

# The radioprotective effect of a new aminothiols (20-PRA)

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## Abstract

We examined the radioprotective effect of aminothiols 2-N-propylamine-cyclo-hexanethiol (20-PRA) on a human leukemic cell line (K562) following various radiation doses (5, 7.5 and 20 Gy) using a source of <sup>60</sup>Co  $\gamma$ -rays. At 5 Gy and 1 nM 20-PRA, a substantial protective effect (58%) was seen 24 h after irradiation, followed by a decrease at 48 h (11%). At the high radiation dose (20 Gy) a low protective effect was also seen (35%). In addition, the antitumorigenic potential of 10 nM 20-PRA was shown by the inhibition of crown gall formation induced by *Agrobacterium tumefaciens*. The radioprotective potency of 20-PRA is 10<sup>5</sup>-10<sup>6</sup> times higher than that of the aminothiols WR-1065 (N-(2-mercaptoethyl)-1,3-diaminopropane) whose protective effect is in the 0.1 to 1.0 mM range.

## Key words

- Aminothiols
- Cytotoxic effect
- Radioprotector
- Antitumorigenic effect

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Research supported by FAPEMIG  
(Nos. CBS 666/91 and CBS 21198/  
95) and CNPq (Nos. 400190/93-1  
and 523304/94-3).

Received April 10, 1997

Accepted April 13, 1998

Aminothiols have been the subject of considerable research because of their reported ability to protect tissues against acute damage induced by either ionizing radiation or alkylating cytotoxic agents (1). However, because of their mutagenic and carcinogenic potential, the clinical application of these agents increases the risk of secondary tumors in treated patients (2).

Aminothiols have also been cited as protective agents against the induction of sublethal mutagenic, transforming and carcinogenic effects by free radicals (3,4). There are reports showing that the aminothiols WR-2721 (S-2-(3-aminopropylamino) ethylphosphorothioic acid) can inhibit the development of radiation-induced sarcoma (5) and that N-acetyl-L cysteine inhibits hepatocar-

cinoma induced by N-2-acetylaminofluorene (6). These results, together with others, support the protective role for aminothiols (7).

Several mechanisms have been proposed to explain radioprotection by aminothiols, including induction of hypoxia (8), scavenging of free radicals (9), protection of nucleic acid against free radicals (10), delayed repair of DNA strand breaks (11-13), increased cystine uptake (14) and glutathione biosynthesis (13). The use of radioprotective drugs in humans has been severely limited by the toxicity of the compounds synthesized earlier (15). This situation prompted the US Army to sponsor a program for the synthesis of compounds with radioprotective potential (16). One of these compounds (WR-2721) has been extensively investigated because of

its reported radioprotective capacity (1).

In the present study, we evaluated the radioprotective properties of a newly synthesized non-phosphorylated aminothiols. 20-PRA (2-N-propylamine-cyclo-hexanethiol) was synthesized by reacting propylamine dissolved in benzene with epithio-cyclo-hexane. The endpoint of the reaction (24 h) was monitored by the exhaustion of epithio-cyclo-hexane using infrared spectrometry. Excess amine and solvent were removed by fractionated distillation. The purity of 20-PRA used in this study was checked by gas chromatography coupled to mass spectrometry. 20-PRA was freshly dissolved in 1 mM dimethylsulfoxide (DMSO) prior to use.

In preliminary experiments, we analyzed the toxicity of 20-PRA using the brine shrimp assay (17). 20-PRA (1 to 100  $\mu\text{M}$ ) dissolved in 10 mM DMSO was added to metanauplius larvae of *Artemia salina* to determine the

survival rate following a 24-h incubation period. The appropriate controls without aminothiols were used to establish the effect of DMSO. The lethal concentration of 50% ( $\text{LC}_{50}$ ) calculated by the PROBIT test (18) was 50  $\mu\text{M}$  (data not shown).

Once the  $\text{LC}_{50}$  was established, we tested the radioprotective effect of 20-PRA on K562 cells, a human leukemic cell line provided by Dr. R. Brentani (Instituto Ludwig de Pesquisa sobre o Câncer) grown in RPMI 1640 supplemented with 5% FBS (fetal bovine serum). The survival rate was determined upon the addition of 20-PRA (1 nM to 10  $\mu\text{M}$ ) to exponentially growing K562 cells ( $1.5 \times 10^4$  cells/ml) between 0-48 h in a cell incubator at 37°C supplied with 3%  $\text{CO}_2$ . The number of viable cells was determined by the Trypan blue dye exclusion test using a Neubauer chamber. 20-PRA (10  $\mu\text{M}$ ) killed most of the K562 cells. Further dilutions showed a gradual decrease in toxicity until no deleterious effects were seen at 1 nM (Figure 1). We therefore chose this concentration as a working concentration for 20-PRA in subsequent experiments.

In radioprotection assays, cells preincubated with 20-PRA for 30 min were subjected to three doses of  $^{60}\text{Co}$   $\gamma$ -ray radiation (5, 7.5 and 20 Gy) and further incubated for various times. The results of these experiments are summarized in Figure 1 and Table 1. Figure 1 shows the effect of 1 nM 20-PRA on the number of viable K562 cells both in irradiated (5 Gy) and non-irradiated samples. As expected, a decay in cell number due to irradiation relative to control without 20-PRA was observed (Figure 1), corresponding to 13%, 57% and 67% at 0, 24 and 48 h, respectively. In cells incubated with aminothiols, there was partial protection of 58% and 11% at 24 h and 48 h, respectively. At 48 h, the radioprotection was low (0-11%), regardless of the radiation dose (data not shown). Although 20-PRA afforded full protection at zero-time (Figure 1), its meaning is questionable since cell lethal effects due to

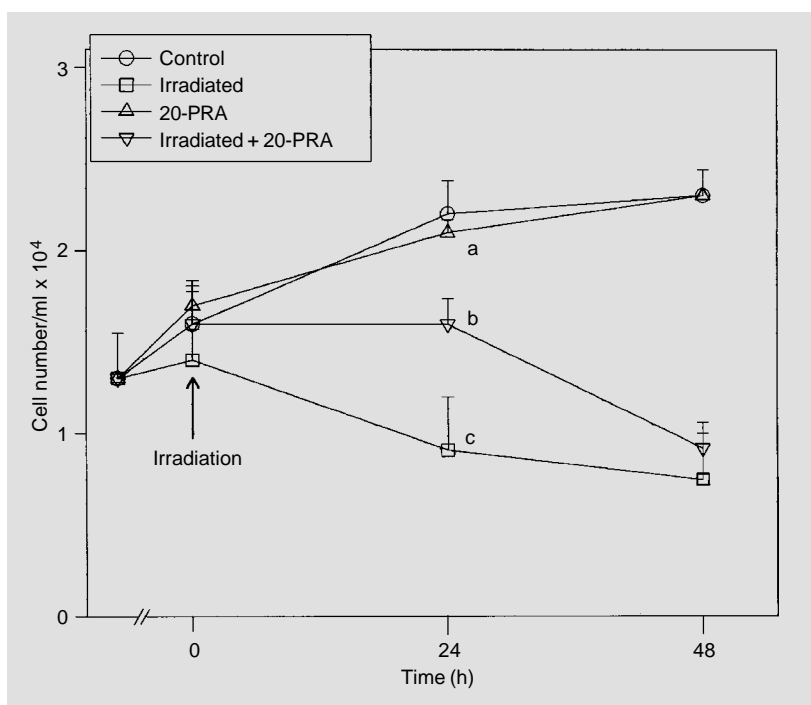


Figure 1 - The radioprotective effect of 20-PRA on K562 cells. Cells were incubated for 30 min with 1 nM 20-PRA and then irradiated with 5 Gy. After irradiation, the cells were incubated for 48 h. The viable cell number was determined by the Trypan blue technique. Data are reported as mean  $\pm$  SEM for N = 3-7 experiments. a vs b, P = 0.07; a vs c, P = 0.005; b vs c, P = 0.02 measured by the Student-t test.

radiation require a minimal period of time to become evident.

The decline in the number of cells observed at 48 h cannot be attributed to the radioprotector or its metabolic products, since controls in which 20-PRA was present at 48 h showed cell counts similar to those of untreated cells (Figure 1).

The drastic reduction in cell viability observed in irradiated cells incubated with 20-PRA at 48 h could be explained by a combined effect of radiation and the radioprotector or its by-products. In this regard, it should be remembered that 20-PRA or its by-products remained in the culture throughout the 48-h period. In previous studies with WR-1065 (N-(2-mercaptoethyl)-1,3-diaminopropane), it was shown that rejoining of single strand breaks following irradiation of CHO cells with 4 Gy was adversely affected when the drug was present in the culture (15). This effect was observed after 60 min of incubation at 37°C following irradiation. Therefore, we can expect a similar effect to occur in 20-PRA-treated cells. The apparent progressive reduction in radioprotection between 0 and 48 h can also be attributed to a different mechanism. Smoluk et al. (19) showed that WR-1065 inhibits DNA replication and cellular viability by binding to DNA under physiological conditions. It remains to be demonstrated whether 20-PRA interacts with DNA under these conditions.

On the other hand, Rubin et al. (13) recently showed that the protective effect of WR-1065 on vascular endothelial cells changes as a function of time. At 2 Gy the protective effect at 24 h was 81%, but dropped to 55% when measured after 48 h. Their data show that the variation in protection depends on the radiation dose.

We also compared the radioprotective effect of 20-PRA at different radiation doses (Table 1) and observed that the protective effect decreased as the radiation dose increased. At 5 Gy, 20-PRA afforded the best protective effect at 24 h but even at higher

doses (20 Gy) the aminothiol exhibited a protective effect during the first 24 h.

Based on these data, we investigated the antitumorigenic capacity of 20-PRA. This effect was measured by the inhibition of crown gall formation on potato discs induced by *Agrobacterium tumefaciens* (20). The results (data not shown) showed that 0.1 µM 20-PRA (LC<sub>50</sub>/100) inhibits 95% of crown gall formation. This high inhibition supports the view that aminothiols also have antitumorigenic activity.

Perhaps the most tempting feature of 20-PRA is the low concentration required for the radioprotective activity to become evident. Thus, while most aminothiols display a radioprotective action at 0.1 to 1.0 mM, 20-PRA is effective at 1 nM. For instance, the aminothiol WR-1065 at 2.0 mM partially suppressed single strand breaks, double strand breaks and restored cell replication of irradiated endothelial cells (13). In this regard, Smoluk et al. (21) observed that WR-1065-mediated γ-radioprotection against cell killing afforded maximum protection when intracellular levels reached 7-10 nM. Assuming free diffusion of 20-PRA across the membrane we would expect intracellular levels similar to those required by WR-1065 to exert its protective action. Free diffusion

Table 1 - Effect of 1 nM 20-PRA as a radioprotective agent at various radiation doses.

K562 cells were seeded into culture flasks at a density of  $1.5 \times 10^4$  cells/ml. One nM 20-PRA was then added and the cells were further incubated for 30 min in a CO<sub>2</sub> incubator at 37°C. Cells were irradiated with 5-, 7.5- and 20-Gy doses and counted after a 24-h incubation period. Data are reported as mean ± SEM (N = 3-7 experiments). a vs b, P = 0.005; a vs c, P = 0.07; b vs c, P = 0.02; a vs d, P = 0.01; a vs e, P = 0.03; d vs e, P = 0.003; a vs f, P = 0.04; a vs g, P = 0.006; f vs g, P = 0.12 (Student t test).

Radiation dose (Gy)	Remaining cell number/ml				Protection (%)
	Control with 20-PRA	Irradiated	Irradiated + 20-PRA		
5	<sup>a</sup> 2.1 ± 0.07 × 10 <sup>4</sup>	<sup>b</sup> 9.1 ± 1.7 × 10 <sup>3</sup>	<sup>c</sup> 1.6 ± 0.08 × 10 <sup>4</sup>		58
7.5	<sup>a</sup> 2.1 ± 0.07 × 10 <sup>4</sup>	<sup>d</sup> 4.1 ± 0.83 × 10 <sup>3</sup>	<sup>e</sup> 1.2 ± 0.08 × 10 <sup>4</sup>		47
20	<sup>a</sup> 2.1 ± 0.07 × 10 <sup>4</sup>	<sup>f</sup> 4.2 ± 0.3 × 10 <sup>3</sup>	<sup>g</sup> 1.0 ± 0 × 10 <sup>4</sup>		35

of the drug across the membrane is possible since 20-PRA bears no phosphate group, in contrast to WR-2721, the precursor of WR-1065. These findings may have relevant implications for 20-PRA regarding its potential protective role when the cell is damaged by anticancer therapy. In this regard, tumoral cells are preferential targets for irradiation

and alkylating agents because of their reduced glutathione levels which impair the removal of free radicals (22). This preliminary report refers to results obtained for 20-PRA while clinical trials are in progress to evaluate radio- and chemoprotection of the aminothiols WR-2721 and WR-151327 (23).

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