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# CHELATION OF LYSOSOMAL IRON PROTECTS AGAINST IONIZING IRRADIATION

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**short (page heading) title:** Iron and ionizing radiation

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**Abbreviations:**
AO, acridine orange; DFO, desferrioxamine; LMP, lysosomal membrane permeabilization; OD, optical density; PF, protection factor; ROS, reactive oxygen species; SIH, salicylaldehyde isonicotinoyl hydrazone

**Key words:** ionizing radiation, iron, lung cancer, lysosomes, oxidative stress, iron chelation

**SYNOPSIS**

Ionizing radiation causes DNA damage and consequent apoptosis, mainly due to the production of hydroxyl radicals that follows radiolytic splitting of water. However, superoxide and hydrogen peroxide also form and induce oxidative stress with resulting lysosomal membrane permeabilization (LMP) arising from iron-catalyzed oxidative events. The latter will significantly contribute to radiation-induced cell death and its degree largely depends on the quantities of lysosomal redox-active iron present as a consequence of autophagy and endocytosis of iron-rich compounds. Therefore, radiation sensitivity might be depressed by lysosome-targeted iron-chelators. Here we show that cells in culture are significantly protected from ionizing radiation damage if initially exposed to the lipophilic iron-chelator salicylaldehyde isonicotinoyl hydrazone (SIH), and that this effect is based on SIH-dependent lysosomal stabilization against oxidative stress. According to its dose-response-modifying effect, SIH is a most powerful radio-protector and a promising candidate for clinical application, mainly to reduce the radiation sensitivity of normal tissue. We propose, as an example, that inhalation of SIH prior to each irradiation session by patients undergoing treatment for lung malignancies would protect normally aerated lung tissue against life-threatening pulmonary fibrosis, while the sensitivity of malignant lung tumors, which usually are non-aerated, will not be affected by inhaled SIH.

## INTRODUCTION

Non-surgical cancer therapy, e.g. chemo- and radiotherapy, is mainly based on the induction of apoptotic cell death following the production of reactive oxygen species (ROS). Proteins combating oxidative stress, such as members of the thioredoxin family of proteins, superoxide dismutases or catalases are often upregulated in tumor cells and associated with resistance to such therapies [1-5]. It is generally assumed that DNA damage, mediated by the hydroxyl radicals ($HO^{\bullet}$) that are formed by radiolytic cleavage of water, is responsible for cell death caused by ionizing radiation [6].

It has previously been pointed out that, in addition to DNA damage and resultant p53-mediated cell death, lysosomal membrane permeabilization (LMP) induced by oxidative stress is a contributing factor in apoptotic cell death caused by ionizing radiation [7]. Such LMP is dependent on intralysosomal redox-active iron that causes peroxidation and fragmentation of the lysosomal membrane secondary to the oxidative stress that radiation induces [7,8]. Fenton-type reactions between hydrogen peroxide and redox-active iron lead to formation of hydroxyl radicals inside the lysosomal compartment. It, therefore, follows that the lysosomal concentration of redox-active iron would be directly related to the extent of LMP. It has been found that irradiation-induced LMP can be abrogated by chelation of lysosomal redox-active iron using desferrioxamine (DFO) [7]. DFO, however, stays within the lysosomal compartment following its endocytic uptake, causes iron-starvation with ensuing cell death, and is obviously not a well-suited chelator.

In the lung alveoli exist a large number of macrophages, many of which have engulfed erythrocytes and, consequently, contain iron-rich lysosomes that may burst as a consequence of ionizing irradiation, induce macrophage death and contribute to the induction of radiation pneumonitis and pulmonary fibrosis. Furthermore, other pulmonary cell types may have iron-rich lysosomes and, interestingly, the reparative autophagy that is initiated by irradiation greatly enhances the amount of lysosomal redox-active iron [7]. Reparative autophaphagy is a way for cells to degrade damaged constituents and involves the breakdown of cellular ferruginous materials, such as ferritin and mitochondria. As a result, autophagolysosomes transiently become rich in low mass redox-active iron, although it is eventually transported out of the lysosomal compartment to be stored in ferritin, or exploited in a variety of anabolic processes within mitochondria and the cytosol [9,10].

Lung cancer is presently the leading cause for cancer-related death worldwide [11]. Many cases of lung cancer require ionizing irradiation as part of the management of this common group of diversified malignancies with a generally poor outcome. A major problem that limits the dose of irradiation is the risk of inducing pulmonary fibrosis which may turn out to be life threatening [12]. Consequently, it is often necessary to apply a dose of ionizing radiation that is less than optimal for effective therapy. Improvement of therapeutic efficiency is, therefore, obviously needed. Here, we suggest a somewhat unorthodox way of handling the situation. Most drugs or treatments aim to enhance the irradiation efficiency on tumors, while we rather suggest strategies for the protection of surrounding normal tissue. So far, only one radioprotector, amifostine, which incidentally also happens to be a lysosomotropic iron chelator, has been explored in clinical trials [13-15].

Here we have assessed the effect of lysosomal iron-chelation by the lipophilic chelator salicylaldehyde isonicotinoyl hydrazone (SIH) on cell survival following irradiation in a variety of cultured cells. Since SIH quickly enters and leaves cells, being in equilibrium with its concentration in the surrounding medium, it can easily be rinsed away and in contrast to DFO, has no long lasting effects [16].

## EXPERIMENTAL

### *Chemicals*

Acridine orange (AO) base was from Gurr (Poole, UK). SIH (a kind gift from Prof. Des Richardson, University of New South Wales, Sydney, Australia) was dissolved in DMSO and then diluted in EtOH in such a way that the final stock solution contained SIH at a concentration of 10 mM in a 10% DMSO/90% EtOH vehicle. Aliquots of this stock solution were added to cell culture medium to obtain final concentrations of 10-100 µM SIH. Since DMSO is a well-known scavenger of hydroxyl radicals, and protects against ionizing irradiation [17], initial experiments were carried out to ensure that the low final concentration of the DMSO/EtOH vehicle had no influence on the cellular sensitivity to radiation or hydrogen peroxide (data not shown). All other chemicals were from Sigma-Aldrich (Stockholm, Sweden).

### *Cell cultures*

Cell lines were originally from ATCC or Uppsala University. HeLa and J774 cells were grown in DMEM (Gibco, USA), U1690 cells in MEM, and the cell lines U2020, U1810, and U1906e in RPMI (Gibco, USA). All media were supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM glutamine, and 100 units$\cdot$ml$^{-1}$ penicillin/streptomycin (PAA, Pasching, Austria). Cells were grown in plastic flasks and 35 mm Petri dishes (Corning, New York, USA) at 37°C in a 90% humidified atmosphere containing 5% $CO_2$. They were subcultivated once or twice a week.

### *Ionizing radiation*

γ-irradiation was performed with a $^{137}$Cs source (Scanditronix, Uppsala, Sweden) at the Karolinska Institute, Stockholm, at a photon dose rate of 0.5 Gy$\cdot$min$^{-1}$. Dosimetry was done with an ionization chamber as well as with ferro sulphate. According to the sensitivity of the used cell lines, doses were in the range of 0-8 Gy. Cells were transported in insulated boxes and irradiated at room temperature. The irradiation was done in fresh medium, with or without SIH. When applied, SIH was added 30 min before irradiation. The irradiated medium was replaced by fresh growth medium (without SIH) when cells were returned to standard culture conditions.

### *Estimation of clonogenic cell survival*

Appropriate cell numbers were plated for survival using the previously described clonogenic assay technique [18]. Single-cell suspensions were plated in 35 mm plastic Petri dishes or 6-well plates in triplicates or quadruplicates in a final medium volume of 3 ml/dish or well and then left in the incubator for 3-4 h to attach before irradiation, which was performed as described above.

Following irradiation, the cultures were incubated for 10-14 days, with a change of medium after 5-7 days. Thereafter, colonies were fixed, stained and counted. Radiation survival curves were constructed from one to four independent experiments.

### *Dose-response models for clonogenic cell survival*

The LQ model [19] was used to fit data with the least square method, where the probability for clonogenic cell survival *S* at a dose *D* is given by [20]:

$$S = exp(-\alpha D - \beta D^2)$$

The doses for 10% survival levels were calculated to estimate the dose-modifying fraction, in this case the protection factor (PF).

***Estimation of growth curves***

Survival of HeLa cells was estimated as described above. Cultures were prepared in numbers that allowed daily counting for 3 days following irradiation. An alternative method to measure cell survival following irradiation was applied to the cell lines U1906e and U1810 as these cell types do not readily form colonies. In those cases, cells were seeded and grown in 25 $cm^2$ culture flasks for 24 h prior to irradiation that was performed under conditions described above. Cells were then routinely sub-cultured in a 1:4 ratio and counted three times during a period of 14-16 days following irradiation. Estimation of cell numbers (cells/ml) was obtained by assaying optical density (OD) at 600 nm on trypsinized single-cell suspensions. The ODs were compared to a standard curve that was previously constructed by counting a series of diluted cell suspensions in a Bürker-chamber. Finally, growth curves were obtained by comparing cell numbers at a number of time-points in relation to the cell number at the previous subcultivation.

***Cell survival following exposure to hydrogen peroxide***

Cells were seeded in 96 well plates at $10^4$ cells/well. After 16 hours, the cells were incubated for 1 h with different concentrations of $H_2O_2$ (0–100 mM) in HBSS with or without 100 µM SIH present. Some cells were incubated with 30 µM $FeCl_3$ for 5 hours before the $H_2O_2$ treatment (when added to culture medium, Fe(III) forms insoluble Fe-phosphates/hydroxides that are taken up by endocytosis and transported to the lysosomal compartment). Following one hour of $H_2O_2$-exposure (during this period of time most of the hydrogen peroxide was degraded by the cells) cells were washed and returned to standard culture conditions. The number of viable cells was determined 24 h later using the Cell Proliferation Kit II (Roche Applied Science, Germany). This assay is based on formation of a colored formazan following mitochondrial oxidation of the tetrazolium salt XTT by metabolically active cells. The dye was quantified using a microplate reader (SpectraMax 340PC, Molecular Devices, CA, USA) at 490 and 650 nm.

***Lysosomal membrane stability assay***

Acridine orange (AO) is a metachromatic fluorophore and a lysosomotropic base (pKa=10.3), which becomes charged ($AOH^+$) and retained by proton trapping within acidic compartments, mainly secondary lysosomes (pH 4.5-5.5). Using blue light excitation, normal cells show bright red lysosomes (indicating high AO concentration) and weak green cytoplasmic and nuclear fluorescence (indicating low AO concentration). The AO relocation technique [16,21] was used to show early lysosomal damage. The lysosomes of cells are pre-loaded with AO before exposure to any treatment that is supposed to cause LMP, which is registered by flow cytofluorometry as an increase of green AO fluorescence that results from AO relocation to the cytoplasm.

One million U1690 cells in 2 ml complete medium were exposed to 10 µg/ml AO for 15 min under otherwise standard conditions. Cells were then washed with complete medium and equilibrated under standard conditions for another 15 min, before they were exposed for 30 min at 37°C to 100 µM $H_2O_2$ in HBSS, with or without 100 µM SIH. After end of the oxidative stress period, cells were kept at standard culture conditions for another 30 min before they were trypsinized and green AO fluorescence was analyzed by flow cytofluorometry (FACScan, Becton-Dickinson, Mountain View, CA, USA) using the FL1 channel.

## RESULTS

***Iron chelation protects cells against radiation-induced cell death***

In order to find out if iron-chelation protects against cell death, several cell-lines were irradiated with or without the iron-chelator SIH present. We tested the mouse macrophage cell line J774, the cervix cancer cell line HeLa and a number of lung cancer cell lines, U1690, U1906e, and U1810. U1690 is a small-cell lung cancer cell line [22], U1810 is a radio-resistant non-small cell lung cancer cell line [23], while U1906e is a radio-sensitive small cell lung cancer sub-cell line [24]. First, growth curves of HeLa, U1906e and U1810 were recorded (Fig. 1). Cells were exposed to a single fraction of ionizing irradiation at 2 Gy (Fig. 1 B), 3 Gy (Fig. 1 A) or 5 Gy (Fig. 1 C, D) +/- 10 µM SIH present during the irradiation period. On HeLa cells, we investigated the direct effect of radiation on cell survival, and on the lung cancer cell lines the ability to repopulate after irradiation. Both immediate protection and repopulation were significantly improved by SIH. SIH-treated, non-irradiated cells grew better than control cells (Fig. 1). Since DMSO is known as a potent scavenger of hydroxyl radicals [17], we ensured that DMSO in the 0.01-0.1 %-range had no protective effect (data not shown). Next, we determined the surviving fractions based on the clonogenic cell survival assay using HeLa, J774 and U1690 cells (Fig. 2). The ability to undergo five or more cell divisions following irradiation is used as an indication of cell survival. A survivor that has retained its reproductive integrity and is able to proliferate continuously to produce a large clone or colony is said to be clonogenic. SIH increased the surviving fractions in all cell lines studied. In line with this result, exposure to an Fe(III) phosphate/hydroxide precipitate (obtained by adding 10 µM $FeCl_3$ to the medium) that was endocytosed by the cells for four hours before irradiation decreased the surviving fractions (Fig. 2 B). Compared to the control cells (irradiated without prior iron exposure), only approximately 30% of the iron-loaded cells survived the radiation doses of 6 Gy (Fig. 2 B) and 8 Gy (results not shown). Protection of cells against radiation was partly dependent on the SIH concentration. The protection factors (PF) were calculated as the ratio of the doses that gave 10% survival with and without SIH-protection, respectively. As shown in Table 1, PF for HeLa cells increased from 1.20 to 1.78 following doubling of the SIH concentration from 10 to 20 µM. This means that, as a consequence of SIH protection, the radiation doses can be increased by 20% and 80%, respectively, without change of the survival rate. The PF for J774 cells was 1.30 at 10 µM SIH, while it was 1.20 for U1690 at 20 µM SIH. When cells were exposed to 2 and 4 Gy, which are reasonable daily doses in the treatment of lung cancers, the PF for U1690 was found to be between 1.40 and 1.80 in the presence of 20 µM SIH.

***Iron chelation protects cells against hydrogen peroxide-induced cell death***

Since it is believed that the effect of ionizing radiation partly depends on intracellular formation of hydrogen peroxide [25] with ensuing LMP [7], we investigated protection by SIH against $H_2O_2$-induced cell death. The small-cell lung cancer lines U2020 [26] and U1690 were exposed to various concentrations of $H_2O_2$ with or without 100 µM SIH present (Fig. 3 A, B) and cell survival was calculated 24 h later. For U2020 cells, the $Ec_{50}$ $H_2O_2$ value increased from 0.22 mM to 7.85 mM (Fig. 3 A) and for the U1690 cells from 93 µM to 580 µM (Fig. 3 B). As was earlier found for ionizing radiation (Fig. 2 B), survival decreased if cells were exposed to an iron phosphate complex prior to the induction of oxidative stress (Fig. 3 B). The $Ec_{50}$ value for U1690 cells dropped to 53 µM following incubation with 30 µM $FeCl_3$ for five hours before ensuing $H_2O_2$ treatment. It should be pointed out that the addition of $FeCl_3$ to culture medium results in formation of an iron phosphate/hydroxide precipitate that is endocytosed by the cells. The lysosomal compartment is thereby enriched with iron.

Using again the clonogenic cell-survival assay, we calculated how many U1690 cells survived exposure to different concentrations of $H_2O_2$ compared to untreated cells. We found that without SIH protection, no U1690 cells survived the one-hour period of $H_2O_2$-exposure at initially 50-150 µM, while 50-100% of the cells that were protected by 20 µM SIH did so (Fig. 3 C).

***Iron chelation influences lysosomal stability under conditions of oxidative stress***

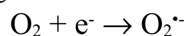
To obtain further insights into the protection mechanism afforded by the iron-chelator SIH, we assayed LMP, given the fact that redox-active iron is mainly found inside lysosomes [21,27-30].

U1690 cells were subjected to the AO-relocation test. Following AO-loading, cells were exposed for 30 min to 100 µM $H_2O_2$ +/- 100 µM SIH in HBSS, and green fluorescence was assayed by flow cytofluorometry (FACS) after another 30 minutes (Fig. 4 A). Compared to the control cells, the mean green fluorescence increased up to 156% following exposure to $H_2O_2$ only, whereas cells exposed to 100 µM $H_2O_2$ under the protection of 100 µM SIH showed only a small increase of the mean green fluorescence; up to 110% of the control cells (Fig. 4 B).

## DISCUSSION

Apart from radiolytic cleavage of water leading to formation of hydroxyl radicals ($HO^•$), the simultaneous production of hydrogen peroxide is a well-known effect of ionizing radiation to tissues [7,25]. However, the possible influence of hydrogen peroxide on radiation-induced cellular damage does not usually seem to be fully taken into account. This is somewhat surprising since in a paper from 1961 Otto Warburg pointed out that the cellular effects of exposure to ionizing radiation or to hydrogen peroxide show substantial similarities [31].

$$H_2O + \text{ionizing radiation} \rightarrow HO^• + H^+ + e^-$$

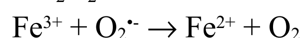


$$O_2 + e^- \rightarrow O_2^{•-}$$

$$O_2^{•-} + O_2^{•-} + 2H^+ \rightarrow H_2O_2 + O_2$$

Following studies on the damaging effects of randomly formed $HO^•$, it has been postulated that these short-lived ($10^{-9}$ sec) and extremely aggressive radicals react with nuclear DNA on the very spot where they are formed, causing adducts, mutations and single and double strand breaks with resulting cellular damage. Even if it is not definitively proven that $HO^•$-induced DNA damage is the main cause of cellular injury following irradiation, there is an overwhelming amount of indirect evidence that this is indeed the case, and there seems to be little reason to question this dogma. However, apart from radiolytic cleavage of water, $HO^•$ can also be produced by Fenton-type (transition metal-mediated) reactions, which gives incentives to examine the occurrence of such reactions during ionizing irradiation.

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO^• + OH^-$$



$$Fe^{3+} + O_2^{•-} \rightarrow Fe^{2+} + O_2$$

Obviously, the presence of redox-active iron in direct contact with DNA would give rise to massive site-specific Fenton-type chemistry, given the radiation-induced presence of hydrogen peroxide and superoxide. Under normal conditions, there are no indications of any significant amount of low mass redox-active iron that is in juxtaposition to DNA [32-34]. However, as was recently demonstrated, under conditions of oxidative stress lysosomal rupture will occur, iron will be relocated, and DNA damage initiated [7,32-34].

Because the lysosomal compartment is the center for normal autophagic turn-over of all organelles and most long-lived proteins, many of which are ferruginous compounds, lysosomes of all cells contain low mass redox-active iron, explaining their vulnerability to oxidative stress [9,10]. An additional way of loading lysosomes with iron is of importance when scavenger cells, for example alveolar macrophages, endocytose erythrocytes and thereby enrich their lysosomal compartment with redox-active iron. The lysosomal compartment is acidic and rich in reducing equivalents, such as cysteine and glutathione, ensuring that any present low mass iron would largely be in $Fe^{2+}$ form [8,35]. That in turn would promote the generation of hydroxyl radicals from hydrogen peroxide diffusing into this compartment.

Lysosomes show widely different sensitivity to oxidative stress [36]. Using vital staining with lysosomotropic fluorochromes, e.g. acridine orange or other available lysotrackers, it was found that after heavy oxidative stress some lysosomes always remain intact, while even low oxidative stress results in the rupture of a small but obviously very sensitive population of lysosomes [36]. The explanation for this phenomenon is probably that lysosomes that are actively engaged in degradation of iron-containg macromolecules are rich in iron, while resting lysosomes may contain little or nothing of this transition metal [37].

Since the hydrogen peroxide that forms throughout the cell during irradiation is highly diffusible, it will enter the lysosomal compartment, meet redox-active iron and induce violent Fenton-type reactions with resultant LMP and release of lysosomal contents to the surrounding

cytosol (Fig. 5). LMP will thus not only allow the escape of low mass iron from lysosomes, but also the relocation of potent lysosomal cathepsins. Dependent on the magnitude of lysosomal rupture, cell proliferation is stimulated or arrested by a minor or a somewhat more pronounced lysosomal destabilization, respectively, while apoptosis or necrosis have been found to follow moderate or major destabilization, respectively [37,38]. Consequently, the amelioration of LMP by chelating lysosomal redox-active iron in a non-redox-active form ought to reduce radiation sensitivity.

This hypothesis was earlier supported by findings following treatment with DFO at high doses for several hours before irradiation [7]. Unfortunately, this hydrophilic and high molecular weight drug has the disadvantage of being taken up only by endocytosis [39,40] and is retained in lysosomes where it causes iron-starvation and, ultimately, cell death [9,10]. Therefore, DFO is not an ideal iron-chelator for cellular protection against oxidative stress. Here we tested the radioprotective effect of the lipophilic iron-chelator SIH that is quickly distributed throughout the cell but can also easily be washed away [16]. That the protective effect reported here is due to the iron-chelating effect of SIH is supported by the experiments showing that addition of iron had a sensitizing effect (Figs. 2B and 3B). While SIH has already been shown to give excellent protection from hydrogen peroxide-induced oxidative stress [10,16], the findings in the present report suggest that SIH also can be used to protect normal tissues from radiation damage and may allow exposure to a higher-than-normal-dose of ionizing radiation without causing damage in the normal tissue that is adjacent to a malignancy.

As an example: in the specific case of lung cancers, the tendency of normal lung tissue to develop radiation-induced pulmonary fibrosis severely limits the use of radiotherapy. Lung cancers usually compress a branch of the bronchus system, leaving most of the lung aerated, while the tumor itself and the lung tissue distal to it are not (Fig. 5). An aerosol containing a powerful iron-chelator might, therefore, protect normal lung tissue against radiation, while the tumor itself should not be affected (Fig. 5). Our data indicate that even low concentrations of SIH (10 or 20 μM) would allow the radiation dose to be increased by 80% without the induction of additional damage to normal tissue. This dose-modifying effect makes SIH one of the most powerful radio-protectors tested so far. Interestingly, cells exposed to SIH-only actually grew better than the control cells, suggesting that SIH protects against damage caused by having cells outside the incubator. Inasmuch as SIH can be readily removed, allowing high concentrations to be used, one might expect striking effects. Indeed, SIH at 100 μM protected between 6- and 35-fold against hydrogen peroxide-induced cell death (Fig. 3). Moreover, doubling the SIH concentration increased its irradiation-dose-modifying effect four-fold (Table 1). All other radio-protective substances, e.g. sulfhydryl compounds, phytochemicals, and aminothiols, which are the most effective of the presently known radioprotectors, must be applied in much higher concentrations (0.5 to 10 mM) in order to reach similar PF [41-47]. To confirm the high effectiveness of SIH, and to compare it to other radioprotectors, animal experiments are needed.

The additive effects of LMP, a consequence of intralysosomal Fenton-type reactions secondary to enhanced cellular amounts of hydrogen peroxide, on top of the effects induced by direct formation of hydroxyl radicals following radiolytic cleavage of water, are dependent on the presence of oxygen that allows formation of superoxide and hydrogen peroxide (see formulas in the beginning of the Discussion section). The importance of this additive effect is illustrated by the well-known fact that hypoxic malignancies, e.g., such that infiltrate bone tissue, respond less well to ionizing radiation. In hypoxic tissues there will be limited formation of superoxide and hydrogen peroxide and, consequently, little LMP will take place.

In this study we propose a new strategy for protection of cells against ionizing radiation and explain its underlying molecular mechanisms. Our results indicate that application of SIH as an

aerosol prior to each irradiation session would allow exposure to a higher-than-normal-irradiation-dose and may increase the survival chance for lung cancer patients, which now show the highest mortality of all cancer patients [11], by protecting normally aerated, and therefore accessible to an aerosol, lung tissue, but not the solid malignancy without airways.

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**TABLE**

**Table 1**. **Protection factors (PF or survival indices) for various cell lines following ionizing irradiation in the presence of the iron-chelator SIH.**
PFs were calculated based on clonogenic cell survival assays (see Fig. 2) as the ratio of the doses with and without SIH given 10 % survival.

| Cell types | SIH concentration | PF | Additional radiation dose without changes in cell survival |
|---|---|---|---|
| HeLa | 10 µM | 1.20 | 20 % |
| HeLa | 20 µM | 1.78 | 78 % |
| J774 | 10 µM | 1.30 | 30 % |
| U1690 | 20 µM | 1.20 | 20 % |

## LEGENDS TO FIGURES

**Figure 1**. **The iron-chelator SIH preserves cell growth following ionizing irradiation**.
HeLa (**A**), U1906e (**B**), and U1810 (**C** and its inset **D**) cells were seeded about 14 h before being exposed for 30–60 min to 10 µM SIH (diamonds) or not (circles) followed by irradiation (filled symbols, solid lines) with 2 (**B**), 3 (**A**) or 5 Gy (**C, D**) or without irradiation (empty symbols, dotted lines). Panel **D** shows survival of U1810 cells two weeks following irradiation under the protection of 10 µM SIH (filled bar) or without such protection (empty bar) compared to non-irradiated cells. Statistically significant differences between irradiated SIH-protected cells and irradiated control cells were calculated using Student's t-test (***P< 0.001, **P< 0.01, *P< 0.05).

**Figure 2**. **The iron-chelator SIH protects cells against ionizing irradiation-induced cell death**.
HeLa (**A** and its inset **B**), J774 (**C**), and U1690 (**D**) cells were seeded about 14 h before being incubated without (circles) or with 10 µM (diamonds) or 20 µM SIH (squares) for 30–60 min and then irradiated with doses between 1 and 8 Gy. The surviving fractions, based on the number of colonies found 10-14 days following the irradiation, were determined and plotted against the radiation dose. Panel **B** demonstrates that enhanced levels of lysosomal iron (a result of endocytotic uptake of Fe-phosphate/hydroxide) increase the damaging effect of ionizing radiation.

**Figure 3**. **The iron-chelator SIH protects U2020 and U1690 cells against hydrogen peroxide-induced cell death**.
U2020 (**A**) and U1690 (**B**) cells were seeded in 96-well-plates ($10^4$ cells/well), while U1690 cells were also seeded in 6-well-plates at a density of 100-800 cells/well (**C**). The cells were then exposed for 1 h to $H_2O_2$ (initially at 0-100 mM) without (empty squares) or together with (filled circles) 100 µM SIH. Cell viability (**A**, **B**) and clonogenic cell survival (**C**) were calculated 24 h and 10-14 days after return to standard culture conditions. The reduced cell survival following the addition of 30 µM $FeCl_3$ to the medium five hours before exposure to $H_2O_2$ is shown by the dashed line with filled diamonds (**B**). The Histogram (**C**) indicates survival of cells exposed to hydrogen peroxide in the presence (filled bars) of 20 µM SIH or not (empty bars). Note that unprotected cells did not survive the $H_2O_2$ exposure. Data represent the average of four to six repeats and two independent experiments.

**Figure 4**. **SIH-protection against the effect of hydrogen peroxide is a function of lysosomal stabilization**.
(**A**). U1690 cells were pre-loaded with AO 16 h after seeding. Cells were then exposed for 30 min to initially 100 µM $H_2O_2$ in Hanks' buffer in the presence of (black histogram) or without (empty histogram) 100 µM SIH, or just kept in Hanks' buffer (grey histogram). After another 30 min at standard culture conditions, the cells were trypsinized and green FL1 fluorescence, being an indicator of AO relocated to the cytosol as a result of lysosomal rupture, was analyzed by flow cytofluorometry (FACS). (**B**). The protection by SIH of lysosomes against $H_2O_2$ was calculated (Student's t-test, ***P< 0.001). The empty bar represents cells exposed to 100 µM $H_2O_2$ only, while the filled bar represents cells exposed to 100 µM $H_2O_2$ in the presence of 100 µM SIH.

**Figure 5**. **Schematic presentation of suggested mechanisms behind SIH-mediated protection against radiation-induced cell death**.
This scenario is based on that lung tumors are non-aerated and therefore not reached by inhaled SIH, while normal lung tissue is aerated and exposed to SIH. Consequently, the normal tissue will be protected against cell death induced by iron-mediated Fenton type reactions following ionizing radiation. If so, a substantially larger dose of irradiation could be applied over a pulmonary field without induction of dangerous fibrosis (for details see text).

**Figure 1**

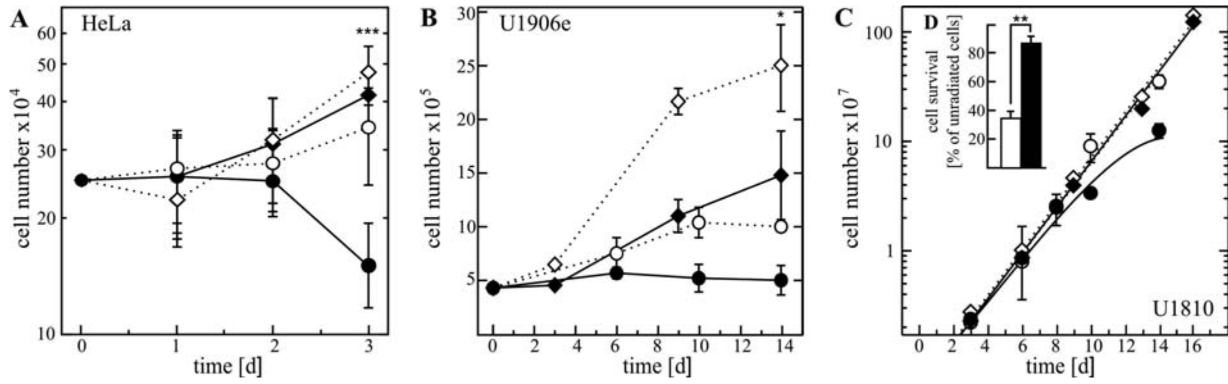

**Figure 2**

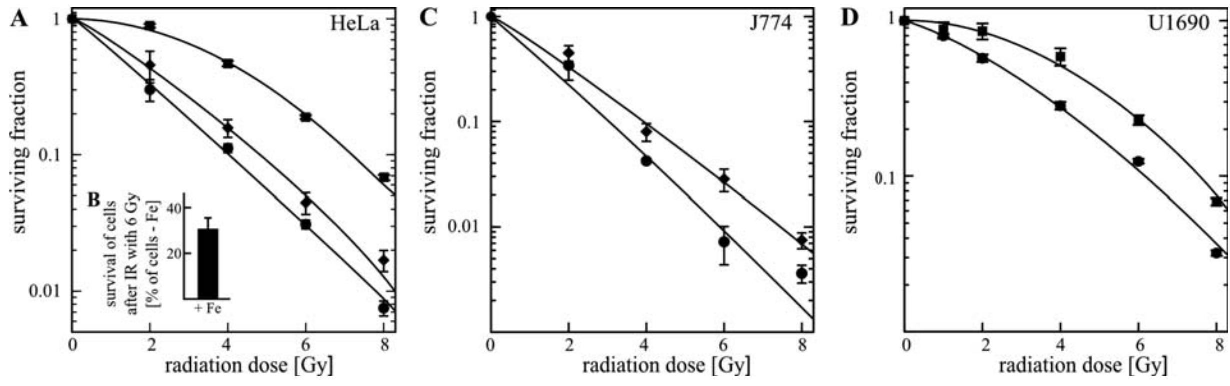

**Figure 3**

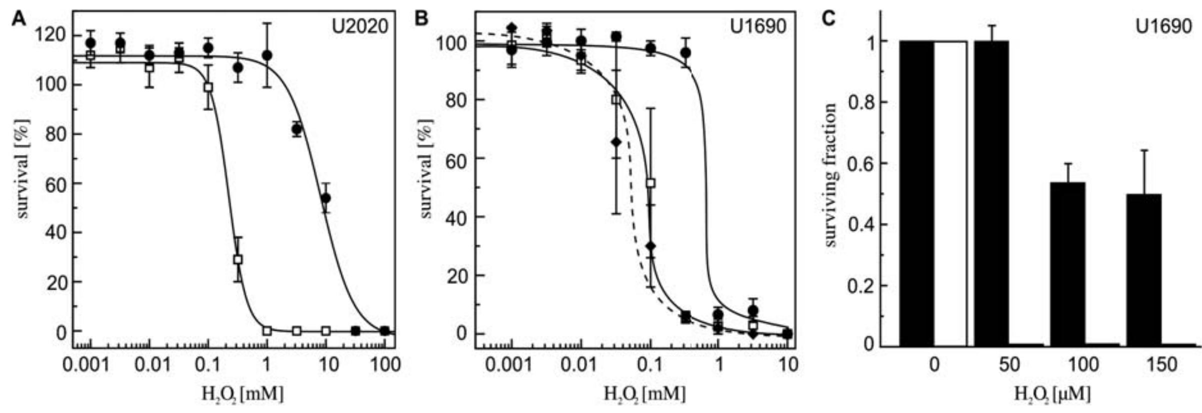

**Figure 4**

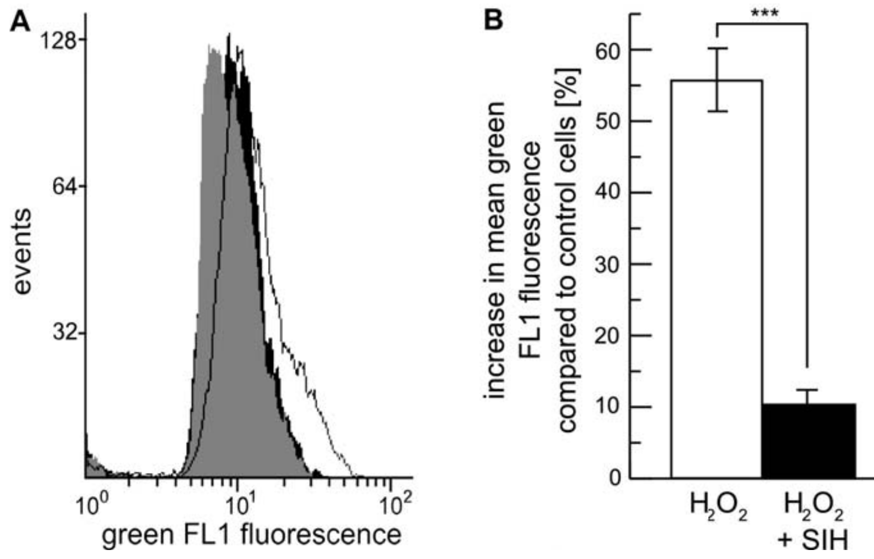

**Figure 5**