ATP Depletion Triggers Acute Myeloid Leukemia Differentiation through an ATR/Chk1 Protein-dependent and p53 Protein-independent Pathway

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Background: Cancer chemotherapeutics often lead to significant toxicities. Identifying alternative strategies that do not cause direct cytotoxicity is desirable. Results: Partial ATP depletion induces AML differentiation without direct cytotoxicity in an ATR/Chk1-dependent fashion. Conclusion: Partial ATP depletion is a promising strategy for AML. Significance: Characterizing therapeutic strategies that do not involve direct cytotoxicity is important to improve cancer therapy.

Despite advances in oncology drug development, most commonly used cancer therapeutics exhibit serious adverse effects. Often the toxicities of chemotherapeutics are due to the induction of significant DNA damage that is necessary for their ability to kill cancer cells. In some clinical situations, the direct induction of significant cytotoxicity is not a requirement to meet clinical goals. For example, differentiation, growth arrest, and/or senescence is a valuable outcome in some cases. In fact, in the case of acute myeloid leukemia (AML), the use of the differentiation agent all-trans-retinoic acid (ATRA) has revolutionized the therapy for a subset of leukemia patients and led to a dramatic survival improvement. Remarkably, this therapeutic approach is possible even in many elderly patients, who would not be able to tolerate therapy with traditional cytotoxic chemotherapy. Because of the success of ATRA, there is widespread interest in identifying differentiation strategies that may be effective for the 90–95% of AML patients who do not clinically respond to ATRA. Utilizing an AML differentiation agent that is in development, we found that AML differentiation can be induced through ATP depletion and the subsequent activation of DNA damage signaling through an ATR/Chk1-dependent and p53-independent pathway. This study not only reveals mechanisms of AML differentiation but also suggests that further investigation is warranted to investigate the potential clinical use of low dose chemotherapeutics to induce differentiation instead of cytotoxicity. This therapeutic approach may be of particular benefit to patients, such as elderly AML patients, who often cannot tolerate traditional AML chemotherapy.

Most clinically used anticancer agents lead to cell death through the induction of significant DNA damage that triggers DNA damage signaling pathways. Although DNA damage can induce cancer cells to die, it can also cause significant toxicities in non-malignant cells. Besides cell death, DNA damage-dependent signaling pathways can lead to other end points, such as growth arrest, differentiation, and senescence (1). The specific cellular response often depends upon the amount and type of stimuli and the cell type. In certain contexts, these non-cell death-mediated pathways may be preferable, as they may offer sufficient efficacy without undesirable side effects.

Acute myeloid leukemia (AML) is an example of a malignancy in which differentiation therapies in contrast to cytotoxic therapies have found significant clinical success. Traditional AML chemotherapeutics (such as cytarabine and anthracyclines) that target cell death exhibit poor efficacy and high toxicities particularly in the adult and elderly populations. For example, only 20% of patients over age 56 survive 2 years, and many elderly patients cannot tolerate the existing AML chemotherapeutics due to their excessive toxicities (2). The use of a regimen containing the differentiation agent all-trans-retinoic acid has dramatically improved AML therapy, leading to the long-term survival and presumed cure of 75–85% of individuals with the rare acute promyelocytic leukemia subtype of AML (3). Even elderly patients with this subtype of AML who cannot tolerate standard AML induction chemotherapy can achieve complete remission. Unfortunately, all-trans-retinoic acid has been found to be clinically efficacious only for the acute promyelocytic leukemia subtype of AML, which has led numerous investigators to search for other clinically useful AML differentiation agents.

We previously reported the identification of 6-benzylthioinosine (6BT) as a novel AML differentiation therapeutic candidate that is currently in preclinical development. During our characterization of its activities, we identified that 6BT induces ATP depletion (4). Utilizing 6BT, we demonstrate here that ATP depletion can induce DNA damage signaling in AML cells.

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3 The abbreviations used are: AML, acute myeloid leukemia; 6BT, 6-benzylthioinosine; NBT, nitro blue tetrazolium.
ATP Depletion Triggers AML Differentiation

and that this DNA damage signaling can play a major role in inducing AML differentiation. Although nucleotide depletion was previously not thought to lead to DNA damage, the use of more sensitive assays has shown that nucleotide depletion can induce limited and reversible DNA damage (5, 6). It has been proposed that the unbalanced nucleotide pools resulting from nucleotide depletion lead to misincorporation during DNA synthesis, activating the mismatch repair process, which includes the creation of a repair patch, a transient form of DNA damage (6, 7). In addition to DNA damage, nucleotide depletion is well known to lead to replication stress that can also initiate DNA damage signaling.

The upstream mechanisms through which ATP depletion leads to differentiation are almost completely unknown (8–10). Here, we found that 6BT induces differentiation from the ATP depletion-mediated activation of an ATR/Chk1-dependent and p53-independent pathway. In addition to elucidating the mechanisms of action of 6BT, this work demonstrates that nucleotide depletion strategies can induce DNA damage signaling-dependent differentiation and that this is a promising therapeutic approach for AML. As many chemotherapeutics induce DNA damage, this work suggests the possibility of revisiting the use of low dose subcytotoxic levels of these agents as differentiation therapies particularly for AML. This approach may provide an alternative regimen for AML patients who are not able to tolerate the existing regimens due to their significant toxicities.

EXPERIMENTAL PROCEDURES

Reagents and Cell Lines—6BT was obtained from the NCI Developmental Therapeutics Program and used at 10 µM. Adenosine, doxorubicin, phifithrin-α, UCN-01, 2-deoxyglucose, methylmercapturine riboside, and caffeine were purchased from Sigma. Antibodies against phospho-H2AX (Ser-139), phospho-ATR, phospho-Chk1 (Ser-345), phospho-Chk2 (Thr-68), phospho-p53 (Ser-15), and p21 were obtained from Cell Signaling Technology Inc. Antibodies against Chk1, Chk2, and p53 were from Santa Cruz Biotechnology. OCI-AML3 cells were from DSMZ, and 293T cells were obtained from Developmental Therapeutics Program and used at 10

Cell Culture—OCI-AML3 cells were cultured in RPMI 1640 medium (HyClone), and 293T cells were cultured in DMEM (HyClone). The media were supplemented with 10% FBS and 1% penicillin/streptomycin. Cells were cultured in a humidified atmosphere of 95% (v/v) air and 5% (v/v) CO2 at 37 °C.

Differentiation—Nitro blue tetrazolium (NBT) reduction assay was performed in the same manner as described previously (4). Immunophenotyping was performed by staining cells with phycoerythin-conjugated CD11b (BD Biosciences). The stained samples were run on a Beckman Coulter Cytomics FC 500 cytometer. For the Chk1-GFP- and GFP-transfected cells, only the GFP-positive cells were gated prior to analysis for CD11b expression. Dead cells were excluded by gating using forward and side scatter.

Cell Proliferation—Cell number was determined by counting the cells after they were treated with 6BT using a Coulter Z2 particle counter.

Transfections and Lentivirus Infections—293T cells were cotransfected with p53 shRNA (provided by Dr. Mark Jackson), Chk1 shRNA, p21 shRNA, or the empty vector pLK (Sigma) and the packaging plasmids pCMVΔR8.74 and pMD.G using Lipofectamine (Invitrogen). OCI cells were infected with the virus-containing supernatant concentrated overnight in PEG in the presence of 6 µg/ml Polybrene for 6 h, and stable cell lines were generated by selection with puromycin (1 µg/ml). OCI cells were transduced with Chk1-GFP or empty vector using X-tremeGENE HP (Roche Applied Science) according to the manufacturer’s instructions. To generate Chk1-GFP, the cDNA encoding full-length human Chk1 was amplified by PCR using a Myc-Chk1 construct that we previously generated as the template (11). The Chk1 cDNA was then ligated into the BglII and HindIII site of the pEGFP-N1 vector to generate Chk1-GFP.

Comet Assay—Cells were treated as indicated, and the assay was performed according to the manufacturer’s protocol (Trevigen). 100 cells were counted from at least two representative fields.

Phospho-H2AX Immunostaining—After treatment with 6BT, cells were washed, immobilized on poly-l-lysine (Sigma)-coated cover glass, fixed in chilled methanol, and permeabilized in 0.01% Triton X-100. Cells were incubated for 2 h with anti-phospho-H2AX primary antibody and for 1 h with Alexa Fluor 488-conjugated anti-mouse IgG secondary antibody (Molecular Probes). The cells were washed and mounted in VECTASHIELD containing DAPI (Vector Laboratories). Pictures were taken using a Nikon Eclipse E800 microscope equipped with a camera using SPOT software (Diagnostic Instruments). Images were taken at magnifications of ×20 and ×40.

Western Blot Analysis—Cells treated with 6BT for the indicated times were washed with PBS, centrifuged, and lysed with Triton-containing lysis buffer. Protein lysates (50 µg/lane) were resolved on the appropriate SDS-polyacrylamide gel and transferred to PVDF membrane (Millipore) using a Bio-Rad transfer apparatus. The membranes were blocked and incubated with the indicated primary antibodies and horseradish peroxidase-conjugated secondary antibodies. Immunoreactive protein bands were detected by enhanced chemiluminescence (Pierce) using XAR-5 film.

ATP Measurement—Cells were treated with 6BT for the indicated times, and the relative ATP concentration was measured using the ATPlite 1step kit (PerkinElmer Life Sciences) according to the manufacturer’s instructions. Briefly, after the indicated treatment time, 3 × 10^5 viable cells were mixed with 100 µl of ATPlite 1step reagent in a 96-well plate, and the luminescence was measured using a SpectraMax L luminometer (Molecular Devices).

RESULTS

6BT Induces Evidence of DNA Damage prior to Differentiation—As we reported previously (4), 6BT treatment of AML cells led to partial ATP depletion and subsequent differentiation (Fig. 1, A and B). To begin to investigate the relationship between 6BT-mediated ATP depletion and differentiation, we assessed whether 6BT induces evidence of DNA damage as has been reported for other nucleotide depletion agents (6, 12). After 6BT treatment, 37 ± 9% ATP depletion was observed in OCI cells after 16 h of treatment (Fig. 1B). The ATP depletion...
that occurs soon after 6BT treatment precedes differentiation, which is a slow process that occurs over several days. To assess whether 6BT-mediated ATP depletion leads to DNA damage, the phosphorylation of the H2A variant H2AX (γ-H2AX), a commonly used marker for DNA damage, was measured. Consistent with ATP depletion leading to DNA damage, H2AX phosphorylation was observed several hours after 6BT treatment by both Western blotting and immunostaining (Fig. 1C). Although limited DNA damage is induced compared with a standard DNA-damaging agent, doxorubicin, 6BT treatment at the doses used does not lead to significant cytotoxicity (4). In addition, to further confirm DNA damage induction by 6BT, the comet assay, which is another sensitive method to measure DNA damage, was performed (Fig. 1D). The results further support that 6BT induces DNA damage. For example, 41% comet-positive cells were seen after 12 h of 6BT treatment. Together, these results suggest not only that 6BT can induce DNA damage, but that this damage occurs following initial ATP depletion prior to significant AML differentiation.

6BT Activates DNA Damage Signaling—Although 6BT induces evidence of DNA damage, it does not lead to significant cytotoxicity at the doses utilized (4). In addition to limited DNA damage, 6BT likely induces replication stress that is well known to occur after nucleotide depletion and to lead to the activation of DNA damage signaling. Therefore, we assessed whether or not the activation of DNA damage signaling pathways in response to DNA damage and/or replication stress is responsible for the ability of 6BT to induce differentiation. 6BT was found to be fully capable of activating downstream DNA damage signaling pathways (Fig. 2). Consistent with the induction of
DNA damage signaling, 6BT led to the rapid phosphorylation of the master checkpoint kinase ATR and the subsequent activation of the checkpoint kinase Chk1. The activation of another master checkpoint kinase, ATM, was not detected after 6BT treatment (data not shown). 6BT activation of ATR is consistent with its known function to respond to DNA damage induced by stalled replication forks that is a known consequence of ATP depletion (13, 14). In addition, p53, another important mediator of DNA damage signaling, was found to be activated as measured by an increase in Ser-15 phosphorylation. Ser-15 on p53 is a common site that is phosphorylated in response to DNA damage stimuli (15). Consistent with p53 activation, 6BT led to a marked up-regulation of the p53-dependent protein p21 (Fig. 2).

**Chemical Inhibition of DNA Damage Signaling Components Abrogates 6BT-mediated Differentiation**—To begin to understand the activation of DNA damage signaling in 6BT-mediated AML differentiation, specific chemical inhibitors of components of this pathway were employed. Caffeine (ATM/ATR inhibitor) and UCN-01 (Chk1) both significantly abrogated 6BT-mediated AML differentiation (Fig. 3A). Interestingly, inhibition of p53 by the chemical inhibitor pifithrin did not interfere with 6BT-mediated differentiation (Fig. 3A).

**Chk1 (but Not p53) Is Essential for 6BT-mediated Differentiation**—As chemical inhibitors are often not specific, we used a genetic approach to further demonstrate that 6BT-mediated differentiation occurs through DNA damage signaling. Utilizing lentiviral shRNA, we stably knocked down the expression of Chk1 and p53 in OCI cells compared with empty vector (pLK)-infected cells (Fig. 3B). After treatment of these cell lines with 6BT, consistent with our studies using chemical inhibitors, 6BT-mediated differentiation was found to be highly dependent on Chk1 (but not p53) as measured by both the NBT reduction assay and the up-regulation of the cell surface differentiation marker CD11b (Fig. 3, C and D). In an opposite approach, Chk1 was overexpressed in OCI cells to assess whether the activation of DNA damage signaling itself could lead to differentiation. This experimental design was based on our previous observation that overexpression of Chk1 induced a transient replication checkpoint even in the absence of DNA damage (11). Thus, this strategy bypasses the requirement of 6BT to activate the checkpoint. A 2-fold increase in the percentage of CD11b-expressing cells was observed in the Chk1-overexpressing cells (Fig. 3E). This result suggests that Chk1 can participate in AML differentiation.

Although p53 is a major downstream effector of DNA damage signaling, the fact that p53 is not essential for 6BT-mediated differentiation is consistent with the fact that pifithrin does not impair 6BT-mediated differentiation and that 6BT can induce differentiation in p53-null AML cell lines (4). As the majority of cancer chemotherapeutics depend upon functional p53 for their optimal activity, this finding suggests that 6BT may be particularly useful for patients whose leukemic cells do not express p53 or exhibit mutant p53 (16, 17).

**Exogenous Adenosine Overcomes 6BT-mediated Differentiation and Induction of DNA Damage Signaling**—To confirm that 6BT-mediated ATP depletion is responsible for the induction of DNA damage signaling and subsequent differentiation, OCI cells were pretreated with adenosine (the cell-permeable nucleoside precursor of ATP) prior to 6BT treatment. The addition of adenosine decreased 6BT-mediated differentiation as measured by both NBT reduction and CD11b expression (Fig. 4A). For example, adenosine pretreatment decreased 6BT-mediated differentiation from 78 ± 7 to 21 ± 3% as measured by NBT reduction, demonstrating that ATP depletion plays an important role in 6BT-mediated differentiation. As expected, cells that received adenosine pretreatment exhibited significantly less 6BT-mediated ATP depletion compared with cells not exposed to exogenous adenosine (Fig. 4B). Importantly, adenosine significantly diminished the 6BT-mediated DNA damage signaling response as measured by reduced H2AX, Chk1, and p53 phosphorylation (Fig. 4, C and D). In addition, the up-regulation of the p53-dependent protein p21 was diminished (Fig. 4D). These results demonstrate that ATP depletion is a key trigger for AML differentiation and is mediated through the induction of DNA damage signaling. Besides differentiation, 6BT also led to the growth inhibition of AML cells. In addition to attenuating 6BT-mediated differentiation, adenosine pretreatment also impaired 6BT-mediated growth inhibition (Fig. 4E).

**p21 Is Important for 6BT-mediated Growth Inhibition, but Not Differentiation**—As p21 is known to play an important role in both growth inhibition and differentiation, the role of p21 in mediating the biological effects of 6BT was analyzed. To assess the importance of p21 in 6BT activities, p21 was knocked down using lentiviral shRNA in OCI cells (Fig. 5A). Surprisingly, as shown in Fig. 5B, knockdown of p21 levels had no significant effect on 6BT-mediated differentiation. In contrast, p21 deficiency impaired 6BT-mediated growth inhibition (Fig. 5C), suggesting that it may play a role in 6BT-mediated growth inhibition, but not differentiation. Although a role for p21 in differentiation cannot be completely excluded, as residual p21 is expressed, it clearly plays a role in 6BT-mediated growth inhibition. As p21 is a p53-dependent gene, these findings are also consistent with the fact that p53 is not required for 6BT-mediated differentiation.

**ATP Depletion Agents Besides 6BT Can Also Induce AML Differentiation through a Chk1-dependent Pathway**—As no small molecules are entirely specific, we utilized two additional
known ATP depletion agents to further demonstrate the role of ATP depletion in DNA damage signaling-dependent AML differentiation. OCI cells were pretreated with 500 μM caffeine (ATM/ATR inhibitor), 25 nM UCN-01 (Chk1 inhibitor), or 10 μM pifithrin (PFT), after which 10 μM 6BT was added, and the percentages of NBT-differentiated cells were determined after 4 days. Values are an average of three independent experiments. UT, untreated. B, p53 and Chk1 knockdown in AML cells. Western blotting demonstrated the stable knockdown of Chk1 and p53 in OCI cells. OCI-PLK represents OCI cells expressing the pLK empty vector. shChk1, Chk1 shRNA. C, Chk1 (but not p53) is important for 6BT-mediated CD11b induction. The indicated cell lines were treated with 6BT for 4 days, the cells were stained with anti-CD11b antibody, and flow cytometric analysis was performed. D, Chk1 (but not p53) is important for 6BT-mediated NBT reduction. Cells were treated as described for C, and the NBT reduction assay was performed. The results are an average of three independent experiments. E, overexpression of Chk1 can induce evidence of AML differentiation. OCI cells were transfected with Chk1-GFP or GFP alone. After 72 h, CD11b expression was assessed by flow cytometry in the viable GFP-positive cells.

**FIGURE 3.** 6BT induces differentiation in a Chk1/ATR-dependent and p53-independent manner. A, chemical inhibition of DNA damage signaling impairs 6BT-mediated differentiation. OCI cells were pretreated with 500 μM caffeine (ATM/ATR inhibitor), 25 nM UCN-01 (Chk1 inhibitor), or 10 μM pifithrin (PFT), after which 10 μM 6BT was added, and the percentages of NBT-differentiated cells were determined after 4 days. Values are an average of three independent experiments. UT, untreated. B, p53 and Chk1 knockdown in AML cells. Western blotting demonstrated the stable knockdown of Chk1 and p53 in OCI cells. OCI-PLK represents OCI cells expressing the pLK empty vector. shChk1, Chk1 shRNA. C, Chk1 (but not p53) is important for 6BT-mediated CD11b induction. The indicated cell lines were treated with 6BT for 4 days, the cells were stained with anti-CD11b antibody, and flow cytometric analysis was performed. D, Chk1 (but not p53) is important for 6BT-mediated NBT reduction. Cells were treated as described for C, and the NBT reduction assay was performed. The results are an average of three independent experiments. E, overexpression of Chk1 can induce evidence of AML differentiation. OCI cells were transfected with Chk1-GFP or GFP alone. After 72 h, CD11b expression was assessed by flow cytometry in the viable GFP-positive cells.

### DISCUSSION

Chemotherapeutic agents have traditionally been used to induce DNA damage and subsequent cell death. Unfortunately, this leads to significant morbidities from toxicities in non-malignant cells and limits the therapeutic options for many patients. One specific malignancy in which the toxicities of the standard agents present an enormous clinical challenge is AML. Although the side effects are troublesome for all patients, elderly patients are quite often not even able to tolerate the chemotherapeutics, leading to no satisfactory therapeutic options. Partly because of the inability of many elderly AML patients to undergo standard AML therapy, the 5-year survival of patients over 65 is only 4% (18, 19).

Besides direct DNA damage induction, many chemotherapeutics also have dramatic effects on intracellular nucleotide
levels, which also impacts their activities (i.e. growth arrest, senescence, and/or differentiation), especially when used at subcytotoxic doses. Interestingly, although nucleotide depletion alone does not lead to large-scale DNA damage, limited and reversible DNA damage, as well as replication stress, can result from nucleotide depletion (5, 6).

Here, we have shown that AML differentiation can be induced through ATP depletion-mediated activation of DNA damage signaling in an ATR/Chk1-dependent but p53-independent fashion. In this case, the DNA damage response is sufficient to trigger downstream signaling but does not induce significant cell death. In addition, we have provided evidence that the overexpression of Chk1 itself can moderately induce AML differentiation. As we reported previously (11), overexpression of Chk1 can only transiently activate the checkpoint. Therefore, it is understandable that Chk1 overexpression showed less effect in inducing differentiation than 6BT. In addition, it is likely that other signals might be necessary for potent differentiation as well.

Although ATP depletion can trigger DNA damage signaling and subsequent differentiation, further work is necessary to characterize the downstream pathways required for differentiation induction. It is well known that Chk1 modulates the activity of cyclin-dependent kinases, which can lead to the growth arrest of cells. In particular, Chk1 is known to directly regulate several Cdc25 family members that control cell proliferation (20). Future work will identify the downstream pathways through which Chk1 leads to AML differentiation.

As many chemotherapeutics require functional p53, and as it is mutated in >50% of all human cancers, therapeutic strategies that exhibit p53-independent effects are highly desirable (21). Furthermore, although p53 and p21 have been associated with both cell growth and differentiation, these results suggest that p53 and p21 may be important in the growth inhibitory effects of 6BT, but they are not essential for differentiation (22, 23).

Although ATP depletion as a therapeutic strategy for solid cancers has been extensively studied, this strategy has not been widely explored for AML. ATP depletion agents, such as meth-
ylmercaptourine riboside and 6-aminonicotinamide, have been tested in clinical trials for several types of cancer without significant clinical efficacy. It is likely that this is due to the fact that it has not been possible to achieve the necessary levels of ATP depletion for cell death in vivo, as partial ATP depletion in most cancer cells (except AML cells) does not trigger differentiation and subsequent irreversible growth arrest. It is generally thought that ATP stores must be depleted to ~15–20% for cell death to occur (24–26). As 6BT does not exhibit this degree of ATP depletion yet still leads to irreversible growth arrest (4), it is likely that the level of ATP depletion required for AML differentiation is significantly lower than that required for cell

FIGURE 5. p21 does not play a crucial role in 6BT-mediated differentiation. A, p21 knockdown in OCI cells. Western blotting demonstrated the knockdown of p21 in OCI cells. As basal levels of p21 are low, the cells were stimulated with doxorubicin (1 μM) to assess p21 knockdown. OCI-shP21, OCI cells expressing p21 shRNA; OCI-PLK, OCI cells expressing the PLK empty vector; UT, untreated. B, p21 knockdown does not affect 6BT-mediated differentiation. OCI cells were treated with 6BT and all-trans-retinoic acid (ATRA; 500 nM) as a control for 4 days, and then the NBT reduction assay was performed. The results are an average of three independent experiments. C, p21 plays a role in 6BT-mediated growth inhibition. OCI cells were treated with 6BT for 72 h, and the cells were counted using a Coulter counter.

FIGURE 6. Multiple ATP depletion agents can induce Chk1-dependent and p53-independent AML differentiation. A, ATP depletion agents can induce AML differentiation. OCI cells were analyzed as described in the legend to Fig. 1A after treatment with 6-methylthioinosine (6MT; 10 μM), 6BT (10 μM), 2-deoxyglucose (6DG), or vehicle (untreated (UT)). B, chemical inhibition of DNA damage signaling impairs ATP depletion-mediated differentiation. The indicated OCI cell lines were treated with all-trans-retinoic acid (ATRA; 1 μM), 6-methylthioinosine (6MT; 10 μM), 2-deoxyglucose (20 μM), or vehicle for 4 days, and the NBT reduction assay was performed. shChk1, Chk1 shRNA.
death induction. This claim is further supported by the fact that only limited and transient DNA damage is sufficient to trigger ATP depletion-mediated AML differentiation. These findings support further testing of ATP depletion strategies for AML. Furthermore, our study suggests that, in certain instances, subcytotoxic levels of chemotherapeutics (such as anthracyclines) may be advantageous, as they can lead to the activation of DNA damage signaling without the necessity for cytotoxic effects.

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