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Article

Radioprotective Efficacy of Phosphorus-Containing Polymer Complexes of Amifostine WR-2721

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Abstract: Background: The aim of this study was to investigate the radioprotective efficacy of polymer complexes constructed from amifostine (WR-2721) and poly(hydroxyoxyethylene phosphate)s with different molecular weights. The use of suitable polymers for the immobilization of radioprotective drugs is aimed at improving or obtaining important new properties. Methods: The radioprotective efficacy of the compounds was investigated by cytotoxicity and the survival of mouse embryonic fibroblasts MEF *LIG4*$^{+/+}$ and MEF *LIG4*$^{-/-}$ cells irradiated with 2, 6 and 12 Gy in the presence of amifostine (WR-2721) and its polymer complexes. Results: The radioprotective efficacy of the polymer complexes constructed of amifostine (WR-2721) and poly(hydroxyoxyethylene phosphate)s with different molecular weights showed promising activity and dose regimens. Conclusions: Cytotoxicity studies for tested cell lines MEF *LIG4*$^{+/+}$ and MEF *LIG4*$^{-/-}$ cells showed that the polymer complexes were not toxic when equivalent doses of the drug amifostine (WR-2721) were applied to the cells. Irradiated MEF *LIG4*$^{+/+}$ cells demonstrated an increase in the surviving fraction when pre-treated with 0.5–5 mM polymer complexes when equivalent doses of amifostine (WR-2721) were applied to the cells and irradiated. The radioprotective efficacy had increased when the cells MEF *LIG4*$^{+/+}$ were irradiated with 12 Gy. These findings demonstrate that poly(hydroxyoxyethylene phosphate)s are suitable carriers of the radioprotective drug amifostine (WR-2721). They further suggest that they may be interesting for researchers seeking new challenges in discovering advanced radioprotective active substances.

Keywords: radioprotective efficacy; cytotoxicity; phosphorus-containing polymer complexes; amifostine (WR-2721); mouse embryonic fibroblasts (MEF)

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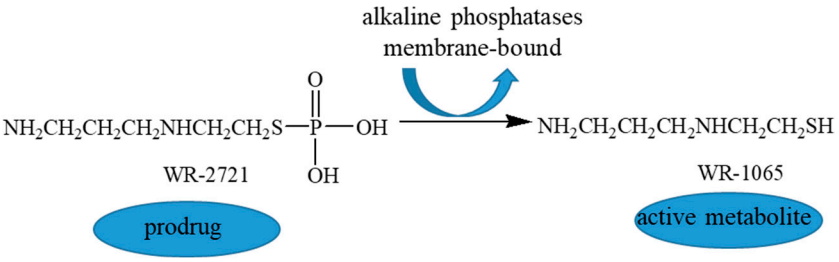
1. Introduction

The search for ideal radioprotectors with high efficacy and low toxicity is still receiving widespread attention in radiation medicine, as ionizing radiation poses an increasing threat to human health. Despite the significant progress have made in conventional radioprotectors, high toxicity and low bioavailability still discourage their application [1]. Compared with traditional radiation injury treatment strategies, radiation stimuli-responsive drug delivery systems have recently been attracted growing interest due to their timely response and excellent efficacy. When the intelligent response carrier materials have been irradiated by a specific dose of radiation, the polymer chains have broken, resulting in degradation and a rapid release of the therapeutic drug for the timely treatment of an injury [2]. In recent years, there has been grown research interest in radioprotectors such as amifostine

(WR-2721), especially with regard to their application in the treatment of human cancer in combination with ionizing radiation [3,4]. Amifostine (WR-2721) is a chemical radioprotector that has been found clinical application and extensive research. Several studies have been shown that amifostine (WR-2721) protects normal tissue from both acute and late radiation damage without protecting tumor tissues, i.e., amifostine (WR-2721) is a selective radioprotector of normal tissues [5–8]. Amifostine (WR-2721) has been usually administered intravenously before chemotherapy or radiotherapy and has been applied in the treatment of head and neck cancer. However, the inconvenient intravenous administration and its toxic side effects, such as hypotension, have severely limited its further clinical application. In order to reduce its toxicity and side effects, scientists are trying to develop a variety of drug administration methods and are devoted to developing a wide application of amifostine (WR-2721) in radiation protection [9]. Due to acidic hydrolysis and decomposition in the gastrointestinal tract, orally administered amifostine (WR-2721) loses its activity [10]. Before radiotherapy for the sick patient, intravenous injection circumvents the problem of hydrolysis; however, its daily administration increases patient discomfort [11]. Amifostine is used in radiotherapy and chemotherapy as a cytoprotective agent to reduce renal toxicity. Alkaline phosphatase can dephosphorylate amifostine to an active free thiol metabolite (WR-1065), which acts as an antioxidant. It is suggested that amifostine's selective cytoprotective effect towards normal tissues is achieved due to its high levels of alkaline phosphatase through DNA stabilization, free radical scavenging, and its regulation of the p53 protein (Scheme 1) [12].



Scheme 1. Molecular structures and enzyme degradation of amifostine (WR-2721) by alkaline phosphatases to active metabolite WR-1065.

Results have been obtained with oral administration of amifostine (WR-2721) immediately before irradiation of the small intestine, which is highly enriched in these activating alkaline phosphatases, resulting in the localized production of the radioprotective WR-1065 in the small intestine, providing protective benefits without significant systemic side effects. The results have been shown that orally administration of amifostine (WR-2721) as effective as intraperitoneal administration in promoting the survival of intestinal crypt clonogens after painful irradiation. Furthermore, orally administered amifostine (WR-2721) has provided complete radioprotection and survival after lethal upper abdominal irradiation of 12.5 Gy × 5 fractions (total 62.5 Gy, EQD2 = 140.6 Gy) [13]. These disadvantages can be overcome by using alternative strategies based on modern drug delivery techniques such as micro/nanoparticle formulations [14–17]. Radiation doses that can be delivered without causing significant damage to surrounding healthy tissue may be insufficient to kill tumor cells. Selective radioprotection is therefore expected to protect healthy tissue from amifostine (WR-2721) and allow for an increase in the dose delivered to the tumor. Despite research and significant progress in improving the efficacy of amifostine (WR-2721) as a radioprotector for acute radiation syndrome, none of the strategies have solved the problems of its toxicity and side effects [6].

One promising approach for improving the radioprotective characteristics of low molecular weight drugs that has already been approved and used in the clinic has been to

bound them to a polymer chain [18]. The design of such polymeric drug delivery systems by physical or chemical conjugation with a polymer offers new properties to drugs, which may include an increase in radioprotective efficacy. Polymer chemistry has contributed to the current progress in biochemistry, biology, medicine, and pharmacy, creating new, highly specific drugs. Synthetic polymer formulations are becoming increasingly attractive as delivery vehicles due to the great flexibility in terms of the degree of carrier loading, the type and size of the delivered bioactive molecules, and the immobilization techniques employed [19–24]. The integration of advanced materials into drug delivery systems has become the modern standard in pharmaceutical knowledge to address the complexity of modern medicine. Advanced materials have revolutionized drug delivery by improving upon conventional systems, and their application in various fields, including oncology and radiotherapy. Although these materials have shown great promise, there are still issues that need to be explored for their application as drug carriers [22].

In order to explore more effective radioprotective agents with minimal toxicity, a mitochondria-targeted nitronyl nitroxide radical with a triphenylphosphine ion and its nanoparticles has been recently synthesized. The protective effect of the nanoparticles against oxidative damage induced by X-ray irradiation has been measured in vitro and in vivo. The results have shown that these nanoparticles are not associated with obvious cytotoxicity to L-02 cells when the concentration is below 1.5×10^{-2} mmol. The nitronyl nitroxide radical nanoparticles with triphenylphosphine ion have been increased the survival rate of L-02 cells significantly at 2, 4, 6, and 8 Gy of X-ray radiation exposure; the survival rate of mice has been obtained highest after 6 Gy of X-ray irradiation [25].

Polyphosphoesters represent an innovative class of biodegradable polymers, with the phosphate ester serving as the core repeating unit of their polymeric backbone. Recently, the use of biomaterials derived from functionalized polyphosphoesters in biomedical applications has garnered significant interest because of their commendable biocompatibility, biodegradability, and the capacity for functional modification [26]. Poly(oxyethylene H-phosphonate)s are especially attractive as carriers of drugs and genes because of the presence of highly reactive H-P groups in the repeating elements, the relative ease of their preparation from commercially available building blocks, their low cytotoxicity, and their ability to be formed from nontoxic segments. Poly(oxyalkylene H-phosphonate)s have a number of advantages as the reactive H-P group in the repeat elements allows for the chemical immobilization of drugs under mild reaction conditions; because of their similarity to biomacromolecules such as nucleic acids, because they are water-soluble, they hold the possibility of a hydrophilic/hydrophobic balance; because their drug carrying capacity is not limited; because the presence of a highly polar O=P group in the repeating unit affords the possibility for the physical immobilization of drugs; because they can be regarded as degradable and biocompatible synthetic polymers; because they can be designed to have nontoxic building blocks; and, finally, because they can be administered over a wider molecular weight range as, after hydrolysis, the low molecular polyethylene glycol will be safely excreted. The most important potential advantage of polyphosphoesters is that they are easy to prepare on an industrial scale [27–34].

A major issue with polymers as drug carriers for medical applications is related to their biodegradability and the safety of their degradation products. In this regard, poly(ethylene glycol)-based polyphosphoesters are promising because they degrade to small molecules: poly(ethylene glycol) and phosphoric acid, which are known to be non-toxic and small enough for natural clearance mechanisms [35].

Efficient treatment requires that the efficacy of the drug be comparable to the extent of injury, so that drug release matches the amount of radiation damage. Therefore, some new

biocompatible, biodegradable and easily introduced functional groups of polymer carriers need to be used in biomedical applications [2].

The goal of the present study is to evaluate the cytotoxicity and radioprotective efficiency of polymer complexes constructed from amifostine (WR-2721) and poly(hydroxyoxyethylene phosphate)s with different molecular weights.

2. Materials and Methods

Polyethylene glycol with an average molecular weight of 600 $g \cdot mol^{-1}$ was purchased from Fluka (Buchs, Switzerland). It was dried prior to use by azeotropic distillation with toluene and a subsequent 4 h heating at 120 °C under dynamic vacuum. Dimethyl H-phosphonate (Fluka) was distilled prior to use. 1,3-Diaminopropane (99%), hydrobromic acid (48%) and ethylene sulfide (98%) were supplied from Aldrich (St. Louis, MO, USA). S-2-(3-aminopropylamino) ethanol was obtained from Fluka. The dichloromethane and carbon tetrachloride were dried and distilled before use, following standard procedures.

Cells: Mouse embryonic fibroblasts MEF *LIG4*$^{+/+}$ and MEF *LIG4*$^{-/-}$ cells were provided by Institute of Medical Radiobiology, University Hospital Essen, Germany.

An X-ray machine (GE-Healthcare (Madison, WI, USA)) operating at a distance of 50 cm, at 320 kV and 10 mA, with a 1.65 mm Al filter, a dose rate of approximately 1.3 Gy/min, and operating at room temperature was used to irradiate the cell cultures.

Statistics analyses: All cell experiments were conducted in triplicate as means ± standard deviation (SD). A difference with a two-sided p-value < 0.05 was considered statistically significant. Data comparison was achieved using t-tests. Plating efficiency (p.e.) equation was as follows: (number of colonies counted/number of cells seeded). The survivals were calculated through the surviving fraction equation: (number of colonies counted/(number of cells seeded × p.e.)) with standard error of the mean error bars.

2.1. Design of Phosphorus-Containing Polymer Complexes of Amifostine (WR-2721)

2.1.1. Obtaining Poly(oxyethylene H-phosphonate)s

The obtainment of the poly(oxyethylene H-phosphonate)s has been described in our previous paper. Briefly, the polymers were synthesized by polytransesterification reaction of dimethyl H-phosphonate with poly(ethylene glycol) with an average molecular weight of 600 Da (PEG 600) [33].

2.1.2. Obtaining the Poly(hydroxyoxyethylene phosphate)s

Quantitative conversion of poly(oxyethylene H-phosphonate)s into poly(hydroxyoxyethylene phosphate)s was achieved through an Atherton–Todd reaction using water as a reagent [33]. Poly(hydroxyoxyethylene phosphate)s were synthesized with three different molecular weights: 2000 Da (PHEP-2000); 5000 Da (PHEP-5000); and 9900 Da (PHEP-9900).

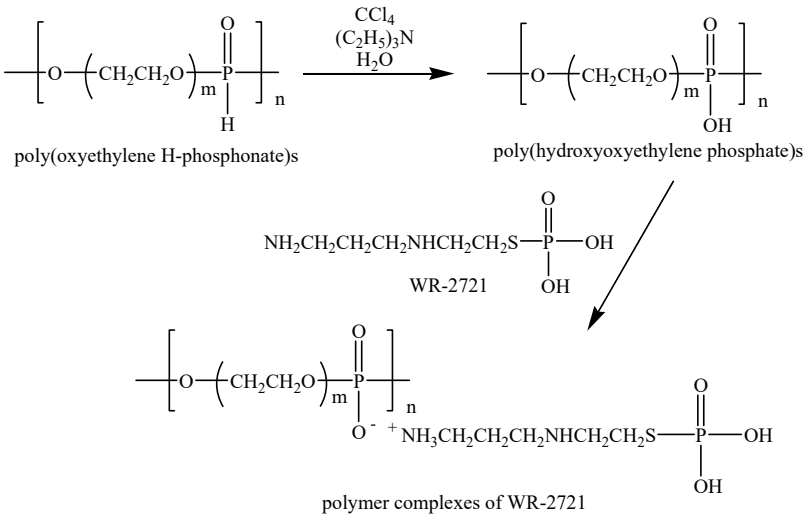
2.1.3. Obtaining Amifostine (WR-2721)

Amifostine (WR-2721) was synthesized in two steps following a previously described procedure [33]. The first step—synthesis of N-(bromoethyl)-1,3-diaminopropane dihydrobromide $C_5H_{15}Br_3N_2$ using 2-(3-aminopropylamino)ethanol and 48% hydrobromic acid—and the second step—synthesis of S-2-(3-aminopropylamino) ethylphosphorothioic acid dihydrate (amifostine, WR-2721) $C_5H_{19}O_5PS$.

2.1.4. Immobilization of Amifostine (WR2721) on Poly(hydroxyoxyethylene phosphate)s

The coprecipitation technique was explored for the immobilization of amifostine (WR-2721) on poly(hydroxyoxyethylene phosphate) for the formation of an ionic bind (Scheme 2) [33]. For the purposes of the study, three phosphorus-containing polymer complexes of amifostine (WR-2721) were obtained depending on the molecular weight of

the poly(hydroxyoxyethylene phosphate), named PHEP-2000/WR-2721; PHEP-5000/WR-2721; and PHEP-9900/WR-2721, respectively.



Scheme 2. Design of phosphorus-containing polymer complexes of amifostine (WR-2721).

2.2. Cells, Cell Culture Conditions and Irradiation

MEFs cells were cultured in Dulbecco's Modified Eagle Medium supplemented with antibiotics and 10% fetal calf serum in a humidified incubator supplied with CO_2 5%, at 37 °C. To maintain cells in an exponential growth state, all cell lines were subcultured every two to three days. Irradiation was carried out with an X-ray machine (GE-Healthcare) operated at 320 kV and 10 mA at a distance of 50 cm, with a 1.65 mm Al filter, a dose rate of approximately 1.3 Gy/min, and at room temperature.

2.3. Clonogenic Survival Assay

Cell cytotoxicity of the pure amifostine (WR-2721) and polymer complexes were determined by the clonogenic assay, 1×10^5 cells were seeded in 60 mm tissue culture dishes with 5 mL growth medium and incubated for 2 to 3 days. For the cytotoxicity study, these exponential growth phase cells were treated with 0, 0.5, 1, 2 and 5 mM pure amifostine (WR-2721) and its phosphorus-containing polymer complexes for 2 h and were trypsinized immediately at 37 °C. Cells were plated into 60 mm dishes, in duplicate, at various densities, aiming for approximately 100 colonies/dish. After one week to 10 days of incubation, cells were stained with crystal violet, and colonies containing approximately 50 cells or more were counted.

To investigate the radiosensitivity of cells, a similar experimental procedure was applied after their X-ray irradiation and their pre-treatment with pure amifostine (WR-2721) and their phosphorus-containing polymer complexes had been determined for 1 h.

3. Results

Figures 1 and 2 show that, for the two cell lines tested, amifostine (WR-2721) was less toxic and its phosphorus-containing polymer complexes (PHEP-2000/WR-2721; PHEP-5000/WR-2721; PHEP-9900/WR-2721) were not toxic when equivalent doses of amifostine (WR-2721) was applied to the cells.

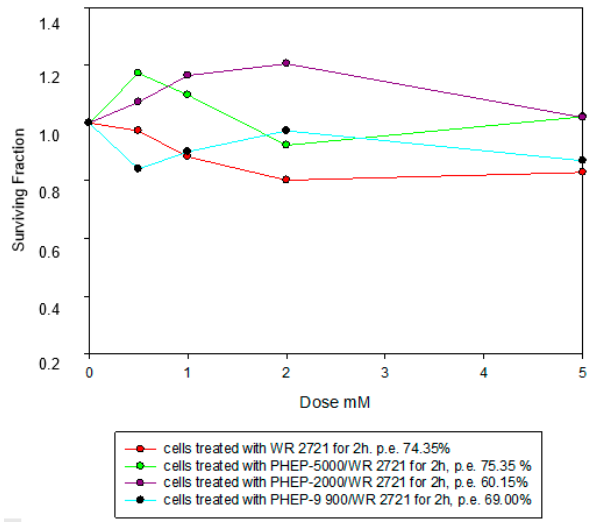


Figure 1. Cytotoxicity of amifostine (WR-2721) and its phosphorus-containing polymer complexes in MEF *LIG4*$^{+/+}$ cells ($n = 3$, $p < 0.05$, *t*-test).

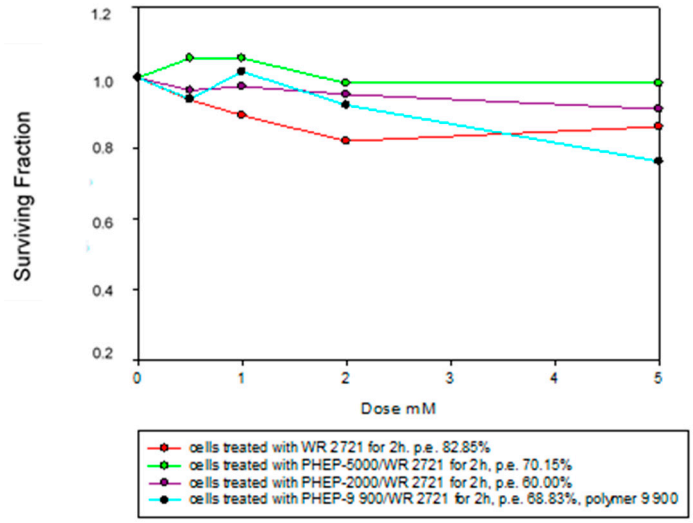


Figure 2. Cytotoxicity of amifostine (WR-2721) and its phosphorus-containing polymer complexes in MEF *LIG4*$^{-/-}$ cells ($n = 3$, $p < 0.05$, *t*-test).

Radioprotective Efficiency

Radioprotective efficacy has been studied with similar experimental procedures after X-ray irradiation of cells that had been pre-treated with pure amifostine (WR-2721) and polymer complexes for 1 h before irradiation via the measurement of cell survival by colony formation. Because of the different radiosensitivities of MEF *LIG4*$^{+/+}$ and MEF *LIG4*$^{-/-}$ cells, varied doses were applied for two cell lines, aiming for similar survival levels. Therefore, 6 and 12 Gy of X-ray were used to irradiate cell line MEF *LIG4*$^{+/+}$ (Figures 3 and 4), and 2 and 6 Gy of X-ray were used to irradiate cell line MEF *LIG4*$^{-/-}$ (Figures 5 and 6).

Irradiated MEF *LIG4*$^{+/+}$ cells demonstrated an increase in the surviving fraction when pretreated with 0.5–5 mM polymer complexes for 1 h, given equivalent doses of amifostine (WR-2721) and when they were irradiated with 6 Gy. This radioprotective efficacy was increased when the same cells were irradiated with 12 Gy (Figures 3 and 4).

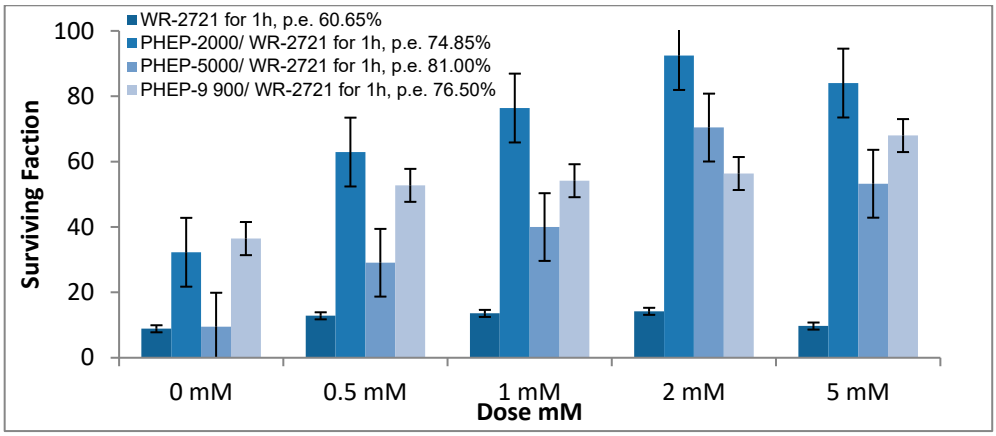


Figure 3. Radiation dose-survival of MEF *LIG4*$^{+/+}$ cells irradiated with 6 Gy in the presence of amifostine (WR-2721) and its phosphorus-containing polymer complexes ($n = 3$, mean ± SD, $p < 0.05$, *t*-test).

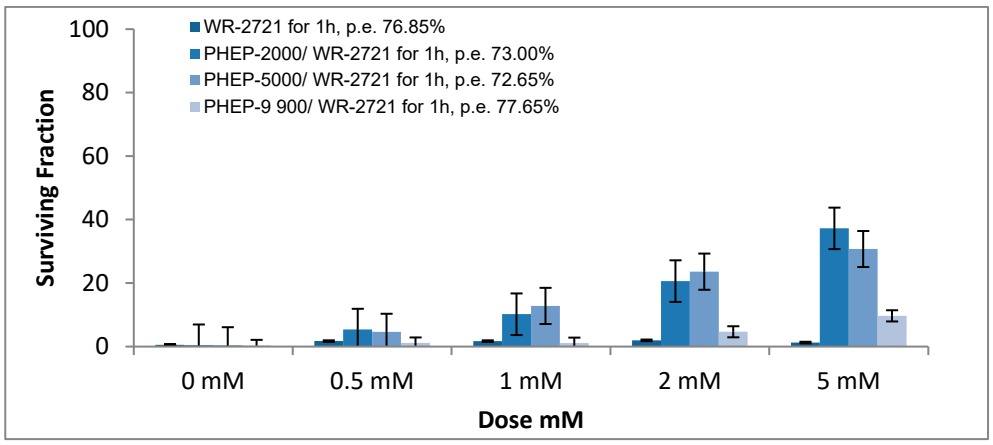


Figure 4. Radiation dose-survival of MEF *LIG4*$^{+/+}$ cells irradiated with 12 Gy in the presence of amifostine (WR-2721) and its phosphorus-containing polymer complexes ($n = 3$, mean ± SD, $p < 0.05$, *t*-test).

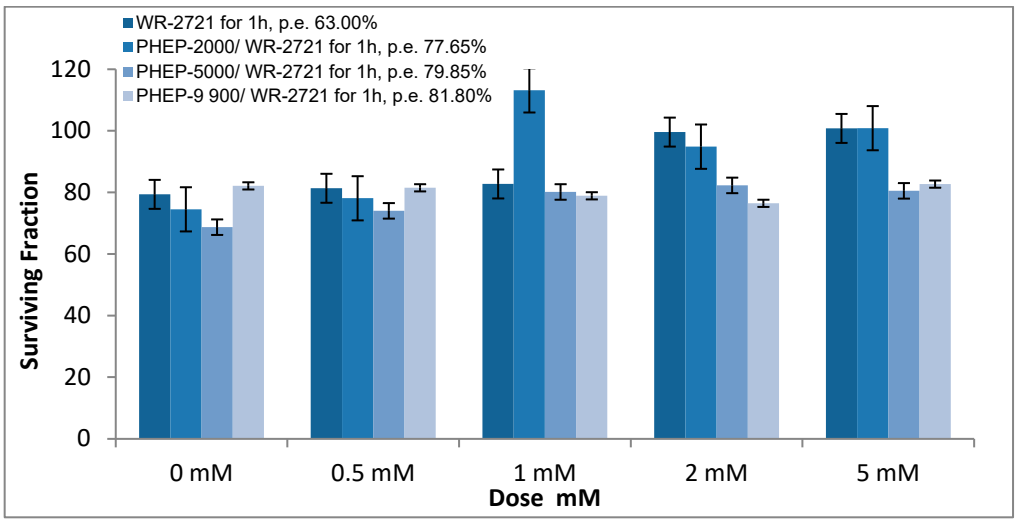


Figure 5. Radiation dose-survival of MEF *LIG4*$^{-/-}$ cells irradiated with 2 Gy in the presence of amifostine (WR-2721) and its phosphorus-containing polymer complexes ($n = 3$, mean ± SD, $p < 0.05$, *t*-test).

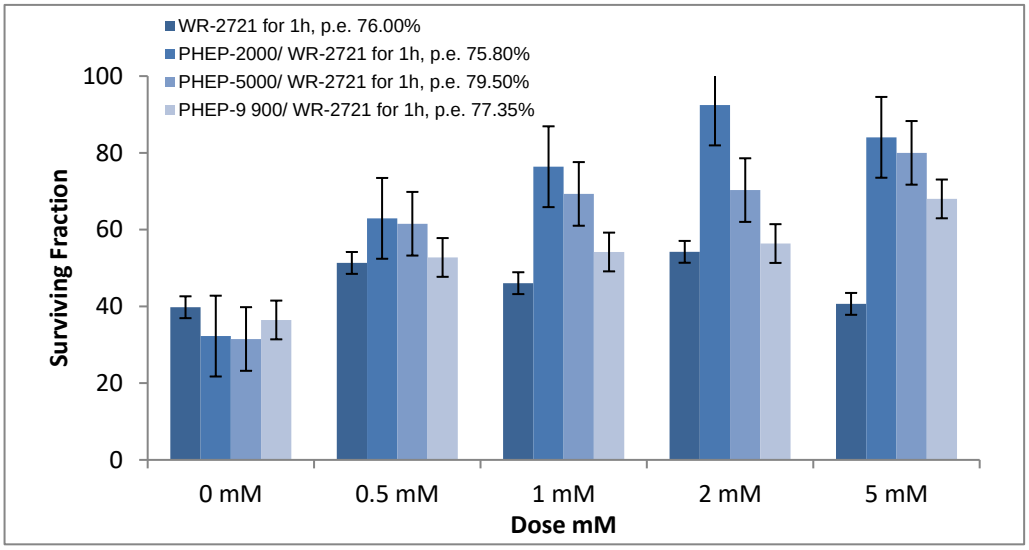
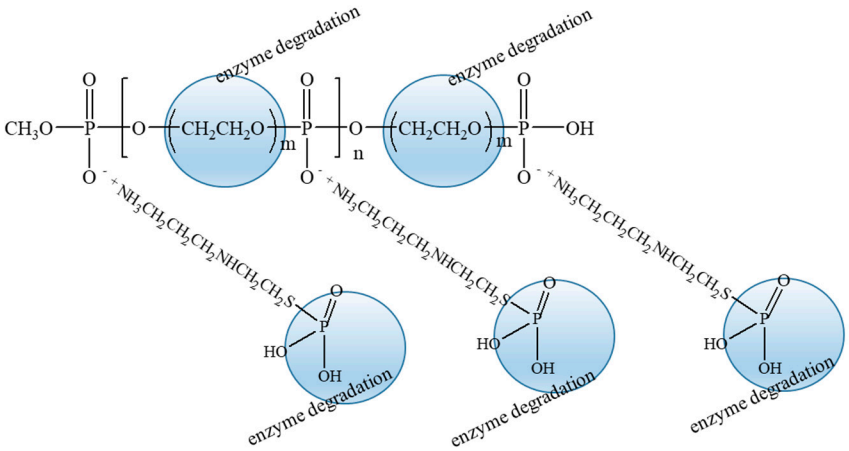


Figure 6. Radiation dose-survival of MEF *LIG4*$^{-/-}$ cells irradiated with 6 Gy in the presence of amifostine (WR-2721) and phosphorus-containing polymer complexes ($n = 3$, mean ± SD, $p < 0.05$, *t*-test).

4. Discussions

The basic strategy for the study was the immobilization of the amifostine (WR-2721) onto poly(hydroxyoxyethylene phosphate)s with different molecular weights to monitor the radioprotective efficiency of the polymer complexes as a function of the molecular weight of the carrier (Scheme 3). For this purpose, polymers with different molecular weights were synthesized as follows: poly(hydroxyoxyethylene phosphate) 2000 Da (PHEP-2000); 5000 Da (PHEP-5000); and 9900 Da (PHEP-9900).



Scheme 3. Mechanistic insights for enzyme degradation of phosphorus-containing polymer complexes of amifostine (WR-2721).

The first step of the research was to determine the cytotoxicity of the synthesized amifostine (WR-2721) and the three obtained phosphorus-containing polymer complexes on the tested cell lines MEF *LIG4*$^{+/+}$ and MEF *LIG4*$^{-/-}$. The cytotoxicity of the polymer carrier is an important factor related to its practical application. The obtained results show low cytotoxicity of the synthesized amifostine (WR-2721) and almost no cytotoxicity for all three phosphorus-containing polymer complexes (Figures 1 and 2). The reason for the lack of cytotoxicity of the phosphorus-containing polymer complexes is most likely due to the type of polymer carrier. Polymers with repeating phosphorus-ester bonds (P-O-C) in the main chain are biocompatible and have structural similarity to biomacromolecules

such as nucleic acids; therefore, they are of exceptional interest for medicine. These polyphosphoesters are biodegradable by hydrolysis or by the enzymatic cleavage of the phosphoester bonds. Among these, poly(alkylene H-phosphonates) are the most attractive class of organophosphorus polymers for pharmacy. They are promising candidates for polymeric drug carriers, as they possess very useful properties such as a suitable functional group for binding drug substances at room temperature; good solubility in aqueous media; the presence of a polar phosphoryl group (P=O), determining the possibility of physical immobilization; the possibility of the immobilization of a low-molecular and biologically active substance to each repeating structural unit of the polymer; its accessibility and ease of preparation; and its biodegradability, biocompatibility, and low toxicity [32].

The second step of the research was the evaluation of the radioprotective efficacy of the synthesized amifostine (WR-2721) and its phosphorus-containing polymer complexes. To evaluate the radioprotective efficacy of the radioprotector and of the application of its phosphorus-containing polymer complexes to the tested cell lines MEF *LIG4*$^{+/+}$ and MEF *LIG4*$^{-/-}$, different doses were used for the two cell lines, aiming at similar survival rates. Therefore, 6 and 12 Gy of X-ray radiation were used for MEF *LIG4*$^{+/+}$ cells (Figures 3 and 4), and 2 and 6 Gy of X-ray radiation were used for MEF *LIG4*$^{-/-}$ cells (Figures 5 and 6).

The obtained results showed that radioprotection was increased to 6.5 times when MEF *LIG4*$^{+/+}$ cells were irradiated with 6 Gy (Figure 3) and treated with polymer complex PHEP-2000/WR-2721 at a concentration of 2 mM compared with pure amifostine (WR-2721). The highest radiation protection efficiency, 8.5 times, was observed when MEF *LIG4*$^{+/+}$ cells were treated with PHEP-2000/WR-2721 at a concentration of 5 mM when compared with pure amifostine (WR-2721).

For the same cell line, MEF *LIG4*$^{+/+}$, under the same experimental conditions but irradiated with 12 Gy (Figure 4), all phosphorus-containing polymer complexes were again found to have higher radioprotective efficacy than pure amifostine (WR-2721). Radioprotection was increased to 29.5 times when compared with pure amifostine (WR-2721) when MEF *LIG4*$^{+/+}$ cells were treated with polymer complex PHEP-2000/WR-2721 at a concentration of 5 mM and irradiated with 12 Gy.

The polymer complexes of amifostine (WR-2721) showed radioprotective efficacy similar to, or slightly lower than, pure amifostine (WR-2721) when MEF *LIG4*$^{-/-}$ cells were irradiated with 2 Gy (Figure 5). The results were shown that the radiation dose of 2 Gy was low for this cell line, and that, in the presence of radioprotective drugs, the survival factor was high.

Radioprotection was increased 1.7 times when MEF *LIG4*$^{-/-}$ cells were irradiated with 6 Gy (Figure 6) using the polymer complex PHEP-2000/WR-2721 at a concentration of 2 mM compared with pure amifostine (WR-2721). The highest radiation protection efficiency, 2 times, was observed when MEF *LIG4*$^{-/-}$ cells were pretreated with the same polymer complex, PHEP-2000/WR-2721, at a concentration of 5 mM compared with pure amifostine (WR-2721).

5. Conclusions

The main objective of this study was to investigate the cytotoxicity and radioprotective efficacy of phosphorus-containing polymer complexes of amifostine (WR-2721) with different molecular weights on mouse embryonic fibroblasts MEF *LIG4*$^{+/+}$ and MEF *LIG4*$^{-/-}$ cells irradiated with 2, 6, and 12 Gy. The obtained results for the evaluation of cytotoxicity showed that all three polymer complexes of amifostine (WR-2721) had weak or almost no toxicity to both cell lines at concentrations up to 5 mM of the active substance. To evaluate the radioprotective efficacy of the synthesized amifostine (WR-2721) and the polymer complexes, both cell lines, MEF *LIG4*$^{+/+}$ and MEF *LIG4*$^{-/-}$ were treated at con-

centrations of 0.5, 1, 2, and 5 mM relative to the active radioprotective drug amifostine (WR-2721) and irradiated with 2, 6, and 12 Gy. The obtained results have shown that all three phosphorus-containing polymer complexes of amifostine (WR-2721) have radioprotective efficacy. The best protective efficacy for all concentrations of the active compound has a polymer complex with a 2000 Da molecular weight PHEP-2000/WR-2721.

The most important conclusion from the study is that phosphorus-containing polymers are suitable carriers of the radioprotective drug amifostine (WR-2721), as the investigated complexes showed no cytotoxicity to MEF *LIG4*$^{+/+}$ and MEF *LIG4*$^{-/-}$ cells and exhibited increased radioprotective efficacy when compared with pure amifostine (WR-2721).

Author Contributions: Methodology, I.T.; formal analysis, I.T.; investigation, I.T.; data curation, D.U.; writing—original draft preparation, I.T. and D.U.; supervision, I.T. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

The following abbreviations are used in this manuscript:

cm	Centimeter
Da	Dalton
Gy	Gray, units used to measure the amount of radiation absorbed
h	Hour
kV	Kilovoltage
mA	Milliamperage
MEF	Mouse embryonic fibroblasts
min	Minute
mM	Millionaire
mm	Millimeter

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