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Degradation of MDM2 by the interaction between berberine and DAXX leads to potent apoptosis in MDM2-overexpressing cancer cells

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Abstract

Berberine, a natural product derived from a plant used in Chinese herbal medicine, is reported to exhibit anticancer effects; however, its mechanism of action is not clearly defined. Herein, we demonstrate that berberine induces apoptosis in acute lymphoblastic leukemia (ALL) cells by downregulating the MDM2 oncoprotein. The pro-apoptotic effects of berberine were closely associated with both the MDM2 expression levels and p53 status of a set of ALL cell lines. The most potent apoptosis was induced by berberine in ALL cells with both MDM2 overexpression and a wild-type (wt) p53, while no pro-apoptotic effect was detected in ALL cells that were negative for MDM2 and wt-p53. In contrast to the conventional chemotherapeutic drug doxorubicin, which induces p53 activation and a subsequent upregulation of MDM2, berberine strongly induced persistent downregulation of MDM2 followed by a steady-state activation of p53. We discovered that downregulation of MDM2 in ALL cells by berberine occurred at a post-translational level through modulation of DAXX, which disrupted the MDM2-DAXX-HAUSP interactions and thereby promoted MDM2 self-ubiquitination and degradation. Given that MDM2-overexpressing cancer cells are commonly chemoresistant, our findings suggest that this naturally-derived agent may have a highly useful role in the treatment of cancer patients with refractory disease.

Keywords

MDM2; DAXX; Berberine; Cancer; Apoptosis

The human MDM2 gene is an oncogene that is amplified in a variety of human cancers [1]. In addition, high levels of MDM2 expression are observed in those malignancies without MDM2 gene amplification, such as in leukemia [2]. MDM2 gained considerable attention following its identification as the protein that negatively regulates p53. The N-terminus of the MDM2 protein binds to p53, restraining p53-mediated transcription [3]; and the C-terminus of MDM2 acts as an E3 ubiquitin ligase, mediating the degradation of p53 [4]. Conversely, the MDM2 gene is a transactivation target of p53; thus, MDM2 and p53 form a

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negative feedback loop [5,6]. In normal growing or unstressed cells, MDM2 regulates the ubiquitination and degradation of p53, but p53 does not regulate expression of MDM2 [7]. However, in response to cellular stress and DNA damage, such as during cancer treatment, p53 is rapidly accumulated and activated, inducing increased expression of MDM2, as well as other p53 target genes such as p21 and PUMA, which induce cell-cycle arrest and apoptosis [7–9]. This p53-mediated increase in MDM2 expression will begin to degrade p53 and repress its function. These last actions are likely part of an important mechanism for the development of chemoresistance, in particular for hematopoietic malignancies and many pediatric cancers in which neoplastic cells have a wt-p53 phenotype and originally overexpressed MDM2.

The MDM2 is itself an unstable protein that is ubiquitinated in an autocatalytic manner [10]. Studies have demonstrated that the self and p53-target ubiquitination functions of MDM2 are regulated by the death domain-associated protein (DAXX) and the deubiquitinating enzyme, HAUSP. HAUSP regulates deubiquitination of both p53 and MDM2 [11], whereas DAXX is an apoptosis-regulating protein that induces cell death or survival, which is closely associated with MDM2 and p53 [12,13]. Recent studies demonstrated that MDM2, DAXX and HAUSP interact with each other to form a tertiary complex. This complex formation reduces self-ubiquitination of MDM2, maintaining MDM2 ligase activity towards p53 under non-stress conditions. During cellular stress and DNA damage such as treatment with the chemotherapeutic drugs doxorubicin and VP-16, DAXX-MDM2-HAUSP complex is disrupted, increasing MDM2 self-ubiquitination and degradation, which leads to the stabilization and accumulation of p53 (14, 15).

Berberine, an alkaloid that was initially isolated from Chinese herbs, is currently used as a traditional medicine that can treat bacterial diarrhea. There is evidence of the anticancer effect of berberine, as it inhibits cell growth and induces apoptosis in a variety of cancer cell lines originating from leukemia [16], epidermoid carcinoma [17], glioblastoma [18], prostate carcinoma [19] and breast cancer [20]. In addition, animal studies have shown that berberine can inhibit chemically-induced carcinogenesis, tumor promotion and tumor invasion [21–23]. Several recent studies have investigated the possible signaling pathways and the molecular mechanisms for the anticancer action of berberine, and they have found that NF- κ B [24] and p53 [25–27] are involved in berberine-mediated cell growth inhibition and apoptosis.

Because many chemotherapeutic drugs such as doxorubicin and VP-16 induce apoptosis in cancer cells through regulation of MDM2 and p53, we investigated whether the signaling pathway of apoptosis induced by berberine is similar or different to that induced by doxorubicin, as we aimed to investigate whether berberine could be used as a chemotherapeutic agent.

Materials and methods

Cell lines and reagents

Seven ALL cell lines used in this study were characterized in prior publications [28]. Four of these cell lines (EU-1, EU-4, EU-6 and EU-8) were established at Emory University. Sup-B13 and UOC-B1 was obtained from Dr. Stephen D. Smith (University of Kansas City) and the CCRC-CEM cell line was obtained from the American Type Culture Collection (ATCC). The berberine was purchased from Sigma-Aldrich (Cat. No: B3251). The detailed information for cell culture and treatment was described in the supplemental data online.

Immunoprecipitation and Western blot assay

Cells were lysed in a buffer composed of 50mM Tris, pH7.6, 150mM NaCl, 1%Nonidet P-40, 10mM sodium phosphate, 10mM NaF, 1mM sodium orthovanadate, 2mM phenylmethylsulfonyl fluoride, 10µg/ml aprotinin, 10µg/ml leupeptin and 10 µg/ml pepstatin. After centrifugation, the clarified cell lysate was incubated with 15µl Protein G plus/Protein A-agarose and 1µg of antibodies overnight at 4°C. Cell lysates or immunoprecipitates were resolved by SDS-PAGE, transferred to nitrocellulose filter and probed with specific antibodies. Protein expression was visualized with a chemiluminescent detection system (Pierce).

mRNA degradation rate and northern blot assay

The degradation rate of mRNA was examined by a standard actinomycin D and Northern blot assay. Briefly, at different times after addition of 5 mg/ml actinomycin D, cells were harvested and their total RNA isolated. RNA was electrophoresed using a 1% agarose/6% formaldehyde gel and transferred to a nylon filter. Probes were prepared by a randomized-labeling approach using α -³²P-dCTP. Following hybridization with probes and washing, the filter was autoradiographed for 24 hours.

Pulse-chase assay

The turnover of protein was tested by a standard protein-synthesis inhibitor cycloheximide (CHX) assay. Briefly, cells were treated with 50µg/ml CHX for different times before lysis in the presence or absence of berberine, and the expression of MDM2, p53 and DAXX was detected by Western blot analysis.

Quantitative RT-PCR

The total RNA was extracted from cells with the RNeasy Mini Kit (Qiagen). First-strand cDNA synthesis was performed with a mixture of random monomers and oligo-dT as primers. The amplification was performed with a 7500 Real-Time PCR System (Applied Biosystems), using the QuantiFast SYBR Green RT-PCR kit (Qiagen), according to the manufacturer's instructions. All specific primers for amplification of specific genes, as well as the house-keeper gene GAPDH, were purchased from Qiagen.

Polysome preparation and analysis

Cells were incubated with 100µg/ml CHX for 15 min to arrest polyribosome migration, and then lysed to isolate cytoplasmic extracts in a buffer containing 20mM Tris-HCl at pH 8.0, 100mM NaCl, 5mM MgCl₂, 0.5% Triton X-100, 500U/ml RNasin, and a cocktail of protease inhibitors, followed by fractionation on 15–45% (w/v) sucrose gradient. The gradient was centrifuged in a SW41Ti rotor at 39,000rpm for 1 hr. Fractions were collected from each gradient tube by up-ward replacement with monitored absorption at OD₂₅₄ by using a fractionator (Brandel, Inc.). The RNA in each fraction was extracted and subjected to quantitative PCR as described below.

Plasmids and transfection

The human MDM2 expression plasmid (pCMV-MDM2) was provided by Dr. B. Vogelstein of Johns Hopkins University. A Quick Change Site Directed Mutagenesis Kit (Stratagene) was used to mutate the MDM2 464 so that cysteine is substituted by alanine, to generate plasmid pCMV-MDM2 C464A, which exhibits loss of E3 ligase activity [29]. The MDM2 and DAXX gene promoters were generated by PCR and cloned into the pGL3 basic vector to produce the MDM2 promoter 1 and DAXX promoter-Luciferase constructs. The MDM2 p2-Luciferase plasmid was kindly provided by Dr. M. Oren (Weizmann Institute of Science,

Israel). Stable and transient gene transfection and reporter assay were described in the supplemental data online.

WST cytotoxicity assay

Cells were cultured for 20 hr in 96-well microtiter plates with different concentrations of reagents. Then, the water-soluble tetrazolium salt (WST) was added and the incubation continued for an additional 4 hr, after which the optical density (OD) of the wells was read with a microplate reader set at a test wavelength of 450 nm and a reference wavelength of 620 nm. Appropriate controls lacking cells were included, to determine background absorbance.

Flow cytometry

Flow cytometry was performed to analyze the cell cycle position and apoptosis. For cell-cycle analysis, cells were collected, rinsed twice with PBS, fixed in 70% ethanol for 1 h at 4°C, washed twice in PBS and re-suspended in 0.5ml PBS containing 20µg/ml of propidium iodide (PI) and 20µg/ml of RNase A. After incubating at 4°C for at least 30 min, the samples were analyzed using a FACScan (Becton Dickinson) with WinList software (Verity Software House, Inc).

For the quantitative detection of apoptotic cells by annexin-V staining, cells with or without treatment were washed once with PBS and stained with FITC-annexin-V and PI, according to the manufacturer's instructions. The stained cells were analyzed either by fluorescence microscopy or by flow cytometry.

Results

Berberine strongly induces cytotoxicity and apoptosis in ALL cells with wt-p53 and MDM2 overexpression

We examined the effect of berberine on the cell viability using the WST cytotoxic assay in 6 ALL lines (Fig. 1A). Berberine exhibited strongly cytotoxic activity in the two wt-p53/MDM2-overexpressing ALL cell lines EU-1 and Sup-B13 (Supplemental Table 1). Berberine showed relatively low cytotoxicity in the mutant-p53 cell lines and the p53-null line that expressed low levels of MDM2, while no cytotoxic effect of berberine was detected in the MDM2-negative line with a p53-null phenotype. To clarify whether the observed cell death induced by berberine was associated with induction of apoptosis, we stained the cells with an Annexin V FITC and viewed them by fluorescence microscopy. As seen in Fig. 1B (upper panel), over 90% of cells were Annexin V positive in berberine-treated wt-p53/MDM2-overexpressing EU-1 cells. As is also seen in this figure (bottom panel), the berberine-induced death of the mutant-p53/MDM2-expressing EU-6 cells was also due to apoptosis. Fig. 1C shows the quantities of apoptotic cells in the six ALL cell lines treated with different doses of berberine, detected by flow cytometry. Results were consistent with the WST results: berberine induced very strong apoptosis in MDM2-overexpressing/wt-p53 cells, but apoptosis was not strong in cells expressing no or low levels of MDM2. This was further confirmed by a western blot assay to evaluate the activation of caspase-3 and the cleavage of PARP during berberine treatment. As shown in Fig. 1D, cleavage of caspase-3 and PARP was detected at 6 hours after berberine treatment of MDM2-overexpressing/wt-p53 cells, whereas cleavage of these proteins was not tested until 24 hours after berberine treatment of MDM2-expressing/p53-mutant cells. No cleavage of caspase-3 and PARP was observed even at 24 hours after similar berberine treatment of MDM2-negative/p53-null cells.

Berberine-induced cell death in wt-p53 cells is mainly through induction of p53

Because berberine induces apoptosis in wt-p53 cells, we tested the effect of berberine on the activation of p53 and its known targets p21, PUMA and Bax. By quantitative RT-PCR and by Western blot assays, we found that berberine could induce the p53 protein, but not p53 mRNA expression in the wt-p53/MDM2-overexpressing cells (Fig. 2A, B and C). The expression of both mRNA and protein for p21 and PUMA was remarkably induced by berberine. Corresponding with the observed accumulation of p53 and induction of p21 and PUMA, there was a G1 cell-cycle arrest and cell death detected in wt-p53 Sup-B13 cells. As shown in Fig. 2D, there were rapid S and G2/M depletion and cell death (sub-G1 DNA content is indicated by an arrow in Fig. 2D), while the number of G1 cells was even increased. Berberine did not induce cell arrest in G1 phase in mutant (EU-6) and null p53 (EU-8) ALL cells with a similar treatment (Fig. 2D).

Berberine promotes p53 activity by downregulating MDM2

We investigated the molecular mechanism by which berberine activates p53, as compared with doxorubicin that induces p53 through DNA damage. Results showed that although berberine and doxorubicin increased expression of p53 in wt-p53 cells in a similar dose-dependent manner, they did not in the mutant-p53 cells (Fig. 3A). Interestingly, berberine downregulated MDM2 expression in all three ALL cell lines studied, regardless of their p53 status, suggesting that berberine-mediated MDM2 downregulation is p53-independent. In contrast, treatment with doxorubicin upregulated MDM2 expression in the wt-p53 cells and the expression of MDM2 remained unchanged in mutant-p53 cells, suggesting that doxorubicin-mediated MDM2 upregulation is p53-dependent. In a detailed kinetic study of the regulation of MDM2 and p53, we found that in berberine-treated cells, MDM2 downregulation occurred as early as 1 hour and persisted until 24 hours after treatment. However, doxorubicin treatment induced a rapid and transient (1–2 hours) reduction of MDM2, followed by a remarkable upregulation of this protein (Fig. 3B). As is also shown in Fig. 3B, berberine treatment induced p53 expression in 4 hours, whereas doxorubicin treatment took only an hour to significantly induce p53.

Berberine downregulates MDM2 at the post-translational level

We wished to investigate further how MDM2 is regulated by berberine. We first performed quantitative RT-PCR for the expression of MDM2 mRNA in berberine-treated cells. Unexpectedly, we found that berberine did not inhibit MDM2 mRNA expression; instead, the MDM2 mRNA level actually increased. Compared to MDM2 mRNA regulation in doxorubicin-treated EU-1 cells, where MDM2 mRNA levels increased as early as 1 hour after treatment, the increase in MDM2 mRNA in the same cells treated with berberine occurred at 4 hours post-treatment (Fig. 4A). This pattern of kinetic induction of MDM2 mRNA was consistent with p53 activation in both the doxorubicin and berberine-treated cells, because significant p53 activation was found to occur at 1 and 4 hours after being given each drug, respectively (Fig. 3B). These results suggest that induction of MDM2 mRNA by berberine and doxorubicin could be attributed to the observed activation of p53, as it is a transactivator of MDM2 [30]. Berberine did not directly regulate MDM2 transcription, which was confirmed by a reporter assay in a p53-null ALL cell line (Fig. S1). Northern blot results showed that the stability of MDM2 mRNA was not affected by berberine (Fig. 4B). Results from polysome profiling showed that berberine also did not regulate MDM2 translation, as no effect of berberine on the polyribosome profile of MDM2 was detected (Fig. 4C).

Furthermore, we measured the turnover of both MDM2 and p53 after berberine treatment by pulse-chase assay. The half-life of MDM2 in untreated cells was larger than 90 min, whereas berberine decreased that MDM2 half-life to < 30 min. In contrast, the half-life of

p53 in untreated cells was less than 30 min, while it was remarkably increased by berberine (Fig. 4D). These results suggested that the berberine-downregulated MDM2 and the berberine-upregulated p53 expression occur only at the post-translational level.

Berberine increases MDM2 self-ubiquitination by disrupting MDM2-DAXX-HAUSP interactions

We investigated whether berberine, like doxorubicin, induces a similar disruption of the MDM2-DAXX-HAUSP complex. Co-immunoprecipitation and western blot assay results showed that berberine abolished the interactions of MDM2-DAXX-HAUSP. As seen in Fig. 5A, the levels of MDM2, DAXX and HAUSP were significantly reduced after only 10 to 20 minutes of treatment, when the immunocomplex was precipitated with DAXX, HAUSP and MDM2 antibodies, suggesting that the three proteins were dissociated. Importantly, we also found that the disruption of MDM2-DAXX-HAUSP interactions resulted in enhanced MDM2 ubiquitination (Fig. 5B).

To further confirm that berberine induces self-ubiquitination of MDM2, we tested the effect of berberine on MDM2 expression and ubiquitination in EU-4 cells that were stably transfected with the mutant MDM2 plasmid pCMV-MDM2 C464A, providing an MDM2 that lacks ubiquitin E3 ligase activity due to the cysteine 464 that is mutated to alanine [29]. Results showed that the mutant MDM2 protein in EU-4 cells transfected with pCMV-MDM2 C464A was indeed resistant to berberine-mediated downregulation (Fig. 5C) and unable to self-ubiquitinate (Fig. 5D), suggesting that berberine downregulated the wt-MDM2 specifically through induction of self-ubiquitination.

Berberine modulates DAXX expression at the transcriptional level

Because berberine induced a similar disruption of the MDM2-DAXX-HAUSP complex as did doxorubicin, the question raised was why berberine induced a persistent degradation of MDM2, whereas doxorubicin only induced a transient downregulation of MDM2 that was followed by an increase in MDM2 expression. We hypothesized that the differential regulation of MDM2 in cells treated with berberine and doxorubicin may likely be associated with some distinct role they may play in regulating DAXX or HAUSP that could help explain the later events. Therefore, we compared the expression levels of DAXX and HAUSP in berberine and doxorubicin-treated cells. Consistent with previous studies [15], doxorubicin does not affect the expression of DAXX nor HAUSP (Fig. 6A, upper panel, right). Interestingly, in berberine-treated cells, the expression of HAUSP remained unchanged, but the drug downregulated the DAXX in a dose- and time-dependent manners (Fig. 6A, upper panel, left and lower panel). To evaluate the mechanism by which berberine regulates DAXX, we first tested the effect of berberine on DAXX transcription. Gene transfection and reporter analysis showed that the DAXX promoter activity was specifically inhibited by berberine (Fig. 6B). The expression of endogenous DAXX mRNA in the same cells was also inhibited by berberine as detected by RT-PCR (Fig. 6B, *insert*), suggesting that berberine suppress DAXX transcription. Northern blot results indicated that the stability of DAXX mRNA was not affected by berberine (Fig. 6C). In additional pulse-chase experiments, DAXX protein turnover was not affected by berberine (Fig. 6D), unlike the drug's effect on MDM2 and p53.

Discussion

In the present study, we sought to test the response of pediatric ALL cells to berberine-induced cytotoxicity and apoptosis, as well as evaluate its underlying mechanism of anti-cancer action. We demonstrated that the response of ALL cells to berberine was tightly associated with both MDM2 expression levels and p53 status. The ALL cell lines having

both MDM2 overexpression and a wt-p53 phenotype were found to be very sensitive to berberine, whereas cell lines lacking MDM2 expression and without wt-p53 did not respond to berberine. The distinct responses to berberine of ALL cells with different MDM2 expression levels and p53 status were attributed to changes in regulation of MDM2 and p53. We discovered that berberine specifically and strongly induced degradation of MDM2 by activating its own self-ubiquitination, which then resulted in the persistent activation of p53, so that potent apoptosis occurred in wt-p53/MDM2-overexpressing cells. Due to their lack of MDM2 and wt-p53, there was no response to berberine in ALL cells with MDM2/wt-p53 null phenotypes. In MDM2-expressing/p53-deficient ALL cells, a moderate apoptosis induced by berberine was most likely attributed to the p53-independent role of MDM2.

A significant finding of the present study is the identification of the unique role of berberine in inducing potent apoptosis in ALL cells that are known to be resistant to conventional chemotherapy. Doxorubicin, the most frequently used chemotherapeutic drug is known to cause DNA damage and kill cancer cells mainly by a p53-dependent mechanism of apoptosis [32,33]. However, resistance to doxorubicin is observed not only in cancer cells having p53 mutations, but also in wt-p53 cancer cells that express high level of the p53 inhibitor MDM2. We previously reported that primary leukemic cells derived from pediatric ALL patients that express wt-p53 and overexpress MDM2 are usually resistant to doxorubicin [34]. The two wt-p53/MDM2-overexpressing ALL cell lines EU-1 and Sup-B13 that were used in the present study are resistant to doxorubicin. However, these two cell lines were quite efficiently killed by berberine, making it a drug candidate of prime interest to investigate further.

Our in-depth mechanistic studies revealed the reason why doxorubicin-resistant wt-p53/MDM2-overexpressing ALL cells were sensitive to berberine. Different regulation of MDM2 in cells treated by doxorubicin and berberine was involved. Prior to this study, advanced studies of MDM2 and p53 interactions had demonstrated that the self and p53-target ubiquitination function of MDM2 is regulated by DAXX and HAUSP [14]. In unstressed cells, MDM2 interacts with DAXX and HAUSP and is stabilized in the MDM2-DAXX-HAUSP complex, which leads to persistent ubiquitination and degradation of the tumor suppressor p53. Under conditions of cellular stress and DNA damage, such when challenged with doxorubicin (Fig. S2A), MDM2 becomes dissociated from the MDM2-DAXX-HAUSP complex resulting in instability, as MDM2 self-ubiquitination and degradation occur. Due to the release of MDM2-mediated ubiquitination, p53 becomes immediately activated, which, in turn, induces MDM2 production, because MDM2 is a transactivation target of p53. In the presence of intact DAXX and HAUSP, the newly synthesized MDM2 could associate with DAXX and HAUSP again, leading to stabilization of MDM2 and new ubiquitination and degradation of p53. It is possible that this is the mechanism for the development of doxorubicin resistance in MDM2-overexpressing cancer cells, as p53 degradation would deny p53-dependent apoptosis.

The MDM2-DAXX-HAUSP interactions were also rapidly disrupted in berberine-treated cells, so a similar reduction of MDM2 as was seen in doxorubicin-treated cells appeared to occur (Fig. S2B). Differences from doxorubicin treatment; however, followed: In the berberine-treated cells, a steady-state downregulation of MDM2 occurred within 1 h and MDM2 became barely detectable by 8–16 h post-treatment, which was the point at which p53 was maximally accumulated. Although new MDM2 was synthesized by the activated p53, as seen by an increase in MDM2 mRNA levels in berberine-treated cells (Fig. 4A), MDM2 protein was rapidly self-ubiquitinated and degraded. The persistent self-ubiquitination of MDM2 in the berberine-treated cells was found to be due to the lack of formation of a new MDM2-DAXX-HAUSP complex. In further studies, we demonstrated that berberine uniquely downregulated the expression of DAXX in a dose- and time-

dependent manner, and because of that DAXX absence in berberine-treated cells, no new formation of the MDM2-DAXX-HAUSP complex was possible, so the p53-induced newly-synthesized MDM2 constitutively underwent self-ubiquitination and degradation. The result of berberine treatment, therefore, was a steady-state activation of p53 and constant potent apoptosis activity if cells were originally of the wt-p53/MDM2-overexpressing phenotype.

Although further studies are required to investigate the mechanism by which berberine inhibits DAXX transcription, our studies so far have demonstrated a critical role for DAXX in promoting berberine-induced cell death. Previous studies have shown that loss of DAXX results in extensive apoptosis in early development [35], and silencing of DAXX expression by RNAi in cancer cells ends up sensitizing them to multiple apoptosis pathways [36,37]. Our own observations in berberine-treated ALL cell lines were consistent with that findings. We also tested to confirm the effect of silencing of DAXX by siRNA on regulation of MDM2 and p53 as well as apoptosis in wt-p53/MDM2-overexpressing cells. Similar to berberine, DAXX siRNA inhibited MDM2 and induced p53 (Fig. S3A), which resulted in cell death (Fig. S3B). Interestingly, DAXX siRNA strongly sensitized wt-p53/MDM2-overexpressing ALL to doxorubicin (Fig. S3C). The fact that berberine can suppress DAXX means the molecule may prove to be of great use in treating resistant cases. The pre-clinical and clinical studies would provide the ultimate answer to the question of berberine's utility.

Because MDM2 is an important inhibitor of p53, and because overexpression of MDM2 occurs in a variety of cancers including leukemia, many efforts have been made so far to target MDM2 or MDM2-mediated signaling, in order to prevent the apparent promotion of cancer and improve the power of anticancer treatment. Direct silencing of MDM2 expression by antisense or siRNA, and blocking of the binding between MDM2 and p53 using small molecule inhibitors such as nutlin-3 and MI-219 are being actively investigated [38–41]. However, targeting MDM2 by increasing MDM2 self-ubiquitination in cancer cells has not been investigated. We believe that discovery of drugs to target MDM2 by disrupting the MDM2-DAXX-HAUSP interaction is an innovative concept in the development of therapies for cancer patients whose neoplastic cells overexpress MDM2. Berberine is one such drug. In the present study, we demonstrated that chemotherapy-resistant MDM2-overexpressing cancer cells are very sensitive to berberine, which suggests that berberine should be developed as a particularly useful agent for the treatment of high risk, refractory cancer patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Oliner JD, Kinzler KW, Meltzer PS, George DL, Vogelstein B. Amplification of a gene encoding a p53-associated protein in human sarcomas. *Nature* 1992;358:80–8. [PubMed: 1614537]
2. Bueso-Ramos CE, Yang Y, deLeon E, McCown P, Stass SA, Albitar M. The human MDM-2 oncogene is overexpressed in leukemias. *Blood* 1993;82:2617–23. [PubMed: 8219216]
3. Momand J, Zambetti GP, Olson D, George D, Levine AJ. The MDM-2 oncogene product forms a complex with the p53 protein and inhibits p53-mediated transactivation. *Cell* 1992;69:1237–45. [PubMed: 1535557]

4. Haupt Y, Maya R, Kazaz A, Oren M. Mdm2 promotes the rapid degradation of p53. *Nature* 1997;387:296–9. [PubMed: 9153395]
5. Juven T, Barak Y, Zauberman A, George DL, Oren M. Wild type p53 can mediate sequence-specific transactivation of an internal promoter within the mdm2 gene. *Oncogene* 1993;8:3411–6. [PubMed: 8247544]
6. Prives C. Signaling to p53: breaking the MDM2-p53 circuit. *Cell* 1998;95:5–8. [PubMed: 9778240]
7. Mendrysa SM, Perry ME. The p53 tumor suppressor protein does not regulate expression of its own inhibitor, MDM2, except under conditions of stress. *Mol Cell Biol* 2000;20:2023–30. [PubMed: 10688649]
8. el-Deiry WS, Tokino T, Velculescu VE, Levy DB, Parsons R, Trent JM, Lin D, Mercer WE, Kinzler KW, Vogelstein B. WAF1, a potential mediator of p53 tumor suppression. *Cell* 1993;75:817–25. [PubMed: 8242752]
9. Nakano K, Vousden KH. PUMA, a novel proapoptotic gene, is induced by p53. *Mol Cell* 2001;7:683–94. [PubMed: 11463392]
10. Fang S, Jensen JP, Ludwig RL, Vousden KH, Weissman AM. MDM-2 is a RING finger-dependent ubiquitin protein ligase for itself and p53. *J Biol Chem* 2000;275:8945–51. [PubMed: 10722742]
11. Li M, Brooks CL, Kon N, Gu W. A dynamic role of HAUSP in the p53-Mdm2 pathway. *Mol Cell* 2004;13:879–86. [PubMed: 15053880]
12. Zhao LY, Liu J, Sidhu GS, Niu Y, Liu Y, Wang R, Liao D. Negative regulation of p53 functions by Daxx and the involvement of MDM2. *J Biol Chem* 2004;279:50566–79. [PubMed: 15364927]
13. Gostissa M, Morelli M, Mantovani F, Guida E, Piazza S, Collavin L, Brancolini C, Schneider C, Del Sal G. The transcriptional repressor hDaxx potentiates p53-dependent apoptosis. *J Biol Chem* 2004;279:48013–23. [PubMed: 15339933]
14. Tang J, Qu LK, Zhang J, Wang W, Michaelson JS, Degenhardt YY, El-Deiry WS, Yang X. Critical role for Daxx in regulating MDM-2. *Nat Cell Biol* 2006;8:855–62. [PubMed: 16845383]
15. Song MS, Song SJ, Kim SY, Oh HJ, Lim DS. The tumour suppressor RASSF1A promotes MDM-2 self-ubiquitination by disrupting the MDM2-DAXX-HAUSP complex. *EMBO J* 2008;27:1863–74. [PubMed: 18566590]
16. Yu FS, Yang JS, Lin HJ, Yu CS, Tan TW, Lin YT, Lin CC, Lu HF, Chung JG. Berberine inhibits WEHI-3 leukemia cells in vivo. *In Vivo* 2007;21:407–12. [PubMed: 17436595]
17. Mantena SK, Sharma SD, Katiyar SK. Berberine inhibits growth, induces G1 arrest and apoptosis in human epidermoid carcinoma A431 cells by regulating Cdk1-Cdk-cyclin cascade, disruption of mitochondrial membrane potential and cleavage of caspase 3 and PARP. *Carcinogenesis* 2006;27:2018–27. [PubMed: 16621886]
18. Eom KS, Hong JM, Youn MJ, So HS, Park R, Kim JM, Kim TY. Berberine induces G1 arrest and apoptosis in human glioblastoma T98G cells through mitochondrial/caspases pathway. *Biol Pharm Bull* 2008;31:558–62. [PubMed: 18379040]
19. Mantena SK, Sharma SD, Katiyar SK. Berberine, a natural product, induces G1-phase cell cycle arrest and caspase-3-dependent apoptosis in human prostate carcinoma cells. *Mol Cancer Ther* 2006;5:296–308. [PubMed: 16505103]
20. Kim JB, Lee KM, Ko E, Han W, Lee JE, Shin I, Bae JY, Kim S, Noh DY. Berberine inhibits growth of the breast cancer cell lines MCF-7 and MDA-MB-231. *Planta Med* 2008;74:39–42. [PubMed: 18203057]
21. Anis KV, Rajeshkumar NV, Kuttan R. Inhibition of chemical carcinogenesis by berberine in rats and mice. *J Pharm Pharmacol* 2001;53:763–8. [PubMed: 11370717]
22. Nishino H, Kitagawa K, Fujiki H, Iwashima A. Berberine sulfate inhibits tumor-promoting activity of teleocidin in two-stage carcinogenesis on mouse skin. *Oncology* 1986;43:131–4. [PubMed: 3081844]
23. Peng PL, Hsieh YS, Wang CJ, Hsu JL, Chou FP. Inhibitory effect of berberine on the invasion of human lung cancer cells via decreased productions of urokinase-plasminogen activator and matrix metalloproteinase-2. *Toxicol Appl Pharmacol* 2006;214:8–15. [PubMed: 16387334]
24. Pandey MK, Sung B, Kunnumakkara AB, Sethi G, Chaturvedi MM, Aggarwal BB. Berberine modifies cysteine 179 of IkappaBalpha kinase, suppresses nuclear factor-kappaB-regulated

- antiapoptotic gene products, and potentiates apoptosis. *Cancer Res* 2008;68:5370–9. [PubMed: 18593939]
25. Choi MS, Yuk DY, Oh JH, Jung HY, Han SB, Moon DC, Hong JT. Berberine inhibits human neuroblastoma cell growth through induction of p53-dependent apoptosis. *Anticancer Res* 2008;28:3777–84. [PubMed: 19189664]
 26. Choi MS, Oh JH, Kim SM, Jung HY, Yoo HS, Lee YM, Moon DC, Han SB, Hong JT. Berberine inhibits p53-dependent cell growth through induction of apoptosis of prostate cancer cells. *Int J Oncol* 2009;34:1221–30. [PubMed: 19360335]
 27. Katiyar SK, Meeran SM, Katiyar N, Akhtar S. P53 cooperates berberine-induced growth inhibition and apoptosis of non-small cell human lung cancer cells in vitro and tumor xenograft growth in vivo. *Mol Carcinog* 2009;48:24–37. [PubMed: 18459128]
 28. Zhou M, Yeager AM, Smith SD, Findley HW. Overexpression of the MDM2 gene by childhood acute lymphoblastic leukemia cells expressing the wild-type p53 gene. *Blood* 1995;85:1608–14. [PubMed: 7888679]
 29. Wawrzynow B, Zyllicz A, Wallace M, Hupp T, Zyllicz M. MDM-2 chaperones the p53 tumor suppressor. *J Biol Chem* 2007;282:32603–12. [PubMed: 17848574]
 30. Barak Y, Juven T, Haffner R, Oren M. MDM-2 expression is induced by wild type p53 activity. *EMBO J* 1993;12:461–8. [PubMed: 8440237]
 31. Zhou M, Gu L, Findley HW, Jiang R, Woods WG. PTEN reverses MDM2-mediated chemotherapy resistance by interacting with p53 in acute lymphoblastic leukemia cells. *Cancer Res* 2003;63:6357–62. [PubMed: 14559824]
 32. Lowe SW, Bodis S, McClatchey A, Remington L, Ruley HE, Fisher DE, Housman DE, Jacks T. P53 status and the efficacy of cancer therapy in vivo. *Science* 1994;266:807–10. [PubMed: 7973635]
 33. Lorenzo E, Ruiz-Ruiz C, Quesada AJ, Hernández G, Rodríguez A, López-Rivas A, Redondo JM. Doxorubicin induces apoptosis and CD95 gene expression in human primary endothelial cells through a p53-dependent mechanism. *J Biol Chem* 2002;277:10883–92. [PubMed: 11779855]
 34. Zhou M, Gu L, Abshire T, Homans A, Yeager AM, Findley HW. Incidence and prognostic significance of MDM-2 oncoprotein overexpression in relapsed childhood acute lymphoblastic leukemia. *Leukemia* 2000;14:61–7. [PubMed: 10637478]
 35. Michaelson JS, Bader D, Kuo F, Kozak C, Leder P. Loss of Daxx, a promiscuously interacting protein, results in extensive apoptosis in early mouse development. *Genes Dev* 1999;13:1918–23. [PubMed: 10444590]
 36. Michaelson JS, Leder P. RNAi reveals anti-apoptotic and transcriptionally repressive activities of DAXX. *J Cell Sci* 2003;116:345–52. [PubMed: 12482920]
 37. Chen LY, Chen JD. Daxx silencing sensitizes cells to multiple apoptotic pathways. *Mol Cell Biol* 2003;23:7108–21. [PubMed: 14517282]
 38. Zhang Z, Li M, Wang H, Agrawal S, Zhang R. Antisense therapy targeting MDM2 oncogene in prostate cancer: Effects on proliferation, apoptosis, multiple gene expression, and chemotherapy. *Proc Natl Acad Sci U S A* 2003;100:11636–41. [PubMed: 13130078]
 39. Guo W, Ahmed KM, Hui Y, Guo G, Li JJ. siRNA-mediated MDM2 inhibition sensitizes human lung cancer A549 cells to radiation. *Int J Oncol* 2007;30:1447–52. [PubMed: 17487365]
 40. Vassilev LT, Vu BT, Graves B, Carvajal D, Podlaski F, Filipovic Z, Kong N, Kammlott U, Lukacs C, Klein C, Fotouhi N, Liu EA. In vivo activation of the p53 pathway by small-molecule antagonists of MDM2. *Science* 2004;303:844–8. [PubMed: 14704432]
 41. Shangary S, Qin D, McEachern D, Liu M, Miller RS, Qiu S, Nikolovska-Coleska Z, Ding K, Wang G, Chen J, Bernard D, Zhang J, Lu Y, Gu Q, Shah RB, Pienta KJ, Ling X, Kang S, Guo M, Sun Y, Yang D, Wang S. Temporal activation of p53 by a specific MDM2 inhibitor is selectively toxic to tumors and leads to complete tumor growth inhibition. *Proc Natl Acad Sci U S A* 2008;105:3933–8. [PubMed: 18316739]

Fig. 1.

Response to berberine-induced apoptosis of ALL cell lines with different levels of MDM2 expression. **A**, the levels of MDM2 protein expression in normal peripheral blood lymphocytes (NPBL) and 6 cultured ALL cell lines with different p53 status were determined by western blot. **B**, representative pictures showing apoptotic cells induced by berberine. Cells were similarly treated with 100 μ M berberine for 24 hr and apoptosis was detected by an Annexin V FITC Kit. The apoptotic cells (Annexin V+, green) and died cells (PI+, red) were examined by fluorescence microscopy. **C**, dose-dependent apoptosis induced by berberine in ALL cell lines. Cells were treated with different concentrations of berberine for 24 hr; apoptotic cells were assessed quantitatively by flow cytometry. **D**, western blot results showing activation of caspase-3 and cleavage of the death substrate PARP in Sup-B13, CCRF-CEM and EU-8 cells treated with 50 μ M berberine for the indicated times.

Fig. 2.

Effect of berberine on induction of p53 and its target genes, as well as resulting cell-cycle arrest in wt-p53/MDM2-overexpressing ALL cells. **A** and **B**, mRNA expression of p53, p21, Bax and PUMA in the cell lines EU-1 (**A**) and Sup-B13 (**B**). Cells were treated with 10 and 50 μ M berberine for 5 h; mRNA levels were determined by quantitative RT-PCR. Data represent the fold induction due to treatment, compared to no treatment. **C**, western blot assay for protein expression of p53, p21, Bax and PUMA in EU-1 and Sup-B13, in response to 50 μ M berberine, for the indicated time points. Numerical labels under each protein band represent expression levels after normalization for GAPDH, compared with untreated (0) samples (defined as 1 unit). **D**, cell-cycle analysis in Sup-B13 (wt-p53), EU-6 (mutant-p53) and EU-8 (p53-null), performed 8 h post-treatment with 50 μ M berberine.

Fig. 3. Comparison of MDM2 regulation as well as p53 induction in berberine and doxorubicin-treated ALL cells with different p53 status. **A**, cells were treated with different doses of berberine and doxorubicin as indicated for 8 hours, and then protein expression of MDM2, p53 were detected by western blot assay. **B**, Sup-B13 cells were treated with 50 μ M berberine and 0.5 μ M doxorubicin, respectively, for different times as indicated; protein expression of MDM2, p53 and Bcl-2 was examined by western blot analysis.

Fig. 4.

Regulation of MDM2 by berberine. **A**, EU-1 cells were treated with 50 μ M berberine or with 0.5 μ M doxorubicin, for different times. The mRNA levels of MDM2 relative to an internal control GAPDH were determined by quantitative RT-PCR. **B**, EU-1 cells were treated with or without berberine for 4 hours, followed by addition of 5mg/ml actinomycin D. At different time points, the cells were harvested and total RNA was isolated. The amount of MDM2 mRNA remaining was determined by Northern blotting and quantified by densitometric analysis. **C**, EU-1 cells were treated with or without 50 μ M berberine for 8 h, and cytoplasmic lysates were fractionated on sucrose gradient. RNA was extracted from each of the fractions and subjected to quantitative RT-PCR for quantitative analysis of the distribution of MDM2 and Actin mRNAs. Data represent percentage of the total amount of corresponding mRNA on each fraction. **D**, turnover of MDM2 and p53 proteins in berberine-treated and untreated cells, as detected by pulse-chase assay. EU-1 cells were treated with or without 50 μ M berberine for 4 hours, followed by addition of cyclohexamide (CHX). At selected times indicated after CHX, cell lysates were prepared and analyzed by western blot assay. The western blot film for p53 was developed at 1 hour (dev 1h) and 2 hours (dev 2h), respectively.

Fig. 5. Effects of berberine on disruption of MDM2-DAXX-HAUSP interactions and self-ubiquitination of MDM2. **A** and **B**, EU-1 cells were treated with 20μM MG-132 for 4 hours and subsequently with 50μM berberine, for the indicated times. MDM2-DAXX-HAUSP interactions (**A**) and MDM2 ubiquitination (**B**) were analyzed by immunoprecipitation (IP)-western blot assays. **C**, a similar berberine treatment and western blot assay, using the MDM2-negative ALL cell line EU-4 that was transfected either with wild-type (wt)-MDM2 or a mutant (mut)-MDM2 C464A. **D**, IP-western blot assay to detect effects of berberine on the ubiquitination of mut-MDM2 C464A, which was expressed following stable transfection of the mutant plasmid into EU-4 cells.

Fig. 6. Modulation of DAXX by berberine. **A**, EU-1 cells were treated with different doses of berberine and doxorubicin, respectively, for 4 hours and then DAXX and HAUSP protein levels were detected by western blot (upper panel). EU-1 cells were treated with 50μM berberine for different times; western blot was performed to detect levels of DAXX and HAUSP (lower panel). **B**, EU-4 cells were transfected with 5μg DAXX promoter luciferase plasmid by electroporation. At 24 hr after transfection, cells were treated with increasing concentrations of berberine (10, 50, and 100μM) or increasing dose of doxorubicin (0.1, 0.5 and 1μM) for another 4 hr, and then cell extracts were prepared for testing of luciferase activity. (*Insert*): cells were treated with 50μM berberine for different times and the expression levels of endogenous DAXX mRNA were detected by RT-PCR. **C**, DAXX mRNA degradation rate in berberine-treated EU-1 cells was detected by northern blot assay as described in Fig. 4B. **D**, turnover of DAXX protein in berberine-treated and untreated EU-1 cells, as detected by pulse-chase assay methods described in Fig. 4D.