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SYNTHESIS AND RADIOPROTECTIVE EFFECTS OF NEW PHOSPHOROTHIOATE ESTERS OF WR-2721, WR-3689 AND WR-151327

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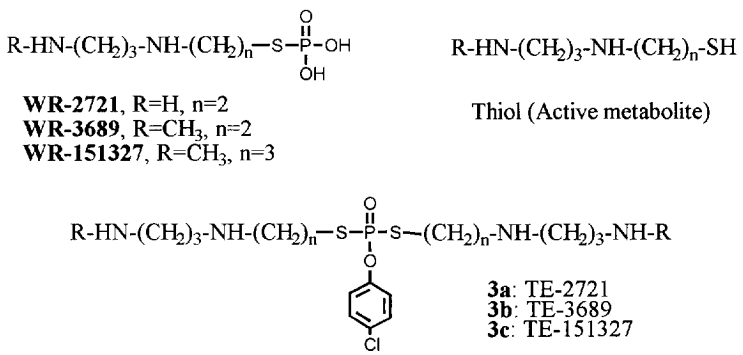
Abstract: Three 4-chlorophenyl diaminoalkylthiophasphate esters of WR -2721, WR-3689 and WR-151327 were synthesized and evaluated for in vitro cytotoxicity and in vitro and in vivo radioprotective efficacy against Chinese hamster V-79 cells and CD2F1 male mice, respectively.

Introduction: A large variety of chemical and biological agents have been investigated in the past for the differential protection of the normal tissues versus neoplastic tissues during radiotherapy, and also for the protection of the military and civilian personnel from exposure to radiation from a variety of environmental factors such as nuclear devices, accidental exposure, space, and radioactive wastes. As a result of these studies several compounds containing phosphorus-sulfur bonds such as phosphorothiols and phosphorothioates have been identified as potential radioprotective agents.[1,2] Specifically, the phosphorothioate drugs S-2(3-aminopropylamino)ethylphosphorothioic acid at (WR-2721), S-2(3-methylaminopropylamino)ethylphosphorothioic acid (WR-3689), and S-2(3-methylaminopropylamino)propylphosphorothioic acid (WR-151327) are the leading radioprotectors currently available.[3,4] These compounds were first synthesized by the Walter Reed Army Institutes of Research, and are still often referred to by their Walter Reed (WR) designation number. WR-2721 has been used for radiotherapy and it also protects against cytotoxic effects of some chemotherapeutic agents.[5] In vitro analysis of these WR agents shows that they are rapidly metabolized by alkaline phosphatase to the active metabolite 'thiol' and an inorganic phosphate (Figure 1).

Figure 1

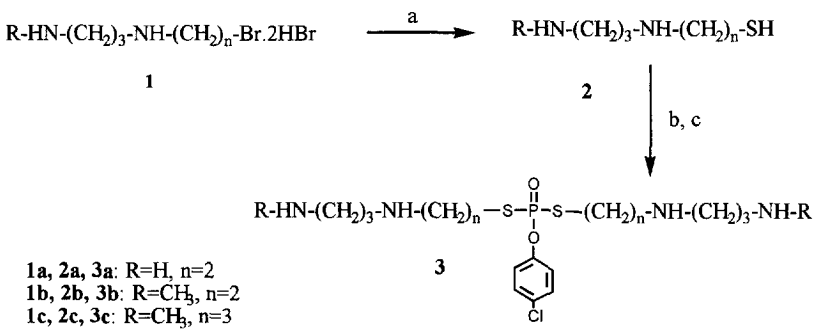


However, these WR compounds are highly water soluble and must be given systemically due to their poor bioavailability upon oral administration. This is due to the ionizable acidic phosphate functional group of

WR-compounds that limits their usefulness in most practical situations.[6] Therefore we hypothesized that chemical masking of the ionizable phosphate group in these WR compounds (2721, 3689, and 151327) with a lipophilic ester functional group may improve its bioavailability and yet release, after hydrolysis, a free WR compound and thiol metabolite following absorption resulting in improved radioprotection. Similar approach of modifying ionizable drugs such as 3'-azidothymidine (AZT) monophosphate to lipophilic ester prodrugs with improved bioavailability has been reported.[7,8] This investigation, therefore, describes the synthesis and radioprotection studies of three new phosphorothioate ester prodrugs TE-2721 (**3a**), TE-3689 (**3b**), and TE-151327 (**3c**). These esters were evaluated in comparison to their respective parent WR compound for in vitro and in vivo radioprotective effects against acute gamma ray irradiation using Chinese hamster V-79 cells and CD2F1 male mice.

Synthesis: Synthesis of triesters (**3a-c**) is shown in Scheme-1. Piper's[9,10] method was adopted to synthesize (3-aminopropylamino)ethyl bromide dihydrobromide (**1a**), 2-(3-methylaminopropylamino) ethyl bromide dihydrobromide (**1b**) and 2-(3-methylaminopropylamino)propyl bromide dihydrobromide (**1c**). These bromides were transformed into thiols by reacting with NaHS/H_2S in methanol. Synthesis of triesters (**3a-c**) was achieved by reacting one equivalent of phosphorylating agent 4-choloropenylphosphorodichloridate with two equivalents of thiol (**2a-c**) in the presence of 1-methylimidazole. Final esters were purified by flash column chromatography and then transformed to a more stable hydrochloride salts by treating with saturated HCl-MeOH solution. The overall yield of final esters were rather low which may be due to the electron withdrawing 4-chloro group of 4-chlorophenyl phosphorodichloridate and we are investigating the effect of other phosphorylating agents such as 4-(methylthio)phenyl phosphorodichloridate on the overall yield of final esters. The structures of final esters (**3a-c**) were confirmed by ^1H NMR and elemental analysis.

Scheme 1



a: NaSH/H_2S; b: 1-methylimidazole, 4-chlorophenylphosphorodichloridate; c: HCl-MeOH

General procedure for the preparation of thiols (**2a-c**)**:** A NaHS solution in methanol was prepared by dissolving NaOMe (162 mg, 3 mmol) in methanol (10 mL) while a slow stream of H_2S was passed through the stirred solution -10 °C. The bromide **1a** (1 mmol, 343 mg) was then added to the NaHS solution at -10 °C. The

solution was stirred at 0 °C for 2 h and at 25 °C for 18 h in a tightly stoppered flask. The methanol was removed and the residue was washed with 2 ml of anhydrous ethanol. The NaBr was filtered out. After removing the ethanol the (3-aminopropylamino)ethylthiol (**2a**) was obtained in 22% yield. Using this general procedure thiols **2b** and **2c** were isolated in 41% and 35% yield.

General Procedure for the preparation of triesters (**3a-c**): A mixture of 1-methylimidazole (287 mg, 3.5 mmol) and 4-chlorophenylphosphorodichloridate (245 mg, 1 mmol) was dissolved in 2 mL of acetonitrile and stirred at room temperature for 10 min. To this solution thiol (**2a-c**) (0.5 mmol) was added in 2 mL of acetonitrile and the reaction mixture was stirred room temperature for 24 h. After removing solvent, the residue was purified by flash column chromatography using ethyl acetate and methanol (4:1) mixture. The chromatographed product (51 mg) was acidified by 3 mL of saturated HCl-MeOH solution and precipitated by addition of 10 mL ether. The white precipitate was collected, dried and isolated as the final triester.

4'-Chlorophenyl bis[2-(3-aminopropylamino)ethylthiophosphate (**3a**): The ester (**3a**) was synthesized as described above in 20% yield. ^{1}H NMR (DMSO-d^6): δ 2.05 (m, 4H, CH_2), 2.98 (m, 4H, NCH_2), 3.05 (m, 4H, CH_2N), 3.50 (m, 4H, CH_2N), 3.85 (t, 4H, SCH_2), 7.25 and 7.50 (2d, 4H, C_6H_4), 8.04 (s, 6H, NH and NH_2), 9.1. *Anal.* calcd. for (C_{16} $H_{30}N_4O_2ClPS_2.2HCl$): C, 37.39; H, 5.88; N, 10.90. Found: C, 37.13; H, 6.21; N,10.52.

4'-Chlorophenyl bis[2-(3-methylaminopropylamino)ethylthiophosphate (**3b**): The ester (**3b**) was synthesized as described above in 21% yield. ^{1}H NMR (DMSO-d^6): δ 1.95 (m, 4H, CH_2), 2.48 (t, 6H, CH_3), 3.04 (m, 8H, NCH_2, CH_2N), 3.35 (m, 4H, CH_2N), 3.71 (m, 4H, SCH_2), 7.21 and 7.45 (2d, 4H, C_6H_4), 8.79 (s, 2H, NH), 9.13 (s, 2H, NH). *Anal.* calcd. for (C_{18} $H_{34}N_4O_2ClPS_2.2HCl$): C, 39.89; H, 6.32; N,10.33. Found: C, 39.74; H, 6.28; N,10.03.

4'-Chlorophenyl bis[2-(3-methylaminopropylamino)propylthiophosphate (**3c**): The ester (**3c**) was synthesized as described above in 25% yield. ^{1}H NMR (DMSO-d^6): δ1.97 (m, 4H, CH_2), 2.16 (m, 4H, CH_2), 2.48 (t, 6H, CH_3), 2.95 (m, 12H, NCH_2, CH_2N), 3.35 (m, 4H, CH_2N), 3.71 (m, 4H, SCH_2), 7.21 and 7.35 (2d, 4H, C_6H_4), 8.75 (s, 2H, NH), 8.93 (s, 2H, NH). *Anal.* Calcd. For (C_{20} $H_{38}N_4O_2ClPS_2.2HCl$): C, 42.14; H, 5.79; N, 9.83. Found: C, 41.82; H, 5.61; N, 9.76.

Radioprotective Activity: The cytotoxicity and radioprotective effects of the esters were initially determined in vitro against Chinese hamster (V-79) cells. The results of these studies are summarized in Table 1. All of the compounds were essentially nontoxic at the concentration of 0.1 mM. Even at the concentration of 1 mM, the esters **3b** and **3c** and their parent compounds (WR-3689 and WR-151327) showed no toxicity against Chinese hamster V-79 cells. In contrast, both the ester **3a** and its parent WR-2721 were highly toxic at 0.5 mM to 1.0 mM concentrations.

Evaluation of in vitro radioprotective effects: Chinese hamster V-79 cells were incubated and allowed to grow in Eagle's minimal medium (MEM) for 24-28 h. They were typsinized and counted. Three plates for each irradiation dose and one triplicate as a control were set up for irradiation. While the cells were attaching, the drug solutions were prepared at 0.1 mM concentration for **3a** and WR-2721 and 1 mM concentration was used for triesters **3b**, **3c**, and WR-3689, WR-151327 compounds. After adding 1.5 mL drug to each plate and incubating for 2 h, cells were irradiated at 2 Gy, 3 Gy, 4 Gy, 8 methylthio Gy, and 11 Gy. Immediately after irradiation, cells were removed by trypsinization for 30 s at room temperature and 3 mL of fresh media was

added after 6 days incubation at 37 °C, in atmosphere of 95% air:5% CO_2, cells were stained and then counted. The cytotoxicities were also tested against Chinese hamster V-79 cells previously to choose the right concentration of drug to use in irradiation experiment. The survival curves were obtained according to survival rate at the various irradiation doses. The in vitro radioprotective effects were evaluated by Dose Modifying Factor (DMF).[11,12]

Table 1: Cytotoxicity and Radioprotective Effects of WR-Compounds and Triesters

Compd.	Cytotoxicity		in vitro	in vivo				
	Conc.	Survival (%)	DMF[a]	Dose (mg/kg)	Survival[b] (%)	MST[b]	T/C	%ILS
Control	-	-	na	-	10	10	na	na
WR-2721	0.1	100	1.4	239	80	27	2.63	130
	0.5	2						
	1	0						
TE-2721 (**3a**)	0.1	92	1.2	200	40	20	1.94	170
	0.5	36						
	1	0						
WR-3689	0.1	100		473	80	27	2.60	81
	0.5	95						
	1	97	2.0					
TE-3689 (**3b**)	0.1	100		400	60	22	1.63	178
	0.5	96						
	1	97	2.0					
WR-151327	0.1	93		469	60	20	1.94	18
	0.5	99						
	1	90	1.5					
TE-151327 (**3c**)	0.1	na[c]		400	50	23	2.26	73
	0.5	na[c]						
	1	94	1.65					

a. The drug concentration used in radioprotective activity test on chinese hamster V-79 cells was 0.1 mM for WR-2721 and **3a**, and 1.0 mM for WR-3689, WR-151327, **3b** and **3c**. The total number of cells used in radioprotective activity test on V-79 cells are 200 at 2 and 3 gy, 400 at 4 Gy, 800 at 8 Gy, and 2000 at 11 Gy respectively (n = 3).

b. Both survivors and Mean Survival Time (MST) were tested at 10 Gy and results were obtained on day 30.

c. The cytotoxicity of **3c** at 0.1 mM and 0.5 mM was not detremined.

Evaluation of in vivo radioprotective effects: The CD2F1 male mice (22-24 g, supplied by Harlan Sprague Dawley, Inc., Indianapolis, IN), in groups of ten, were given the compounds at day zero. The esters in an isotonic saline solution were injected ip 30 min preirradiation at the doses in Table 1. The whole body irradiation was performed on a Nordion Gammacell 40 Laboratory ^{137}Cs Irradiator (Nordion International, Inc., Ontario, Canada). The dose rate was equivalent to 1.16 Gy/min. The total irradiation dose was 10 Gy. Five mice held in a 5" x 5" x 1.2" plastic box were placed in the middle of the chamber for irradiation. Then the animals were observed in a regular animal care room for 30 days, keeping a record of deaths and survivors.[13]

The radioprotective effects were evaluated by comparing the mean survival time of treated with that of the control animals (i.e., T/C, where T represents the mean survival time of the treated group and C the mean survival time of the control group). The percent Increase in Life Span (%ILS) was calculated by the formula [(T-C)/C]x100].

Results and Discussion: In vitro radioprotective effects of WR-2721 and its triester **3a** are at the 0.1 mM concentration, and WR-3689 and WR-151327 and their esters (**3b** and **3c**) are at the 1 mM concentration respectively. These radioprotective studies were performed on Chinese hamster V-79 cells. The total number of cells used in radioprotective activity on V-79 cells are 200 at 2 Gy and 3 Gy, 400 at 4 Gy, 800 at 8 Gy and 2000 at 11 Gy irradiation respectively. The results of this study indicated that all of the esters (**3a**, **3b**, and **3c**) provided significant in vitro radioprotective effects on Chinese hamster V-79 cells. The radioprotective effects of esters are similar to their parent compounds. The ester of WR-3689, **3b** provided the best radioprotection with a dose modification factor (DMF) of 2.0. The other esters also showed almost or better radioprotection compare to the parent compounds (Table-l).

The results of in vivo radioprotection showed that all of the compounds afforded significant radioprotective effects against acute gamma irradiation to mice. The mice treated with the esters showed a significant increase in 30 day survival after exposure to 10 Gy irradiation compare to control. For example, the nontreated control group of animals mean survival time (MST) is only 10 days. In contrast to MST for esters (**3a-c**) varied from 20-23 days. Although the MST for the parent WR-compounds WR-2721 and WR-3689 was better than the esters **3a** and **3b**, the MST for **3c** was slightly higher than its parent compound WR-151327 (Table-l).

In summary, the esters provided almost similar in vivo radioprotective effects compare to their parent compounds when given systemically 30 min preirradiation. Follow up studies are underway to determine if these esters have radioprotective effects when given orally, since the parent compounds are inactive when given orally.

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