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Article in Journal of Medicinal Chemistry · April 2016
DOI: 10.1021/acs.jmedchem.5b01628

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Two New Faces of Amifostine: Protector from DNA Damage in Normal Cells and Inhibitor of DNA Repair in Cancer Cells

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ABSTRACT: Amifostine protects normal cells from DNA damage induction by ionizing radiation or chemotherapy, whereas cancer cells typically remain unaffected. While confirming this phenomenon, we have revealed by comet assay and currently the most sensitive method of DNA double strand break (DSB) quantification (based on γH2AX/53BP1 high-resolution immunofluorescence microscopy) that amifostine treatment supports DSB repair in γ-irradiated normal NHDF fibroblasts but alters it in MCF7 carcinoma cells. These effects follow from the significantly lower activity of alkaline phosphatase measured in MCF7 cells and their supernatants as compared with NHDF fibroblasts. Liquid chromatography–mass spectrometry confirmed that the amifostine conversion to WR-1065 was significantly more intensive in normal NHDF cells than in tumor MCF cells. In conclusion, due to common differences between normal and cancer cells in their abilities to convert amifostine to its active metabolite WR-1065, amifostine may not only protect in multiple ways normal cells from radiation-induced DNA damage but also make cancer cells suffer from DSB repair alteration.

1. INTRODUCTION

Amifostine (ethanethiol, 2-[(3-aminopropyl)amino]dihydrogen phosphate), also known as WR-2721, is an organic thio-phosphate agent; it is rapidly dephosphorylated by alkaline phosphatase (ALP) at the cell surface of healthy tissues, giving rise to its clinically active metabolite, WR-1065.1−3 When activated, amifostine protects cells from radiation- and chemotherapy-induced DNA damage, mostly by competing with oxygens and preventing their interactions with DNA radicals and donating hydrogen to repair the already existing DNA damage.4−8 Currently, amifostine is the only radio-protective drug approved for clinical use.

Concerning the practical application of amifostine in human medicine, a key role has been ascribed to its differential effect on cancer and normal cells, respectively: Whereas in normal cells or tissues amifostine clearly acts as a radio- and chemoprotective agent, this property of the drug is lost in cancer cells.6−12 This cell type-specific behavior of amifostine has been largely attributed to low levels of ALP in cancer cells as compared with normal cells,13 however, the situation is still not that clear because a variety of human cancers ectopically express high levels of ALP, thus leading some scientists to the (opposite) suggestion that ALP might be critically involved in tumor development.14−16 Indeed, a comprehensive comparison of ALP mRNA and protein expression and activity in cancer cells in the literature is missing. This uncertainty points to the caution with which each model of disease (e.g., different cell types) should be tested.

In addition, it is still not obvious how the four main classes of ALP (tissue nonspecific TNAP, intestinal IAP, placental PLAP, and placental-like GCAP)15 participate in amifostine conversion in various normal and especially cancer cells where expression of ALP isoenzymes may be altered.17 ALP genes are also highly inducible by many agents.15 Finally, though covalently anchored to the outer surface of the plasma membrane,16 ALP can be released into the serum (or extracellular medium) by the GPI-dependent phospholipase D under stress and some medical conditions, such as cancer. While previous reports showed that the membrane-bound ALP...
has different enzymatic kinetics and molecular properties as compared to the soluble enzyme, the question remains of how this finding is reflected in amifostine metabolism in normal and cancer tissues.

The most serious radiation-induced DNA damage is the double strand break (DSB) that causes a loss of the DNA molecule integrity. As DSB formation is also modulated by amifostine, DSBs represent the most relevant type of DNA lesions in the context of amifostine-mediated cell radioprotection and radiosensitization. Molecular events following the ionizing radiation-induced DNA breakage include an immediate phosphorylation of histone H2AX (ser139), which can be nowadays used to immunologically visualize the extent of DSB damage induction in intact cells as the so-called γH2AX foci within minutes after the DSB induction. On the other hand, assessment of γH2AX foci disappearance during the postirradiation (PI) time allows monitoring of DSB repair.

In this work, we advantageously employ this method, combined with a high-resolution confocal microscopy, to quantify currently with the maximum sensitivity the induction and processing of DSBs in intact (3D-preserved) cells (Figure 1) irradiated with γ-rays in the presence or absence of amifostine or WR-1065. To further increase the fidelity of DSB evaluation, we simultaneously detect γH2AX together with 53BP1 DSB repair protein that colocalizes with γH2AX foci both in very early and late periods of time PI. To our best knowledge, this study is the first to use this approach for exploring the effects of amifostine on the DSB damage induction and repair.

We compare the effects of amifostine and its clinically active metabolite WR-1065 in two human cell lines, normal dermal fibroblasts (NHDF) and breast adenocarcinoma cells (MCF7). MCF7 cells were selected due to a high prevalence of breast cancer while NHDFs were chosen because of their inevitable direct irradiation during the classical radiotherapy (especially with γ- or X-rays).

To explore whether different cellular and/or extracellular levels of ALP in fibroblasts and MCF7 cells may explain the cell type-specific effects of amifostine on DSB induction and repair, we used currently the most sensitive method to detect DNA double strand breaks (DSBs) and monitored their induction and consequent repair during the postirradiation (PI) time in γ-irradiated normal NHDF fibroblasts and cancer MCF7 cells treated with amifostine and WR-1065 radioprotective drugs, respectively. The results of our approach are demonstrated in Figure 1. γH2AX and 53BP1 foci, markers of DSBs, were immunodetected in the cell nuclei of spatially fixed cells (i.e., cells with the structure of nuclei preserved in 3D space by paraformaldehyde) and visualized for scoring by high-resolution confocal microscopy. Figure 1A shows the maximum images of an illustrative nucleus computationally reconstructed from forty 0.2 μm-thick confocal slices in all three planes. Figure 1B then displays a single confocal slice through the same nucleus in x-y, x-z, and y-z planes (indicated by dashed lines); γH2AX, 53BP1, and chromatin stainings are presented overlaid (top-left image) and separately for each of these signals (remaining images). It is evident from the panels A and B that almost all individual γH2AX/53BP1 foci can be easily distinguished in space and precisely identified. For the analyses, we considered as DSB only γH2AX foci colocalizing with 53BP1 and fulfilling the criterion of a minimum size, arbitrarily determined separately for each period of time PI. Immediately after irradiation (5 min PI), we obtained the average numbers of DSBs per nucleus per Gray (Figures 2 and 3) that were comparable with the already reported results obtained in a research context different from that followed in this study with a similar method of DSB foci detection.

Our earlier results demonstrated, both for NHDFs and MCF7 cells studied in this work, that a 5 min period after irradiation is sufficient to develop 53BP1 foci and their colocalization with γH2AX, detectable by high-resolution confocal microscopy. This is in agreement also with several other reports.

2. RESULTS

2.1. Codetection of γH2AX and 53BP1 Foci by Immunofluorescence High-Resolution Confocal Microscopy in Spatially (3D) Preserved Cells To Monitor DSB Induction and Repair. The response of normal and cancer cells to amifostine and WR-1065 was analyzed by various approaches in the past, usually in terms of DNA damage formation; however, the repair of DNA lesions has not been systematically studied yet. In this work, we used currently the most sensitive method to detect DNA double strand breaks (DSBs) and monitored their induction and consequent repair during the postirradiation (PI) time in γ-irradiated normal NHDF fibroblasts and cancer MCF7 cells treated with amifostine and WR-1065 radioprotective drugs, respectively.

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2.2. Immediate Radioprotection by Amifostine and Its Effect on DSB Repair in Irradiated Normal NHDF Fibroblasts and MCF7 Cancer Cells. Figure 2 summarizes the median numbers of colocalizing γH2AX and 53BP1 foci per nucleus in normal NHDF fibroblasts and MCF7 cancer cells irradiated with a dose of 1 Gy of γ-rays and treated or not treated with 4 mM amifostine 15 min before irradiation. In both cell types, material samplings were performed 5, 60, and 120 min PI. The first time interval reflected the extent of the radiation-induced damage “immediately” after the irradiation, and the other two allowed us to quantify the efficiency and kinetics of DSB repair processes. Panel A in Figure 2 shows the maximum images (see Experimental Section) of illustrative
nuclei of NHDF and MCF7 cells for each treatment and the first two periods of time PI analyzed (data for 120 min PI are presented and discussed in more detail later in section 2.3, Figure 5). In normal NHDF fibroblasts, amifostine induced a significant ($P < 0.001$) decrease in the median numbers of $\gamma$H2AX/53BP1 foci per nucleus in all the time intervals, for 5 and 60 min PI, namely up to 75.8% and 61.9% of the values in untreated cells, respectively. The repair efficiency determined as the difference between the median DSB values at 5 and 60 min PI (Figure 4) corresponded to 7 DSBs in cells irradiated in the plain medium and 8 DSBs in cells irradiated in the medium supplemented with amifostine. This result reflects an ongoing DSB repair slightly accelerated (about 10%) by amifostine (and/or its active metabolite WR-1065, discussed later; see Figure 4).

On the other hand, cancer MCF7 cells provided a totally different picture as compared with normal fibroblasts. Amifostine did not significantly decrease the median numbers of $\gamma$H2AX/53BP1 foci per nucleus in MCF7 cells at 5 min PI.

Figure 2. Numbers of ionizing radiation-induced repair foci ($\gamma$H2AX, green; 53BP1, red) demonstrating the effects of amifostine on the extent of DSB induction (5 min PI) and efficiency of DSB repair (60 min PI) in normal human skin fibroblasts (NHDF) and mammary carcinoma cells (MCF7). A. Maximum images composed of 40 confocal slices (0.2 $\mu$m thick) through spatially (3D) fixed cells by paraformaldehyde are shown; chromatin counterstaining with TO-PRO-3 (artificial blue). B. Median DSB values with standard errors; statistical significance determined by the Mann–Whitney U test is indicated (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

Figure 3. Numbers of ionizing radiation-induced repair foci ($\gamma$H2AX, green; 53BP1, red) demonstrating the effects of WR1065 on the extent of DSB induction (5 min PI) and efficiency of DSB repair (60 min PI) in normal human skin fibroblasts (NHDF) and mammary carcinoma cells (MCF7). A. Maximum images composed of 40 confocal slices (0.2 $\mu$m thick) through spatially (3D) fixed cells by paraformaldehyde are shown; chromatin counterstaining with TO-PRO-3 (artificial blue). B. Median DSB values with standard errors; statistical significance determined by the Mann–Whitney U test is indicated (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

Figure 4. Kinetics of DSB repair in irradiated (1 Gy of $\gamma$-rays; 1 Gy/min) NHDF and MCF7 cells treated or not with 4 mM amifostine or WR-1065 for 15 min prior to irradiation. The graph shows average fractions of $\gamma$H2AX/53BP1 foci persisting in cells at 60 min PI, determined as % of corresponding values measured at 5 min PI. Mean values with SEM are shown.

(Figure 2). In addition, as we report here for the first time for $\gamma$-irradiated intact cells, a significantly ($P < 0.001$) higher number of the foci (137.5% of the value in untreated cells) surprisingly
2.3. Immediate and Later Effects of Amifostine Metabolite WR-1065 in Irradiated Normal NHDF Fibroblasts and MCF7 Cancer Cells. In the next step, we used the same experimental approach to reveal the effects of an isomolar concentration of WR-1065 (Figure 3), an amifostine metabolite that is supposed to mediate the radioprotective effects of amifostine. Unlike in cells treated with amifostine, WR-1065 in the cultures reduced the numbers of γH2AX/53BP1 foci per nucleus in both normal and cancer cell types and both the time intervals of 5 and 60 min PI. The percentage decreases produced by WR-1065 for MCF7 cells amounted to 75.0% (P < 0.001) and 82.9% (P < 0.05) at 5 min PI and 60 min PI, respectively; for NHDF fibroblasts, these values corresponded to 65.2% (P < 0.05) and 50.0% (P < 0.001) at 5 min PI and 60 min PI, respectively.

Altogether, the immediate (5 min PI) and late (60 min PI) response to the irradiation was comparable for normal NHDF and cancer MCF7 cells only in cultures incubated with WR-1065 (Figure 3). For amifostine, the response basically depended on the normal or cancer cell status. Though amifostine at 5 min PI induced a response similar to that of WR-1065 (radioprotection) in the case of NHDF fibroblasts, we observed no effect of this prodrug in MCF7 cells (Figure 2). At 60 min PI, as compared with the averaged value for untreated irradiated controls, we observed a small acceleration (about 9%) of DSB repair in NHDF fibroblasts incubated with amifostine (Figure 4). On the other hand, the speed of repair markedly decreased (−20.7%) in cancer MCF7 cells relative to the average control. WR-1065 then also speeded up (17.7%) DSB repair in NHDF fibroblasts relative to the averaged untreated control but exerted slightly opposite (−7.7%) changes in MCF7 cells.

Figure 5 shows how DSB foci continued until 120 min PI in NHDF and MCF7 cells irradiated with 1 Gy of γ-rays and treated or not treated with amifostine, as described previously. As follows from both the inserted images and graphs showing the distributions of γH2AX/53BP1 foci per nucleus, amifostine supported DSB repair in normal NHDF fibroblasts while it had the opposite influence on cancer MCF7 cells. The differences from the untreated cells were statistically significant in both cell types though the enhancement of DSB repair in NHDF fibroblasts was less prominent (P = 0.19) than its deceleration in MCF7 cells (P < 0.001). The repair of untreated NHDF and MCF7 cells proceeded with similar efficiency (P = 0.579).

2.4. DSB Induction and Repair Analyzed by Comet Assay. To verify our results obtained above with γH2AX/53BP1 foci immunostaining, we quantified DSB induction and repair in irradiated normal NHDF and cancer MCF7 cells treated or not treated with amifostine or WR-1065 also by the comet assay, the method accepted in radiobiology as a “gold standard” for this purpose. While the comet assay provides a more direct view on DNA breakage and rejoining than γH2AX/53BP1 foci immunodetection, it suffers from lower sensitivity. Therefore, we irradiated the cells with 6 Gy of γ-rays (1 Gy/min) instead of 1.0 Gy used in the immunodetection experiments.

As demonstrated by Figure 6 and Figures S1-A and S1-B in Supporting Information, the measurements of comet tail moments (TM = L(T) × IF(T)/100, where L(T) = length of the tail and IF(T) = the fluorescence intensity of the tail given as a percentage; Figure 6B) well correlated at all the periods of time postirradiation with the results that followed from γH2AX/53BP1 foci immunodetection. Shortly (5 min) after irradiation, WR-1065 markedly decreased DSB induction in NHDF (p < 0.01) and MCF7 (p < 0.001) cells but amifostine only protected the first cells (p < 0.01 for NHDF vs p = 0.553 for MCF7). In later periods of time PI (60 and 120 min PI)
amifostine and WR1065 supported DSB repair in normal NHDF cells (p < 0.001 for amifostine, p < 0.01 for WR1065) but decreased the kinetics of this process in MCF7 cancer cells. When compared as a difference between the average tail moments of untreated irradiated controls and cells treated with amifostine or WR1065, the delay of DSB repair was significant (p < 0.001) for amifostine in both periods of time but insignificant (P = 0.722, 60 min PI; P = 0.063, 120 min PI) for WR1065. This lack of significance in case of WR1065 in MCF7 cells clearly follows from the "averaging" of the two opposite effects observed with this drug in this cell type: the slower repair kinetics and the (already described) lower induction of DSBs at 5 min PI (p < 0.001) in MCF7 cells treated with WR1065 but not amifostine (Figure 6). Hence, the repair in MCF7 cells treated with WR-1065 proceeds with a similarly slower kinetics to that observed for cell cultures with amifostine. The obtained results agreed well with both analytical strategies used in this work (see Experimental Section, section 4.6.2): the high-throughput analysis of "low-resolution" comets (Figures S1-A and S1-B) and the comparison of detailed comets acquired with a high resolution (63x oil immersion lens; Figure 6A).

Our results coming from DNA comet measurements and γH2AX/53BP1 foci counting thus correspondingly support the hypothesis that while amifostine administration protects normal cells from radiation damage through its metabolite WR-1065, it is rather toxic for cancer cells, where it negatively influences DSB repair. Moreover, WR-1065 in normal cells has a double-positive effect; it protects them from radiation-induced DSB lesions and consequently supports their repair.

2.5. Activity of ALP in Cell Lysates of Normal NHDF Fibroblasts and MCF7 Cancer Cells and in Their Extracellular Environment. To confirm that our observations described above correlate with the metabolic conversion of amifostine to WR-1065, we determined the activity of alkaline phosphatase (ALP) in NHDF and MCF7 cells. To reveal a potential importance of ALP localization, we compared the enzyme activity in both the cells themselves (the membrane-bound ALP) and in the supernatants of their cultures (excreted ALP). This approach was used because of uncertainty in the literature about some data showing that rather extracellular ALP is responsible for the conversion of amifostine to WR-1065 in in vivo conditions. The results are summarized in Figure 7. It is evident that when we measured the ALP activity in the cells themselves, its value in the normal NHDF cells was 124.8% of that in the tumor MCF7 cells (P < 0.01); when determining its values in the supernatants, the ALP activity in NHDF cells was 148.9% of that in MCF7 cells (P < 0.001).

Next, we addressed (ALP-mediated) conversion of amifostine to WR-1065 in NHDF and MCF7 cells directly by quantifying the ratio of these compounds in cells and cell supernatants by liquid chromatography–mass spectrometry (LC-MS/MS). Figure 8A proves a high percentage (>90%) of amifostine to WR-1065 in in vivo conditions.37 The results are
WR-1065 in both NHDF and MCF7 cells directly treated with this compound (4 mM, 37 °C, pH 7.1, at the beginning of incubation) and at both periods of time studied, i.e. at 20 and 75 min after the drug administration. Figure 8B then compares percentages of WR-1065 in cell cultures incubated with 4 mM amifostine under the same experimental conditions. It is evident from the figure that the levels of WR-1065, the amifostine metabolite, are substantially higher in normal NHDF fibroblasts and their culture supernatants than in MCF7 cells and their culture supernatants, both at 20 min (2.56× for the cells; 1.62× for the supernatants) and 75 min (1.47× for the cells; 2.17× for the supernatants) of incubation.

WR-1065 in both NHDF and MCF7 cells after 20 min incubation (C); total-ion chromatogram of MCF7 cells in plain DMEM medium (D).

Figure 8C shows illustrative LC-MS/MS total-ion compound chromatograms for amifostine and WR-1065 in NHDF and MCF7 cells after 20 min incubation. It should be noted that the chromatograms show the raw data before their correction for cell concentrations and vitalities. As determined by flow cytometry, the concentration of MCF cells was 2.1 times higher as compared with NHDF fibroblasts, and this ratio was 1.8 when only nonapoptotic cells were considered (the later conversion factor was used to obtain Figure 8A,B). The total-ion chromatogram of MCF7 cells in plain DMEM medium shown in Figure 8D (with x-axis resolution increased by several orders of magnitude as compared with Figure 8C) revealed no signals in amifostine and WR-1065 positions.

The LC-MS/MS results thus correlate with our colorimetric measurements on ALP activity (Figure 7) and directly prove conversion of amifostine to WR-1065, which is high in NHDF cells and their supernatants and substantially lower (but still measurable) in MCF7 cells and MCF7 cells. The lower level of WR-1065 in NHDF cells observed for the 75 min treatment as compared with the 20 min treatment probably reflects WR-1065 decomposition into its metabolites (see Discussion).

2.6. Transcription Levels of ALP Isoenzyme Genes in Normal NHDF Fibroblasts and MCF7 Cancer Cells. At least four main classes of ALP, namely tissue nonspecific ALP (TNAP), intestinal ALP (IAP), placental ALP (PLAP), and
placental-like ALP (GCAP), have been distinguished, but their involvement in amifostine conversion in normal and cancer cells remains to be systematically studied. The expression patterns of ALP isoenzymes may be altered in cancer cells, and ALP genes are highly inducible by a variety of agents. Hence, in addition to the overall ALP activity and WR-1065 levels in cells and supernatants of their cultures, we also determined mRNA expression for individual ALP isoenzymes in both the tumor MCF7 and normal NHDF cells. The values for TNAP, IAP, and PLAP are shown in Figure 9; the GCAP isoenzyme mRNA expression was too low to allow for quantification. Unexpectedly and in contrary to the ALP activity, the mRNA expression levels of all three quantifiable isoenzymes including IAP, which has been suggested as predominantly responsible for the conversion of amifostine to WR-1065, were always higher in MCF7 cells than in NHDF cells, the differences being significant (P < 0.05) in the case of the TNAP and IAP isoenzymes.

3. DISCUSSION AND CONCLUSIONS

3.1. Mysterious Amifostine as a Still Controversial Paradigm in Radiotherapy and Radioprotection. Cancer therapy always balances on a sharp edge between the killing of the tumor and the survival of (critically important) adjacent normal tissues. Chemical compounds specifically interacting with altered biochemical and genetic processes in cancer cells in the way they support cancer cell eradication while leaving normal cells uninfluenced or even protected may thus significantly shift therapeutic outcomes to benefits. However, there is still only one radioprotectant fulfilling these criteria that has been approved for clinical use: amifostine (see ref 41 for a comprehensive review).

Not surprisingly, amifostine is the best characterized radioprotective agent; however, reassessment of its mechanism of action by newly emerged molecular-genetic methods brought about interesting findings, showing that biological effects of amifostine are more complex and difficult to explain than previously thought. This is documented by a number of conclusions published in the literature on various aspects of amifostine and WR-1065 and on the activities of their metabolites (e.g., ref 42 and citations therein; comprehensively reviewed in ref 41). Hence, our better understanding of the amifostine paradigm may pave the way for a more efficient search of new selective radiomodifiers, so needed in light of the still increasing incidence of cancers. Current reports and also our results discussed here suggest that amifostine could be (at least) in some therapeutic circumstances more advantageous than it seemed previously. On the other hand, some discoveries warn that the clinical use of amifostine might also be counterproductive. For instance, Andreassen et al.33 demonstrated undesirable radioprotection of some tumors upon amifostine treatment. Thus, what remains unclear is the phenomenon of why amifostine selectively protects normal cells, and some transformed cells while it does not eliminate the cytotoxicity delivered by ionizing radiation to other cancer cells.

3.2. Radioprotection versus Radiosensitization by Amifostine in Normal and Cancer Cells. With the use of two independent methods, the comet assay and γH2AX/53BP1 foci immunofluorescence microscopy, we systematically compared under the same conditions the effects of amifostine and its active metabolite WR-1065 on the induction and repair of DNA double strand breaks (DSBs) in normal human skin fibroblasts and mammary carcinoma MCF7 cells irradiated with γ-rays.

It follows from our results obtained by both the methods (the first representing a gold standard in radiobiology and the second the most sensitive method of DSB detection at present) that, in contrast to the earlier opinion simply considering (activated) amifostine as a selective radioprotector of normal cells, the drug has also pronounced negative effects on irradiated cancer (MCF7) cells. We have also found that both amifostine and WR-1065, its metabolite, not only protect normal (NHDF) cells from acute radiation damage but also significantly support DSB repair in these cells. Because the DSB repair-supporting effects were higher for WR-1065 than for amifostine, it could be supposed that amifostine was not completely converted to WR-1065 even in normal cells, though the “equilibrium” was largely shifted to WR-1065. These assumptions have been confirmed in our study by a direct assessment of the amifostine-to-WR-1065 conversion by LC-MS/MS in the two cell lines (see Figure 8).

Contrary to normal cells, the radioprotective effect of amifostine on DSB induction (5 min PI) was absent in cancer MCF7 cells as detected by comet assay (see Figure 6) as well as by γH2AX/53BP1 foci immunodetection (see Figure 2). Even more, the addition of the drug increased the persistence of DSBs in the nuclei at 60 and 120 min PI (Figures 2 and 6). Therefore, amifostine (or its metabolites) not only missed its radioprotective effect in cancer (MCF7) cells, but also caused the DSB repair process and acted in a radiosensitizing manner.

The lower DSB induction measured at 5 min PI in normal cells incubated with amifostine prior to their irradiation (relative to untreated cells) can be easily explained by the radical scavenging ability of the metabolized drug (reviewed in ref 44). However, the necessity of converting amifostine into its active thiolic form (WR1065) can account (at least partially, Mitchell et al.55,44) for the absence of the amifostine-mediated radioprotection in cancer cells; the activation is mediated by alkaline phosphatase which is in low abundance in the majority of cancer cells including the MCF7 cells, as we also show in this study.

3.3. What is the Mechanism of How Amifostine Exerts Its Negative Effects on DSB Repair in Cancer Cells?
However, how amifostine exerts its negative effect on DSB repair in cancer cells remains disputable (Figure 10). We suppose that amifostine and/or its metabolites interfere in malignant cells in some detrimental way with the processes of DNA repair, having no significant influence on the process of the radiation-induced formation of DSBs itself. On the other hand, amifostine in normal cells is converted to WR-1065, its “Good” active metabolite. WR-1065 primarily ensures protection of normal cells against immediate cytoplasmic and DNA radiation-induced damage by scavenging free radicals (ROS). However, as also shown here, it supports the repair of DSBs too, directly by (physicochemical) interactions with damaged DNA and/or indirectly by modifying gene expression and biochemical cell regulatory pathways (see main text for more detailed discussion). C: As for (B) but the negative effect on cancer cells is exerted by WR-1065 (instead of amifostine). Low amounts of WR-1065 in cancer cells cannot protect these cells from DSB induction but are sufficient to negatively influence their DSB repair (and potentially other functions). The opposite effects of WR-1065 on DSB repair in normal and cancer cells follow from different WR-1065 levels and/or genetic backgrounds of these cells. WR-1065 thus only shows its “Mr. Hyde” face in cancer cells but “Mr. Jekyll” face in normal cells. D: As for B and C but varying mixtures of amifostine, WR-1065 and their metabolites are produced in normal and cancer cells, respectively; these mixtures interact with processes in normal and cancer cells in specific ways.

Figure 10. The original hypothesis (A) and the current “Good and Bad” (B), “Jekyll and Hyde” (C), and “Third Player” (D) hypotheses on amifostine effects in normal and cancer cells. B: In cancer cells, amifostine is almost not converted to WR-1065 (because of low levels of ALP and acidic pH) and behaves as “Bad”. While amifostine was considered as biologically inactive in previous works (panel A), some authors show that this prodrug per se is rather toxic, with direct and/or indirect negative effects on DSB repair and cell survival. On the other hand, amifostine in normal cells is converted to WR-1065, its “Good” active metabolite. WR-1065 primarily ensures protection of normal cells against immediate cytoplasmic and DNA radiation-induced damage by scavenging free radicals (ROS). However, as also shown here, it supports the repair of DSBs too, directly by (physicochemical) interactions with damaged DNA and/or indirectly by modifying gene expression and biochemical cell regulatory pathways (see main text for more detailed discussion). C: As for (B) but the negative effect on cancer cells is exerted by WR-1065 (instead of amifostine). Low amounts of WR-1065 in cancer cells cannot protect these cells from DSB induction but are sufficient to negatively influence their DSB repair (and potentially other functions). The opposite effects of WR-1065 on DSB repair in normal and cancer cells follow from different WR-1065 levels and/or genetic backgrounds of these cells. WR-1065 thus only shows its “Mr. Hyde” face in cancer cells but “Mr. Jekyll” face in normal cells. D: As for B and C but varying mixtures of amifostine, WR-1065 and their metabolites are produced in normal and cancer cells, respectively; these mixtures interact with processes in normal and cancer cells in specific ways.

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Normal, however, amifostine exerts its negative effect on DSB repair in cancer cells remains disputable (Figure 10). We suppose that amifostine and/or its metabolites interfere in malignant cells in some detrimental way with the processes of DNA repair, having no significant influence on the process of the radiation-induced formation of DSBs itself. Some authors reported that only the active metabolite of amifostine, WR1065, was taken up into the cells. This finding opens up a paradox: Nonmetabolized amifostine, if considered responsible for the alteration of DSB repair, cannot penetrate into the cells; WR1065, when suspect, is not produced in cancer cells in larger amounts.

Theoretically, low amounts of amifostine itself might enter the cells, which could be enough to exert negative effects of this drug on DSB repair in cancer cells. Inside the cells, amifostine may influence the repair directly or indirectly by interfering with vital cell processes. Differences in signaling pathways in normal and cancer cells, and their cell type-specific interactions with amifostine, can explain how the negative influence on DSB repair is mediated in cancer cells. Because the benefits for normal cells, the immediate radioprotection and improvement of DSB repair, are exerted by amifostine metabolites, we termed this scenario The Good and the Bad Hypothesis (Figure 10B).

Though this mechanism might be supported by our LC-MS/MS data (see Figure 8) revealing non negligible concentrations of amifostine also inside the cells, the sensitivity of the method does not exclude the possibility that amifostine contained in the cell medium contributed to the values measured, despite our careful cell washing. In any case, because we observed the alteration of DSB repair also in MCF7 cells directly incubated with WR1065 and a reverse conversion of this metabolite back to amifostine has not been described, we consider as more probable The Jekyll and Hyde Hypothesis, where WR1065 supports DSB repair in normal cells but disturbs it in cancer
cells (Figure 10C). Only low amounts of WR-1065 produced in cancer cells, which does not allow for efficient radioprotection, are probably sufficient to modify the expression of genes that alter directly or indirectly DSB repair.

Alternatively, other metabolites of amifostine or WR-1065 might cooperate in a plethora of different effects, including the deceleration of DSB repair observed in this work (The Third-Player Effect Hypothesis; Figure 10D). In support of this hypothesis, McKibbin et al. showed that amifostine and WR-1065 are rapidly metabolized and distributed in the tissues, whereas the excretion of their metabolic products is very slow. WR1065 could be eliminated by several pathways and, in turn, the metabolites might interact with various cellular compounds and processes in their specific ways. In vitro studies showed that oxidation of WR-1065 to its polyamine-like disulfide metabolite (WR-33278) is followed by a rapid consumption of oxygen in the culture medium; through this hypoxia, WR-33278 may up-regulate the expression of a variety of proteins participating in DNA repair and apoptosis, such as Bcl-2 and the hypoxia-inducible factor-1α. Bcl-2 may then initiate or inhibit apoptosis depending on its cell type- and cell status-specific interactions (reviewed in refs 51 and 52). Similar dual effects might thus also influence DSB repair.

Concerning all the presented hypotheses, the different response of normal (NHDF) and cancer (MCF7) cells to amifostine treatment most probably and primarily reflects (1) a different rate of ALP-mediated conversion of amifostine to WR-1065 (and consequently other metabolites), resulting in qualitatively and quantitatively varying “mixtures” of biologically active compounds influencing the cells, and (2) a different genetic background of the cells that is responsible for cell type- and cell status-specific interactions of cells with these mixtures. In in vivo conditions, physiological parameters (hypoxia, pH, etc.) play an additional important role (reviewed in ref 41).

3.4. What is the Mechanism of How Amifostine Exerts Its Positive Effect on DSB Repair in Normal Cells? A further question remains, namely how amifostine metabolites stimulate DSB repair in normal cells. As already discussed in the previous paragraphs, (metabolized) amifostine influences normal cells via multiple pathways. It has been known that amifostine/WR-1065 can directly support DNA repair (Figure 10) by donating hydrogen atoms and depleting oxygen to a singlet state. Further, Almeida et al. revealed that the protection by amifostine against radiation-induced genotoxicity in Escherichia coli cells depends on the functional recN gene that is essential for the SOS response and repair of DSBs by homologous recombination. Dziegielewski et al. showed that WR-1065 prevents delayed genomic instability though, contrary to Almeida et al., these authors suppose that WR-1065 disrupts homologous recombination and prevents its dangerous hyperstimulation by ionizing radiation. In any case, direct interactions of the drug with p53, NFkB, ATM, and Tip60 (Xu et al.), together with the modified expression of genes involved in DNA repair, cell cycle regulation, and apoptosis, confirm a more general influence of amifostine (and its metabolites) on DSB repair. In amifostine-treated cells, this influence is also evidenced by a reduced frequency of chromosomal aberrations which represent late effects of ionizing radiation.

Multiple direct and indirect mechanisms therefore seem to cooperate on the final positive effect of WR1065 on DSB repair in normal cells (Figure 10). However, it remains yet undisclosed how individual processes interconnected in this amifostine response network interplay in providing the final effect. For instance, because cancer cells are usually resistant to cell-cycle checkpoint arrests, only normal cells can profit from prolonged times available for DSBs repair (reviewed in ref 61). At the same time, DSB repair can be stimulated directly and by initiating other (still unknown) processes. The more pronounced effect on DSB repair observed in this work at 60 min PI for WR-1065 as compared with amifostine (Figures 2-4) could be explained by an incomplete amifostine metabolic conversion, though in normal cells.
Hypothesis} discussed in section 3.3. Taken together, the levels of active radioprotective compounds in the cell nucleus might be much higher in normal (NHDF) cells and much lower in cancer (MCF7) cells than indicated by our ALP-activity and LC-MS/MS measurements, in accordance with the pronounced radioprotective effect in NHDF fibroblasts but its absence in MCF7 cells.

The measurement of ALP activity (and WR-1065 levels) also in the culture supernatants and the finding of high ALP levels also in the cell milieu suggest that the conversion of amifostine to WR-1065 can, in principle, take place both extracellularly and intracellularly and during the passage through the cell membrane (Figure 10).

Of interest is also the observation that whereas the ALP activity is significantly higher in normal cells compared with cancer cells, the opposite is true for the levels of ALP mRNAs. The finding of a high ALP mRNA concentration in the cancer MCF cells suggests that some malfunction of the translation of ALP mRNA into the respective functional protein exists in these cells, which unsuccessfully try to compensate for it by an increased mRNA production. This is in good agreement with the fact that ALP is suspected to play an important role in carcinogenesis, because some tumors showed a strong activity of alkaline phosphatase. ALP ensures the cellular absorption of complex molecules, regulates the activity of other enzymes, and provides phosphate groups for various cellular functions. Cancer cells would be expected to preferentially suffer from ALP insufficiency because they are fast proliferating and have a high metabolic activity. In our study, the mRNA levels of tissue nonspecific ALP (TNAP) were the highest and 1 order higher in cancer MCF7 cells than in normal fibroblasts. Nevertheless, further studies are needed to understand the role of individual ALP isoenzymes in amifostine processing and in carcinogenesis.

3.7. Reassessment of Old Problems with New Tools. Methodological Progress in the Research of Radioprotective Agents and Novelty of This Work. Concerning our methodology, we followed DSB damage induction and repair in intact normal and cancer cells treated with amifostine or WR-1065 by double immunodetection of DSB repair foci in combination with high-resolution fluorescence microscopy. To our best knowledge, the only similar work was published by Kataoka et al. (discussed in ref 69) who, however, used flow cytometry to quantitatively fractionate cells positive for DSB damage at 1 h PI. Instead, we scored individual γH2AX and 53BP1 foci (Figure 1) at 3D-high resolution microscopic images immediately (5 min) postirradiation and then at 1 and 2 h PI. This allowed us to separately evaluate the effect of amifostine or WR-1065 on DSB induction and repair. Our method also currently provides the highest sensitivity of DSB detection and to distinguish unrepaired DSBs from the background or “relicts” from already rejoined lesions.

DSB induction in amifostine-treated cells was already evaluated by a number of techniques, including neutral comet assay also employed in this work, pulsed-field gel electrophoresis (PFGE), counting of chromosomal aberrations, measuring of apoptosis activation, cytokinesis-blocked micro-nucleus assay, or methods assessing genomic instability. All these methods have their benefits but also suffer from some underlying limits. The immunofluorescence microscopy that we used here allowed us to quantify DSBs from minutes up to hours postirradiation, in intact cells, with the maximum sensitivity, and upon the (relatively low) radiation doses typically used in radiotherapy (reviewed in ref 76). Comet assay enabled us to study DSB repair more directly, though higher doses (6 Gy of γ-rays) were required to obtain more prominent differences between the samples. The results of both methods well correlated; nevertheless, our results still have to be generalized only with caution: Kataoka et al. found that while an increased γH2AX signal corresponded with a subsequent cell survival in the case of WR-1065, this was not true for some other radioprotectors included in their study. Leaving thus the detection of γH2AX as a method of searching for novel radioprotectors as disputable, these findings indicate that amifostine/WR-1065 influences more aspects of the cellular response to radiation than only DSB induction; this is retrospectively in accordance with our results.

3.8. Conclusions. Taken altogether, our results put amifostine in an entirely new light. In the treatment of some malignancies, this drug can not only selectively protect normal tissues but can also act as a radiosensitizer which in parallel improves the killing of the cancer cells by disrupting DNA DSB repair. This finding highlights separated effects of amifostine on DSB induction and repair. Nevertheless, the complexity of cellular processes potentially specifically influenced by amifostine and its metabolites in various normal and cancer cells prevents a simple extension of our results to normal and cancer cells in general. Hence, the action of amifostine and other radiomodifiers should be studied carefully for each particular cell type. The rapidly growing repertoire of new molecular-genetic, genomic, and other “omic” methods now opens new dimensions of further research on radiomodifying drugs and the mechanisms of their action.

4. EXPERIMENTAL SECTION

All materials were obtained from common commercial suppliers and used without further purification. Amifostine and WR-1065, the compounds studied in this article, were of ≥97% purity determined by TLC and ≥98% purity determined by HPLC, respectively.

4.1. Cell Lines and Their Cultivation. Certified normal human dermal fibroblasts (NHDF) and human breast adenocarcinoma cells (MCF7) were obtained from CLS Cell Lines Service GmbH (Eppelheim, Germany), cultivated in DMEM medium (PAN Biotech, Aidenbach, Germany, cat. no: P03-0710), supplemented with 10% fetal calf serum (PAA Laboratories GmbH, Pasching, Austria) and 1% penicillin + streptomycin (stock solution mixture 10 000 U/mL penicillin + 10 mg/mL streptomycin; PAN Biotech, cat. no.: P06-07100), at 37 °C in a humidified atmosphere of 5% CO2. The cells obtained (at passage 2) were multiplied and frozen, and the “young” passages 5 and 6 used for experiments to prevent possible accumulation of chromosomal aberrations and mutations and their potential effects on results.

4.2. Amifostine and WR-1065. Amifostine (Sigma, St. Louis, MO, product no.: A5922) was added to the cell cultures 15 min before irradiation. The concentration of amifostine in the cultures was 4 mM. This concentration was chosen as the most effective on the basis of literature data. WR-1065 (Tocris, Abingdon, UK, catalogue no.: 3356) was used in an isomolar concentration and applied in the same time interval as amifostine.

4.3. Irradiation. The cells were irradiated in the culture medium at 37 °C from a 60Co source (Gliwice, Poland) with a dose of 1 Gy (γH2AX/53BP1 foci immunofluorescence) or 6 Gy (comet assay); the dose rates were 0.5 Gy/min and 1.0 Gy/min, respectively. During the irradiation, the cells were kept in a thermostable box, ensuring a constant temperature and prevention from infection during the whole procedure. After irradiation, the cells
were immediately placed back into the incubator (37 °C/5% CO₂) until the fixation.

4.4. h2AX and 53BP1 Foci Immunostaining. Nonirradiated cells (0 min PI) and cells irradiated with 1 Gy of γ-rays (5°C; 0.5 Gy/min) were washed in PBS, spatially (3D) fixed with 4% paraformaldehyde (10 min, room temperature (RT)) in early and later time intervals (5 and 60 min PI), permeabilized in 0.2% Triton X-100/PBS (14 min, RT), and immunosassayed as described in refs 26 and 75. The primary mouse antibodies, antiphospho-h2AX (serine 139) (Upstate Biotechnology, Lake Placid, NY, cat. no.: 05-636) and rabbit anti-53BP1 (Cell Signaling Technology, Danvers, MA, cat. no.: 4937), were used simultaneously to detect the γh2AX and 53BP1-irradiation-induced DSB-repair foci (IRIFs). The antibodies bound were visualized with the secondary FITC-conjugated donkey antimouse and Cy3-conjugated donkey antirabbit antibodies (both Jackson Laboratory, West Grove, PA, cat. no.: 715-095-150 and 711-165-152); nuclear chromatin was counterstained with 1 μM TO-PRO-3 (Molecular Probes, Eugene, OR) in 2× saline sodium citrate (SSC) prepared fresh from a stock solution. After brief washing in 2× SSC, Vectashield medium (Vector Laboratories, Burlington, Canada) was used for the final mounting of the samples.

4.5. Evaluation of DNA Double Strand Break (DSB) Damage and Repair by Comet Assay. Cell cultures were washed with fresh DMEM medium (described in section 4.1) to remove dead cells and supplemented with new medium containing or not 4 mM amifostine (Sigma) or 4 mM WR1065 (Tocris), both prepared fresh from 100 mM stock solution. After 15 min treatment with these compounds in an incubator (37 °C, 5% CO₂), cells were irradiated as described (section 4.3) and immediately harvested (5 min PI) or allowed to repair their DNA for next 60 or 120 min. For the comet assay, cells were deprived of medium and scratched (by cell scrapers, Biotech) in 800 μL of 1× PBS. The cell suspension (100 μL) was mixed with 400 μL of melted 0.7% LMT-agarose (Ultra Pure Low Melting Point Agarose; Luria, Buchs, Switzerland). The incubation took place in a 96-well plate at 37 °C for 30 min, as described previously.64 The optical densities were measured at 405 nm (DigiScan Reader). The same approach was used for the measurement of ALP activity in the cell lysates and supernatants of the cell cultures.

4.6. Confocal Microscopy and Image Analysis. 4.6.1. Imaging and Quantification of h2AX and 53BP1 Foci. An automated high-resolution confocal fluorescence microscopic system Leica DM BX51 equipped with a CSU10a Nipkow disc (Tokyogawa, Tokyo, Japan), an oil immersion Plan Fluor objective (100×/NA1.3), a CoolSnap HQ CCD camera (Photometrics, Tucson, AZ), and an Ar/Kr laser (Innova 70C Spectrum, Coherent, Santa Clara, CA), was used for image acquisition.40 Forty serial optical sections were captured at 0.2 μm intervals along the z-axis to reconstruct 3D images of the nuclei,56 at a constant temperature of 26 °C. The maximal images represent computational superimpositions of individual confocal slices. The exposure time and the dynamic range of the camera in the red, green, and blue channels were adjusted to the same values for all slides to obtain quantitatively comparable images. Automated exposure, image quality control, and other procedures were performed using Aquaculture software.62

4.6.2. Imaging and Quantification of DNA Comets (comet assay). A Leica SP5 microscopy system equipped with UV-lasers (355 and 405 nm), a white laser, and sensitive hybrid detectors (Leica) was used. A Leica SP5 microscopy system equipped with UV-lasers (355, 4937), were used simultaneously to detect the fluorescence microscopy, DNA comets were stained with Gel Red (Biotium). Two kinds of confocal image (cations images) or 30 μm (detailed comets) were captured and superimposed to maximum images for consequent analyses. The same conditions were kept for all slides (image size 1024 × 1024, scanning frequency 400 Hz in bidirectional mode, pinhole 208.07 μm for low-magnification images and 95 μm for high-magnification images, constant laser power, and hybrid detector acquisition range 606–708 nm). Two hundred to 1000 comets per sample and about 50 comets per sample were used for the “high-throughput” and the “detailed” comets analyses, respectively.

Maximum images were computed and converted to TIFF files by Leica LAS AF software (ver. 2.6.0, Leica Microsystems). CASP Lab software (ver. 1.2.3beta1, Comet Assay Software Project Lab; Krzysztof Kožná, 2003)65 was consequently used to quantify comets parameters with the same settings for all samples compared. The slides were prepared and analyzed in duplicates. The data were analyzed in Sigma Plot Scientific Software (SPSS, ver. 12.5, Systat Software); statistical significance of tail moment differences was determined using the Mann–Whitney U test.

4.7. Flow Cytometry. Flow cytometry was used to quantify cell concentrations and vitality prior to LC-MS/MS and colorimetric measurements. The Muse Cell Analyzer (Merck Millipore) and Muse Annexin V & Dead Cell Assay Kit (MCH100105, Millipore) were used according to the manufacturer’s instructions. The status of the cells was analyzed in three measurements.

4.8. Alkaline Phosphatase (ALP) Activity. The ALP activity in NHDF and MCF7 cells was determined in a lysate of sonicated cells (5 × 10^5 per sample) and cell supernatants, respectively, after incubation with the ALP substrate (4-nitrophenyl phosphate, Fluka, Buchs, Switzerland). The incubation took place in a 96-well plate at 37 °C for 30 min, as described previously.64 The optical densities were measured at 405 nm (DigiScan Reader). The same approach was used for the measurement of ALP activity in the cell lysates and supernatants of the cell cultures.

4.9. Liquid Chromatography–Mass Spectrometry (LC-MS/MS). Preparation of cells and cell supernatants: The cells in culture flasks were provided with fresh DMEM medium (section 4.1) supplemented (37 °C, pH 7.1 at the beginning of incubation) with 4 mM amifostine (Sigma) or 4 mM WR1065 (Tocris) prepared fresh from 100 mM stock solution. The cells were treated were placed into a cell incubator (37 °C) for 20 or 75 min until harvested for the measurement (i.e., scratched, resuspended in 800 μL of 1× PBS, and disintegrated by sonication (2 min) and 3× repeated freezing in liquid nitrogen. Filtered suspension with disintegrated cells was used for LC-MS/MS.

LC-MS/MS instrumentation and conditions: The analyses were performed on a Waters 1200 series (Agilent, Waldbronn, Germany) equipped with a G1312B binary pump, a G1367D-HiPALS SL autosampler, and a G1361B column oven. The sample was separated using a Zorbax Poroshell 120 EC 18 column (1D. 3.0 mm × 50 mm, 2.7 μm particle size; Agilent, Santa Clara, CA) and the column temperature was 25 °C. Isocratic elution was applied with 0.2% acetic acid and methanol (2:98, v/v). The flow rate was 0.60 mL min⁻¹, and the injection volume was 1 μL.

Determination was performed using an Agilent Technologies 6460 Triple Quadrupole LC/MS/MS system with Jet Stream Technologies with electrospray ionization (ESI). The compounds were ionized in the positive ion polarity mode. The ionization source conditions were as follows: capillary voltage 400 V, gas temperature 350 °C, gas flow 12 L min⁻¹, nebulizer pressure 50 psi, sheath gas temperature 350 °C, sheath gas flow 12 L min⁻¹. Quantification was performed using multiple reaction (MRM) modes. Precursor ion → product ion, fragmentor voltage and collision energy were selected for each compound individually for amifostine: 215 → 135, 90, 8 eV; for WR 1065: 135 → 58, 90, 16 eV.

4.10. Quantitative Real-Time Reverse Transcription–PCR (qRT-PCR) analyses. cDNA was prepared using the Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics, Mannheim, Germany). One microgram of RNA was used per each reverse transcription. The mRNA levels of selected genes (four isotypes of ALP) were measured by a real-time reverse-transcription-polymerase
chain reaction (qRT-PCR) in triplicates on a RotorGene 6000 cycler (Cobett Research, Sydney, Australia) using the FastStart SYBR Green Master (Rox) (Roche Diagnostics, Mannheim, Germany). The final reaction volume (20 μL) included 10 μL of FastStart SYBR Green Master (Rox), 2 μL of cDNA, and 100 nM concentration of each primer. The primer sequences (see Table S1, Supporting Information) were obtained from Schär et al. The initial reaction cycle carried out at 95 °C for 10 min was followed by 40 cycles, each consisting of 15 s denaturation at 95 °C, 20 s annealing at 60 °C, and 20 s extension at 72 °C. To determine the relative gene expression levels, we used the delta–delta Ct method based on the difference of the threshold cycles (Ct) of the target gene and β-actin housekeeping.

4.11. Statistics. For the testing of statistical significance of the differences in the numbers of γH2AX foci between pairs of experimental groups, the Mann–Whitney U test was used. For the testing of statistical significance of the differences in the ALP activity or the mRNA expression of the individual ALP isoenzymes, the two-sample t test was used; these analyses were performed on data of merged experiments after standardization using z-score. All the statistical analyses were performed using the IBM SPSS Statistics 19 for Windows software (Release 19.0.1, IBM Corporation 2010).

ASSOCIATED CONTENT

Supporting Information
The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jmedchem.5b01628.

Sequence of primers used in RT-PCR for amplifying alkaline phosphatase (ALP) isoenzyme mRNAs; DSB induction and repair quantified by the neutral comet assay–wide-field images (extending Figure 6); HPLC-MS/MS fragmentation spectra of WR-1065 and amifostine (for Figure 8) (PDF)

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Author Contributions
Michal Hofer and Martin Falk contributed equally. They designed the experiments, analyzed data, and prepared the manuscript. Martin Falk summarized and proposed the hypotheses on amifostine action displayed in Figure 5. Martin Falk also participated in confocal microscopy. Denisa Komůrková, Lenka Weitérova, and Lenka Štefančíková performed γH2AX/53BP1 foci immunostaining, quantification, and confocal microscopy; Denisa Komůrková also analyzed the ALP activity and mRNA levels. Iva Falková, Alena Bačíková, and Bořivoj Klejdus determined amifostine conversion to WR-1065 by LC-MS/MS. Iva Falková, Alena Bačíková, Karel Angelis, and Štefan Galbavý performed comet assay experiments, confocal image acquisition, and data analyses. IF also contributed to manuscript preparation. Eva Pagáčová took part in immunofluorescence experiments and image acquisition and analyses; she also maintained cell cultures. Stanislav Kožubek participated in confocal microscopy, data acquisition and analyses, and in manuscript preparation. Ladislav Dušek performed statistical evaluations.

Notes
The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This work was supported by the Czech Science Foundation (projects 16-12454S, P302/12/G157, and 16-01137S), Ministry of Health of the Czech Republic, AZV grant no. 16-29835A (all rights reserved) and from the Czech Republic contribution to the Joint Institute for Nuclear Research, Dubna (Project of the Czech Plenipotentiary and the 3 + 3 Project for 2015, 2016). LC-MS/MS experiments were realized in CEITEC-Central European Institute of Technology with research infrastructure supported by the project CZ.1.05./1.1.00/02.0068 financed from European Regional Development Fund.

ABBREVIATIONS USED

ALP, alkaline phosphatase; DSB, DNA double strand breaks; GCAP, placental-like ALP; IAP, intestinal ALP; IR, ionizing radiation; MCF7, human mammary carcinoma cell line; NHDF, normal human dermal fibroblasts; PI, postirradiation; PLAP, placental ALP; TNAP, tissue nonspecific ALP

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DOI: 10.1021/acjmedchemlett.0b01628


