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Journal Title: Molecular Cancer Therapeutics
Volume: Volume 17, Number 2
Publisher: American Association for Cancer Research | 2018-02-01, Pages 497-507
Type of Work: Article | Post-print: After Peer Review
Publisher DOI: 10.1158/1535-7163.MCT-17-0566
Permanent URL: https://pid.emory.edu/ark:/25593/tnfc0

Final published version: http://dx.doi.org/10.1158/1535-7163.MCT-17-0566

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Accessed August 14, 2019 8:26 PM EDT
Inhibition of MDM2 by a Rhein-Derived Compound AQ-101 Suppresses Cancer Development in SCID Mice

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Abstract

A novel small-molecule anthraquinone (AQ) analogue, AQ-101, which was synthesized through chemical modification of the core structures of rhein, exhibited potent anticancer activity. In the present study, we evaluated the cancer-inhibiting mechanism of AQ-101 and tested the therapeutic potential of this compound for treating cancer in mice. We found that AQ-101 was able to induce MDM2 protein degradation through a self-ubiquitination and proteasome-mediated mechanism. This AQ-101-induced MDM2 downregulation led to activation of p53, which contributed to apoptosis of acute lymphoblastic leukemia (ALL), especially those with a wild-type p53 phenotype and MDM2 expression in vitro and in vivo. When given for a period of 2 weeks (20 mg/kg/day, 3x/week), AQ-101 inhibited development of ALL in nude or SCID mice with a human ALL xenograft and achieved cure by the end of the 5-month experiment. Importantly, AQ-101 showed minimal or no inhibitory effect on normal human hematopoiesis in vitro and was well tolerated in vivo in animal models. Given that MDM2-overexpressing cancers are commonly refractory to current treatment options, our study results suggest that further development of
AQ-101 is warranted, as it represents a potentially new, safe anticancer drug with a novel strategy for targeting MDM2.

Introduction

The human MDM2 gene is an oncogene that is amplified and/or overexpressed in diverse human cancers (1, 2). The oncogenic function of MDM2 is mainly to inhibit the p53 tumor suppressor (3, 4); thus, p53 function is inactivated in MDM2-overexpressing cancer cells, leading to aberrant cell proliferation and growth. Clinically, there is a correlation between overexpression of MDM2 and poor treatment outcome (5–7).

The MDM2 protein is unstable and is degraded by a self-ubiquitination and proteasome-mediated mechanism. MDM2, a member of the RING-finger-type family of E3 ubiquitin ligases, is also a substrate of its own RING domain E3 ligase; thus, MDM2 regulates ubiquitination of both p53 and MDM2 itself (8, 9). The E3 ubiquitin ligase activity of MDM2 is tightly regulated by its partner MDM4 (MDMx). Both MDM2 and MDM4 proteins contain a similar C-terminal RING domain, through which dimerization occurs (10). Although the RING domain of MDM4 has no E3 ligase activity, binding of the RING domains between MDM4 and MDM2 reduces the E3 ligase activity of MDM2 for MDM2 self-ubiquitination, enabling stabilization of MDM2 protein (11–13). In contrast, the E3 ligase activity of MDM2 to catalyze p53 for polyubiquitination in the MDM2/MDM4 heterodimer is greatly increased (14–16).

Because MDM2 is an important inhibitor of p53, efforts have been made to target the MDM2-p53 interaction as a therapeutic strategy. Small-molecule inhibitors such as nutlin-3, which are able to efficiently block the MDM2/p53 interaction, resulting in activation of p53 and cancer-cell apoptosis, have been actively investigated in preclinical studies and clinical trials for the treatment of cancer (17, 18). In addition, previous studies have found that several natural products can directly downregulate MDM2 expression, which leads to p53 activation and cancer cell apoptosis. The downregulation of MDM2 by these reagents is through different mechanisms. For example, a dietary component curcumin inhibits MDM2 through the PI3K/mTOR pathway (19). The natural product berberine can induce MDM2 degradation through disrupting the MDM2-DAXX-HAUSP interaction (20).

Rhein, a natural product (isolated from rhubarb), has an AQ scaffold. This scaffold is the key structural feature required for the cytotoxic activity in treating cancer patients with existing AQ drugs, such as doxorubicin. However, these currently used AQ drugs are all DNA intercalators and have many side effects (21, 22). Studies show that rhein has low-to-moderate anticancer activity against various tumor cell lines (23–25). The mechanisms by which rhein induces cancer cell death are not known, although previous studies suggest a link with the p53 and caspase pathways (26, 27). The low-to-moderate antitumor activity of the natural rhein limits its therapeutic potential. However, an important feature of rhein is low toxicity to normal cells and very well tolerated in humans in its use as a laxative (28). Due to severe side effects of the currently used AQ drugs, we had previously explored whether an effective but less toxic drug could be developed using AQ, the basic structure of...
rhein. We chemically modified the rhein structure, synthesized and screened a panel of rhein analogues, and found one (AQ-101) that exhibited potent cytotoxicity against cancers (29).

In the present study, we evaluated the cellular signaling pathway regulated by AQ-101 for cancer cell death. We demonstrate that AQ-101 can downregulate MDM2. We have further characterized the molecular mechanism of action of AQ-101 to inhibit MDM2 and evaluated whether AQ-101 induced downregulation of MDM2 is associated with the cytotoxicity of AQ-101 in cancer cells. Additionally, we evaluated the inhibitory effect of AQ-101 on normal human hematopoiesis in vitro, its toxicity and tolerance in mice as well as the therapeutic potential of this compound in ALL in an SCID mouse model.

**Materials and Methods**

**Cells and compounds**

This study used five established cell lines derived from children with ALL. Four of these cell lines (EU-1, EU-3, EU-6, and EU-8) were established at Emory University (Atlanta, GA), and one (SUP-B13) was obtained from Stephen D. Smith (University of Kansas Medical Center, Kansas City, KS). All ALL cell lines were authenticated and their p53 phenotypes were as follows: Sup-B13, EU-1, and EU-3 cells (wt-p53), EU-6 (mutant p53), and EU-8 (p53-null). Four of the p53-positive cell lines, including the p53-mutant EU-6, expressed MDM2, whereas MDM2 was not expressed in the EU-8 cells (30). The 293T cell line, purchased from the ATCC was used for gene transfection assays.

Following informed consent, fresh leukemia samples and normal human bone marrow mononuclear (NBMM) cells were selected from 16 ALL patients and 5 donors, respectively, as described in detail in Supplementary Materials and Methods online.

AQ-101 was a synthesized rhein analogue, which was reported in our previous publication (29). Nutlin-3a was purchased from Cayman Chemical and its structure was shown in a previous publication (31).

**Plasmids**

The MDM2 and MDM4 constructs were generated by polymerase chain reaction (PCR) and cloned into either pCMV-Myc or pCMV-HA expression vectors. A “Quick Change Site-Directed Mutagenesis Kit” (Stratagene) was used to mutate MDM2 at codon 464 (C464A), generating the corresponding plasmid. The GST-tagged MDM2 and MDM4 C-terminal RING domain constructs were generated by PCR and then cloned into the bacterial pGEX expression vector. The primers used for construction of these plasmids are listed in Supplementary Table S1. The p53 plasmid was provided by Dr. B. Vogelstein (Johns Hopkins University). The pCI-His-hUbi plasmid was purchased from Addgene. Transfections were performed using Lipofectamine 2000 reagents (Invitrogen) according to the manufacturer’s instructions.

**Immunoprecipitation and Western blot assay**

Cells were lysed in a buffer composed of 50 mmol/L Tris, pH 7.6, 150 mmol/L NaCl, 1% Nonidet P-40, 10 mmol/L sodium phosphate, 10 mmol/L NaF, 1 mmol/L sodium...
orthovanadate, 2 mmol/L phenylmethylsulfonfyl fluoride (PMSF), 10 μg/mL aprotinin, 10 μg/mL leupeptin and 10 μg/mL pepstatin. After centrifugation, the clarified cell lysate was separated from the pellet of cell debris, and then incubated with 15 μL Protein G/Protein A-agarose and 1 μg of antibodies, overnight at 4°C. For the Western blot, the resulting cell lysates or immunoprecipitates were resolved by SDS-PAGE. They were then transferred to a nitrocellulose filter and probed with the specific antibodies as listed in the Supplementary Materials section. Finally, proteins were visualized with a chemiluminescent detection system.

**Ubiquitination assay**

Ubiquitination assay was performed as described previously (32). Briefly, gene-transfected cells were treated with or without selected reagents and MG132, and then collected in two aliquots. One aliquot (10%) was used for conventional Western blotting. The remaining cells (90%) were used for the purification of His6-tagged protein, with the aid of Ni2+-nitrilotriacetic acid beads. Purified His-tagged protein was then eluted and analyzed by Western blot assay.

**Compound-protein binding assays**

The binding activity of AQ-101 to either MDM2 or MDM4 protein was examined by fluorescence titration and isothermal titration calorimetry (ITC) assays, as described in detail in Supplementary Methods online.

**Clonogenic assay**

Aclonogenic assay was used to determine the effect of AQ-101 on in vitro growth of cancer and normal human hematopoietic cells, respectively. Briefly, a bottom layer of low-melting-point, 0.5% agarose (in RPMI1640 medium plus 10% FBS) was poured into gridded 35-mm dishes and allowed to gel. Cells were cultured in a top layer of 0.35% agarose/medium at 37°C in a humidified atmosphere containing 5% CO2. After 2 to 3 weeks, cultures were fixed with formalin and colonies scored.

**In vitro cytotoxicity assay**

The cytotoxic effect of AQ-101 on ALL cells was determined using the water-soluble tetrazolium salt (WST) assay. Briefly, cells cultured in 96-well microtiter plates were treated with different concentrations of AQ-101 for a 20-hour period. WST (25 μg/well) was then added and incubation continued for an additional 4 hours, after which optical density (OD) was read with a microplate reader (test wavelength of 450 nm; reference wavelength of 620 nm).

**Flow cytometry**

Flow cytometry was performed to analyze cell-cycle position and state of apoptosis. For cell-cycle analysis, cells were collected, rinsed, and fixed in 70% ethanol, and then washed again and resuspended in PBS solution containing propidium iodide. The samples were analyzed using a FACScan. For quantitative detection of apoptosis, cells with or without treatment were washed with PBS and stained with FITC-annexin V and 7-aminoactinomycin.
(7-AAD) using an FITC Annexin V Apoptosis Detection Kit 1 (BD Pharmingen) following the manufacturer’s instruction and analyzed by flow cytometry.

**Animal studies**

Animals were housed, maintained, and treated at Emory University Children Center in accordance with protocols approved by the IACUC at Emory University. For the leukemic xenograft assay, the human ALL cell lines EU-1 (parental and luciferase-transfected) were engrafted into nude and SCID mice, respectively. Cells (10^7 cells/mouse) were injected into the tail vein of 5-week-old female nude mice (Hsd:Athymic Nude-Foxn1^nu^) or SCID mice (C.B-17/IcrHsd-Prkdc^scid^Lyst^bg^). At 72 hours after injection (tumor xenograft was confirmed by bioluminescence imaging in all SCID mice), mice were then randomly divided into control and different treatment groups. Mice were injected intraperitoneally (i.p.) with either AQ-101 (in a formula containing 10% DMSO and 10% Tween 80) at dose levels of 10, 15, and 20 mg/kg. Injections were performed at 24-hour intervals for a period of 3 days (one course). After a 4-day rest, a second course was administered. The development of leukemia and the efficacy of AQ-101 treatment in SCID mice were monitored by bioluminescence imaging at least twice a week for SCID mice engrafted with luciferase-transfected EU-1. Xenografted recipients were euthanized following development of hind-limb paralysis, and then evaluated for the presence of human leukemic cells by histopathology and by PCR detection of a DNA sequence in exon 1 of the human β-globin gene. Using survival as the test endpoint, the probability of event-free survival (EFS) for treatment and control groups was analyzed by constructing EFS curves using the Kaplan-Meier product limit method. The effect of treatment with AQ-101 on EFS was determined by the log-rank test.

**Results**

**AQ-101 induces MDM2 protein degradation**

We performed Western blot assays for possible effects of AQ-101 (Fig. 1A) on several survival or antiapoptotic factors MDM2, MDM4, survivin, cIAP-2, and Bcl-2 in ALL cell lines. Results showed that AQ-101 induced a remarkable downregulation of MDM2 but other tested proteins (Fig. 1B). AQ-101 inhibited MDM2 in a dose-dependent manner and inhibition of MDM2 occurred at approximately 2 hours after treatment, followed by steady-state suppression of MDM2 levels (Fig. 1C). Downregulation of MDM2 was accompanied by increased expression of p53.

To investigate how AQ-101 inhibits MDM2, we first tested the effect of AQ-101 on MDM2 protein stability. We treated cells with MG132 and performed CHX pulse-chase assays in AQ-101-treated EU-1 cells. The observed downregulation of MDM2 by AQ-101 was blocked by the protein-degradation inhibitor MG132 (Fig. 1D). CHX pulse-chase results showed that the half-life of MDM2 in untreated EU-1 cells was >90 minutes, whereas AQ-101 treatment decreased the MDM2 half-life to <60 minutes (Fig. 1E). In contrast, the half-life of p53 in untreated cells was <30 minutes, and was increased to >90 minutes by treatment with AQ-101.
We next examined whether AQ-101 regulates MDM2 at the transcriptional and translational levels. We performed gene transfection and reporter assays and results showed that AQ-101 did not regulate MDM2 promoter activity (Supplementary Fig. S1A). RT-PCR results demonstrated that AQ-101 did not inhibit MDM2 mRNA expression, and instead, the MDM2 mRNA level was significantly elevated by AQ-101 (Fig. 1F). We believe that this enhanced MDM2 mRNA expression in AQ-101-treated cells was a result of p53 activation, because MDM2 is a transactivational target of p53 (33). When we performed actinomycin D pulse-chase and polysome profiling, our results showed that AQ-101 did not regulate MDM2 mRNA stability (Supplementary Fig. S1B) and translation (Fig. 1G). These results suggest that AQ-101 downregulate MDM2 expression only at the posttranslational level, indicating an effect on protein stability.

AQ-101 induces MDM2 self-ubiquitination and reduces p53 polyubiquitination by binding to MDM2 and blocking MDM2-MDM4 interaction

It is known that MDM2 degradation is regulated by a self-ubiquitination manner. Thus, we tested whether AQ-101 has a role to increase MDM2 E3 ligase activity for MDM2 self-ubiquitination. We performed IP-Western blot assay and results showed that AQ-101 induced self-ubiquitination of endogenous MDM2 followed by a decreased p53 polyubiquitination in EU-1 cells (Supplementary Fig. S1C and S1D). As a control, MDM2 self-ubiquitination did not increase in doxorubicin-treated EU-1 cells.

Because MDM2 self-ubiquitination is inhibited by its partner MDM4 that forms a heterodimer with MDM2, we evaluated whether AQ-101 induces MDM2 self-ubiquitination by preventing or disrupting the formation of MDM2-MDM4 heterodimer. We performed co-IP and Western blot assays and results showed that the corresponding levels of endogenous MDM2 or MDM4 were significantly reduced after only 20 to 40 minutes of treatment with AQ-101, as shown by immunoprecipitation with either MDM4 or MDM2 antibodies (Fig. 2A). In contrast, the levels of MDM2 and MDM4 were not reduced after a similar treatment with doxorubicin, suggesting that AQ-101, but not doxorubicin, was able to disrupt the binding of these two proteins. In addition, we performed a pulldown assay using GST-fused MDM4 or MDM2 RING protein and EU-1 cell extracts. We confirmed that AQ-101, but not doxorubicin, prevented the interaction between MDM2 and MDM4 (Fig. 2B). Furthermore, we performed an in vivo bimolecular fluorescence complementation (BiFC) assay, where the MDM2 RING domain (415–491) and MDM4 RING domain (421–490) were fused to the N- (1 to 154) and C- (155 to 238) terminal halves of YFP, respectively. The MDM2 and MDM4 RING domain-mediated dimerization of two YFP fragments should reconstitute a fluorescent protein, when coexpressed in cells. As expected, the YN-MDM2 RING or YC-MDM4 RING transfections alone did not generate a signal (Supplementary Fig. S2A and S2B), whereas cotransfection of the YN-MDM2 RING and YC-MDM4 RING produced strong fluorescence with a diffuse localization in 293T cells (Fig. 2C). AQ-101 significantly decreased the fluorescence generated by the interaction of the YN-MDM2 RING and YC-MDM4 RING (Fig. 2D). The fluorescence signal was not reduced by doxorubicin, further suggesting that AQ-101 prevents the formation of MDM2-MDM4 heterodimer.
We also performed an \textit{in vivo} ubiquitination assay to confirm that AQ-101 induced MDM2 self-ubiquitination and reduced p53 polyubiquitination through the disruption of MDM2-MDM4 dimerization. MDM2 self-ubiquitination was indeed reduced following cotransfection of MDM2 and MDM4 compared with MDM2 transfection alone (Fig. 2E, lane 4 vs. lane 2). Cotransfection of MDM2 and MDM4 induced greater ubiquitination of p53 than did transfection of MDM2 alone (lane 4 vs. lane 2, p53 panel). Such results are consistent with literature reports (11, 16). Addition of AQ-101 to the cotransfection experiment remarkably induced MDM2 self-ubiquitination and inhibited p53 polyubiquitination in a dose-dependent manner (lanes 5–7). In addition, we tested the MDM2 RING-domain mutation C464A, which causes loss of ubiquitin ligase activity (34). Results of similar cotransfection of p53, C464A, and MDM4 and AQ-101 treatment showed that self-ubiquitination of MDM2 and p53 polyubiquitination were almost completely inhibited by the MDM2 C464A mutation (lanes 8–10), further confirming the effect of AQ-101 on ubiquitination of MDM2 and p53.

Finally, we tested whether AQ-101 binds to the C-terminal RINGs of MDM2 or MDM4 to block their interaction. We performed fluorescent titration assays for binding activity. The GST-MDM2 RING protein has natural fluorescence with an excitation at 280 nm and emission of 335 nm (Supplementary Fig. S2C). We titrated AQ-101 to the GST-MDM2 RING protein (Fig. 2F): Results showed a binding $K_d = 0.31 \mu\text{mol/L}$ (Supplementary Fig. S2D). Because AQ-101 contains a Michael acceptor and is expected to form a covalent bond with a cysteine in a protein, we tested whether the binding between AQ-101 and MDM2 is covalent. Results from a time dependency of fluorescent binding assay showed no time-dependent inhibition (Supplementary Fig. S2E), suggesting that AQ-101 does not form a covalent bond with MDM2. We also titrated the GST-MDM2 RING protein with doxorubicin and did not detect doxorubicin binding (Supplementary Fig. S2D). In addition, we performed ITC assays and results showed that while AQ-101 bound to MDM2 ($K_d = 0.37 \mu\text{mol/L}$; Supplementary Fig. S2F), it failed to bind to MDM4 (Supplementary Fig. S2G).

\textbf{AQ-101-mediated downregulation of MDM2 results in p53 activation}

Results as shown in Figs. 1 and 2 indicate that following MDM2 self-ubiquitination and degradation by AQ-101, there were decreased p53 polyubiquitination and increased p53 stabilization. Activation of p53 can occur through multiple mechanisms. For example, cellular stress and DNA damage, such as treatment with doxorubicin, induce immediate p53 activation through the ATM signaling pathway. A recently developed activator of p53, nutlin-3, induces p53 activation through blocking the interaction between MDM2 and p53. We therefore compared the effect of AQ101, doxorubicin, and nutlin-3a on p53 activation. All three reagents increased p53 protein expression. From the data in Fig. 3A, we found that doxorubicin and nutlin-3a induced p53 at an early time (2–4 hour posttreatment), while AQ101-induction of p53 occurred at 4 to 8 hours after treatment. Doxorubicin induced significant activation of ATM phosphorylation, which is an endpoint of DNA damage, while an obvious induction of this protein was not observed with either AQ-101 or nutlin-3a. Importantly, AQ-101 inhibited MDM2, while both doxorubicin and nutlin-3a induced a large
increase in MDM2 expression. These results suggest that AQ-101 induces p53 activation through a mechanism distinct from that induced by doxorubicin and nutlin-3a.

To compare the effects of AQ-101 and doxorubicin on DNA damage, we performed comet assays. The results showed that doxorubicin but not AQ-101 induced DNA damage at an early (4 hours) time point, although nuclear fragmentation (but not comets) was observed at a later time (8 hours) following AQ-101 treatment (Fig. 3B).

We further investigated the effect of AQ-101 on p53 functionality by evaluating expression of its downstream factors. Using Western blot assays, we found that p53 targets p21 and PUMA were induced in AQ-101-treated cells (Fig. 3C). Corresponding with the changes, AQ-101 induced G_1 cell-cycle arrest in wt-p53 EU-1 cells (Fig. 3D, left and E). In contrast, G_1 cell-cycle arrest did not occur in AQ-101-treated EU-1 cells in the presence of sip53 (Fig. 3D, right). Similarly, AQ-101 failed to induce G_1 arrest in mutant-p53 EU-6 cells; instead, a G_2-M arrest was detected in the latter cells (Supplementary Fig. S3A). In addition, we detected a dose- and time-dependent activation of caspase-3, -7, and -9 as well as cleavage of PARP in AQ-101-treated ALL cells (Fig. 3C; Supplementary Fig. S3B and S3C).

**AQ-101 induces cancer cell apoptosis in an MDM2-dependent manner**

We previously reported that AQ-101 has potent cytotoxic activity against cancers (29). In the present study, we evaluated whether the cytotoxic activity of AQ-101 is associated with the expression of MDM2 and p53 status of the cancer cells. We tested five ALL cell lines with different MDM2/p53 phenotypes (Fig. 4A). AQ-101 exhibited strong cytotoxic effects on three wt-p53/MDM2-overexpressing cell lines: Sup-B13, EU-1, and EU-3 (Fig. 4B). AQ-101 showed less cytotoxicity for the mutant-p53/MDM2-expressing cell line EU-6 and much less cytotoxicity for the p53/MDM2/MDM4-null cell line EU-8. Colony formation assay results also showed that AQ-101 potently inhibited EU-1 cell growth. We observed a significant reduction of both colony number and size in AQ-101-treated EU-1 cells, as compared with the control (Fig. 4C). AQ-101 had less inhibitory effect on EU-6 and EU-8 colony formation.

To clarify whether the observed cell-growth inhibition and cell death induced by AQ-101 were associated with induction of apoptosis, we stained cells from these ALL lines with Annexin-V FITC and 7-AAD, and quantitated the results by flow cytometry. Supplementary Fig. S4A shows representative flow histographs. Most cells are Annexin-V positive, an indication of apoptosis in the treatment (after 8–12 hours of AQ-101). Consistent with the WST cytotoxicity results, AQ-101 induced strong apoptosis in Sup-B13, EU-1, and EU-3 cells, less apoptosis in EU-6 and EU-8 (Fig. 4D). These results suggested that AQ-101-induced cancer cell death is by apoptosis and is closely associated with the level of MDM2 expression and p53 status of the cancer cells.

We also tested the cytotoxicity of AQ-101 for freshly isolated ALL cells from patients. We selected 16 bone marrow (BM) samples from pediatric ALL patients (Supplementary Table S2) having peripheral total white blood cell (WBC) counts higher than 100 × 10^9/mL, which is a poor prognosis indicator for this disease. Western blot assays for MDM2 and MDM4...
expression showed that 13 of the 16 samples (81%) had detectable levels of MDM2; furthermore, 7 of these 16 (43%) had high levels of MDM2 and 10 of the 16 cases (63%) had high levels of MDM4. Almost all samples with high levels of MDM2 also showed high levels of MDM4 (Fig. 4E). One patient’s ALL cells (UPN 4) had a p53 mutation in exon 7, codon 248. The WST assay results showed that AQ-101 exhibited cytotoxicity for all 16 samples studied, with IC_{50} values ranging from 0.3 to 2.35 μmol/L (Fig. 4F); moreover, there was a high correlation between potent cell killing by AQ-101 and elevated levels of MDM2 expression (Supplementary Fig. S4B and Supplementary Table S2). The cytotoxicity of AQ-101 was less correlated with MDM4 expression (Supplementary Fig. S4C).

To further validate that AQ-101 induced cell apoptosis/death is MDM2 and p53 dependent, we performed knockdown (KO) of MDM2 and p53 using siRNAs in EU-1 cells and treat the cells with AQ-101. We found that inhibition of MDM2 and p53 led to resistance to AQ-101 (Supplementary Fig. S4D), suggesting that the expression of MDM2 and activation of p53 contributes to AQ-101-induced cell death.

**AQ-101 suppresses ALL cell proliferation in animal models**

We evaluated the anticancer activity of AQ-101 in vivo, in an animal model. First, we determined the maximum tolerated dose (MTD) of AQ-101, in order to define the appropriate in vivo dosage. AQ-101 doses at 12.5, 25, 50, 100, and 200 mg/kg were given i.p. to ICR normal mice (5 mice per group), at 24-hour intervals for a period of 3 days (1 course). After a 4-day rest, this dosage schedule was repeated for a second course. The 200 mg/kg dose was lethal within 30 days for three out of five mice, whereas the dose range between 12.5 and 100 mg/kg was well tolerated, with no death at day 30 (Supplementary Fig. S5A).

To test whether AQ-101 is active in vivo against cancer cells, we administrated AQ-101 in immune-deficient mice, including nude and SCID mice previously inoculated with parental EU-1 and EU-1 having luciferase transfection, respectively. We treated the mice (5–8 mice per group) with three doses of AQ-101 (10, 15, and 20 mg/kg), using the same schedule as for the MTD test. Our results showed that all control nude mice (without treatment) had a significant loss of body weight after EU-1 inoculation, while the AQ-101-treated mice continued to gain body weight throughout the period of observation (Fig. 5A). Similar results were observed in AQ-101-treated and control SCID mice (Supplementary Fig. S5B).

Bioluminescence imaging of luciferase-tagged EU-1 was used in SCID mice to allow quantification of cancer cells throughout the entire mouse. As shown in Fig. 5B, massive infiltration of EU-1 cells was seen in the control group, while no detectable leukemia was seen in the group treated with AQ-101 at dose of 20 mg/kg.

We also examined nude mice for presence of EU-1 cells (blasts) in the peripheral blood. We found that all control mice had leukemia at time of death. Supplementary Fig. SSC, left, is a representative histological analysis showing human ALL blasts in the blood of pretreatment mice, but not in AQ-101-treated mice (Supplementary Fig. SSC, right). Furthermore, we performed PCR to examine the human β-globin gene in nude mice. We detected this gene in
untreated control mice but not in mice treated with 20 mg/kg AQ-101 (Supplementary Fig. S5D).

All eight of the untreated control nude mice died within 50 days after inoculation, while all eight of the 20 mg/kg AQ-101-treated mice still survived at study termination (150 days) (Fig. 5C). Three of eight of the 10 mg/kg AQ-101-treated mice and two of eight of the 15 mg/kg AQ-101-treated mice died within 97 to 145 days after inoculation and treatment. For SCID mice, all five controls died within 45 days, while two of five of the 10 mg/kg AQ-101-treated mice and one of five 15 mg/kg AQ-101-treated mice died within 125 to 143 days. Significantly, all five mice treated with 20 mg/kg AQ-101 were alive after 150 days (Fig. 5D).

**AQ-101 exhibits minimal inhibitory and cytotoxic effects on normal cells**

Because human NBMM (normal bone marrow mononuclear) cells express no or very low levels of MDM2 (Fig. 4E), we examined the inhibitory effects of AQ-101 on these cells. Results of clonogenic assays for CFU-GM and BFU-E of human NBMM cells showed that AQ-101 had minimal inhibitory effect on the CFU-GM and BFU-E, as compared with doxorubicin, which showed severe inhibitory activity. As seen in Fig. 6A and B, CFU-GM and BFU-E colony numbers and size in AQ-101-treated samples were similar to the control, whereas both colony number and size were significantly reduced in the doxorubicin-treated samples.

In addition, we evaluate potential AQ-101 cytotoxicity for NBMM, as compared with doxorubicin and nutlin-3a. The results showed greater than 90% cell survival of NBMM treated with AQ-101 at a dose of 2 μmol/L for 24 hours. In contrast, NBMM cell survival following similar treatment with doxorubicin was only about 50%. At a high concentration (8 μmol/L), over 90% of cells survived treatment with AQ-101, whereas only 60% survival was detected with nutlin-3a (Fig. 6C).

Using NBMM cells treated with AQ-101 or doxorubicin, we performed Western blot assays and found that p53 was significantly induced by doxorubicin, but not by AQ-101 (Fig. 6D). We also performed comet assays to evaluate whether p53 induction in the doxorubicin-treated NBMM was due to DNA damage; and similarly, whether the lack of p53 induction in AQ-101-treated NBMM was due to absence of DNA damage. As expected, doxorubicin induced DNA damage in NBMM cells. In contrast, no DNA damage was detected in AQ-101-treated NBMM, even at a dose 1 μmol/L (Supplementary Fig. S6A), capable of inducing DNA fragmentation and cell death of malignant EU-1 cells.

We also evaluated the possible toxic effects of AQ-101 when administered in animals. We treated ICR normal mice with AQ-101 at dose of 40 mg/kg, using a similar schedule as for the MTD test. We performed histopathology on harvested tissues of the heart, liver, and kidney, and found no evidence of toxicity after treatment with AQ-101 (Fig. 6E). In contrast, doxorubicin treatment even at a much lower dose (10 mg/kg) led to pronounced heart tissue damage, with increased cytoplasmic vacuolization and myofibrillar loss (Fig. 6E, top right).
Discussion

In an attempt to discover effective and less harmful anticancer drugs, we previously synthesized and screened a panel of rhein analogues and identified one of the analogues (AQ-101) as having potent cytotoxicity against cancers (29). In the present study, we have further tested the cytotoxicity of AQ-101 in ALL and demonstrated that the response of ALL cells to AQ-101 is associated with their expression of MDM2 and p53 status. We found that ALL cells having MDM2 overexpression and a wt-p53 phenotype were very sensitive to AQ-101, whereas cells lacking MDM2 expression and without wt-p53 (naturally or KO) were less sensitive. The distinct responses to AQ-101 of ALL cells with different MDM2 expression levels and p53 status were correlated with changes in the regulation of both MDM2 and p53. We found that AQ-101 downregulates MDM2, resulting in activation of p53, which is a major contributor to the anticancer activity of this compound.

Activation of p53 can be induced through many mechanisms. The mechanism by which AQ-101 induces p53 is distinct from that of either doxorubicin or nutlin-3: AQ-101 activates p53 by ultimately inducing degradation of MDM2, while doxorubicin and nutlin-3 induce p53 either through DNA damage or by blocking the interaction between MDM2 and p53, respectively. Furthermore, in cells treated with doxorubicin or nutlin-3, a significant induction of MDM2 follows after activation of p53. However, activation of p53 in AQ-101-treated cells results from a decrease in MDM2 levels. Additionally, because AQ-101 induces MDM2 degradation, the p53-independent role of MDM2 becomes inhibited in turn, thus inducing a more potent suppression of cancer cell growth than either doxorubicin or nutlin-3.

Results of a series of experiments suggested that blockage or disruption of the interaction between MDM2 and MDM4 mediated by the AQ-101-MDM2 binding contributes to AQ-101 induced cell death. Furthermore, experiments utilizing cotransfection of MDM2 and MDM4 showed that coexpression of these proteins inhibited self-ubiquitination of MDM2 while greatly increasing ubiquitination of p53. Importantly, addition of AQ-101 to the MDM2/MDM4-cotransfected cells significantly increased MDM2 self-ubiquitination and decreased p53 polyubiquitination. These biological and functional studies further confirmed the mechanism of action by which AQ-101 induces p53 and cancer cell apoptosis through the degradation of MDM2.

A significant finding of the present study suggests that AQ-101 shows very little toxicity for normal human hematopoiesis. Similarly, AQ-101 shows minimal toxicity for normal tissues/cells in animal studies. In mice, the MTD (100 mg/kg/d) of AQ-101 is 5-fold higher than the 20 mg/kg/d dose that effectively inhibits cancer cells. There were virtually no hematological and pathological abnormalities found in mice treated with AQ-101 at 40 mg/kg/d. Although AQ-101 and doxorubicin contain a similar AQ scaffold, these two compounds have different toxicities and tolerances, which is most likely due to the different pathways for p53 activation induced by the two compounds. Doxorubicin has been well characterized to induce p53 through intercalation with DNA, leading to DNA damage (35, 36) in both malignant and normal cells, whereas AQ-101 activates p53 by inhibiting MDM2-MDM4 heterodimerization, which leads to MDM2 degradation. Because normal cells express no or
very low levels of MDM2 and MDM4, lack of p53 activation after treatment with AQ-101 is likely a major reason for the very little toxicity of this compound.

In summary, we have characterized a small-molecule inhibitor, AQ-101, that can target MDM2 for protein degradation by binding to MDM2 and blocking the interaction between MDM2 and MDM4. This represents a novel mechanism for induction of apoptosis specifically in cancer cells that express MDM2, while sparing normal cells. The ability of AQ-101 to block the interaction between MDM2 and MDM4 leads to MDM2 self-ubiquitination and degradation; this in turn causes p53 activation and cancer cell apoptosis, particularly in cancer cells expressing high levels of MDM2. In contrast, due to typically low levels of MDM2 in normal tissues/cells, AQ-101 exhibits minimal toxicity for these cells and is thus very well tolerated in animals. Thus, AQ101 may represent an excellent candidate for targeting refractory cancers expressing high levels of MDM2.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by R01 grant (CA180519) to B. Wang and M. Zhou and grants CA123490 and CA143107 to M. Zhou; a research grant (279706) from ST. Baldrick to M. Zhou; and research grants from CURE to M. Zhou and L. Gu.

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References


Figure 1.
The effect of AQ-101 on MDM2 expression. A, Structure of AQ-101. B, Western blot assays for expression of proteins, as indicated, in four ALL cell lines treated with 1 μmol/L AQ-101 for 24 hours. C, control; T, treatment. C, Western blot assays showing the dose-response (left) and time-course (right) of MDM2 and p53 expression in EU-1 cells treated by AQ-101. D, EU-1 cells with or without AQ-101 treatment (1 μmol/L for 8 hours) were treated with 10 μmol/L MG132 for additional 4 hours and then Western blots performed for expression of proteins as indicated. E, CHX pulse-chase assay for detection of protein turnover in EU-1 cells treated with or without 1 μmol/L AQ-101 for 4 hours. Numerical labels under each band of Western blots represent the expression levels after normalization for GAPDH, compared with untreated (0) samples (defined as 1 unit). F, EU-1 cells were treated with 1 μmol/L AQ-101 for different times, as indicated. The mRNA levels of MDM2, MDM4, and p53 relative to GAPDH were determined by quantitative RT-PCR. G, EU-1 cells were treated with or without 1 μmol/L AQ-101 for 4 hours and cytoplasmic lysates fractionated on a sucrose gradient. RNA was extracted from each fraction and subjected to quantitative RT-PCR for analysis of the distribution of MDM2 and GAPDH mRNAs. Data show the percentage of the total amount of corresponding mRNA in each fraction and represent mean ± SD of three independent experiments.
Figure 2.
AQ-101 induces MDM2 self-ubiquitination and reduces p53 polyubiquitination by binding to MDM2 and blocking MDM2-MDM4 interaction. A, EU-1 cells were treated with 20 μmol/L MG132 for 4 hours and subsequently with 1 μmol/L AQ-101 or doxorubicin (control), for the indicated times, and then IP-Western blot assays were performed. B, Pulldown assay to confirm the effect of AQ-101 on the MDM2-MDM4 interaction. GST-MDM4 (top) or GST-MDM2 (bottom) fusion proteins were incubated with EU-1 cell lysate, in the presence or absence of AQ-101 or doxorubicin. Bead-purified proteins were detected by Western blot using antibodies as indicated. The GST served as control for equal protein loading. C, Representative photographs of YFP fluorescence signal in 293T cells cotransfected with YFP N/C fragments fused to the MDM2 and MDM4 RING domain, respectively (YN-MDM2 RING and YC-MDM4 RING). D, The percentage YFP-positive cells (following cotransfection of YN-MDM2 RING and YC-MDM4 RING) after 2-hour treatment with the indicated compounds. E, Results of in vivo ubiquitination assay for...
MDM2 and p53 in 293T cells either transfected with p53 alone or cotransfected with p53, MDM2 (or MDM2 C464A mutation), and MDM4, in the presence or absence of AQ-101. Western blots for the transfected proteins were performed using either Ni-NTA purified or direct cell lysates. The amount of each plasmid used for each transfection assay is 5 μg. AQ-101 was used at concentrations from 0.25 to 1 μmol/L for 4 hours. F, A representative fluorescent-titration assay of AQ-101 binding to the MDM2 RING domain.
Figure 3.
MDM2 degradation by AQ-101 induces p53 activation. **A,** AQ-101, doxorubicin, and nutlin-3a differentially regulate MDM2 and p53 expression. EU-1 cells were treated with 1 μmol/L AQ-101, doxorubicin, or 5 μmol/L nutlin-3a, for different times as indicated; protein expression was detected by Western blot. **B,** Typical comet images of EU-1 cells treated with 1 μmol/L of either AQ-101 or doxorubicin, or 5 μmol/L nutlin-3a, for different times. Gray arrow: comet assay indicating DNA damage; white arrow: nuclear fragmentation, indicating apoptosis. Scale bars = 10 μm. **C,** The expression levels of the p53 targets p21 and PUMA, as well as activation of caspase-3 and cleavage of death substrate PARP, in EU-1 cells treated with AQ-101, as indicated. **D,** Cell-cycle distribution analysis of EU-1 and EU-1 (+sip53) cells treated with AQ-101 for 8 hours and data represent mean of three independent experiments, bars ± SD. **E,** Representative flow-cytometry histograms for EU-1 cells treated with 1 μmol/L AQ-101 for 8 hours.
Figure 4.
AQ-101 inhibits proliferation and induces apoptosis in MDM2-positive leukemic cells. **A,** Expression of MDM2 and MDM4, and status of p53 (w, wild-type; m, mutation; n, null), in five ALL cell lines, as detected by Western blot. **B,** Dose-dependent cytotoxic response to AQ-101 in 5 ALL cell lines tested in A. Cells were incubated with different doses of AQ-101 for 24 hours and cell viability detected by WST assay; data represent mean ± SD of three independent experiments. **C,** EU-1, EU-6, and EU-8 cells (5 × 10^3) were treated with different concentrations of AQ-101 and cell growth analyzed by clonogenic assay; data show number of colonies after 10-day incubation; inset: size comparison of representative colonies of EU-1 cells treated with 0.5 μmol/L AQ-101 vs. untreated controls (0). **D,** Apoptotic response for five cell lines shown in A, treated with the indicated AQ-101 doses for 24 hours, as detected by flow cytometry; data represent mean of three independent experiments, bars ± SD. **E,** Western blot assay for MDM2 and MDM4 expression in fresh childhood ALL cells from high risk (WBC > 100 × 10^6/mL) patients (n = 16). UPN, unique patient number; NBMM, normal bone marrow mononuclear cells. **F,** AQ-101 IC_{50} values for fresh ALL cells analyzed by WST.
Figure 5.
AQ-101 inhibits proliferation of MDM2-positive leukemic cells in vivo, leading to long-term survival of xenografted mice. A, Weight of nude mice xenografted with EU-1 human leukemia cells and treated with AQ-101 vs. control; data represent mean ± SD of 8 mice. 
*Insert:* Representative mice from treatment and control groups at 40-days posttreatment. B, Bioluminescence imaging showing intensity of total body leukemia developing in SCID mice treated without (control) or with different doses of AQ-101 for 24 days. C, Comparison of EFS curves for nude mice from AQ-101-treated vs. control groups. D, Comparison of EFS curves for SCID mice from AQ-101-treated vs. control groups.
Figure 6.
AQ-101 has minimal inhibitory and toxic effects on normal cells. A and B, Comparison of inhibitory effects of AQ-101 and doxorubicin on CFU-GM and BFU-E in NBMM cells, using in vitro colony formation analysis. NBMM cells (1 × 10^5) were incubated with GM-CSF or Epo, in the absence or presence of either 1 μmol/L AQ-101 or doxorubicin. Colonies were counted after 14 days of culture. A, Comparison of colony numbers; data represent mean of three independent experiments, bars ± SD (*, P < 0.01). B, Comparison of representative colony sizes. Scale bars = 50 μm. C, WST assay for cell survival of NBMM after 24-hour treatment with either AQ-101, doxorubicin, or nutlin-3a, as indicated. D, Western blot showing expression of proteins as indicated in NBMM cells after treatment with either 1 μmol/L doxorubicin or AQ-101, respectively. E, Representative histopathology of the heart, liver, and kidney of mice treated with 40 mg/kg/day AQ-101 or 10 mg/kg/day doxorubicin, following same schedule as in A. Scale bars = 50 μm.