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PROTECTION FROM CELLULAR OXIDATIVE INJURY AND CALCIUM INTRUSION BY *N*-(2-MERCAPTOETHYL)-1,3-PROPANEDIAMINE, WR 1065

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Abstract—The radioprotective agent WR 2721 has been shown to prevent deterioration of renal function in a rodent model of chronic renal failure, and it has been suggested that WR 2721 may exert these protective effects by scavenging free radicals. To test this hypothesis, we investigated the effects of WR 2721 and its dephosphorylated, active metabolite, WR 1065, in an *in vitro* model of oxidative injury. By using the classical assay for superoxide production (reduction of ferricytochrome *c*), we first demonstrated that WR 1065 may act as a reducing agent. To establish the biological relevance of this reducing ability, U937 cells (a human premonocytic line) were incubated with or without WR 1065 or 2721, then exposed to increasing concentrations of H_2O_2. Cell viability was measured by Trypan blue exclusion and [^{3}H]-thymidine incorporation into DNA, and cytosolic free calcium determined by fura-2 fluorescence. WR 1065 protected U937 cells from H_2O_2-induced cell death in a dose-dependent manner, and more efficiently than WR 2721. WR 1065 also prevented the rise in cytosolic free calcium induced in these cells by H_2O_2. These *in vitro* molecular and cellular events may account, at least in part, for the *in vivo* protective effects of WR 2721.

The organic phosphorothioate WR 2721 [*S*-,2-(3-aminopropylamino)-ethylphosphorothioic acid], initially developed as a radioprotective agent, was subsequently shown to prevent progression of chronic renal failure (CRF) associated with hyperparathyroidism in the rat [1], and to modulate calcium metabolism in this *in vivo* model of CRF as well as in hypercalcemia of malignancy [2, 3].

Proposed protective mechanisms from irradiation and alkylating agents include alterations in oxygen transport, a decrease in oxygen availability, consumption and/or delivery, conversion to cysteamine, hydrogen donation and free radical scavenging [4–7]. These latter effects may indeed also be involved in the protection of the kidney from the deleterious effects of calcium accumulation [1].

N-(2-mercaptoethyl)-1,3-propanediamine, usually named WR 1065 [$H_2N(CH_2)_3NH(CH_2)_2$-SH] is the dephosphorylated form of WR 2721 [$H_2N(CH_2)_3NH(CH_2)_2$-SPO_3H_2] and is generally considered to be the active form of the drug. In order to directly establish the reducing potential of WR 1065, we used, in the absence of cells, the classical assay for superoxide (O_2^-) production, i.e. the reduction of ferricytochrome *c* (Fe^{3+}-cyt) [8]. We have previously characterized the fate of oxidative injury in the human premonocytic line U937 [9], and we therefore used this model system to demonstrate the protective effects of WR 1065 and WR 2721 from cellular oxidative injury. Since in the U937 cell model (as well as in rat renal mitochondria, [10]) oxidative injury was shown to be associated with—and potentiated by—an increase in cytosolic free calcium concentration ([Ca^{2+}]i) [9–11], we also evaluated in U937 cells exposed to hydrogen peroxide (H_2O_2) the effect of WR 1065 and WR 2721 on [Ca^{2+}]i.

Here we report that WR 1065 directly reduced ferricytochrome *c*, protected U937 cells from the toxic effects of exposure to H_2O_2 and prevented the rise in [Ca^{2+}]i induced in these cells by oxidative injury. These molecular and cellular mechanisms could explain, at least in part, the protective effects of WR 2721 in CRF.

MATERIALS AND METHODS

Reagents. WR 1065 and WR 2721 were kindly provided by the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute (Bethesda, MD, U.S.A.), stored at −20°, and diluted before use in 50 mM Tris–HCl buffer, pH 7.5. Phorbol 12-myristate, 13-acetate (PMA), zymosan A, ferricytochrome *c*, catalase and superoxide dismutase (SOD) were obtained from the Sigma Chemical Co. (St Louis, MO, U.S.A.). Zymosan A was opsonized with human serum as described [12]. Opsonized zymosan (OZ) was stored at −70° and thawed just before use (final concentration 3 mg/mL). Catalase (specific activity of 14,000 units/mg) was stored at −20° in aliquots of 10 mg/mL in distilled water. 1,25-Dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$) (provided by Dr U. Fischer, Hoffmann La Roche, Basel, Switzerland) was dissolved in ethanol as a stock solution of 1.6 mg/mL and diluted with medium before addition to U937 cells (final concentration 10 ng/mL). Fura 2-AM (acetylmethoxy ester of fura-2) was obtained from

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Molecular Probes, Inc. (Junction City, OR, U.S.A.).

Cells and media. U937 cells were maintained in stationary suspension in RPMI 1640 (Gibco, Paisley, U.K.) with 10% fetal calf serum (FCS) (Gibco) and 1% glutamine (Gibco). As undifferentiated U937 cells do not have the capacity to produce O_2^- but acquire a functional NADPH oxidase upon differentiation, cells were preincubated with 1,25-$(OH)_2D_3$ (10 ng/mL) for 3 days to assess the effects of WR on O_2^- production. The cells were maintained at 37° in a humidified atmosphere containing 5% CO_2.

Reduction of ferricytochrome c. Reducing properties of WR 1065 and WR 2721 were assayed in a cell-free system by minor modifications of the previously described assay used to measure O_2^- production (Fe^{3+}-cyt reduction) [8]. The reaction buffer (RB) contained 138 mM NaCl, 6 mM KCl, 1 mM $MgSO_4$, 1.1 mM $CaCl_2$, 100 μM EGTA, 1 mM KH_2PO_4, 5 mM $NaHCO_3$, 5.5 mM glucose, 20 mM HEPES, at pH 7.4. Serial dilutions of WR 1065 and WR 2721 were added to the RB with 50 mM of Fe^{3+}-cyt and with or without catalase (2000 units/mL). The final volume of each sample was 1 mL. For each sample, reduction of Fe^{3+}-cyt was determined over time (0–180 min) by reading the absorbance at 550 nm against a blank (RB with 50 mM Fe^{3+}-cyt) in a Uvikon 810 Kontron spectrophotometer.

The SOD inhibitable reduction of Fe^{3+}-cyt was also used as described before [12, 13] to determine the effect of WR 1065 or WR 2721 on O_2^- production by 1,25$(OH)_2D_3$-treated U937 cells. Briefly, 1,25$(OH)_2D_3$-treated U937 cells were washed in phosphate buffered saline (PBS) (Gibco), resuspended in RPMI with 10% FCS, 1% glutamine and 25 mM HEPES, and further incubated for 1 hr with WR 1065 (5 mM), WR 2721 (5 mM), or vehicle alone. Cells were then washed again in PBS, resuspended in RB buffer containing 50 mM Fe^{3+}-cyt, and stimulated with PMA or OZ for 30 min at 37°, with SOD (30 μg/mL final concentration) added to control samples.

Cell viability. Cell viability was assayed using both Trypan blue exclusion and [^{3}H]thymidine incorporation into DNA. In all experiments presented here, WR 1065 or WR 2721 were added to the cells 1 hr prior to H_2O_2 and the cells exposed to H_2O_2 in the presence of the drug. After treatment with or without WR, and exposure to H_2O_2 for 1 hr, Trypan blue-positive cells were counted among 200 cells in two individual microscopic fields. To avoid potential bias brought about by disappearance of non-viable cells, total cell number was sampled at various times during the viability studies. Results presented in the form "% viable" reflect data collected from experiments where the total number of cells did not change. Cell viability was also determined by [^{3}H]thymidine uptake as previously described [9]. Briefly, cells were counted before exposure to H_2O_2, exposed to H_2O_2 for 30 min, and 200 μL of a cell suspension of 10^5 cells/mL for each condition were added to wells of 96-well plates. After overnight culture, cells were labelled with 1 μCi/well of [^{3}H]thymidine (sp. act. 2 Ci/mmol) (Amersham, Bucks, U.K.) for 6 hr, then washed and collected with an automated cell harvester (Skatron AS, Lier, Norway) on glass filter strips (Filtermats, Skatron), which were dried and counted in scintillation liquid (Kontrogel, Kontron Analytic, Zürich, Switzerland).

Determination of cytosolic calcium levels by fura 2 *fluorescence.* Cells were loaded with fura 2-AM for 30–35 min at 37° as previously described [12]. The culture medium was removed, the cells were washed with PBS and incubated at 20×10^6/mL in a buffer containing 136 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 5 mM $NaHCO_3$, 20 mM HEPES, 5.5 mM glucose, 0.2 mM EGTA, 1.2 mM $CaCl_2$, pH 7.4 with 0.1% BSA (buffer A). Fura 2-AM was then added at a concentration of 2 μM. Other cells were treated in the same manner as the loaded cells and incubated in the same buffer except that the DMSO, the vehicle for fura 2-AM, was added alone, without fura 2-AM. These cells were used for determination of autofluorescence. At no time did the DMSO concentration of the incubation medium exceed 0.5%. Prior to the measurement of fluorescence, cells were washed twice by centrifugation and resuspended at 5×10^6 cells/mL in buffer A without BSA. Fluorescence was measured with a Perkin-Elmer spectrofluorimeter at excitation wavelength 340 nm and emission wavelength 505 nm. Cells were equilibrated at 37° in a thermostated cell holder, and the suspensions were gently mixed. $[Ca^{2+}]_i$ was determined using the following expression: $[Ca^{2+}]_i = K_d \ ((F - F_{min})/(F_{max} - F))$. Calibrations were performed at the end of each tracing and the appropriate corrections made. K_d was assumed to be 224 nm at 37° [14].

Statistical analysis. Two-way analysis of variance, one-way analysis of variance and multiple comparisons with the methods of Bonferroni or Tukey were used where appropriate for the analysis of Trypan blue exclusion and [^{3}H]thymidine uptake assays. When necessary, inequality of variances was taken into consideration, using logarithmic values or *t*-tests with separate estimation of variances.

RESULTS

Reducing potential of WR 1065 *and WR* 2721

In order to assess directly the reducing potential of WR 1065 and WR 2721, reduction of Fe^{3+}-cyt was measured over time at given time-points, in the absence of cells, and in the presence of WR 1065 or WR 2721, with or without catalase. WR 1065 rapidly reduced Fe^{3+}-cyt whereas WR 2721 had no effect (Fig. 1). Catalase increased the Fe^{3+}-cyt reduction induced by WR 1065, suggesting that H_2O_2 was formed during the reaction (Fig. 1). On the other hand, incubation of 1,25-$(OH)_2D_3$-treated U937 cells with WR 1065 or WR 2721 (5 mM, 1 hr) had no effect on PMA-stimulated or OZ-stimulated O_2^- production by these cells, indicating that these agents do not interfere with the respiratory burst enzyme NADPH oxidase (data not shown).

Effects of WR 1065 *and WR* 2721 *on U*937 *cell viability after exposure to* H_2O_2

The effects of WR 2721 and WR 1065 on U937

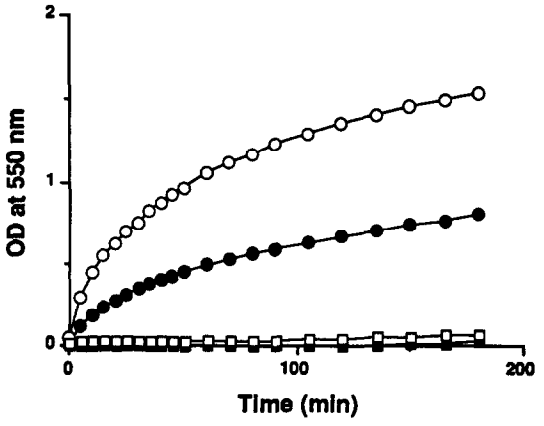

Fig. 1. Reduction of ferricytochrome *c* by WR 1065. The reduction of Fe^{3+}-cyt by WR 1065 (circles) and WR 2721 (squares) was measured as described. The drugs were used at 1 mM in presence (open symbols) or absence (closed symbols) of catalase (2000 units/mL). The results are reported in OD at 550 nm as a function of time.

Table 1. Effects of WR 1065 and WR 2721 on U937 cell viability (Trypan blue exclusion) after exposure to H_2O_2

	Viability (%)		
	H_2O_2 (mM)		
WR added	0	1	5
None	99.8 ± 0.2	56.0 ± 9.2	41.8 ± 2.3
WR 2721	99.3 ± 0.2	63.8 ± 3.1	68.3 ± 0.9†
WR 1065	98.8 ± 0.8	82.5 ± 1.8*	83.8 ± 0.8†

* $P < 0.01$ compared to WR 2721-treated group.

† $P < 0.001$ compared to the two other groups.

U937 cells were incubated with or without 5 mM WR 2721 or WR 1065 for 1 hr, then exposed H_2O_2 for another hour in the presence of the drugs. Cell viability was estimated by Trypan blue exclusion as described.

Numbers represent means ± SE, N = 4. Significance levels were determined by multiple comparisons with the method of Bonferroni.

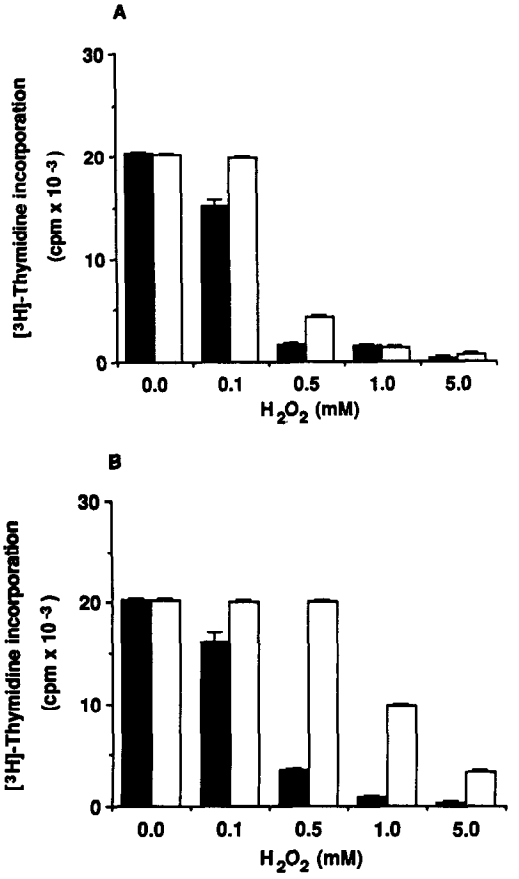

Fig. 2. Protection from H_2O_2-induced cell death in U937 cell line by WR 2721 or WR 1065. (A) Effects of WR 2721: U937 cells were preincubated for 1 hr with 5 mM WR 2721 (open columns) or vehicle alone (shaded columns), then exposed for 30 min to H_2O_2 at the indicated concentrations and [^{3}H]thymidine incorporation determined as described. Results are expressed as means ± SE (N = 5). (B) Effects of WR 1065: U937 cells were treated as described under (A) but with WR 1065. In contrast with WR 2721, the protective effects of WR 1065 were observed even at high concentrations of H_2O_2. Results are expressed as means ± SE (N = 5).

cell viability upon exposure to H_2O_2 were first assessed by Trypan blue exclusion. Whereas both WR 1065 and WR 2721 significantly protected U937 cells from H_2O_2-induced cell death, this protective effect was more pronounced with the former (Table 1).

When we used [^{3}H]thymidine incorporation into DNA, a more sensitive assay for cell viability, similar results were found (Fig. 2, A and B). For these experiments, two-way analysis of variance showed a strong interaction ($P < 0.001$) between the two factors (H_2O_2 and WR). We therefore used a one-way analysis of variance for each concentration of H_2O_2; when the differences were significant we performed multiple comparisons with the method of Bonferroni for the four predefinite comparisons (control A vs control B, WR 1065 vs WR 2721, WR 1065 vs cumulated controls and WR 2721 vs cumulated controls). In the absence of H_2O_2, cell viability of the four groups (control A, control B, WR 2721 and WR 1065) was the same. The effects of WR 2721 are shown on Fig. 2A and those of WR 1065 on Fig. 2B. With WR 2721, the protective effects were observed in particular for the lower concentrations of H_2O_2 tested ($P < 0.001$ for 0.1 and 0.5 mM H_2O_2), whereas WR 1065 prevented cell death upon exposure to all concentrations of H_2O_2 tested ($P < 0.001$).

Whereas both WR 2721 and WR 1065 completely prevented cell death at low concentrations of H_2O_2 (0.1 mM) (cell viability = 100% in both cases), the WR 1065 was significantly more effective in protecting cells from H_2O_2 0.5 mM and above ($P < 0.001$) (compare Fig. 2, A and B).

The dose-dependence of WR 1065 for protection from high concentrations of H_2O_2 (0.5 mM and

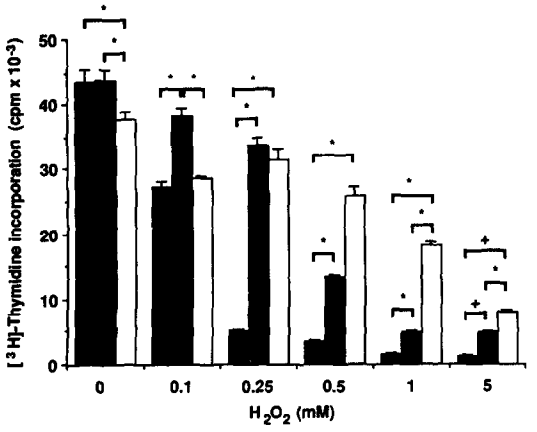

Fig. 3. Dose-dependent protection from H_2O_2-induced cell death in U937 cells by WR 1065. U937 cells were pre-incubated for 1 hr with WR 1065 or vehicle alone (■ = vehicle, ▨ = 2.5 mM, □ = 5.0 mM) and exposed to H_2O_2 (0–5 mM) for 30 min, and [^{3}H]thymidine uptake determined as described. Results are expressed as means ± SE, N = 5. Since means and SE showed a linear dependence, logarithm of the variables were used for statistical analysis. Significance levels were determined by Tukey's method for pairwise comparisons on the transformed variables (+$P < 0.05$, *$P < 0.001$).

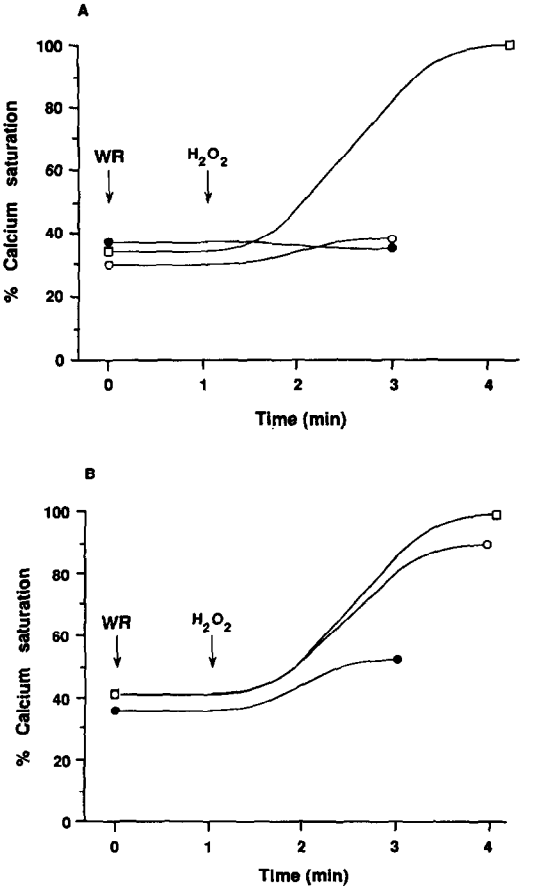

Fig. 4. Effects of WR 1065 on [Ca^{2+}]i. Cytosolic calcium variation was determined by fura-2 cytofluorometry as described. WR 1065 (○: 2.5 mM; ●: 5.0 mM) or vehicle alone (□) were added 1 min before H_2O_2 [125 μM (A) or 250 μM (B)]. Results are expressed as percentage of calcium saturation. The end points of the curves represent the maximal levels of [Ca^{2+}]i at the times they were reached.

above) is shown on Fig. 3. In some experiments (as those shown in Fig. 3) we observed that WR 1065, when used at high concentrations and without further exposure to H_2O_2, by itself actually decreased [^{3}H]thymidine incorporation into DNA (Fig. 3, 0 H_2O_2, open bar). Such a cytotoxic effect of the drug when used in cell cultures had been described before by Mori *et al.* in mouse L cells [15].

Effects of WR 1065 *and WR* 2721 *on* [Ca^{2+}]i *upon exposure of U*937 *cells to* H_2O_2

H_2O_2 induces in U937 cells a rapid and massive influx of [Ca^{2+}]i from the extracellular medium [9, 11], and calcium influx is associated with cell death [16]. It was therefore of interest to measure the effects of WR, in particular WR 1065, on [Ca^{2+}]i upon exposure of U937 cells to H_2O_2. A representative experiment is shown in Fig. 4. For both concentrations of H_2O_2 used (125 or 250 μM), [Ca^{2+}]i increased to levels which saturated the dye in the absence of WR 1065, suggesting permeabilization of U937 cell membranes. Characteristics of this rise were that it began after only a few seconds following the addition of H_2O_2 to the cells, that the maximal fluorescence was reached within 2–4 min (depending on the concentration of H_2O_2 used) and that it was not reversible, suggesting that it resulted from calcium influx rather than from specific mobilization of calcium from intracytoplasmic stores.

When H_2O_2 was added to the U937 cells in the presence of WR 1065, the rise in [Ca^{2+}]i induced by H_2O_2 was decreased, or even prevented at high concentrations of WR 1065 (Fig. 4, A and B). For the lower concentration of H_2O_2 (125 μM), the increase in [Ca^{2+}]i was completely prevented at both concentrations of WR 1065 used (2.5 as well as 5 mM) (Fig. 4A), whereas for 250 μM H_2O_2, WR 1065 only partially prevented this increase. Table 2 reports the results of a set of separate experiments, in which prevention of calcium entry into U937 cells exposed to H_2O_2 was complete at both concentrations tested.

DISCUSSION

The organic thiophosphate WR 2721, initially developed as a radioprotective agent, subsequently proved to inhibit PTH secretion, and to prevent progressive deterioration of renal functions in a CRF model associated with hyperparathyroidism in the rat [1]. It has been suggested that both radioprotection and protection from progression of CRF could be mediated by hydrogen donation [1]. In this report, by applying the classical assay used for measurements of superoxide [8] we assessed the reducing potential of WR 1065 and established that WR 1065 has strong reducing potential, which

Table 2. Effects of WR 1065 on calcium rise induced by H_2O_2 in the U937 cells

H_2O_2 (μM)	WR 1065 (mM)	Calcium saturation (%) Basal	Maximal
125	0.0	35.63 ± 0.79	64.15 ± 5.62
	2.5	33.21 ± 1.02	30.01 ± 2.70
250	0.0	35.41 ± 0.59	85.53 ± 1.79
	2.5	33.87 ± 1.09	34.95 ± 1.75
	5.0	33.42 ± 0.96	28.85 ± 1.32

U937 cells were prepared for fura-2 fluorescence as described in Materials and Methods. WR 1065 was added after the stabilization of basal fluorescence and 1 min before H_2O_2. Each measure was performed with 5×10^6 cells. Results are expressed as means ± SE (N = 4).

appears to be related to the free thiol group (RSH), since the phosphorylated thiol WR 2721 ($RSPO_3H_2$) had no such effect *per se*. The reducing ability of WR 1065 was increased and prolonged in the presence of catalase, indicating that, in the presence of oxygen, formation of H_2O_2 will limit the reducing potential of WR 1065. It has been reported before that radioprotection by aminothiols is dependent on tissue oxygen concentrations, higher protection occurring at lower O_2 concentrations [17].

We then investigated the ability of WR 1065 to protect cells from oxidative injury. Since we have previously characterized the fate of oxidative damage in the human premonocytic line U937 [9, 11], we used this same cell line to characterize the effects of WR 1065 and WR 2721 on cell survival after exposure to H_2O_2. WR 1065 and WR 2721 protected U937 cells from oxidative injury induced by H_2O_2. Viability of U937 cell exposed to H_2O_2 was significantly increased by both WR 1065 or WR 2721, WR 1065 being more effective than WR 2721. Previous studies dealing with radioprotection showed that in order to exert its protective effects against irradiation, WR 2721 has to be dephosphorylated to its free thiol WR 1065 [15, 18]. Thus, since WR 2721 has no reducing ability *per se*, the fact that it could interfere with oxidative injury in U937 cells suggest that WR 2721 was at least partially metabolized into WR 1065 in these cells. In normal human peripheral blood monocytes, the protective effects of WR 2721 were even greater [Polla, unpublished data], which is in agreement with the preferential uptake and metabolism of the drug by normal as compared to transformed cells [19, 20].

Oxidative injury to cells and tissues is mediated by oxygen derived reactive species (O_2^-, $^{\cdot}OH$) and lipid peroxides (LOOH) [21, 22]. In contrast to O_2^-, which requires an anion channel to enter the cells because of its negative charge, H_2O_2 can easily cross cell membranes, and even though it is not by itself a free radical, its metal ion-dependent transformation (Fenton reaction) into hydroxyl radical leads to major cell toxicity [21]:

$$H_2O_2 \xrightarrow{\text{Iron}} {}^{\cdot}OH + OH^-$$

$^{\cdot}OH$ is a highly reactive free radical and the most likely oxygen species responsible for DNA strand breaks [23]. Because of the direct reduction of Fe^{3+}-cyt by WR 1065, it is possible that WR 1065 exerts its protective activity by directly scavenging hydroxyl radicals:

Reaction 1: $2RSH + {}^{\cdot}OH \rightarrow H_2O + {}^{\cdot}RSSR^- + H^+$

An alternative possibility is that WR 1065 scavenges H_2O_2:

Reaction 1': $2RSH + H_2O_2 \rightarrow RSSR + 2H_2O$

Direct measurements of H_2O_2 and $^{\cdot}OH$ will be required to solve this issue.

H_2O_2 toxicity was previously demonstrated to be associated in the U937 cells with a massive increase in $[Ca^{2+}]i$ [9, 11]. This increase in cellular calcium may initiate a cascade of events leading to cell death, among which activation of phospholipase A_2, subsequent breakdown of cell membranes, ATP depletion and uncoupling of oxidative phosphorylation in mitochondria [10]. WR 1065 prevented both calcium entry and cell death in U937 cells exposed to H_2O_2. These effects of WR 1065 are likely to be related to interruption of membrane lipid peroxidation, the inhibition of calcium entry being secondary to preservation of membrane integrity; the possibilities that WR 1065 acts primarily on calcium entry or directly inhibits phospholipase A_2 have however also to be considered.

On the other hand, a rise in $[Ca^{2+}]i$ is known to be related to many different cellular activation processes and in this case will lead to cell activation [24, 25]. In particular, calcium influx is an essential component of concavalin A-induced lymphocyte mitogenesis [26]. In our experiments however, the massive influx of calcium did not appear to play a role as second messenger for cellular activation, but rather was related to cell death. Indeed, non-cytotoxic H_2O_2 concentrations did not increase $[Ca^{2+}]i$ and no concentration of H_2O_2 tested induced any increase in $[^3H]$thymidine incorporation. Moreover, H_2O_2 toxicity is prevented when U937 cells are exposed to H_2O_2 in the absence of extracellular calcium [9, 11].

In conclusion, and although the precise molecular reactions involved in these phenomena warrant further investigation, the data presented here provide direct evidence for a protective effect of WR 1065 against cellular oxidative injury and associated calcium intrusion. This mode of action may explain, at least in part, the mechanism whereby WR 2721 prevented the progressive deterioration of kidney

functions in the rodent model of chronic renal failure previously described [1]. In this model, the progression of renal failure was directly related to high plasma level of parathyroid hormone. This hormone increases both the calcium uptake and $[Ca^{2+}]i$ in renal tubular cells [27, 28] and can induce, when circulating at high concentration, nephrocalcinosis [29]. Alterations in either intracellular calcium metabolism or free radical generation could be involved in renal tissue damage observed after acute ischemia [10, 30–33] or chronic exposure to nephrotoxic agents, including high level of parathyroid hormone [34–36]. The present demonstration that WR 1065 can prevent both calcium entry and oxidative injury in U937 cells should encourage further *in vivo* evaluation of this compound, or other analogs sharing the same *in vitro* pharmacological properties, in models of acute and chronic renal failure.

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