Published in final edited form as:

Radiat Res. 2012 September ; 178(3): 150–159.

Identification of Druggable Targets for Radiation Mitigation Using a Small Interfering RNA Screening Assay

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Abstract

Currently, there is a serious absence of pharmaceutically attractive small molecules that mitigate the lethal effects of an accidental or intentional public exposure to toxic doses of ionizing radiation. Moreover, cellular systems that emulate the radiobiologically relevant cell populations and that are suitable for high-throughput screening have not been established. Therefore, we examined two human pluripotent embryonal carcinoma cell lines for use in an unbiased phenotypic small interfering RNA (siRNA) assay to identify proteins with the potential of being drug targets for the protection of human cell populations against clinically relevant ionizing radiation doses that cause acute radiation syndrome. Of the two human cell lines tested, NCCIT cells had optimal growth characteristics in a 384 well format, exhibited radiation sensitivity ($D_0 = 1.3 \pm 0.1$ Gy and $\tilde{n} = 2.0 \pm 0.6$) comparable to the radiosensitivity of stem cell populations associated with human death within 30 days after total-body irradiation. Moreover, they internalized siRNA after 4 Gy irradiation enabling siRNA library screening. Therefore, we used the human NCCIT cell line for the radiation mitigation study with a siRNA library that silenced 5,520 genes known or hypothesized to be potential therapeutic targets. Exploiting computational methodologies, we identified 113 siRNAs with potential radiomitigative properties, which were further refined to 29 siRNAs with phosphoinositide-3-kinase regulatory subunit 1 (p85 α) being among the highest confidence candidate gene products. Colony formation assays revealed radiation mitigation when the phosphoinositide-3-kinase inhibitor LY294002 was given after irradiation of 32D cl 3 cells ($D_0 = 1.3 \pm 0.1$ Gy and $\tilde{n} = 2.3 \pm 0.3$ for the vehicle control treated cells compared to $D_0 = 1.2 \pm 0.1$ Gy and $\tilde{n} = 6.0 \pm 0.8$ for the LY294002 treated cells, $P = 0.0004$). LY294002 and two other PI3K inhibitors, PI 828 and GSK 1059615, also mitigated radiation-induced apoptosis in NCCIT cells. Treatment of mice with a single intraperitoneal

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Editor's Note. The online version of this article (DOI: 10.1667/RR2810.1) contains supplementary information that is available to all authorized users.

LY294002 dose of 30 mg/kg at 10 min, 4, or 24 h after LD_{50/30} whole-body dose of irradiation (9.25 Gy) enhanced survival. This study documents that an unbiased siRNA assay can identify new genes, signaling pathways, and chemotypes as radiation mitigators and implicate the PI3K pathway in the human radiation response.

INTRODUCTION

Recent events at the Fukushima Nuclear Reactor illustrate the enormous risk human populations have of being exposed accidentally to potentially lethal doses of irradiation. Exposure from intentional malicious use of radioactivity also cannot be discounted. Regrettably, there are almost no available effective therapeutic countermeasures against such events.

Radiation damage leads to a host of time-, dose- and cell-type-dependent cellular responses that frequently result in death (1). The array of signaling responses to the radiation-induced damage suggests that multiple targets should exist for therapeutic interdiction of the ultimate toxic effects of radiation. Indeed, experimental radiomitigators have been reported (2–6), such as tetracycline, captopril, enalapril, ramipril, genistein, simvastatin, pravastatin, lovastatin, palifermin, oltipraz, carbamazepine, PD0332991, and the hemigrammidin isostere-linked nitroxide JP4-039, but none have yet been established as being clinically useful. Thus, it is extremely important to define additional radiomitigators as well as new molecular targets for radiation mitigation. We posit that high-throughput small interfering RNA (siRNA)-based screening assays will permit the identification of novel cellular signaling pathways involved in cellular survival after irradiation and help identify potential pharmacological targets. Regrettably, no cell line has been reported to be suitable for high-throughput siRNA screening, which has been shown to have radiobiological sensitivity relevant to the key targeted human populations, namely hematopoietic and gut progenitor cells.

There is a portion of the human genome that has been identified to encode proteins with functions predicted to be targets or are targets for drugs clinically used for human diseases. This so-called druggable genome comprises between 3,000–10,000 genes (7). The products of these genes include protein classes such as kinases, G-protein coupled receptors, phosphatases, proteases, transcriptional regulators, peptidases, cytokines, transporters, transmembrane receptors, and ion channels. By narrowing searches to these proteins, it seems plausible that we would increase the likelihood of identifying useful drug targets for human diseases using siRNA screening methodologies.

Recently, we (8) and Kim *et al.* (9) established the utility of siRNA screening to identify small molecules with radiation protection properties, which are effective when administered prior to irradiation. This is in contrast to radiation mitigators, which are administered after irradiation but before clinical syndromes manifest. In previously published work (8), we optimized an siRNA transfection process using a highly radiation-resistant cell population and screened for radioprotective effects using an siRNA library targeting 5,520 genes known to encode gene transcripts that are actual or potential drug targets. We found 116 candidate protective genes and discovered that the commonly used second generation hypoglycemic

agent, 5-chloro-N-(4-[N-(cyclohexylcarbamoyl)sulfamoyl]phenethyl)-2-methoxybenzamide (also known as glyburide or glibeclicimide), and an inhibitor of the sulfonylurea receptor type 1 (SUR1), protected multiple cell lines against γ radiation. Significantly, glyburide was radioprotective *in vivo* (8). Although these results illustrate the power of an unbiased combined approach of high-throughput siRNA screening and conventional cell-based assay to identify new radioprotectors, regrettably, glyburide and almost all known radioprotectors do not function as radiomitigators (3, 8). This may not be surprising because the temporal biological processes involved in protection against the toxic actions of radiation are likely to be different from those involved in mitigating the damage (3). The most important cellular populations requiring protection after accidental or intentional human radiation exposure are the pluripotent stem cells. Therefore, in this study we developed an assay with pluripotent cells that would empower us to identify therapeutically attractive molecular targets and small molecules for radiation mitigation.

MATERIALS AND METHODS

Reagents

DharmaFect2 transfection reagent and 5X siRNA resuspension buffer were obtained from Dharmacon (Lafayette, CO). CellTiter-Blue® viability and CaspaseGlo® 3/7 caspase activity assays were purchased from Promega (Madison, WI). White and black tissue culture treated 384-well microtiter plates were from Greiner Bio-One (GmbH, Frickenhausen, Germany). OptiMem was purchased from Life Technologies (Grand Island, NY), and RPMI-1640 and fetal bovine serum (FBS) were from MediaTech, Inc. (Manassas, VA). The Silencer Druggable Genome siRNA Library (version 1.1), GAPDH siRNA and negative control siGenome Non-Targeting siRNA (#1) was purchased from Ambion (Austin, TX), and the positive control “AllStars HS Cell Death” siRNA was purchased from Qiagen (Germantown, MD). Caspase 3 siRNA was from Dharmacon (OnTarget Plus SmartPool). Independent secondary siRNA for AGT, DCK, PRIM2A and PI3KIR were obtained from Dharmacon. LY294002 (2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one) was purchased from Enzo Life Sciences (Farmingdale, NY) and PI 828 (2-(4-morpholinyl)-8-(4-aminophenyl)-4H-1-benzopyran-4-one) and GSK105961 (5-[[4-(4-pyridinyl)-6-quinolinyl]methylene]-2,4-thiazolidinedione) were obtained from Tocris (Ellisville, MO). Annexin V, propidium iodide and annexin V buffer were purchased from Becton Dickinson (Franklin Lakes, NJ).

Cell Culture

Human pluripotent embryonal carcinoma NCCIT and NTERA2 cells were purchased from American Type Culture Collection (Manassas, VA). Cell lines were maintained in RPMI-1640 (for NCCIT cells) or DMEM (for NTERA2 cells) medium supplemented with 10% fetal bovine serum, penicillin, streptomycin and L-glutamine. Murine hematopoietic progenitor 32D cl 3 cells were passaged in 15% WEHI-3 cell-conditioned medium (as a source of Interleukin 3), and 10% fetal bovine serum and McCoy's supplemented medium according to published methods (2, 8). All cell lines were maintained at 37°C with 5% CO₂.

Automated High-Throughput siRNA Delivery of siRNA

We optimized NCCIT transfection for vehicle (0.7 µl/well DharmaFect2), siRNA concentration (15 nM), and total volume (50 µl) using reverse transfection of Cell Death siRNA in 384-well microtiter plates. The siRNA complexes were prepared by mixing DharmaFect2 in OptiMem serum-free medium and were dispensed into assay plate with a Zoom bulk dispenser (Titertek-Berthold, Huntsville, AL). The siRNA library was transferred to 384-well microtiter plates with Velocity 11 Bravo (Agilent, Santa Clara, CA). The NCCIT cell suspension (3×10^5 cells/ml) was exposed to γ irradiation (4 Gy) using the Gammacell 1000 Elite (Best Theratronics, Ottawa, ON, Canada). The cell suspension was diluted and 800 cells were added to each well in a volume of 50 µl. After 48 h incubation, the medium was replaced, and after an additional 48 h incubation, the medium was replaced with CellTiter-Blue® reagent. After 4 h incubation at 37°C, the fluorescence was read on the SpectraMax M5e plate reader (Molecular Devices, Sunnyvale, CA). Control plates with nonirradiated cells transfected with both positive control (Cell Death) siRNA (for transfection) and negative control (scrambled) siRNA (for day-to-day variability) were also included in each experiment to control for quality. Data from experimental plates when the positive control caused <80% death or negative controls caused >15% death were not included. Data from the siRNA screening was obtained from experiments performed on 4 independent days. The resulting data were analyzed using our previously described methods (10, 11). Briefly, relative fluorescence units from each targeted siRNA well were normalized to in-plate scrambled negative control values, which allowed for plate-to-plate comparisons. The Z'-score for each gene was used to measure the statistical difference from the in-plate scrambled control. This was followed by a Median Absolute Deviations analysis for outlier detection (11). The top siRNAs from these analyses (192 siRNAs) had a Z'-score >5. As a parallel analysis, the number of cells in the siRNA treated population was compared to the number of cells in the population exposed to scrambled siRNA to calculate the magnitude of radiation-induced mitigation. The top siRNAs from this analysis (225 siRNAs) had a percentage of in-plate control value of >10% that was reproducible in at least 3 of the 4 replicates of the screen. The two analysis methods had an overlap of 113 siRNAs, which we defined as our initial highest confidence siRNA radiomitigators.

Colony Formation and Western Blotting Assays

Colony formation was determined as previously described (2, 8, 12). Briefly, 32D cl 3 cells (5×10^5 cells/ml) were exposed to 0–8 Gy using a Shepard Mark I model 68 cesium irradiator (dose rate 75 cGy/min) and 1 h later vehicle or test compound was added to the Interleukin 3-supplemented semisolid methycellulose-containing medium in which cells were incubated for 7 days at which time colonies >50 cells were counted. NCCIT and NTERA2 cells were similarly irradiated (0–8 Gy) and 1 h later plated in 4-well Linbro plates (MP Biomedicals, Solon, OH) in RPMI-1640 (for NCCIT cells) or DMEM (for NTERA2 cells) medium supplemented with 10% fetal bovine serum, penicillin and streptomycin. Cells were incubated at 37°C for 5 days, at which time the cells were stained with crystal violet and colonies of >50 cells were counted. The data for all cell types were fitted using linear quadratic and single-hit, multitarget models as previously described (2, 8, 12).

For irradiation protection experiments, 32D cl 3 cells were preincubated for 1 h with LY294002 (0–10 μ M), irradiated with 0–8 Gy, plated in methylcellulose and incubated at 37°C for 7 days. For irradiation mitigation experiments, cells were irradiated, plated in methylcellulose medium containing LY294002 (0–10 μ M) and incubated at 37°C for 7 days. Colonies of >50 cells were counted and the data were analyzed using linear quadratic and single-hit, multitarget models (2, 8, 12).

Protein lysates from NCCIT cells (~30 μ g/well) were isolated as previously described (10) and, after separation on a 4–20% acrylamide gradient gel, the proteins were transferred to a nitrocellulose membrane with iBlot (Life Technologies). The membranes were blotted with caspase 3 antibody (Stressgen, New York, NY) at a 1:1000 dilution in 5% milk/Tris buffer saline (50 mM Tris • HCl, pH 7.4, 150 mM NaCl) with 0.1% Tween 20 at 4°C overnight. Positive antibody reactions were visualized using peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA) and chemiluminescence by ECL Western blotting.

Apoptosis Assays

Apoptosis was measured by caspase activation and annexin V staining. For caspase activation, an NCCIT cell suspension (~3 $\times$ 10⁵ cells/ml) was exposed to 0 or 4 Gy γ irradiation and cells were then diluted and plated (final 3,500 cells/well) with 40 nM siRNA and 0.7 μ l/well DharmaFect2 in 384-well plates in 50 μ l total volume. In some assays cells were treated 1 h after irradiation with various concentrations of LY294002, PI 828 or GSK 1059615. After 48 h of growth, the medium was replaced with a 1:5 dilution of CaspaseGlo 3/7 reagent (Promega) in phenol red-free RPMI 1640 medium with 1% fetal bovine serum. After 1 h incubation at room temperature, the luminescence was measured on an Envision (Perkin Elmer, Waltham, MA) or Molecular Devices SpectraMax M5 (Sunnyvale, CA) plate reader. For annexin V staining, NCCIT cell suspension (~3 $\times$ 10⁵ cells/ml) was exposed to 0 or 4 Gy γ irradiation, plated in 25 cm² flasks (3 $\times$ 10⁵ cells/flask) and 1 h later was treated with LY294002. After 48 h detached cells in the medium were combined with cells removed from the interior flask surface by trypsin treatment, centrifuged at 100g for 5 min, washed twice with cold PBS, and resuspended in a solution of 10 mM HEPES (pH 7.4), 140 mM NaCl₂, 2.5 mM CaCl₂ at the density of 1 $\times$ 10⁶ cells/ml. From this cell solution, 100 μ l (~1 $\times$ 10⁵ cells) was transferred to 5 ml culture tubes with 5 μ l annexin V-FITC and 2 μ l propidium iodide. Cells were then gently mixed and incubated for 15 min at room temperature in the dark and then analyzed by flow cytometry (Becton Dickinson/Cytek FACSCalibur Benchtop Analyzers).

In Vivo Mitigation Assays

C57BL/6NTac female mice (15 mice per treatment group) were irradiated to 9.25 Gy total body irradiation and injected intraperitoneally 10 min, 4 h, 24 h, or 48 h later with 30 mg/kg LY294002 or vehicle control and were followed for development of hematopoietic syndrome at which time they were sacrificed (8, 12). For complete blood counts, blood was collected from mice 3 days after irradiation in EDTA treated MiniCollect tubes (Greiner-bio-one, Germany). Samples were analyzed using a scil Vet abc hematology analyzer (scil Animal Care Company, Gurnee, IL). Each sample was analyzed for the following: total

white blood cells, total red blood cells, lymphocytes, granulocytes, monocytes, and neutrophils. For the hematopoietic cell colony-forming assays, tibial bone marrow cells were plated in triplicate in methylcellulose medium supplemented with mouse stem cell factor, IL-3, IL-6 and erythropoietin (Stem Cell Technologies, Inc., Vancouver, Canada) for assessment of hematopoietic cells within the marrow capable of forming colonies in semisolid medium *in vitro*. Colonies were scored on day 11 for colony forming unit-granulocyte macrophage, burst forming unit erythroid, and the colony-forming unit: granulocyte-erythroid-megakaryocyte-monocyte.

Statistics

Unless otherwise noted, data were expressed as means $\pm$ SEM of at least three determinations. Data from the *in vitro* radiation survival curves were analyzed with the Student's *t* test, while mouse survival data were analyzed by a log rank test (2). Statistical comparisons between different groups were performed with Student's *t* test or ANOVA, and $P = 0.05$ was considered significant. Hematopoietic end points and the colony-formation assays were analyzed with a two-sided two-sample Student's *t* test to compare treatment groups.

Animal Welfare

All animal protocols were approved by the University of Pittsburgh Institutional Animal Care and Use Committee and were performed under the supervision of the Division of Laboratory Animal Research of the University of Pittsburgh. The Division of Laboratory Animal Research of the University of Pittsburgh provided veterinary care.

RESULTS

Selection of NCCIT Cells as the Model System

We first quantified the radiosensitivity of two human pluripotent embryonal carcinoma cells, NCCIT and NTERA2, using a homogeneous, fluorometric cell viability assay that was previously found to be suitable for high-throughput screening (13). Ninety-six hours after exposure to 4 Gy, NCCIT cells displayed an approximate 50% decrease in viability (See Supplementary Material, Fig 1A: <http://dx.doi.org/10.1667/RR2810.1.S1>). By comparison, NTERA2 cells were more sensitive to irradiation, showing an approximate 50% decrease in viability after only 2 Gy exposure. These data were supported by survival results using NCCIT and NTERA2 clonogenic assays (See Supplementary Material, Fig 1B: <http://dx.doi.org/10.1667/RR2810.1.S1>). Specifically, NCCIT cells had a D_0 value of 1.3 ± 0.1 Gy and an $\tilde{n}$ value of 2.0 ± 0.6 , while NTERA2 cells had a D_0 value 1.1 ± 0.3 Gy and an $\tilde{n}$ value of 1.0 ± 0.1 , respectively (See Supplementary Material, Fig 1B: <http://dx.doi.org/10.1667/RR2810.1.S1>). Both cell lines were more radiosensitive than murine hematopoietic progenitor 32D cl 3 cells, which had a D_0 value of 1.3 ± 0.1 and an $\tilde{n}$ value of 2.3 ± 0.3 . We found, however, that NTERA2 cells required an exceptionally high plating density for reproducible growth, which was not suitable for high-throughput screening. Moreover, treatment of NCCIT cells 20 min after irradiation with the previously reported radiomitigators, JP4-039 and amifostine (14) resulted in a significant reduction of caspase 3 activation (Fig. 1A), which validated the assay. We also found NCCIT cells had a colony

formation efficiency of 14.2 ± 0.8 ($n = 8$). Therefore, NCCIT cells were used for all further studies.

siRNA Transfection Efficiency after Ionizing Radiation

To ensure that ionizing radiation did not negatively alter NCCIT transfection efficiency with siRNA, we subjected NCCIT cells to reverse siRNA transfection 90 min after mock or 4 Gy γ irradiation. Cell death siRNA was equally effective in reducing cell viability (~97%) in both control and 4 Gy treated cells (See Supplementary Material, Fig 2A: <http://dx.doi.org/10.1667/RR2810.1.S2>). Similarly, caspase 3 siRNA retained its ability to suppress caspase 3 activity (See Supplementary Material, Fig 2B: <http://dx.doi.org/10.1667/RR2810.1.S2>) and protein levels (See Supplementary Material, Fig 2C: <http://dx.doi.org/10.1667/RR2810.1.S2>) following 4 Gy. These data indicated ionizing radiation did not impede siRNA transfection or the depletion of targeted proteins within NCCIT cells.

Prioritization of Genes and Signaling Pathways for Potential Radiation Mitigation

We therefore used this assay format and examined a 5520 siRNA druggable genome library for potential radiation mitigators using a two-tiered data analysis scheme. We identified 113 siRNAs, which represented an approximate 2% hit rate (See Supplementary Material, Table 1: <http://dx.doi.org/10.1667/RR2810.1.S4>). These targets ranged in functional category from transcriptional activators to cytokines (See Supplementary Material, Fig 3: <http://dx.doi.org/10.1667/RR2810.1.S3>). The frequency of target classes being identified would be expected to reflect the composition of the siRNA library used. For example, the library we screened was enriched for kinases as they are frequently viewed as being good drug targets and 25% of the targets identified were indeed kinases.

To evaluate the siRNAs as mitigators of apoptosis induction, we used a caspase 3 secondary assay and found that 29 siRNAs suppressed caspase 3 activation after γ irradiation (Table 1). Of particular note are four gene products: i.e., DNA primase 2 (PRIM2A), angiotensinogen (AGT), phosphatidylinositol 3-kinase regulatory protein (PIK3R1) and deoxycytidine kinase (DCK) for which there are known small molecule inhibitors. Reexamination of these targets with new independent siRNAs (different sequences from an alternative source) confirmed mitigation of radiation-induced apoptosis as measured by caspase 3 activation (Fig. 1B). We also subjected the 29 gene products to computational interrogation, using the web-based Ingenuity Pathway Analysis tool (Ingenuity Systems®, www.ingenuity.com), to identify biological and molecular networks involved in regulating cellular survival after γ irradiation. This web-based tool employs functional annotation and known molecular interactions found in published peer-reviewed scientific publications stored in the Ingenuity Pathways Knowledge Base, which is continuously updated. A molecular network of direct or indirect physical, transcriptional, and enzymatic interactions between mammalian orthologs was computed from this knowledge base and we identified one prominent signaling pathway, which surprisingly contains a majority (13/29) of the siRNA gene products (Fig. 2). In this network, we also noted engagement of PRIM2A, AGT, and PIK3R1.

PI3K Inhibitors as a Radiomitigator in Cells

We were particularly intrigued with the observation that PIK3R1 siRNA mitigated cell survival in response to γ irradiation and the apparent central role of PI3K in the Ingenuity Pathway Analysis because PI3K is generally considered a pro-survival pathway and a target for experimental cancer chemotherapy (15, 16). It is widely appreciated, however, that the cytotoxic stress from exposure to ionizing radiation causes the activation of multiple intracellular signaling pathways, including PI3K (17). Therefore, we tested the ability of the prototype PI3K small molecule inhibitor, LY294002, to enhance cell survival after γ -irradiation exposure. 32D cl 3 cells were treated with LY294002 (0–10 μ M) prior to or after 0–8 Gy γ irradiation. Remarkably, we observed enhanced survival when cells were incubated with a low LY294002 concentration (i.e., 0.1 or 1 μ M) after irradiation as indicated by the increased shoulder on the radiation survival curve [$\bar{n}$ = 6.0 ± 0.8 or 6.4 ± 2.1 , respectively, compared to 2.3 ± 0.3 (P = 0.0004 or 0.011, respectively) for the control irradiated cells] (Table 2, Fig. 3). There was no significant difference in the D_0 (1.2 ± 0.1 Gy) compared to the cells incubated with vehicle alone (1.3 ± 0.1 Gy) nor did we observe statistically significant differences in either $\bar{n}$ or D_0 at higher LY294002 concentrations. However, pre-treatment with 10 μ M LY294002 also protected 32D cl 3 cells against radiation-induced cell death (Table 2). We next examined the ability of LY294002 and two other PI3K inhibitors to mitigate NCCIT radiation-induced apoptosis (Fig. 4). Cells treated 1 h after 4 Gy with 3.12 and 6.25 μ M LY294002 exhibited reduced apoptosis, while concentrations of LY294002 12.5 μ M alone caused apoptosis as anticipated for a PI3K inhibitor (Fig. 4A). We also observed a decreasing trend in the percentage of cells that were annexin V positive after treatment with LY294002 (Fig. 5). Concentrations of PI 828 ranging from 0.78 to 3.12 μ M also decreased caspase 3 activation; higher concentrations of PI 828 alone caused apoptosis (Fig. 4B). We next tested other known PI3K inhibitors as potential radiation mitigators. The potent PI3K inhibitor GSK 1059615, which has a chemical structure that is distinct from either LY294002 or PI 828, also was a radiation mitigator and produced significant reduction in caspase 3 activation at 0.2–0.8 μ M without causing apoptosis alone (Fig. 4C). These results revealed that at least two different PI3K inhibitory chemotypes could mitigate the apoptotic effects of radiation.

LY294002 as an In Vivo Radiomitigator against the Hematopoietic Syndrome after Total-Body Radiation

Prior work indicated loss of hematopoietic progenitors is responsible for the lethality caused by the LD_{50/30} dose of total-body radiation. Therefore, we assessed whether our cell culture result with pluripotent embryonal cells could guide mitigation studies in whole organisms.

Mice were exposed to 9.25 Gy total-body irradiation, and 10 min, 4, 24 or 48 h later they were injected intraperitoneally with vehicle or LY294002 (30 mg/kg). The mice were then monitored for development of bone marrow failure and subsequent death (Fig. 6). More than half of the mice injected with LY294002 10 min after the lethal dose of radiation survived for at least 60 days, while 50% of mice receiving vehicle alone died by 14 days. Therefore, there was a significant increase in survival (P < 0.0001) with LY294002 treatment 10 min after irradiation. Significant radiation mitigation was also seen when LY294002 was given 4 h (P = 0.001) or 24 h (P = 0.017) after 9.25 Gy total whole body radiation (Fig. 6).

Intraperitoneal LY294002 treatment at 48 h did not yield statistically significant radiomitigation. We examined mice 3 days after irradiation and observed a marked decrease in white blood cell counts that was not altered by LY294002 treatment 4 h after 9.25 Gy ($8.8 \times 10^3/\text{mm}^3 \pm 2.8 \times 10^3/\text{mm}^3$ for 0 Gy, $0.7 \times 10^3/\text{mm}^3 \pm 0.5 \times 10^3/\text{mm}^3$ for 9.25 Gy alone, $0.5 \times 10^7/\text{mm}^3 \pm 0.1 \times 10^3/\text{mm}^3$ for 9.25 Gy plus LY294002 treatment: $n = 5$ mice). Similarly, we saw a decrease in red blood cells, lymphocytes, mononuclear granulocytes and neutrophils, but no obvious mitigation with a single LY294002 treatment 4 h after irradiation. We also conducted hematopoietic cell colony-forming assays 3 days after irradiation. Total colony formation was markedly reduced in mice exposed to 9.25 Gy and LY294002 treatment (30 mg/kg) mitigated this effect (See Supplementary Material, Table 2: <http://dx.doi.org/10.1667/RR2810.1.S5>). Therefore, it is possible that radiomitigation effect of LY294002 reflects actions on the critical, but relatively rare, hematopoietic stem cells or other cellular populations important in mouse survival.

DISCUSSION

Unbiased loss-of-function experiments using siRNA libraries and phenotypic high-throughput mammalian cell screening have enabled investigators to determine essential viability genes (13, 18, 19), chemosensitivity and chemo-resistance genes (20–22), and novel interactive pathways (23–25). Indeed, we previously employed the same siRNA library and phenotypic end point as used in the current study to successfully identify novel radioprotective genes and small molecules (8). Importantly, in the current study, we focused on discovering radiomitigators, which by definition are effective when administered after irradiation but before clinical syndromes are detected, and have used cells that emulate the most important population to protect after irradiation: the pluripotent stem cells. In our previous study (8), we used the highly radioresistant T98G glioblastoma cells, which require a 25 Gy dose to achieve a 50% decrease in cell survival in the high throughput screening format. In contrast, in the current study we identified two pluripotent cell lines that respond radiobiologically similar to human hematopoietic and gut progenitor cell populations, which are the targets of organ failure leading to the hematopoietic and gastrointestinal syndromes, respectively. The sensitivity range of NCCIT cells (2–8 Gy) encompasses the human-relevant LD₁₀, LD₅₀, and LD₁₀₀ doses that cause death at 30 days after human total-body irradiation (1). Drugs that could reduce cellular death after these doses of radiation are of considerable importance because of the possible applications in clinical radiotherapy, after accidental exposure to radiation, and in radiation counterterrorism.

Ionizing irradiation generates nuclear DNA strand breaks. Quite rapidly afterward, a robust molecular damage response ensues that includes mitochondrial transport and activation of both apoptotic and antiapoptotic signal transduction (12, 26, 27). The resulting cell population has a markedly different gene expression profile, signaling network, and metabolic status from that of the pre-irradiated population. Therefore, it is possible that different proteins would play important roles for cell survival before and after irradiation. Indeed, the results from our study support this hypothesis. We previously used the same siRNA library and end point, and none of the reported 116 radioprotective siRNAs were seen among the 29 radiomitigative siRNA with the exception of the siRNA targeting the EPH receptor B2 and the mitochondrial solute carrier family 25 member 22. We should

acknowledge, however, that we previously examined a highly radioresistant glioblastoma cell line rather than a radiosensitive pluripotent embryonal carcinoma cell line. It is interesting that among the top 29 radiation mitigation genes found in Table 1, eighteen have been associated with apoptotic processes based on a PubMed search of the literature. It seems likely that other genes could participate in radiation mitigation. However, that they were not identified in our screen because we used a siRNA library that targeted gene product viewed as historically druggable, which usually are enzymes. Such a library would not be enriched in macromolecules, which participate in protein-protein interactions and are critical for the apoptotic process.

An intrinsic limitation of siRNA methodology for drug discovery is that it depletes the entire protein while pharmacological agents usually disrupt the enzymatic activity of the protein, a property that makes them so valuable. Therefore, we selected from the collection of radiomitigative siRNAs candidates for which there were readily available and previously reported small molecule inhibitors: AGT, DCK, PRIMA2A and PI3KR1. Angiotensinogen is a serum α 2-globulin secreted in the liver that is hydrolyzed by renin to generate angiotensin-1, a potent regulator of blood pressure, body fluid and electrolyte homeostasis. Angiotensin-1 is a substrate of angiotensin converting enzyme, which removes a dipeptide to yield the physiologically active peptide angiotensin-2 (angiotensin 1–8). It is of particular interest that the angiotensin converting enzyme inhibitors, captopril and losartan, have already been shown to mitigate renal injury caused by single-dose total-body irradiation (4). So, our unbiased siRNA screen provided independent verification that the angiotensin-renin axis can have a critical role in cell survival after ionizing radiation.

Human DNA primase synthesizes short RNA primers that DNA polymerase α then elongates during the initiation of all new DNA strands. Primase is an essential enzyme in all well characterized systems of DNA replication, although to our knowledge it has not been firmly associated with any DNA repair process after ionizing radiation. We evaluated the radiomitigation actions of the primase inhibitor decitabine (1–100 μ M) in 32D cl 3 cells, but found it inactive at low concentrations and toxic at higher concentrations. Deoxycytidine kinase phosphorylates deoxycytidine, deoxyguanosine, and deoxyadenosine and plays an important role in the salvage pathway of nucleoside metabolism. The enzyme appears to have a role in immune function, but there is no firm evidence that it participates in DNA repair. We investigated the deoxycytidine kinase inhibitor fludarabine (1–100 μ M) but found no evidence for mitigation of radiation-induced cell death in 32D cl 3 cells.

It is intriguing that five of the initial 113 highest confidence siRNA radiomitigators identified from 5520 siRNAs in the human druggable genome library were members of the phosphatidylinositol axis: PI3KR1, IMPK, PIK3C2B, PIP5-K2A, and PIP5K2B (See Supplementary Material, Table 1: <http://dx.doi.org/10.1667/RR2810.1.S4>). Phosphatidylinositol 3-kinase phosphorylates the inositol ring of phosphatidylinositol at the 3-prime position. The enzyme comprises a 110 kD catalytic subunit and a regulatory subunit of 85, 55 or 50 kD. The PI3KR1 gene encodes the 85 kD regulatory subunit. PI3KR1 binds to activated (phosphorylated) phosphatidylinositol 3-kinase and acts as an adapter, mediating the association of the p110 catalytic unit to the plasma membrane. Phosphatidylinositol 3-kinase plays an important and complex role in responses to

extracellular signals, in cell cycle control, and in cell survival. Interestingly, PI3KR1 has been reported to act as a signal transducer mediating a proapoptotic response to UVR, not through activation of PI3K, but by the induction of tumor necrosis factor alpha gene expression (28). Recently it has been shown that depletion of PI3KR1 in mouse embryonic fibroblasts significantly impairs UVB-induced apoptosis, as well as p53 transactivation and acetylation at Lys370 (29). In contrast, inhibitors of the PI3K/Akt signaling pathways generally induce apoptosis in tumor cells (30). The specific PI3K inhibitor LY294002 inhibits PI3K/Akt signaling and potentiates the radiation-induced apoptosis (30). So, it was quite unexpected that LY294002 significantly mitigated the loss of colony formation and induction of apoptosis in both murine and human cells after ionizing irradiation. We also observed mitigation of radiation toxicity with PI 828 and GSK 1059615. The pathways regulated by PI3K and PI3KR1 are, however, quite complex and we do not yet have a satisfying mechanistic explanation for the radiomitigation. It is noteworthy that LY294002 has been shown to inhibit apoptosis induced by the phosphatase inhibitor okadaic acid in normal renal epithelial cells (31). This antiapoptotic effect of LY294002 was associated with an increase in the antiapoptotic protein Bcl-2 (31). While we found NCCIT cells expressed basal levels of Bcl-2, we were unable to detect any increase in Bcl-2 after treatment of cells with LY294002 (data not shown). Therefore, mechanistic studies of the radiomitigative actions of LY294002 are worthy of further investigation.

In summary, this study documents that an unbiased siRNA screen can provide information about genes and pathways involved in cell death after irradiation, and reveal new chemotypes that can guard pluripotent cells against death after radiation. Pathway analyses of our screen implicated several genes in the mitigation process including the PI3K pathway. Indeed, our screen identified a promising radiomitigator, the PI3K inhibitor LY294002, which when administered to mice after a lethal dose of irradiation reduced mortality.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

We are grateful for the assistance of Fang Zhang for her intellectual input in the design and experimental methods for the siRNA high-throughput screen. We also thank Sébastien Coquery of the University of Virginia Flow Cytometry Core Facility for his assistance in the annexin V determination. This work was supported by a grant from the National Institutes of Health National Institute of Allergy and Infectious Diseases [U19 AI068021].

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FIG. 1.

Radiomitigation with NCCIT cells. Panel A: NCCIT cells were exposed to 0 Gy or 4 Gy γ irradiation and 20 min later treated with either 0.1% DMSO, 50 μ M JP4-039 or 100 μ M amisfostine. After 48 h, caspase 3 activity was quantified and was normalized to cell number: $N = 4$, bars = SEM. Panel B: NCCIT cells were exposed to 4 Gy γ irradiation and plated in 384-well plates containing scrambled siRNA or siRNA targeting caspase 3, PRIMA2, AGT, DCK or PIK3R1. Medium was replaced after 48 h and 24 h later apoptosis was measured by caspase 3 activation: $N = 4$, bars = SEM.

FIG. 2.

Mapping of potential radiation mitigation networks. The 29 siRNA gene products that were observed as radiation mitigators were functionally analyzed using Ingenuity Pathway Analysis (www.ingenuity.com). The most prominent identified network is illustrated containing 13 siRNA (shaded) from the 29 siRNA. The biological relationship between two nodes is represented as a solid line for a direct protein-protein interaction and dashed line for a potentially indirect relationship. Self-interactions are not shown. All relationships are supported by at least one reference as stored in the Ingenuity Pathways Knowledge Base. Shaded nodes indicate that the siRNA targeting the gene product was exhibited as radiation mitigation in our assay. Nodes are displayed using various shapes that represent the functional class of the gene product: (diamond) enzyme; (inverted triangle) kinase; (square)

cytokine; (elongated diamond) peptidase; (oval) transcription factor; (rectangle) GPCR, and (circle) others.

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FIG. 3.

Radiation mitigation by LY294002 with 32D c13 cells. Cells (5×10^5 cells/ml) were exposed to 0–8 Gy (dose rate 75 cGy/min) and 1 h later vehicle or LY294002 was added to the interleukin 3-supplemented semisolid methycellulose-containing medium in which cells were incubated at 37°C for 7 days at which time colonies >50 cells were counted. The data were fitted using the linear quadratic as well as single-hit, multitarget models as previously described (2, 8, 12). Vehicle control (●), 0.1 μM LY294002 (△), 1 μM LY294002 (◆), or 10 μM LY294002 (▼).

FIG. 4.

Reduction in radiation-induced apoptosis by PI3K inhibitors. NCCIT cells were irradiated with 0 Gy (black bars) or 4 Gy (open bars), and 1 h later treated with DMSO vehicle, LY294002, PI 828 or GSK 1059615. Cells were incubated for an additional 48 h and caspase 3 activation determined: $N = 5 \pm \text{SEM}$.

FIG. 5.

Reduction in radiation-induced apoptosis by LY294002. NCCIT cell suspension ($\sim 3 \times 10^5$ cells/ml) was exposed to 0 Gy (black bars) or 4 Gy (open bars) γ irradiation, plated and 1 h later cells were treated with LY294002. After 48 h the detached cells in the medium were combined with cells detached from the flask, centrifuged, washed with PBS, resuspended in a buffered solution with annexin V-FITC and propidium iodide. Cells were gently mixed, incubated and analyzed for apoptosis by flow cytometry.

FIG. 6.

In vivo radiomitigation with LY294002. Female C57BL/ 6NHsd mice (15 per group) were exposed to an LD_{50/30} dose (9.25 Gy) whole-body irradiation and then received an intraperitoneal injection (0.3 ml) of vehicle alone 10 min later (●) or LY294002 (30 mg/kg) 10 min (■), 4 h (▲), 24 h (▼) or 48 h (◆) later. Mice were followed for development of radiation-induced hematopoietic syndrome.

TABLE 1**siRNA Mitigators of Radiation-Induced Apoptosis**

Gene symbol	Full gene name	Percentage mitigation
PRIM2A	Primase, polypeptide 2A, 58kDa	31.5%
GRIK3	Glutamate receptor, ionotropic kainite 3	26.5%
AGT	Angiotensinogen proteinase inhibitor, clade A (α -1 antiproteinase antitrypsin, member 8)	24.6%
HYAL2	Hyaluronoglucosaminidase 2	22.0%
POLD3	Polymerase (DNA-directed), delta 3, accessory subunit	21.3%
NKTR	Natural killer-tumor recognition sequence	21.2%
ACLY	ATP citrate lyase	21.1%
GBA	Glucosidase, beta; acid (includes glucosylceramidase)	20.2%
TNFRSF1A	Tumor necrosis factor receptor superfamily, member 1A	19.8%
DAB1	Disabled homolog 1 (Drosophila)	19.8%
SUV39H2	Suppressor of variegation 3–9 homolog 2 (Drosophila)	19.5%
GPR62	G protein-coupled receptor 62	19.3%
DCK	Deoxycytidine kinase	18.0%
SI	Sucrase-isomaltase (alpha-glucosidase)	17.5%
SLC25A22	Solute carrier family 25 (mitochondrial carrier:glutamate), member 22	17.5%
CHKB	Choline kinase beta	16.6%
PIK3R1	Phosphoinositide-3-kinase, regulatory subunit 1 (p85 α)	16.4%
FLT3LG	Fms-related tyrosine kinase 3 ligand	16.4%
EPHB2	EPH receptor B2	15.7%
MC3R	Melanocortin 3 receptor	15.4%
P2RY8	Purinergic receptor P2Y, G-protein coupled, 8	15.3%
ATF1	Activating transcription factor 1	15.2%
KHSRP	KH-type splicing regulatory protein (FUSE binding protein 2)	15.2%
CSNK2A2	Casein kinase 2, alpha prime polypeptide	15.1%
NRIP1	Nuclear receptor interacting protein 1	14.8%
MAN2B1	Mannosidase, alpha, class 2b, member 1	14.5%
PMVK	Phosphomevalonase kinase	14.3%
CHUK	Conserved helix-loop-helix ubiquitous kinase	14.1%
RNASET2	Ribonuclease T2	14.1%

Note. The 29 genes silenced by the top siRNAs as described in the Materials and Methods section are listed in descending order of their effectiveness in inhibiting radiation-induced caspase activation.

TABLE 2

Radiomitigative Effects of LY294002

LY294002 (μM)	Preirradiation		Postirradiation	
	D_0 (Gy)	$\bar{n}$	D_0 (Gy)	$\bar{n}$
0	1.3 ± 0.1	2.3 ± 0.3	1.3 ± 0.1	2.3 ± 0.3
0.1	1.4 ± 0.2	4.1 ± 1.7	1.2 ± 0.1	6.0 ± 0.8 ($P = 0.0004$)
1.0	1.5 ± 0.3	3.7 ± 1.8	1.2 ± 0.2	6.4 ± 2.1 ($P = 0.011$)
10	1.4 ± 0.1	3.9 ± 0.4 ($P = 0.03$)	1.2 ± 0.1	3.3 ± 0.7

Notes. 32D cl 3 cells were irradiated with 0–8 Gy and 1 h later vehicle or LY294002 was added to the Interleukin 3-supplemented semisolid methycellulose-contain medium in which the cells were incubated for 7 days at which time colonies with ≥ 50 cells were counted. Mean values of three determinations from a single experiment $\pm$ SEM. These results were confirmed in two other independent studies.