Breaking the Invulnerability of Cancer Stem Cells: Two-Step Strategy to Kill the Stem-like Cell Subpopulation of Multiple Myeloma

Agnieszka Morgenroth, Andreas T.J. Vogg, Boris D. Zlatopolskiy, Monika Siluschek, Caroline Oedekoven, and Felix M. Mottaghy

Abstract

In multiple myeloma, the presence of highly resistant cancer stem cells (CSC) that are responsible for tumor metastasis and relapse has been proven. Evidently, for achieving complete response, new therapeutic paradigms that effectively eradicate both, CSCs and bulk cancer populations, need to be developed. For achieving that goal, an innovative two-step treatment combining targeting of thymidine de novo synthesis pathway and a nanoirradiation by the Auger electron emitting thymidine analogue \( 125/123\text{I-5-iodo-4′-thio-2′-deoxyxuridine (125/123I-ITdU)} \) could be a promising approach. The pretreatment with thymidylate synthase inhibitor 5-fluoro-2′-deoxyuridine (FdUrd, 1 \( \mu \)mol/L for 1 hour) efficiently induced proliferation and terminal differentiation of isolated myeloma stem-like cells. Moreover, FdUrd stimulation led to a decreased activity of a functional CSC marker, aldehyde dehydrogenase (ALDH). The metabolic conditioning by FdUrd emerged to be essential for enhanced incorporation of \( 125\text{I-ITdU} \) (incubation with 50 kBq/2 \( \times 10^6 \) cells for 4 days) and, consequently, for the induction of irreparable DNA damage. \( 125\text{I-ITdU} \) showed a pronounced antimyeloma effect on isolated tumor stem-like cells. More than 85% of the treated cells were apoptotic, despite activation of DNA repair mechanisms. Most important, exposure of metabolically conditioned cells to \( 125\text{I-ITdU} \) resulted in a complete inhibition of clonogenic recovery. This is the first report showing that pretreatment with FdUrd sensitizes the stem-like cell compartment in multiple myeloma to apoptosis induced by \( 125\text{I-ITdU} \)-mediated nanoirradiation of DNA. Mol Cancer Ther; 13(1); 1–10. ©2013 AACR.

Introduction

Multiple myeloma is a plasma cell malignancy characterized by accumulation of malignant, terminally differentiated B cells in the bone marrow. In fact, the current available conventional therapies (e.g., dexamethasone and melphalan) followed by autologous stem cell transplantation (ASCT) or immunomodulatory drugs (thalidomide, lenalidomide, and bortezomib) efficiently eliminate the bulk of rapidly dividing terminally differentiated tumor cells but fail to eradicate a subpopulation of cancer stem cells (CSC; ref. 1). This small fraction of tumor cells characterized by increased resistance to chemotherapy and radiotherapy persists after treatment and drives the recurrence of the disease even in patients who achieved a complete clinical remission. Several \textit{in vitro} and \textit{in vivo} studies have attempted to isolate and characterize myeloma stem cells. The first \textit{in vivo} model using severe combined immunodeficient (SCID) mice implanted with human fetal bone fragments identified clonogenic properties of CD38\textsuperscript{+} but CD19\textsuperscript{+} CD45\textsuperscript{−} cells (2). The capacity of these plasma cells to self-renewal was additionally shown by engrafting secondary SCID-hu recipient mice. In contrast to these studies, Pilarski and colleagues indicated that the clonogenic subpopulation consists of both CD38\textsuperscript{+} plasma cells and CD19\textsuperscript{+} B cells (3). A subsequent study showed that clonotypic CD19\textsuperscript{+} CD138\textsuperscript{−} cells are myelomagenic in NOD/SCID mice and give rise to both CD19\textsuperscript{+} and CD138\textsuperscript{+} tumor cells (4). Another study revealed that only CD138\textsuperscript{−}, but not CD138\textsuperscript{+} cells, were able to engraft in mice following intravenous injection and give rise to mature CD138\textsuperscript{+} plasma cells functionally capable of producing circulating M protein (5). In addition, an \textit{in vitro} clonogenic assay confirmed that myeloma stem cells are not mature CD138-expressing plasma cells but instead resemble CD19\textsuperscript{+} CD27\textsuperscript{+} CD138\textsuperscript{−} memory B cells (6). Finally, clinical studies have found that circulating B cells can overcome systemic chemotherapeutic treatments and their frequency increases during clinical relapse (7). Opposing these studies, Hosen and colleagues reported that both CD138\textsuperscript{−} and CD138\textsuperscript{+} myeloma cells have potential to propagate and maintain myeloma clones (8).
Interestingly, recent studies suggest that CD138 expression on myeloma cells may be reversible and is dependent on the microenvironment (9). Thus, the significance of CD138 expression needs to be carefully interpreted and for precise characterization of myeloma stem cells further qualities have to be considered. Generally, CSCs were shown to share several properties with normal stem cells, like the high expression level of ATP-binding cassette (ABC) drug pumps, intracellular detoxification enzymes, and cell quiescence (6). On the basis of these cellular analogies, several therapeutic strategies have been developed to kill tumor-initiating cells. Potential approaches include blocking essential self-renewal signaling, inhibition of cell survival mechanisms, or targeting of tumor stem cells surface markers (10, 11). Another strategy relies on the sensitization of tumor stem cells. For this proposal, the quiescent CSCs need to be awakened to enter the cell cycle. Because some of CSC properties resemble those of stem cells, it is likely that multiple myeloma stem cells can be activated by “danger signals.” It was shown that normal stem cells enter cell cycling in response to stimulation with 5-fluorouracil (5-FU), the prodrug of 5-fluoro-2'-deoxyuridine (FdUrd; ref. 12). As proliferating cells are potential targets, we decided to investigate the efficiency of thymidine analogue 5-iodo-4'-thio-2'-deoxyuridine (FdUrd; ref. 12). As proliferating cells are potential therapy targets, we decided to investigate the efficiency of the Auger electron emitting thymidine analogue 123I-5-iodo-4'-thio-2'-deoxyuridine (ITdU; refs. 13–15) for eradication of myeloma cell population displaying stem cell properties. 123I-ITdU is known to be efficiently incorporated into the DNA via the salvage pathway, thus providing a promising access for delivering Auger radiation emitters such as 125I and 125I into tumor DNA (15). Auger electrons emitted within DNA are extremely toxic to the cell. This effect is due to the generation of high ionization density clusters at the location of emission, which predominantly induce double-strand breaks (DSB), representing highly cytotoxic forms of DNA damage (16). As shown previously in a leukemia SCID mouse model, pretreatment with a nontoxic dose of FdUrd resulted in increased incorporation of 125I-ITdU into the leukemia cells by blocking the key enzyme thymidylate synthase followed by a depletion of the cellular thymidine triphosphate pool (14, 15). The predominant increase in 125I-ITdU incorporation was observed in tumor tissue leading to extensive tumor damage, whereas only moderate injury occurred in normal tissues (14).

Encouraged by these recently published results, in the current study, we evaluated a 2-step strategy for an efficient eradication of an FdUrd-activated myeloma stem-like cell subpopulation using the radiotherapeutic drug 125I-ITdU.

Materials and Methods

Chemicals

Chemicals and solvents were purchased from Sigma-Aldrich and Merck or otherwise as indicated. All reagents and solvents were of the highest commercially available purity grade. No-carrier-added (n.c.a.) sodium 125I-iodide was obtained from PerkinElmer. The precursor of 125I-ITdU, precursor 5-(trimethylstannyl)-4'-thio-2'-deoxyuridine, CAS Nr. 444586-71-4, and the unlabeled reference standard, 5-iodo-4'-thio-2'-deoxyuridine (ITdU) CAS Nr. 134699-95-9, were synthesized as previously reported (17).

Radiochemistry

Chloramine T (CAS Nr. 144-86-5, 16μL; 2 mg/mL in H2O:CH3CN = 2:1) was added to a mixture of 17 μL phosphate buffer (0.2 mol/L, pH 2.0, in H2O: methanol = 7:3), 3 μL precursor solution (123 mmol/L in H2O: methanol = 1:2), and 10 μL n.c.a. 125I-NaI solution in 0.05 mol/L NaOH. Labeling reaction completed within 10 minutes at room temperature and was stopped by addition of 40 μL 25 mmol/L sodium thiosulfate. The product was purified by high-pressure liquid chromatography (HPLC) with UV and gamma detection using a MultoKrom 100-5 C4 (250 × 4 mm²) reverse-phase column (CS-Chromatography) eluted with 15% ethanol aq at a flow rate of 1 mL/min. Retention times were 3.8 minutes for 125I-iodide and 7.0 minutes for 125I-ITdU. Product volume was 1.0 ± 0.2 mL. Quality control was conducted by analytic HPLC with UV and gamma detection [column: LiChrosphere 100 18-5 μm; 125I-iodide, eluent: 0.05 mol/L phosphate buffer (pH 3.6) containing 12% methanol by volume, and flow rate: 1 mL/min, retention times: 1.9 minutes for 125I-iodide, 9.0 minutes for 125I-ITdU]. Total radiochemical yields were 83 ± 8%. Radiochemical purities were more than 98%. Molar concentrations of prepared n.c.a. 125I-ITdU solutions in 15% ethanol aq. were 3 mmol/L (± 10%) and with typical activity concentrations of 60 MBq/mL (with batch variations ±10 MBq/mL) the corresponding mean specific activity was 20 GBq/µmol.

Cell lines and culture conditions

The human multiple myeloma cell line KMS12BM was obtained from DSMZ. Dex-sensitive MM1.S and resistant MM1.R cell lines were kindly provided by Dr. Steven Rosen (Northwestern University, Chicago, IL). Each cell line was cultured in its standard medium (RPMI-1640 medium; Biochrom), and no further cell line authentication was conducted. The multiple myeloma stem-like cell populations were isolated by CD138 MicroBeads depletion followed by positive selection using CD27 MicroBeads (Miltenyi Biotec). The isolated cell fractions were CD27⁺ CD138⁻ with a purity of more than 97%.

Flow cytometric analyses

The phenotype was investigated using flow cytometry (FACS, Cytomics FC 500, Beckman Coulter) by staining with fluorochrome-labeled CD27, CD45, CD138, and corresponding Ig control antibodies (Miltenyi Biotec). For measurement of ALDH activity, the isolated myeloma stem-like cells were stained with Aldefluor reagent (Stem Cell Technologies). For investigation of FdUrd-mediated effect on cell-cycle status, cells were fixed in 70% ethanol at 4°C for 30 minutes and incubated with RNase and propidium iodide (2.5 μg/mL) for 30 minutes at 4°C. To
The proliferation status of CD138 analyzed by fluorescence-activated cell sorting (FACS). Media (DMEM) supplemented with 10% FBS to allow dye incubated for 45 minutes in Dulbecco's Modified Eagle's medium (DMEM) at 37°C for 48 hours. Cells were recovered by centrifugation, cell pellets were resuspended in 40 mM mercaptoethanol. Equal amounts of protein were subjected to electrophoresis (10% Tris-HCl gel, Bio-Rad) and blotted onto polyvinylidene difluoride (PVDF) membranes. Detection was conducted with polyclonal antibodies at dilutions recommended by the supplier (Cell Signaling Technology). The binding of secondary horse-radish peroxidase (HRP)-coupled antibodies was visualized with enhanced chemiluminescence (ECL+, GE Healthcare). Equal protein loading was controlled using GAPDH-specific antibody (Cell Signaling Technology).

Clonogenic assay

After treatment, 1,000 viable myeloma stem-like cells were plated into 24-well culture dishes in complete medium supplemented with methylcellulose-based medium (R&D Systems). After incubation at 37°C and 5% CO2 atmosphere for 14 days, colonies were fixed in 10% neutral-buffered formalin and stained with 0.5% crystal violet. Colonies containing ≥50 cells were counted on an invert microscope (IT400 Trino Plan, VWR International).

Statistical analysis

Cellular uptake with DNA incorporation experiments, all FACS analyses and clonogenic assays were conducted in triplicate and by repeating independent blocks of experiments, including all appropriate controls. Data are presented as mean ± SD. The percentage of specific cell death was calculated as 100% × (experimental dead cells (%) – spontaneous dead cells in medium (%))/100% – spontaneous dead cells in medium (%).

Results

Isolated myeloma cell populations resemble memory B cells and exhibit stem cell properties

Multiple myeloma cell lines KMS12BM, MM1.S, and MM1.R cell lines were shown to contain distinct populations of CD138− cells that express markers reminiscent of B cells (13, 18). A minor cell fraction ranging from 4% to 7% was determined as CD138− within the terminally differentiated MM1.S and MM1.R cells, whereas more than 95% of KMS12BM cells lack this surface marker (Fig. 1A). The analysis of Pgp expression by staining with rhodamine 123 revealed 2 cell populations, Rho(low) and Rho(high). The Rho(low) fraction with 8.0%, 4.2%, and 6.2% of total cells for KMS12BM, MM1.S, and MM1.R cell lines, respectively, contained CSCs. After isolation, the phenotype of enriched subpopulations was verified as CD27+ and CD138− (Fig. 1B). Moreover, this population displayed an increased capacity of Rho 123 extrusion (76.1%, 64.6%, and 84.1% of isolated cells for KMS12BM, MM1.S, and
MM1.R cells, respectively). The dye efflux was blocked by the Pgp inhibitor verapamil indicating that differences in the intensity of Rho 123 fluorescence were due to Pgp activity. To determine whether the isolated CD138^-CD27^+ cells were dormant, the CFSE staining was monitored over a 7-day period (Fig. 1C). In comparison
with CD138+ cells, the separated cells proliferated very slowly verifying that the addressed CD138+ CD27+ cell population is relatively quiescent.

**FdUrd induced differentiation and proliferation of myeloma stem-like cells**

The isolated CD27+ CD138− multiple myeloma cell fractions were incubated with FdUrd for 1 hour. The effects of FdUrd treatment on ALDH activity, mitosis, and phenotype were studied by flow cytometry (Fig. 2). As stem cells exhibit higher levels of ALDH activity than the differentiated progeny, the fluorescently labeled ALDH substrate, Aldefluor, can be used for identification of tumor-initiating cells (19). Staining of isolated CD27+ CD138− multiple myeloma cell fractions revealed ALDH+ cells accounting for 69.8%, 89.0%, and 80.2% for KMS12BM, MM1.S, and MM1.R cells, respectively. Treatment with FdUrd strongly decreased the percentage of ALDH+ cells and increased the CD138-expressing population in all 3 cell lines (Fig. 2A and B). Importantly, at 16 hours after treatment, more than 50% of FdUrd-stimulated cells were induced to proliferate (Fig. 2C).

**FdUrd stimulation increased the cellular uptake and incorporation into the DNA of 125I-ITdU in myeloma stem-like cells**

Without FdUrd pretreatment, only a small portion of isolated myeloma cells (<6%) was labeled with 125I-ITdU after 96 hours of incubation with the thymidine analogue (Fig. 3A). However, already short-term pre-exposure to FdUrd considerably increased the cellular uptake and retention of 125I-ITdU (4.9% ± 1.0% vs. 20.9% ± 1.6%, 4.9% ± 0.8% vs. 28.5% ± 2.6%, and 5.6% ± 1.2% vs. 21.6% ± 1.9% for KMS12BM, MM1.S, and MM1.R stem cells, respectively). Correspondingly, nonactivated slowly proliferating myeloma stem-like cells only marginally incorporated 125I-ITdU into DNA. Here, less than 10% of the intracellularly accumulated tracer was used as a substrate for DNA polymerase (Fig. 3B). In contrast, the FdUrd-induced proliferation led to a significantly increased incorporation rate of 125I-ITdU into the DNA (9.2% ± 1.3% vs. 40.3% ± 2.9%, 2.5% ± 0.6% vs. 43.6% ± 3.1%, and 1.4% ± 0.4% vs. 14.6% ± 1.5% for KMS12BM, MM1.S, and MM1.R stem cells, respectively) as shown by Fig. 3B.

**FdUrd sensitized myeloma stem-like cells to 125I-ITdU-emitted nanoirradiation**

An extensive DNA fragmentation was detected in cells stimulated with FdUrd before 125I-ITdU incubation using a DNA ladder assay (Fig. 4A, left). The adjacent gel exposure to phosphorimager confirmed that the massive DNA damages were induced by DNA-incorporated 125I-ITdU (Fig. 4A, right). The FdUrd-promoted and 125I-ITdU–emitted DNA fragmentation in myeloma cells led to an induction of programmed cell death as assessed by Annexin V staining. The basal incidence of apoptosis among untreated control cells and cells treated with FdUrd only varied between 1.5% and 2.5% in all cell lines (data not shown). After exposure to 125I-ITdU alone, only a minor portion of myeloma stem-like cells was detected as apoptotic (35.2% ± 2.6%, 11.4% ± 1.2%, and 24.8% ± 1.8% for KMS12BM, MM1.R, and MM1.S cells, respectively; Fig. 4B) reflecting the low rate of 125I-ITdU incorporation into the DNA. As anticipated, stimulation with nontoxic concentrations of FdUrd causing a remarkable higher DNA incorporation of 125I-ITdU (see above) resulted in a strong increase of the apoptotic fraction (86.1% ± 3.6%, 85.5% ± 2.2%, and 89.7% ± 3.1% for KMS12BM, MM1.R, and MM1.S cells, respectively).

**Myeloma stem-like cells activated the DNA damage checkpoint in response to treatment 125I-ITdU**

As the DNA damage checkpoints are essential for induction of radiosensitivity responses (20, 21), we determined the activation of the ATM as well as the expression of ribonucleotide reductase p53R2 in treated myeloma stem-like cells. An exposure to 125I-ITdU resulted in a significant activation of ATM in cells sensitized by FdUrd stimulation (Fig. 5). Moreover, expression of p53R2, a protein involved in the repair of damaged DNA, was clearly increased in the FdUrd-conditioned myeloma stem-like cells before 125I-ITdU treatment.

**125I-ITdU induced apoptosis through a caspase-dependent mechanism**

Because apoptotic cell death initiated by Auger electron emitters occurs through the caspase-3–mediated pathway, we examined proteolytic cleavage of caspase-3 and its cellular substrate, the PARP enzyme, to determine whether the 125I-ITdU–mediated decrease in viability in myeloma stem cells was due to apoptosis. The intrinsic apoptotic pathway activation by 125I-ITdU was found to depend on FdUrd stimulation (Fig. 5). The exposure to 125I-ITdU alone was not sufficient to trigger the cell death in either myeloma stem-like cell population.

**Conditioning with FdUrd was essential for inhibition of clonogenic potential of myeloma stem-like cells**

The 2-step treatment involving radiosensitization by exposure to FdUrd followed by incubation with 125I-ITdU completely inhibited the clonogenic growth in all tested myeloma stem-like cell fractions (Fig. 6). In contrast, exposure to 125I-ITdU as a single treatment did not affect the clonogenic potential of either cell line. Pretreatment with FdUrd alone was completely nontoxic.

**Discussion**

Our in vitro study shows for the first time that the Auger electron emitting thymidine analogue 125I-ITdU can be a potential anti-CSC agent for multiple myeloma, provided that CSCs were presensitized to therapeutic intervention by an induction of the cell-cycle entrance. The combination of FdUrd as radiosensitizer and DNA nanoirradiation mediated by incorporated 125I-ITdU was able to break the...
resistance and to achieve a complete inhibition of clonogenic recovery of myeloma stem-like cell populations.

Current developments strengthen the focus on an evaluation of novel CSC-targeted strategies that may complement conventional cancer therapies. In fact, diverse preclinical CSC targeting approaches have highlighted the therapeutic promise of considering the CSC subpopulation and some of them are already being translated to the clinic routine (22). Despite the controversial results regarding the precise marker definition, the myeloma-initiating cells are considered important therapeutic targets. In our experimental setting, the properties of the isolated myeloma stem-like cell populations largely overlap with the criteria for

Figure 2. Effects of stimulation with FdUrd on the differentiation and proliferation of myeloma stem-like cells. A, dot blots represent percentage of ALDH-positive stem cells before and after exposure to FdUrd. B, histograms represent CD138 expression prior (white) and FdUrd stimulation (gray). C, cell fractions in G0–G1, S, and G2–M phases before and after exposure to FdUrd treatment. Data are representative of 3 independent experiments.
FdUrd-Induced Radiosensitization of Myeloma Stem-like Cells

CSCs. Here, we propose a 2-step strategy for targeting the stem-like cell population of multiple myeloma. In step one, the slowly proliferative myeloma stem-like cells would be activated, leading to their sensitization to nanoirradiation using the Auger electron emitting thymidine analogue 125I-ITdU in step 2. In our setting, the cells were primed using nontoxic concentrations of FdUrd, the direct precursor of FdUrd-5’-monophosphate (FdUMP), being the biologic active anabolite of the chemotherapeutic agent 5-FU. FdUMP affects DNA synthesis by irreversible inhibition of thymidylate synthase thymidylate synthase, which catalyzes an important step of the de novo synthesis by formation of thymidine-5’-monophosphate (TMP; ref. 23). Several clinical studies have shown that the protein and mRNA levels of thymidylate synthase are highly elevated in diverse tumor entities (24). Moreover, thymidylate synthase exhibits oncogene-like activity, and the thymidylate synthase overexpression was shown to induce neoplastic transformation in NIH/3T3 fibroblasts (25).

As 5-FU was shown to activate the dormant hematopoietic stem cells (HSC) to switch to self-renewal by induction of myeloid depletion (‘injury signals’; ref. 26), we recognized the potential of FdUrd for priming of quiescent CSCs. The fluoropyrimidine exhibits selective tumor toxicity due to the preferential metabolic activation by tumor cells (27). Clinically, bolus administration of FdUrd in patient with meningeal dissemination of malignant tumors was well-tolerated also at high doses (28, 29). Evaluation of the results showed a favorable antitumor effect paired with apparently no adverse affects. In this study, the short-term stimulation with a nontoxic concentration of FdUrd induced phenotypic and functional changes in isolated myeloma stem-like cell populations. As a consequence, more than 50% of the slowly proliferative cells were activated to enter cell cycling. Moreover, the exposure to FdUrd stimulated the cell differentiation. Similar effects were shown for HSCs after treatment with 5-FU and 5-bromo-2-deoxyuridine (BrdUrd; ref. 26). For chronic myeloid leukemia (CML), stimulation with cytokines like granulocyte colony-stimulating factor (G-CSF) and IFN-α was presented to induce proliferation of the dormant stem cell compartment (30, 31).

In this study, due to FdUrd stimulation, myeloma stem-like cells appeared to acquire an injury- and repair-activated state characterized by cell cycling accompanied with high metabolic turnover rates. The FdUrd-mediated thymidylate synthase blockade results in a significant depletion of intracellular thymidine triphosphate (TTP), which strongly affects the production and balance of DNA precursor pools and subsequently induces cell danger signals. This depletion and the intracellular imbalances impair DNA repair fidelity and capability (32). In addition, the interaction of FdUrd with thymidylate

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**Figure 3.** Effect of stimulation with FdUrd on (A) cellular uptake [% of incubated dose ID/2 x 10^6 cells] and (B) DNA incorporation of 125I-ITdU (% DNA association relative to total cellular uptake) in myeloma stem-like cells. Data are representative of 3 independent experiments.

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**Figure 4.** Apoptosis-inducing activity of 125I-ITdU in myeloma stem-like cells. A, DNA fragmentation assay with cells pretreated with FdUrd and exposed to 125I-ITdU (left) and after exposure to a phosphorimager screen (right). B, dot blots represent apoptosis extent using Annexin V/PI staining in untreated, only 125I-ITdU-treated, and FdUrd/125I-ITdU-treated cells. Data are representative of 3 independent experiments.
synthase leads to a cell synchronization in the S-phase and consequently—as cells during the G2–M/S-phase are more susceptible to radiation than cells in G0–G1 phase—increases their radiosensitivity.

Auger electron therapy is a promising form of targeted endoradiotherapy, which allows an irradiation of single cells due to the unique physical characteristics of emitted Auger electrons, namely, the short range and high local toxicity. Several preclinical and clinical studies emphasize the benefit of the use of Auger electron emitting radiopharmaceuticals (33). In this study, short-term exposure to FdUrd effectively increased the extent of the Auger electron emitter125I-ITdU incorporation into the cells as well as into the DNA. This effect was less pronounced in the stem-like cell subpopulation of drug-resistant MM1.R cells, probably due to their lowest proliferation rate and sophisticated resistance mechanisms (34). The DNA-incorporated 125I-ITdU induced extensive DNA damages in all tested myeloma stem cells. Generally, the massive DNA fragmentation in myeloma cells is, to some extent, due to the impaired nonhomologous end joining (NHEJ) function, which contributes to the karyotypic instability observed in myeloma (35). In this context, the fact that deficits in NHEJ confer an inability to efficiently conduct DNA repairs may be relevant in a 125I-ITdU–based therapy, given that NHEJ is activated during the DNA damage response as the predominant mechanism in DSB repair (36). Furthermore, FdUrd is known to block the DSB repair mechanisms (37), thus amplifying apoptotic effects of DNA damages induced by incorporated 125I-ITdU. Accordingly, the Annexin V/PI (propidium iodide) assay revealed a dramatic increase of apoptotic cells after the 2-step regimen comprising conditioning of cells with FdUrd followed by exposure to 125I-ITdU. In this regimen, FdUrd stimulation efficiently potentiated the therapeutic efficiency of 125I-ITdU, yielding an apoptotic rate of more than 85%.

DNA damage checkpoint responses play an essential role in cellular radiosensitivity, and their enhanced activation in response to radiation-induced DNA damage was observed within the CSC subpopulation (38). Conditioning of myeloma stem-like cells with FdUrd before incubation with 125I-ITdU led to distinctly increased ATM kinase activity by efficient protein phosphorylation. A single treatment with 125I-ITdU was barely able to activate the checkpoint response, most likely due to the low cellular and DNA incorporation rate of the tracer in this setup. Given that cytosolic and extracellular decay of Auger emitters is 100-fold less radiotoxic than decay within the DNA (39) and that more than ~20 DSBs per cell are required for ATM activation (40), the incubation

![Figure 5](image_url)

**Figure 5.** Effects of pretreatment with FdUrd on activation of DNA damage checkpoint response and repair mechanisms and of induction of the intrinsic apoptosis signal pathway. SDS-PAGE/Western blot analysis of ATM, pATM, p53R2, cleavage of PARP and caspase-3 in myeloma stem cells after exposure to FdUrd and 125I-ITdU.

![Figure 6](image_url)

**Figure 6.** Clonogenic survival in percent of clonogenic recovery of myeloma stem-like cells after four different treatment regimens as indicated. Data are representative of 3 independent experiments.

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with $^{125}$I-ITdU without FdUrd pretreatment seemed to be insufficient to induce the DNA repair signal cascade. Importantly, the observed FdUrd-potentiated radiotoxicity is related not only to cell synchronization and inhibition of thymidylate synthase–mediated nucleotide deiodination (15) but also to a reduction of dNTPs pools (41). The latter effect was shown to cause the replication fork stalling during the S-phase, leading to DNA lesions (42). The ribonucleotide reductase p53R2, involved in DNA damage repair, catalyzes the conversion of ribonucleoside diphosphates to the corresponding deoxyribonucleotides to provide a balanced supply of precursors for DNA synthesis (43). The massive DNA damage by $^{125}$I-ITdU following FdUrd stimulation led to a significantly induced p53R2 transcription. Notably, despite the activation of DNA damage repair mechanisms, the majority of cells were primed for apoptosis, which yielded complete inhibition of clonogenic growth.

**Conclusion**

In this preclinical in vitro study, we present a promising approach involving radiosensitization of myeloma stem-like cells by FdUrd pretreatment followed by a DNA nanoirradiation by incorporated $^{125}$I-ITdU. As CSCs represent translationally relevant targets for cancer therapy, this 2-step strategy may complement the conventional multiple myeloma therapies especially in patients with relapsed and refractory multiple myeloma.

**References**


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