Caspase-dependent cell death mediates potent cytotoxicity of sulfide derivatives of 9-anilinoacridine

Sang-ki Park, Hyerim Kang and Chul-Hoon Kwon

9-Anilinoacridine contains a tricyclic and planar aromatic structure that enables DNA intercalation and inhibition of topoisomerase II. Two recently developed sulfide derivatives of 9-anilinoacridines, 2-({4-[4-(acridin-9-ylamino)phenylthio]phenyl}(2-hydroxyethyl)amino)ethan-1-ol (CK0402) and 3-({4-[4-(acridin-9-ylamino)phenylthio]phenyl}(3-hydroxypropyl)amino)propan-1-ol (CK0403), displayed potent cytotoxic activity in multiple cancer cell lines. In-vitro enzymatic assay demonstrated that CK0402 and CK0403 directly inhibit decatenation reaction of topoisomerase IIα. Cells exposed to CK0403 showed DNA fragmentation, and activation of caspase-3 and caspase-2, indicating that it triggers caspase-dependent apoptosis. This was further supported by the fact that cytotoxicity of these drugs is attenuated by pharmacological inhibition of caspases with z-VAD-FMK. Studies with wild-type and $p53^{-/-}$ primary mouse embryonic fibroblasts demonstrated that p53 does not play a significant role in cell death process initiated by this kind of drug. In addition, pharmacological inhibition of poly(ADP-ribose) polymerase-1activity moderately enhanced cytotoxic activity of sulfide 9-anilinoacridine, suggesting that poly(ADP-ribose) polymerase-1 may have a protective function against 9-anilinoacridine-induced cell death process. *Anti-Cancer Drugs* 19:381–389 © 2008 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Introduction

Uncontrolled proliferation of transformed cells is a fundamental feature of carcinogenesis and hence commonly targeted by anticancer drugs. Various aspects of DNA metabolism, ranging from replication to chromosome segregation and condensation to maintenance of genomic stability, pose topological challenges for any rapidly dividing cells and requires DNA topoisomerase II (Topo II) that catalyzes DNA double-strand breaks (DSBs) and ligation [1]. Topo II has served as a target of several different classes of chemotherapeutic agents [2]. 4′-(9-Acridinylamino)methanesulfon-*m*-anisidide (amsacrine or *m*-AMSA) is currently used for the treatment of acute adult myelogenous leukemia. In numerous studies, *m*-AMSA showed desirable results against hematological cancers, such as nonlymphocytic leukemia, but it was considered relatively ineffective in solid tumors [3,4]. Potent anticancer activity of *m*-AMSA has been attributed to its ability to interact with DNA, which is facilitated by DNA intercalation of the tricyclic acridine chromophore between the neighboring bases in the duplex DNA, and additional interaction between the aniline ring of drug and DNA minor groove [5,6]. It is widely accepted that this type of physical interaction between drug and DNA leads to the inhibition of Topo II [7,8]. One of the immediate consequences of Topo II inhibition by *m*-AMSA and other related drugs is generation of DNA strand breaks, which eventually results in cell demise [9,10]. Owing to these reasons, anilinoacridine has been subjected to various structural modifications on both the acridine backbone and the aniline ring, in an attempt to develop better chemotherapeutic agents [2,6]. Unexpectedly, these new drugs and other Topo II inhibitors helped reveal novel cellular molecules that mediate antineoplastic action of this class of drugs. For instance, triazoloacridone (C-1305), a highly effective Topo II inhibitor, kills poly(ADP-ribose) polymerase-1 (PARP-1) deficient cells more efficiently than wild type cells [11], whereas doxorubicin produces opposite results [12]. On the other hand, methyl-*N*-[4-(9-acridinylamino)-2-methoxyphenyl] carbamate hydrochloride, exhibits a better cytotoxic profile toward nondividing cells, independent of poly(ADP-ribosyl)lation and S-phase [13]. Moreover, it was proposed that DNA intercalation of acridine-based drugs produces oxidative stress by directly participating in electron transfer reactions that donate electrons to DNA molecules independent of Topo [14,15]. The apparent incongruence among the mechanisms proposed to explain the drug actions argues that cytotoxicity of acridine or acridinone-related drugs does not necessarily hinge solely on Topo II inhibition, but rather uses multiple cellular molecules. We recently reported the synthesis of a series of sulfur-containing 9-anilinoacridine derivatives related to *m*-AMSA [16]. Among several structurally related compounds, two diol-containing 9-anilinoacridine analogs,

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2-({4-[4-(acridin-9-ylamino)phenylthio]phenyl}(2-hydroxyethyl)amino)ethan-1-ol (CK0402) and 3-({4-[4-(acridin-9-ylamino)phenylthio]phenyl}(3-hydroxypropyl)amino)propan-1-ol (CK0403), showed the most potent cytotoxic activity in Chinese hamster lung transformed V-79 cells [16]. Anticancer activity of CK0402 was also demonstrated *in vivo* in the study, in which it significantly increased the lifespan of the cancer-bearing animals [16]. In this study, we expanded the cytotoxic profile of CK0402 and CK0403 to multiple cancer cell lines and explored their cytotoxic mechanisms.

Materials and methods

Chemicals

m-AMSA and wortmanin were purchased from Sigma Chemical Co. (St Louis, Missouri, USA). Staurosporine and NU1025 were obtained from EMD Biosciences, Inc. (San Diego, California, USA). Ac-DEVD-CHO and z-VAD-FMK were obtained from Axxora, LLC (San Diego, California, USA). All compounds were dissolved in dimethylsulfoxide (DMSO) to make $\times 1000$ stock solutions and stored at -20°C until use.

Cell culture

HepG2 (ATCC HB8065), T98G (ATCC CRL-1690), and *ras*-transformed fibroblasts were cultured in DMEM (Invitrogen, Carlsbad, California, USA). MCF-7 (ATCC HTB-22) cells were cultured in MEM (Invitrogen). Both types of media were supplemented with 10% fetal bovine serum, 100 units/ml of penicillin G, 100 µg/ml streptomycin and all cells were cultured in a 37°C humidified incubator under an atmosphere of 5% CO_2. Wild-type and $p53^{-/-}$ mouse embryo fibroblasts (MEFs) were prepared from C57Bl/6 and $p53^{-/-}$ mice (kind gifts from Dr Randy Johnson, University of California, San Diego, California, USA).

Clonogenic assay

In-vitro clonogenic assay was carried out with HepG2 and MCF-7 cells as previously [16]. Five hundred cells per well were seeded in six-well plates. Following treatment for 48 h with a series of concentrations of drugs, the cells were provided with fresh media and allowed to grow for 4–6 days in the incubator. The cells were stained with crystal violet, and the colonies with more than 10 cells were counted with a stereomicroscope.

Cell proliferation assay

Cells were grown in tissue culture-treated, white-walled 96-well plates and treated with increasing doses of drugs for 48 h before cell viability assay with the Cell-Titer Glo assay kit according to a manufacturer's protocol (Promega, Madison, Wisconsin, USA). Emission of luminescence was detected with a microplate luminescence reader (GENios Plus, Tecan, Durham, North Carolina, USA) with an integration time of 0.5 s. To determine the effects of *m*-AMSA and CK0403 on T98G cell growth, equal number of T98G cells were seeded in 60-mm dishes and cultured for up to 72 h in the presence of 0.1% DMSO, 50 µmol/l *m*-AMSA, or 10 µmol/l CK0403. Following treatment, viable cells were stained with Trypan blue and counted with a hemacytometer.

DNA fragmentation assay

Fragmented DNA of apoptotic cells was isolated according to the procedure of Wang *et al.* [17] with the following modification. Cells (2×10^6) treated with tested drugs in 60-mm dish were harvested and washed with phosphate-buffered saline. The pelleted cells were resuspended with 100 µl cell lysis buffer (50 mmol/l Tris–HCl, pH 8.0, 0.01 mol/l EDTA·Na, and 0.5% SDS), and treated with 0.1 mg/ml RNase A for 30 min and then with 0.5 mg/ml proteinase K for 60 min at 50°C. DNA fragments were collected by phenol–chloroform–isoamyl alcohol extraction and analyzed by 1.5% agarose gel electrophoresis.

Caspase-3 and caspase-2 assay

Enzymatic activity of caspase-3 and caspace-2 were determined with Caspase-3/CPP32 and Caspase-2 Colorimetric Assay Kit (BioVision, Mountain View, California, USA) according to the manufacturer's suggested protocol. Briefly, the cells were grown in a 10-cm dish and treated with drugs indicated for 8 h. Following cell lysis with the buffer provided by the manufacturer, an equal amount of protein was incubated at 37°C for 1 h with either DEVD-pNA (for caspase-3) or Ac-VDVAD-pNA (for caspase-2) (BioVision Inc., Mountain View, California, USA) in a 96-well plate. At the end of incubation, absorbance changes at 405 nm were measured by using a microtiter plate reader (GENios Plus, Tecan, Durham, North Carolina, USA), and used to determine fold increase in enzymatic activity over the vehicle-treated control.

Topoisomerase II assay

Inhibition of Topo II catalytic activity by 9-anilinoacridines was determined by measuring decatenation reaction of kinetoplastid DNA (kDNA) with purified human Topo IIα (TopoGEN) according to the manufacturer's protocol. In brief, 200 ng of kDNA was incubated with 2 units of Topo IIα in a reaction buffer provided by the manufacturer at 30°C for 20 min. The reaction products were electrophoresed in a 1% agarose gel containing 0.5 µg/ml of ethidium bromide and visualized by ultraviolet transluminator.

Western blot analysis

Cell extracts were prepared with cell extraction buffer (BioVision) and equal amount of protein (40 µg) was loaded onto 4–12% Tris-Bis Acrylamide gel (Invitrogen). Transfer of separated proteins to the membrane and western blotting were carried out as described [18]. The membrane with the transferred proteins was cut in between 60 kDa, and the size of the protein bands were estimated based on the locations of the protein size

markers coloaded on the same gel (BenchMark Prestained Protein Ladder, Invitrogen).

Results

CK0402 and CK0403 inhibit topoisomerase II enzymatic activity

CK0402 and CK0403 are distinguished from *m*-AMSA by the presence of 4′-diol substituted phenyl sulfide at the *para* position of the aniline ring instead of methanesulfonamide [16] (Fig. 1a). The only difference between CK0402 and CK0403 is the length of the carbon chain of 4′-diol (two carbons for CK0402 and three carbons for CK0403) (Fig. 1a). The presence of the tricyclic acridine chromophore and aniline ring of this kind of drug is thought to confer their DNA intercalation and subsequent inhibition of Topo II [7,8]. In-vitro enzymatic assay with purified human Topo IIα confirmed that CK0402 and CK0403 directly inhibit the decatenation reaction of kDNA by Topo IIα (Fig. 1a). CK0403 is the most effective among three 9-anilinoacridine derivatives tested in inhibiting Topo IIα, about 10 times more potent than CK0402 and *m*-AMSA. The ability of CK0402 to inhibit Topo IIα-catalyzed decatenation reaction was similar to that of *m*-AMSA (Fig. 1b).

CK0402 and CK0403 strongly inhibit cell survival and growth

The ability of CK0402 and CK0403 to inhibit survival of HepG2 and MCF-7 cells was determined by clonogenic assay (Table 1). Overall, CK0402 and CK0403 were at least 10 times more potent than *m*-AMSA in inhibiting colony formation of both types of human cancer cell lines (Table 1). CK0403 was slightly more effective than CK0402 in inhibiting clonal expansion of HepG2 cells, whereas CK0402 and CK0403 were equally potent in MCF-7 cells (Table 1).

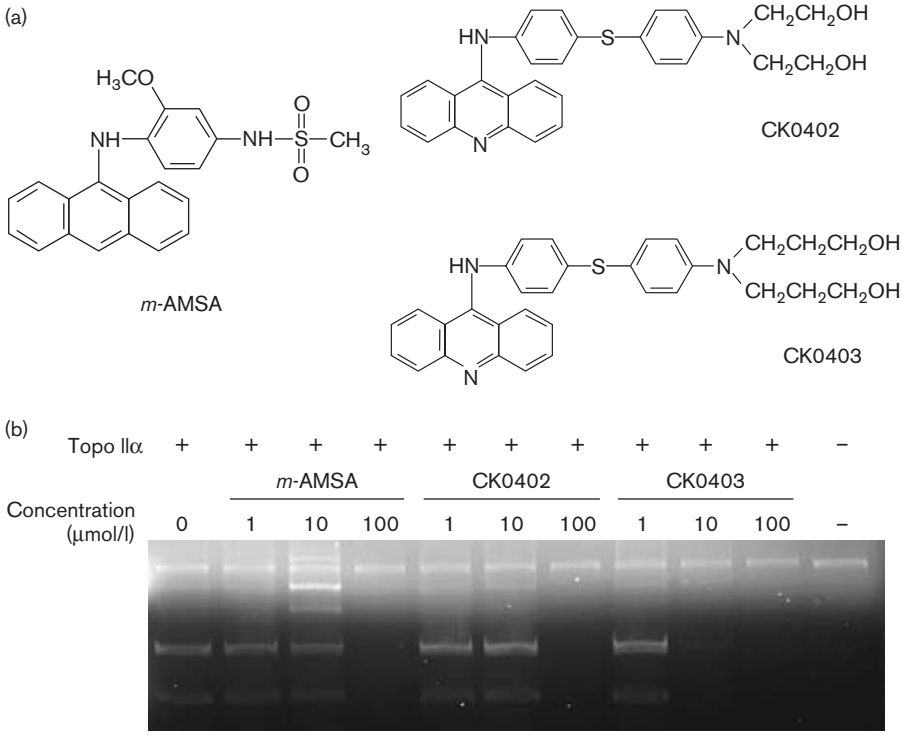
We also tested the effects of these two drugs on proliferation of two additional cancer cell lines, transformed mouse fibroblasts and human glioblastoma

Table 1 **Inhibition of clonogenic survival by 9-anilinoacridines**

IC_{50} (μmol/l)	HepG2	MCF-7
m-AMSA	1.65 ± 0.04	1.73 ± 0.03
CK0402	0.12 ± 0.01	0.18 ± 0.03
CK0403	0.03 ± 0.02	0.14 ± 0.01

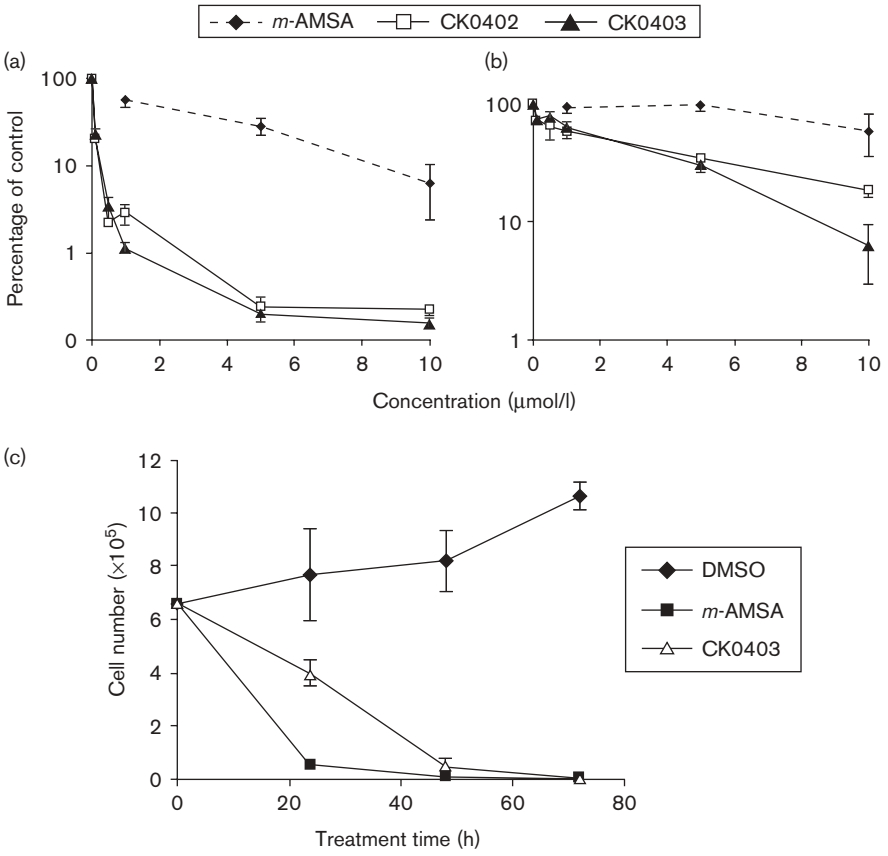
In-vitro colony formation assay was carried out as described in Materials and methods with HepG2 and MCF-7 cells. IC_{50} for each drug is shown below. Each datum represents the mean ± standard error of triplicate determinations.
m-AMSA, 4′-(9-acridinylamino)methanesulfon-*m*-anisidide.

Fig. 1



(a) Structures of 9-anilinoacridine analogs. 4′-(9-acridinylamino)methanesulfon-*m*-anisidide(*m*-AMSA also known as amsacrine): *m*-AMSA, CK0402: 2-(4-[4-(acridin-9-ylamino)phenylthio]phenyl(2-hydroxyethyl)amino)ethan-1-ol, and CK0403: 3-(4-[4-(acridin-9-ylamino)phenylthio]phenyl(3-hydroxypropyl)amino)propan-1-ol (b) Inhibition of topoisomerase II (Topo II) catalytic activity by sulfide-containing 9-anilinoacridines. Decatenation activity of Topo II was assayed with kinetoplastid DNA and purified human Topo IIα with 1, 10, and 100 μmol/l of *m*-AMSA, CK0402, and CK0403.

Fig. 2



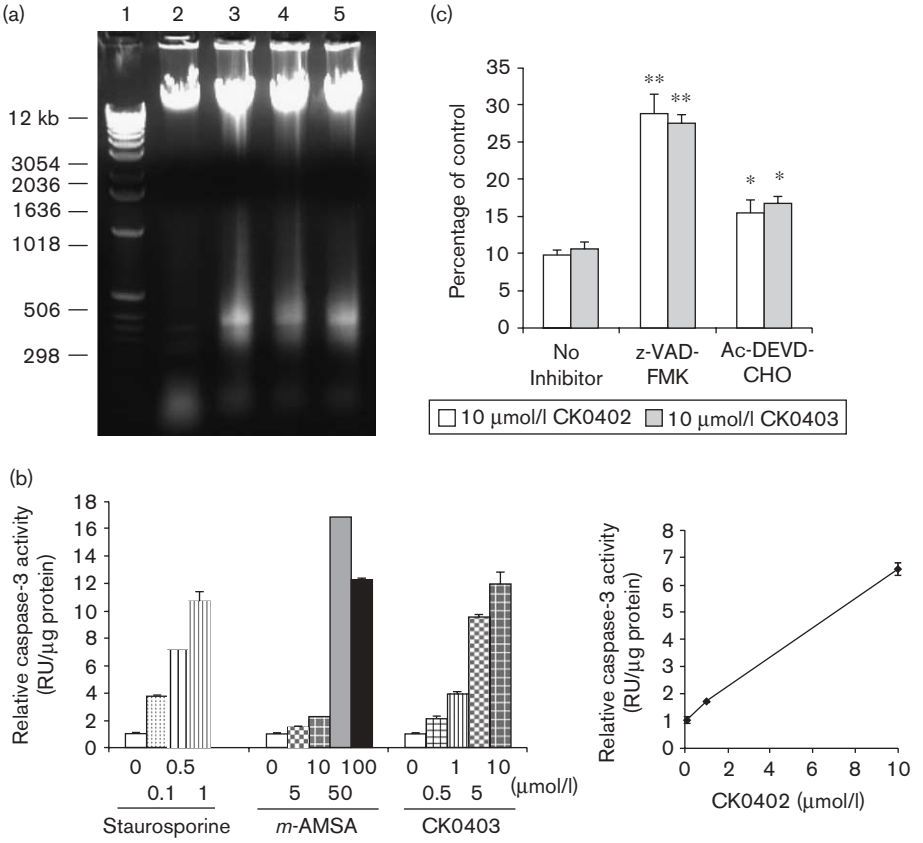
(a and b) Dose-dependent cell proliferation inhibition by CK0402 and CK0403. Transformed fibroblasts (a) and T98G (b) cells were treated in white-walled 96-well plates with increasing doses of drugs for 48 h before cell viability assay with Cell-Titer Glo assay kit (Promega). (c) Kinetics of cytotoxic effects of 4′-(9-acridinylamino)methanesulfon-*m*-anisidide (*m*-AMSA) and CK0403 in T98G cells. Equal number of T98G cells were seeded in 60-mm dishes and cultured for the indicated times on the x-axis in the presence of 0.1% dimethylsulfoxide, 50 μmol/l *m*-AMSA, or 10 μmol/l CK0403. Following treatment, viable cells were stained with Trypan blue and counted with a hemacytometer. Each datum point represents the mean ± standard deviation of triplicate determinations.

(T98G) cell lines. Fibroblasts used in this study were derived from MEFs, which were, first, immortalized with Simian vacuolating virus-40 large T antigen and then transformed with *H-ras* [19]. This cell line showed extreme sensitivity to antiproliferative effects of CK0402 and CK0403 (Fig. 2a). In this cell line, the concentrations of drugs needed to inhibit cell proliferation by 50% (IC_{50}) were 2.54, 0.08, and 0.03 μmol/l for *m*-AMSA, CK0402, and CK0403, respectively. Although much less effective than in fibroblasts, CK0402 and CK0403 were two- to three-fold more potent inhibitors of cell proliferation of T98G (IC_{90}s for *m*-AMSA, CK0402, and CK0403 in T98G cells were 8.6, 2.9, and 2.3 μmol/l, respectively, Fig. 2b). Results in Figs 1 and 2 also suggest that despite potent inhibition of cell survival and growth, there is no strong association between cytotoxicity of CK0402 and CK0403 and their ability to inhibit Topo IIα. Next, we examined the effects of *m*-AMSA and CK0403 on cell growth rates. Exponentially growing cells were treated with 0.1% DMSO, 50 μmol/l *m*-AMSA, or 10 μmol/l CK0403 for up to 72 h. Regardless of the drugs tested, the total number of viable cells was significantly decreased after 48 h of treatment below the level of 0 h (about 6×10^5 cells), indicating effective cell killing by these drugs (Fig. 2c). Maximum cell loss in *m*-AMSA-treated cells was observed within 24 h, whereas it needed an additional 24 h in CK0403-treated cells (Fig. 2c). These data suggest that the cell death process initiated by CK0403 is executed at a slower pace than the *m*-AMSA-induced cell death process.

Caspase-dependent apoptotic cell death of CK0403-treated cells

Decline in the cell number following treatment with *m*-AMSA or other Topo I and II inhibitors is known to mainly result from apoptosis, which can be supported by the presence of internucleosomal DNA fragmentation [10,20]. Similarly, low-molecular-weight fragmented

Fig. 3



CK0403 induces caspase-dependent apoptotic cell death. (a) DNA fragmentation of drug-treated cells. HepG2 cells were treated with 0.1% dimethylsulfoxide (lane 2), 1 μmol/l staurosporine (lane 3), 50 μmol/l 4′-(9-acridinylamino)methanesulfon-*m*-anisidide (*m*-AMSA) (lane 4), or 1 μmol/l CK0403 (lane 5) for 8 h. Following treatment, genomic DNA was extracted from the cell lysates and analyzed on 1.5% agarose gel. Lanes 1: 1-kb DNA ladder (Invitrogen). (b) Dose-dependent activation of caspase-3 by sulfide derivatives of 9-anilinoacridine. Transformed fibroblasts were treated with various concentrations of staurosporine (a positive control for caspase-3 activation), *m*-AMSA, CK0403, or CK0402 for 8 h before cell lysis. Equal amount of protein from each treatment group was assayed with colorimetric casapse-3 substrate. Each datum point represents the mean ± standard deviation of triplicate determinations. (c) Inhibition of CK0402 and CK0403-induced cell death by caspase inhibition. Transformed fibroblasts were treated with 10 μmol/l CK0402 or 10 μmol/l CK0403 for 24 h in the presence or absence of 100 μmol/l z-VAD-FMK (a pan-caspase inhibitor) or 100 μmol/l Ac-DEVD-CHO (a caspase-3 inhibitor) before cell viability assay with the Cell-Titer Glo assay kit (Promega). Each datum point represents the mean ± standard deviation of triplicate determinations. *Significantly different from the no inhibitor counterpart with $P<0.05$. **Significantly different from the no inhibitor counterpart for each drug with $P<0.005$.

DNA was detected in cells treated with staurosporine and *m*-AMSA (Fig. 3a, lanes 3 and 4, respectively), as well as in cells treated with CK0403, suggesting that apoptotic cell death may underlie CK0403 cytotoxicity (Fig. 3a, lane 5). Next, we determined the activation status of an effector caspase that is known to mediate intrinsic apoptosis pathways induced by many cytotoxic agents in cells treated with CK0403. Treatment of transformed fibroblasts with CK0403 significantly increased the activity of caspase-3 in a dose-dependent manner (Fig. 3b). Relative potency of the drug to induce caspase-3 strongly correlated with its cytotoxicity; CK0403 was 10 times more efficient in stimulating caspase-3 activity than *m*-AMSA (Fig. 3b). Dose-dependent activation of caspase-3 was also observed in CK0402-treated cells (right panel, Fig. 3b). To further verify the involvement of caspase-dependent apoptosis in the CK0403-induced cell killing process, cell viability was determined following treatment with CK0403 for 24 h in the presence or absence of two different caspase inhibitors, z-VAD-FMK (a pan-caspase inhibitor) and Ac-DEVD-CHO (caspase-3 inhibitor). As expected, 10 μmol/l CK0403 alone decreased the number of viable cells to 10% of DMSO-treated control within 24 h (Fig. 3c, closed bar above no inhibitor). Cotreatment of cells with 100 μmol/l z-VAD-FMK, however, significantly attenuated the cell killing effect of CK0403 (Fig. 3c). Selective inhibition of caspase-3 with Ac-DEVD-CHO also reduced CK0403 cytotoxicity, although it was not as effective as z-VAD-FMK (Fig. 3c). Similar results were obtained with

cells simultaneously treated with CK0402 and caspase inhibitors (Fig. 3c). Taken together, these data indicate that CK0403 induces cell death via a caspase-dependent manner.

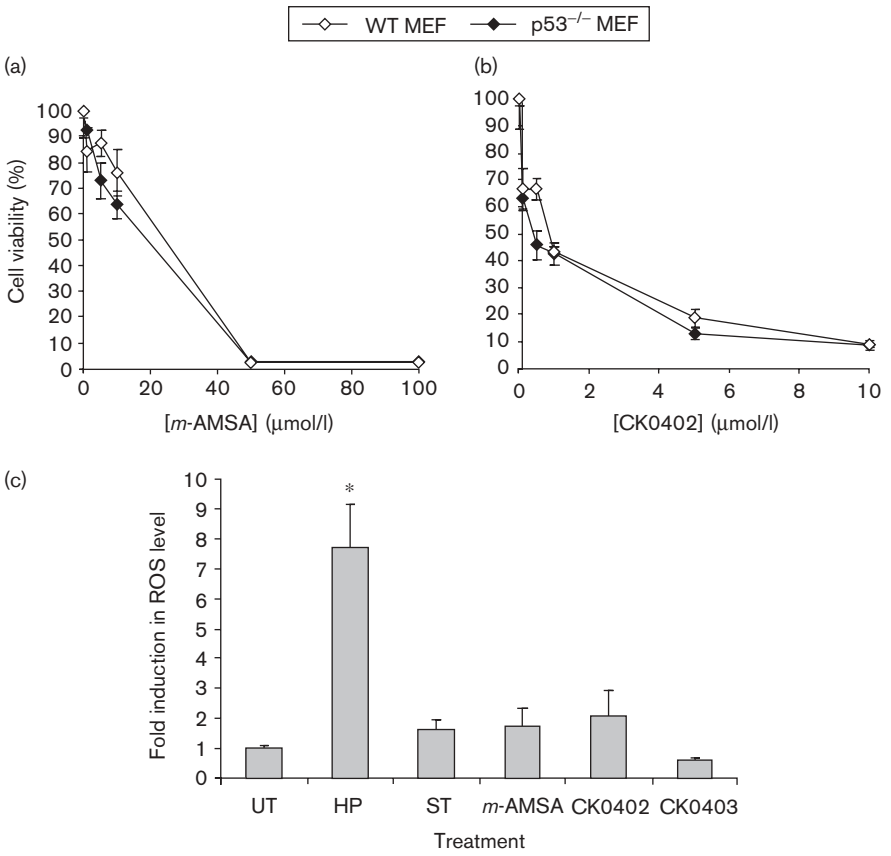
Cellular sensitivity to CK0402 is independent of p53 and reactive oxygen species generation

T98G cell line, which shows at least 50 times more resistance to CK0402 and CK0403 than transformed fibroblasts, is deficient of wild-type p53 [21]. This led to a question whether p53 determines cellular sensitivity to this kind of drug. To address this issue, we compared cytotoxicity of CK0402 in $p53^{-/-}$ primary MEFs with that in the wild-type counterpart. Both *m*-AMSA and CK0402 showed comparable cytotoxicity in wild-type and $p53^{-/-}$ fibroblasts, suggesting that p53 does not play a major role either in promotion of or protection against CK0402-induced cell death (Fig. 4a and b, respectively). Oxidation of *m*-AMSA to the quinine diimine was experimentally demonstrated, and believed to be accountable for the increase in reactive oxygen species (ROS) and Topo-independent cytotoxic action of the drug [14,15]. To test whether oxidative stress underlies CK0402 cytotoxicity, the intracellular ROS level was monitored in cells immediately following treatment with *m*-AMSA and CK0402. No significant change, however, was observed in the ROS level of cells treated either CK0402 or *m*-AMSA, implicating that oxidative stress is not the major cause of cell death induced by these drugs (Fig. 4c). Similar results were obtained with cells treated with CK0403 (Fig. 4c).

CK0402 and CK0403 strongly activate caspase-2

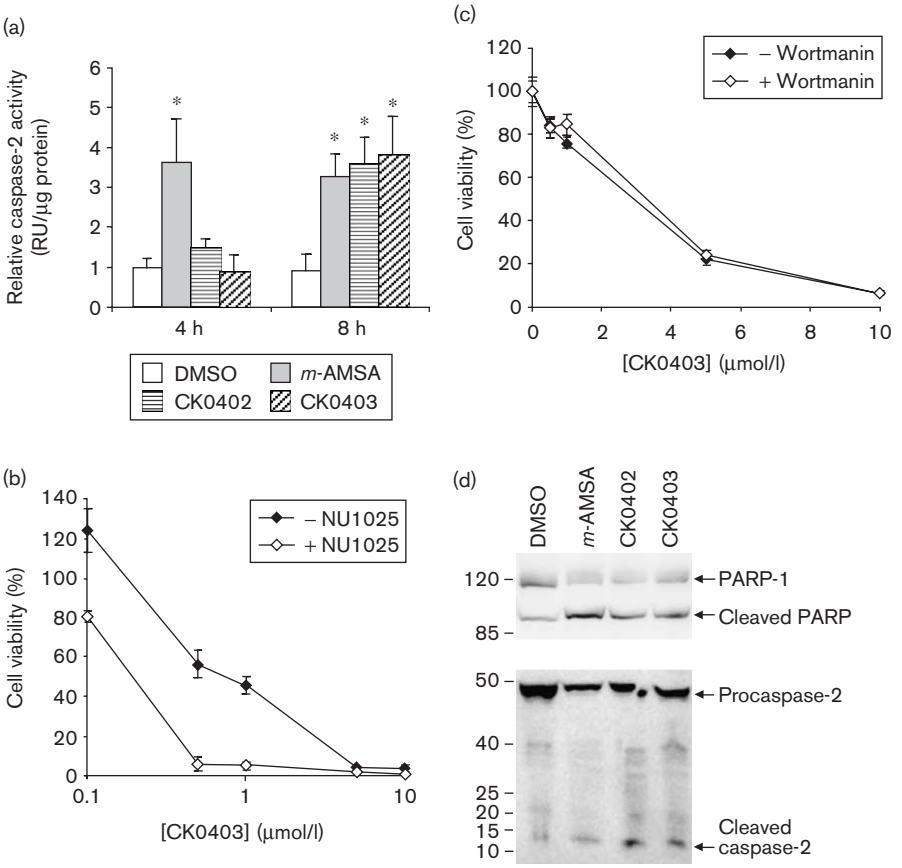
To gain further understanding of cell death mediators in CK0402 and CK0403 exposed cells, we checked the activation status of other caspases. Caspase-2 is regarded as one of the initiating caspases, and known to become

Fig. 4



(a and b) Inhibition of cell proliferation by 4′-(9-acridinylamino)methanesulfon-*m*-anisidide (*m*-AMSA) and CK0402 is independent of p53. Both wild-type (WT) and $p53^{-/-}$ primary mouse embryo fibroblasts were treated with various concentrations of *m*-AMSA (a) and CK0402 (b) for cell viability assay. (c) Effect of CK0402 and CK0403 treatments on cellular reactive oxygen species (ROS) level. Fibroblasts were pre-loaded with 2′,7′-dichlorodihydrofluorescein diacetate and then treated with either 1 μmol/l staurosporine (ST), 50 μmol/l *m*-AMSA, 5 μmol/l CK0402, or 5 μmol/l CK0403. 1 μmol/l H_2O_2 (HP) was used as a positive control for increased ROS generation in the cells. Each datum point represents the mean ± standard deviation of triplicate determinations. *Significantly different from the dimethylsulfoxide-treated control with $P < 0.05$.

Fig. 5



(a) Induction of caspase-2 enzymatic activity by 9-anilinoacridines. Cell lysates were prepared from transformed fibroblasts treated with 0.1% dimethylsulfoxide (DMSO), 50 μmol/l 4′-(9-acridinylamino)methanesulfon-*m*-anisidide (*m*-AMSA), 5 μmol/l for CK0402, or 5 μmol/l CK0403 for 4 and 8 h. Equal amount of protein (40 μg) was incubated with colorimetric casapse-2 substrate, Ac-VDVAD-pNA. Each datum point represents the mean ± standard deviation of triplicate determinations. *Significantly different from the DMSO-treated control with $P < 0.05$. (b) Inhibition of poly(ADP-ribose) polymerase-1 (PARP-1) enhances cell proliferation inhibition by CK0403. Transformed fibroblasts were treated with increasing concentrations of CK0403 in the presence or absence of 100 μmol/l NU1025 for 48 h. Following treatment, cell viability was measured with the Cell-Titer Glo assay kit (Promega). (c) Effect of wortmanin cotreatment on sensitivity to CK0403. Each datum point represents the mean ± standard deviation of triplicate determinations. (d) Western blot analysis of PARP-1 and caspase-2. Cell extracts were prepared from fibroblasts treated with DMSO (lane 1), *m*-AMSA (lane 2), CK0402 (lane 3), and CK0403 (lane 4) for 8 h. Equal amount of protein was separated by SDS–PAGE, and transferred to the membrane, which was probed with anti-PARP-1 and anti-caspase-2 antibodies.

activated in response to DNA damage [22]. To determine whether sulfide derivatives of 9-anilinoacridines stimulate protease activity of caspase-2, protein extracts were prepared from cells treated with 50 μmol/l *m*-AMSA, 5 μmol/l CK0402, or 5 μmol/l CK0403. Enzymatic caspase-2 assay of these exacts showed that all three chemicals significantly increase caspase-2 activity (Fig. 5a). Interestingly, CK0402 and CK0403 showed the delayed onset of caspase-2 activation compared to *m*-AMSA. In *m*-ASMA-treated cells, elevation of caspase-2 activity was observed at 4 h, whereas that was not evident until 8 h in CK0402 and CK0403-treated cells (Fig. 5a). Activation of caspase-2 by CK0402 and CK0403 was further substantiated by western blot analysis with anti-caspase 2 antibody that shows an increased level of cleaved caspase-2 in cells treated with these drugs for 8 h (Fig. 5d).

NU1025 sensitizes cells to CK0402 and CK0403

Another factor that has been repeatedly implicated in mediating cytotoxic activity of Topo inhibitors is PARP-1 [11,23]. NU1025 [8–hydroxy-2-methyl-quinazolin-4-(3H) one] is a potent and highly specific PARP-1 inhibitor that displays selective potentiation of cytotoxicity of certain types of DNA-damaging drugs [23]. To determine whether PARP-1 plays any role in cell killing effects of the sulfide derivatives of 9-anilinoacridines, transformed MEFs were treated with various concentrations of CK0403 in the presence or absence of 100 μmol/l NU1025. Cell viability assay showed that cells exposed to NU1025 are much more sensitive to cytotoxic effect of CK0403 (Fig. 5b). NU1025 alone was not toxic to this cell line (cell viability of untreated vs. 100 μmol/l NU1025-treated; 100.0 ± 10.0 vs. 123.4 ± 10.5%, $P < 0.005$). This suggests that PARP-1 may

provide cells with protection against cell killing effects of CK0403. One suggested mechanism for the NU1025-mediated potentiation of cytotoxic activity of certain DNA-damaging agents is its inhibitory effect on DNA repair, which was further supported by similar results obtained by inhibition of DNA-protein kinase with wortmanin [24]. In contrast, cotreatment with wortmanin failed to enhance CK0403 cytotoxicity (Fig. 5c). This implicates that DNA damage response and repair *per se* may not be important for cellular resistance against the cell death process induced by CK0403. In agreement with pharmacological inhibition study, the level of the activated PARP-1 protein was elevated in cells treated with *m*-AMSA, CK0402, or CK0403, as demonstrated by western blot analysis for cleaved PARP-1 product (Fig. 5d).

Discussion

CK0402 and CK0403 are newly developed antineoplastic agents that are at least 10 times more effective than *m*-ASMA in inhibiting clonogenic survival of HepG2 and MCF-7 cell lines (Table 1) and proliferation of transformed fibroblasts (Fig. 2a). One of the cell lines tested, T98G, showed relative resistance to CK0402 and CK0403-induced cell death. Initially, it was suspected that lack of functional p53 might be responsible for the resistance, as its involvement has been implicated in cytotoxicity of various Topo inhibitors. For instance, p53 expression status differentially affects cellular sensitivity to different Topo II inhibitors in HCT-116 colon carcinoma cells [25]. Studies with various acridine derivatives including *m*-AMSA have showed that acridine structure is responsible for their ability to induce cell death, which correlates with the activation of p53 and subsequent induction of Bax [26]. Despite their extreme sensitivity to CK0402, however, fibroblasts do not necessitate p53 for induction of cell death upon exposure to these drugs (Fig. 4a). This apparent disagreement may represent one of the examples of cell-type-specific drug sensitivity to 9-anilinoacridine analogs.

Both direct enzymatic assay and western blot analysis for cleavage products of procaspase-2 clearly showed that caspase-2 is activated in CK0402 and CK0403-treated cells (Fig. 5d). The fact that the timeline of *m*-AMSA and CK0403 to induce caspase-2 activity nicely correlates with their cytotoxic kinetics strongly suggests that caspase-2 is involved in the 9-anilinoacridine-induced cell death process and is probably responsible for activation of caspase-3. It should be noted that the potency of 9-anilinoacridine drugs to block the Topo IIα catalytic reaction is poorly associated with their overall efficacy to inhibit cell proliferation (Fig. 1b). This suggests that the antineoplastic target of this kind of drug is not limited to Topo IIα. It has been shown that the PARP inhibitor exerts differential effects on the cytotoxicity of Topo I and II inhibitors [11,23]. One of the important functions of PARP related to cell protection upon DNA damage is induction of repair machinery for DNA single-strand breaks and DSBs [24]. Owing to the same reason, wortmanin, a kinase inhibitor that broadly inhibits phosphatidylinositol 3-kinase members, potentiates cytotoxicity associated with DSBs by inhibiting DNA-protein kinase and DSB repair [27]. Our studies, however, showed that only NU1025 was able to enhance cellular sensitivity to 9-anilinoacridine analogs (Fig. 5b and c). This implies that DNA damage repair response may not be a primary function of PARP-1 in protecting cells from the 9-anilinoacridine-induced cell death process. It was recently shown that the double-strand breaking enzymatic activity of Topo IIβ, in collaboration with PARP-1, is required for the estrogen-responsive promoter to shed repression complex and recruit activation complex, and to initiate estrogen receptor-mediated gene expression [28]. This raises a possibility that cell-type dependence of CK0402 and CK0403 cell death effects might involve inhibition of the collaborative function of Topo IIβ with PARP-1, specifically relating to regulation of cell-type-specific gene expression. Further studies are needed to test this hypothesis and determine whether CK0402 and CK0403 activate additional cell death signaling, such as the caspase-independent cell death pathway.

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