Additional putrescine was shown to be available for this reaction in the WR1065-treated cells as a consequence of the observed increase in ODC activity. It was not obvious, however, why the addition of WR1065 would enhance ODC activity.

Tissue ODC has been shown to be regulated at the transcriptional, translational and post-translational levels. Though product feedback might exert some influence on ODC translation [23,41,45,46], the predominant mechanism involves control of the rate of degradation of ODC protein [23]. As the level of free intracellular spermidine increases, it enhances the translational frameshift required to synthesize the regulatory protein antizyme [42]. This protein binds and inactivates ODC and induces its degradation by the 26 S proteasome [47,48]. Thus a cell stimulation event such as medium replacement will induce an initial increase in ODC activity. However, as putrescine and spermidine concentrations begin to increase, antizyme levels also increase, shortening the half-life of ODC and causing its activity to return to the background level, even though ODC mRNA levels remain elevated. In our experiments, the enhanced peak of ODC activity in the presence of WR1065 was associated with a longer protein half-life. This in turn suggests a delay in the spermidine-induced increase in antizyme levels in treated cells compared with controls. Because spermidine is required in the induction of the translational frameshift allowing antizyme synthesis, it might be that WR1065, as a polyamine analogue, interferes with this unusual frameshift process. However, at present it is not possible to eliminate enhanced antizyme turnover or altered cellular location as possible mechanisms for the effect of WR1065 on antizyme activity.

Although these results do not explain WR1065-induced radioprotection, they do suggest that the antimutagenic properties of the aminothiol might be associated with its unexpected ability to increase cellular spermidine levels. This study also shows for the first time that a polyamine analogue can have a stimulatory effect on polyamine levels through its interference in the synthesis of the critical regulatory protein antizyme. This discovery illustrates the possibility of designing compounds for the control of cellular polyamine levels by targeting the unusual frameshift step required in antizyme synthesis.

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