Involvement of the aldo–keto reductase, AKR1B10, in mitomycin-c resistance through reactive oxygen species-dependent mechanisms

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The human aldo–keto reductase (AKR) 1B10 is suggested as a tumor marker in various solid tumors. Using colon cancer cells, we found that AKR1B10 was induced with acquisition of resistance to the anticancer drug mitomycin-c (MMC). In the resistant cells, treatment with an AKR1B10 inhibitor decreased their MMC tolerance. In the nonresistant cells, overexpression and silencing of AKR1B10 decreased and increased, respectively, susceptibility to cytotoxic effects of MMC and 4-hydroxy-2-nonenal, which was formed as a product of lipid peroxidation by MMC treatment. These results suggest a role of AKR1B10 in the development of MMC resistance, which may be mediated by its ability to detoxify cytotoxic aldehydes including 4-hydroxy-2-nonenal. *Anti-Cancer Drugs* **22:402–408 © 2011 Wolters Kluwer Health | Lippincott Williams & Wilkins.**

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Introduction

Mitomycin-c (MMC) is one of the most widely used chemotherapeutic drugs for patients with advanced and metastatic cancers of the stomach and colon [1]. A major mechanism underlying the anticancer effect of the agent is the intercalation of its hydroquinone metabolite to DNA, which inhibits the replication and repair of DNA in tumor cells [2,3]. The active hydroquinone metabolite is the product of a two-electron reduction reaction by reductases such as nicotinamide adenine dinucleotide phosphate [NAD(P)H]:quinone oxidoreductase and NADPH-cytochrome *c* reductase. In addition, the generation of reactive oxygen species (ROS) is also believed to partially participate in the MMC-induced cell toxicity [4]. ROS are generated under aerobic conditions through the so-called 'redox cycle of MMC', which is composed of one-electron reduction of MMC by enzymes such as NADPH-cytochrome *c* reductases and subsequent oxidation [5]. The ROS-dependent apoptotic signaling including mitochondrial dysfunction and caspase activation is shown to be involved in the toxic events induced by MMC [5,6]. Despite the profound anticancer effects of MMC, its clinical usage, similar to that of other chemotherapeutic agents, is limited because of the development of drug resistance by tumors [7]. Various studies have suggested that MMC resistance is mediated by increased drug efflux, the repair of DNA lesions, or a deficiency in the bioreductive enzymes such as NAD(P)H:quinone oxidoreductases [8,9]. In addition, other MMC-metabolizing enzymes have been reported to exist in elevated levels and to be involved in the drug resistance [10–12], suggesting other mechanisms underlying the drug resistance.

Recently, aldo–keto reductase (AKR) 1C1, AKR1C2, and AKR1C3 belonging to the AKR superfamily have been shown to be overexpressed and linked to resistance against anticancer drugs such as anthracyclines, cisplatin, and methotrexate [13–15]. The enzymes are hydroxysteroid dehydrogenases with a broad substrate specificity for other carbonyl compounds including 4-hydroxy-2-nonenal (HNE), a major product of lipid peroxidation through oxidative stress, but the mechanism(s) by which the enzymes confer drug resistance remain unknown. In addition, AKR1B10, a human aldose reductase (AKR1B1)-like protein, was found to be overexpressed in the medulloblastoma cell lines resistant to cyclophosphamide [16]. AKR1B10 is also upregulated in many types of solid tumors [17–21], and its gene silencing results in growth inhibition of colorectal cancer cells [22] and suppression of tumor growth *in vivo* [21]. AKR1B10 reduces a variety of lipidic aldehydes including all-*trans*-retinal [23], acrolein [22], and HNE [24], and is thereby thought to regulate retinoic acid homeostasis [23] and lipid metabolism [25], being closely associated with tumor development. Thus, AKR1B10 has been recognized to a potential molecular target for preventing cancer proliferation and as a diagnostic tumor marker. In this paper, we describe that, among the AKRs, AKR1B10 is significantly overexpressed in colon cancer HT29 cells

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as they were forced to survive in increasing concentrations of MMC. We also show a novel role of AKR1B10 in drug resistance by studying the effects of transient overexpression and knockdown of the enzyme in colon cancer cells on the toxicity of HNE that was generated during the exposure to MMC. Furthermore, we show a reversal of MMC resistance in the drug-resistant cells by a potent AKR1B10 inhibitor, and suggest the usefulness of AKR1B10 inhibitors in restoring sensitivity to MMC in patients with cancer.

Materials and methods

Materials

MMC was kindly supplied by Kyowa Hakko Chemical (Tokyo, Japan). TRIzol reagent, Superscript III reverse transcriptase, oligo $(dT)_{12–18}$ primer, and Lipofectamine 2000 were obtained from Invitrogen (Carlsbad, California, USA); acetyl Asp-Glu-Val-Asp *p*-nitroanilide was from Sigma-Aldrich (St Louis, Missouri, USA). HNE was synthesized as described by Esterbauer and Weger [26]. All other chemicals were of the highest grade that could be obtained commercially.

Cell culture and transfection

Two human colon cancer cells (HT29 and HCT15) were from the American Type Culture Collection (Manassas, Virginia, USA). These cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 μg/ml) at 37°C in a humidified incubator containing 5% CO_2.

To obtain a subpopulation of HT29 cells resistant to MMC, the parental cells were continuously treated in the growth medium supplemented with MMC, the concentration of which was increased in a stepwise manner. At each MMC concentration of 0.01, 0.02, 0.05, 0.1, 0.2, and 0.5 μmol/l, the cells were passaged three times.

The resistance to MMC toxicity was estimated by monitoring the cell viability, which was evaluated by a tetrazolium dye-based cytotoxicity assay using 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium monosodium salt [27]. The value of 50% lethal dose (LD_{50}) for MMC was calculated from the viabilities of cells treated for 24 h with increasing concentrations of the agent.

The transfection of the pGW1 expression vector harboring the cDNA for AKR1B10 was carried out according to the method described earlier [28]. For the AKR1B10 silencing, the small interfering RNAs (siRNAs) [22] were transfected into the culture cells using Lipofectamine 2000 when the cells were grown to 60% confluence in microplates or dishes. After a 48-h culture, the cells were washed twice with Dulbecco's modified Eagle's medium containing 2% fetal bovine serum and antibiotics, and then subjected to treatment with agents.

Reverse transcription polymerase chain reaction

Total RNA was isolated from cells using the TRIzol reagent, and single-stranded complementary DNAs (cDNAs) were prepared from the total RNA samples by incubation for 50 min at 42°C with Superscript III reverse transcriptase and oligo $(dT)_{12–18}$ primer. The cDNAs for human AKRs (1B1, 1B10, 1C1, 1C2, and 1C3) were amplified from the single-stranded cDNA sample (5 μg) by polymerase chain reaction (PCR) using gene-specific primers as described earlier [28–30]. The PCR products were analyzed by agarose gel electrophoresis and stained with ethidium bromide. The cDNA for human β-actin was also amplified as an internal control with specific primers (Toyobo, Osaka, Japan).

Western blot and dot blot analyses

The cells were washed twice with Dulbecco's phosphate-buffered saline (DPBS), suspended in DPBS containing 0.5% Triton × 100 and 0.3 mmol/l phenylmethanesulfonyl fluoride, and subjected to sonication. The cell extract was isolated by centrifuging at 12 000 × *g* for 15 min. Protein concentration was determined with a Pierce bicinchoninic acid protein assay reagent. For western blotting, the proteins in the extracts of the cells were electrophoretically separated on a 12% SDS polyacrylamide gel under reducing conditions, and then transferred to a Millipore polyvinylidene fluoride membrane by electroblotting. To detect the HNE bound to proteins, the cell extracts (100 μg) were bound to the polyvinylidene fluoride membrane using a Sanplatec dot blot instrument (Sanplatec, Osaka, Japan). After blocking with 0.5% bovine serum albumin, the membrane was allowed to react with primary monoclonal antibodies against HNE (Alexis, San Diego, California, USA) and AKR1B10 [31]. The immunoreactive proteins were visualized using a peroxidase-conjugated secondary antibody and an enhanced chemiluminescence substrate system (GE Healthcare, Little Chalfont, UK). The densities of the bands and dots were estimated using Bio-Rad GelDoc 2000 (Bio-Rad Laboratories, Segrate, Italy) and the attached program, Quantity One.

Assay of enzyme activity

The reductase activities toward pyridine-3-aldehyde (P3A) and HNE of the cell extracts were determined by monitoring the oxidation rate of NADPH at 340 nm. The cells were washed twice with ice-cold DPBS, suspended in 10 mmol/l tris–HCl, pH 7.4, containing 5 mmol/l 2-mercaptoethanol and 20% glycerol, and homogenized by passing the cell suspension through a 26-gauge needle (20 strokes). The homogenate was centrifuged at 12 000 × *g* for 15 min, and the supernatant was subjected to the assay of enzyme activity. The reaction mixture consisted of 0.1 mol/l potassium phosphate, pH 7.4, 0.1 mmol/l NADPH, substrate (0.2 mmol/l P3A or 10 μmol/l HNE), and the cell extract in a total volume of 2.0 ml. One unit (U) of enzyme activity was defined as the amount of enzyme that catalyzes the oxidation of 1 μmol NADPH per minute at 25°C.

The activities of caspase 3 in the cell extracts were measured using acetyl Asp-Glu-Val-Asp *p*-nitroanilide as the substrate [32]. The cell extracts were prepared as mentioned above, except that the cells were suspended in 50 mmol/l (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid–NaOH, pH 7.4, containing 5 mmol/l 3-[(3-cholamidopropyl) dimethylammonium]-1-propanesulfonate and 5 mmol/l dithiothreitol.

Statistical analysis

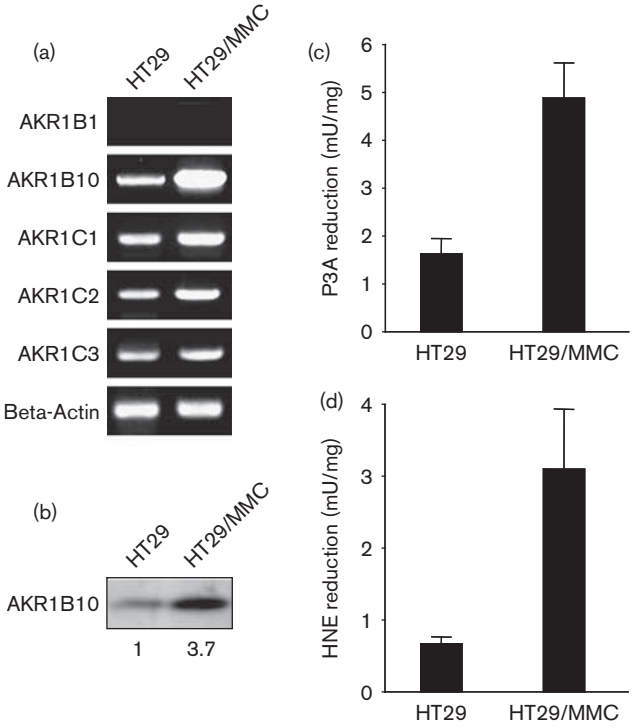
Data are expressed as means ± standard deviation of at least three independent experiments. Statistical evaluation of the data was conducted using the unpaired Student's *t*-test and analysis of variance followed by Fisher's test. A *P* value of less than 0.05 was considered statistically significant.

Results

By continuous exposure of HT29 cells to increasing concentrations (from 0.01 to 0.5 μmol/l) of MMC, we established the MMC-resistant variant (HT29/MMC) cells, which showed a 2.5-fold higher LD_{50} value for MMC than parental cells (30 vs. 12 μmol/l). Reverse transcriptase PCR analyses showed that the transcripts for AKR1B10, AKR1C1, AKR1C2, and AKR1C3 were detected in the parental cells, but that for AKR1B1 was below the detectable level (Fig. 1a). Compared with the parental cells, HT29/MMC cells exhibited high expression levels of the messenger RNAs for the four AKR enzymes, of which AKR1B10 was the most highly upregulated one. At the protein level estimated by western blot analysis, the HT29/MMC cells also expressed AKR1B10 more highly (3.7 fold) than the parental cells (Fig. 1b), although increases in the expression levels of AKR1C1, AKR1C2, and AKR1C3 during the MMC resistance were slight (data not shown). Furthermore, the reductase activities toward AKR1B10 substrates, P3A, and HNE [23,24], in the HT29/MMC cells were approximately four fold higher than those of the parental cells (Fig. 1c and d).

AKR1B10 effectively reduces a reactive lipid aldehyde HNE produced during exposure of lipids to ROS [24], and the MMC treatment increases the production of ROS [4]. Considering the high HNE reductase activity in the HT29/MMC cells (Fig. 1d), it is possible that HNE is the target molecule of AKR1B10 induced in the HT29/MMC cells, leading to the anticancer drug resistance. To test this possibility, we used HCT15 cells that hardly expressed the messenger RNAs for both AKR1B10 and AKR1B1 (data not shown), and prepared their AKR1B10-overexpressing phenotypes by transfection of the expression vector harboring the cDNA for AKR1B10. The overexpression of AKR1B10 was confirmed by a 20-fold higher P3A reductase activity than the control HCT15 cells transfected with the vector alone. The AKR1B10-overexpressing cells were clearly resistant to the toxicity of MMC, compared with the control cells (Fig. 2a). The

Fig. 1



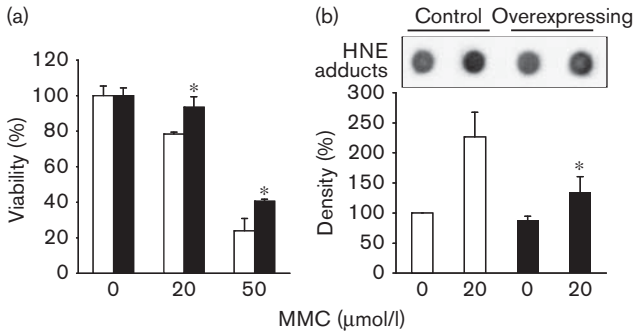
Upregulation of aldo–keto reductase (AKR) 1B10 by long-term exposure to mitomycin-c (MMC) for tolerance induction. (a) Reverse transcription PCR analyses of expression of messenger RNAs for five human AKRs in the control HT29 cells and HT29/MMC cells. (b) Western blotting of AKR1B10. The proteins (40 μg) in the extracts of HT29 and HT29/MMC cells were subjected to western blot analyses probed with the anti-AKR1B10 antibodies. The mean of the band density is shown at the bottom of the panel. (c and d) Reductase activities toward pyridine-3-aldehyde (c) and 4-hydroxy-2-nonenal (HNE) (d).

significant production of HNE by the MMC treatment was evident from a 2.2-fold higher level of the cellular HNE-bound proteins compared with that in untreated control HCT15 cells. The forced expression of AKR1B10 also resulted in a significant decrease in the production of the HNE-bound proteins by the MMC treatment (Fig. 2b). Furthermore, the AKR1B10-overexpressing cells were markedly resistant not only to cytotoxicity of HNE (Fig. 3a), but also to those of 4-oxo-2-nonenal and acrolein (Fig. 3b and c), which are also known as reactive aldehydes generated from lipid peroxidation [33,34].

To verify the above-mentioned role of AKR1B10 in MMC resistance through detoxification of reactive aldehydes, we prepared AKR1B10-silenced cells by the transfection of the siRNA for the enzyme into wild-type HT29 cells, and examined the effect of the silencing of AKR1B10 on the cytotoxicity of MMC and HNE. Western blot analysis showed a 68% decrease in AKR1B10 expression in the cells treated with siRNA. Despite the incomplete silencing, the siRNA treatment resulted in significant increases in susceptibility to the toxicity of MMC and HNE (Fig. 4a and b). A similar increase in sensitization to

the toxicants was also observed when the wild-type HT29 cells were pretreated for 2 h with (*Z*)-2-(4-methoxyphenylimino)-7-hydroxy-*N*-(pyridin-2-yl)-2*H*-chromene-3-carboxamide, a potent inhibitor of AKR1B10 [35] (Fig. 4c and d). Furthermore, pretreatment with polyethylene glycol-conjugated catalase, a potent inhibitor of ROS, significantly reduced the susceptibility to MMC in the AKR1B10-silenced (Fig. 4a) and AKR1B10-inhibited cells (Fig. 4c). The AKR1B10 inhibitor decreased the growth of the MMC-resistant HT29/MMC cells in a dose-dependent manner (Fig. 5a). Although the MMC treatment was shown to induce the activation of caspase 3, a member of apoptotic signaling, in cultured cells [6], we found that the MMC-induced activation of the enzyme in the resistant HT29/MMC cells was significantly low compared with that in the parental HT29 cells sensitive to MMC (Fig. 5b). The addition of the AKR1B10 inhibitor to the HT29/MMC cells resulted in a high MMC-induced activation of caspase 3 that was almost of same level as that in parental HT29 cells.

Fig. 2

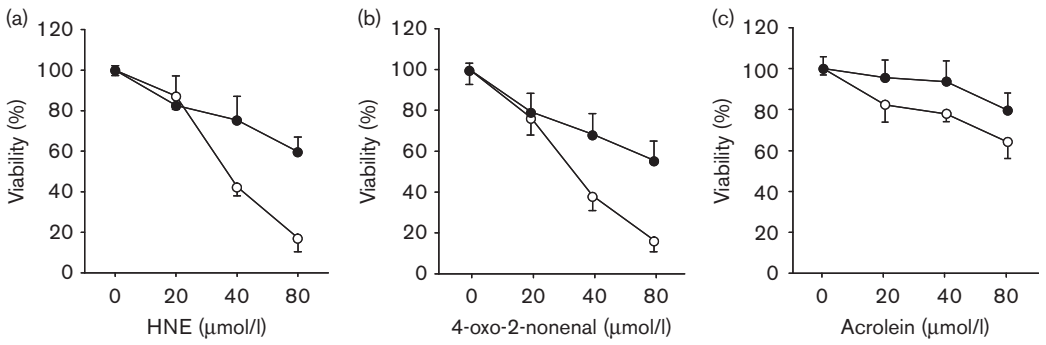


Overexpression of aldo–keto reductase (AKR) 1B10 in HCT15 cells lessens cell damage and 4-hydroxy-2-nonenal (HNE) formation induced by mitomycin-c (MMC). The cells transfected with the empty vector (open bar) and the vector harboring complementary DNA for AKR1B10 (closed bar) are referred to as the control cells and overexpressing cells, respectively. (a) Effect of the overexpression on viability against MMC treatment. The control and overexpressing cells were treated for 24 h with 0, 20, or 50 μmol/l MMC. The cell viabilities are normalized to those in the cells treated with the vehicle dimethylsulfoxide alone. (b) Effect of the overexpression on the accumulation of the HNE-protein adducts. The cells were treated for 24 h with 0 or 20 μmol/l MMC, and the HNE-protein adducts in the cell extracts were detected by dot blot analyses using the anti-HNE antibodies (upper panel). The dot densities are represented as the percentage of those in the control cells treated with the vehicle dimethylsulfoxide alone, and shown in the lower bar graph. *Significant difference from the control cells treated with the same concentration of MMC, $P<0.05$.

Discussion

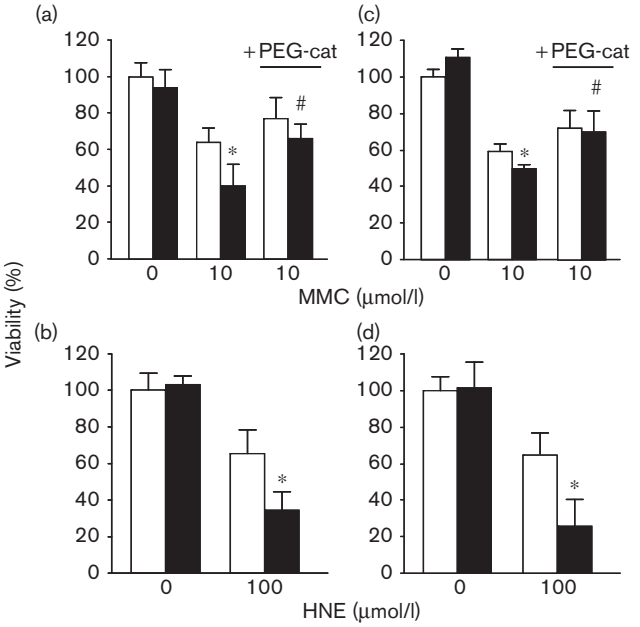
AKR1B10 has been suggested as a diagnostic tumor marker, particularly in lung cancer [17,18], and has been implicated in the proliferation of cancerous cells [21,22]. In this study, we found that induction of MMC resistance in colon cancer HT29 cells significantly elevates the expression of AKR1B10, compared with those of the AKR1C isoforms that are known to be overexpressed with resistance to other anticancer drugs [13–15]. Thus, we propose that AKR1B10 is also a potential marker for MMC resistance in colon cancer cells. This hypothesis was supported by the reversal of MMC resistance after suppression of AKR1B10 expression (Fig. 4) and inhibition of its activity by the inhibitor (*Z*)-2-(4-methoxyphenylimino)-7-hydroxy-*N*-(pyridin-2-yl)-2*H*-chromene-3-carboxamide (Fig. 5). There have been few reports on AKR1B10 overexpression associated with anticancer drug resistance. AKR1B10 is one of the upregulated genes in the medulloblastoma cell lines resistant to preactivated cyclophosphamide [16] and in HT29 colon cancer cells resistant to methotrexate [15], in which the most highly upregulated aldehyde dehydrogenase or AKR1C1 plays a key role in the development of resistance. In addition, an overexpression of AKR1B10 makes hepatocellular carcinoma more resistant to anticancer drugs with the carbonyl group, such as MMC [36], but molecular mechanisms to explain how upregulation of AKR1B10 induces MMC resistance in colon cancer cells

Fig. 3



Effect of aldo–keto reductase (AKR) 1B10 expression in HCT15 cells on cytotoxicity of 4-hydroxy-2-nonenal (HNE) (a), 4-oxo-2-nonenal (b), and acrolein (c). The control (open circle) and AKR1B10-overxpressing cells (closed circle) were treated for 24 h with the indicated concentrations of the four compounds. The viability values are normalized to those in the cells treated with dimethylsulfoxide alone.

Fig. 4



Effects of knockdown and inhibition of aldo–keto reductase (AKR) 1B10 in HT29 cells on cytotoxicity of mitomycin-c (MMC) and 4-hydroxy-2-nonenal (HNE). (a and b) Effect of AKR1B10 silencing. The wild-type cells (open bar) and AKR1B10-silenced cells (closed bar) were treated for 24 h with 10 μmol/l MMC (a) or 100 μmol/l HNE (b). *Significant difference from the wild-type cells treated with MMC or HNE, $P < 0.05$ (c and d). Effect of AKR1B10 inhibitor, (*Z*)-2-(4-methoxyphenylimino)-7-hydroxy-*N*-(pyridin-2-yl)-2*H*-chromene-3-carboxamide. The wild-type cells were pretreated for 2 h without (open bar) or with 5 μmol/l inhibitor (closed bar) before treating for 24 h with 10 μmol/l MMC (c) or 100 μmol/l HNE (d). In the MMC + polyethylene glycol-conjugated catalase (PEG-cat) group, the cells were pretreated for 2 h with 200 U/ml PEG-cat before 24 h treatment with MMC. *Significant difference from the control cells (without the inhibitor) treated with MMC or HNE, $P < 0.05$. #Significant difference from the cells treated with MMC alone, $P < 0.05$.

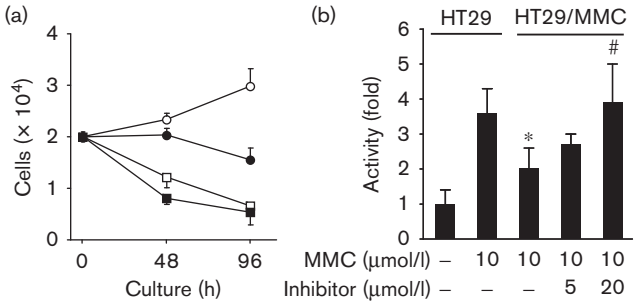
remain unknown. We also detected the overexpression of AKR1B10 in some colon cancer cells that gain resistance to doxorubicin (our unpublished results). Considering the mechanism underlying the MMC resistance (described below), AKR1B10 may be a marker and a therapeutic target candidate for resistance development to MMC and to other anticancer drugs that generate ROS.

It was suggested that cell damage evoked by MMC is in part mediated by ROS generation and by the resultant formation of reactive aldehydes such as HNE and 4-oxo-2-nonenal through peroxidation of polyunsaturated fatty acids [4]. This study indeed showed the increase in HNE-bound proteins in colon cancer cells by MMC treatment (Fig. 2b). In addition, pretreatment of cells with polyethylene glycol-conjugated catalase prevented their viability loss by MMC (Fig. 4a and c). These results are direct evidence showing the involvement of ROS and lipid peroxides in the MMC-induced apoptosis of colon cancer cells. AKR1B10 is capable of converting HNE and 4-oxo-2-nonenal into less-toxic corresponding primary alcohols *in vitro* [24]. The function was shown in this study using AKR1B10-overexpressing cells, which suppressed both accumulation of HNE-protein adducts by MMC treatment (Fig. 2b) and cell damage induced by HNE (Fig. 3a), and AKR1B10-silencing cells, which increased its sensitization to HNE-elicited cell death (Fig. 4b). Thus, AKR1B10 induced by MMC may mainly detoxify reactive aldehydes derived from lipid peroxidation because of ROS production through the redox cycle of MMC. Similar to MMC, anticancer drugs such as anthracyclines and platinum-containing drugs generate ROS through their redox cycles. Thus, it is possible that the proposed mechanism underlies the drug resistance, which induces the expression of AKR1B10 or other lipid aldehyde-metabolizing enzymes.

It has recently been shown that, in addition to antioxidant enzymes [e.g. NAD(P)H:quinone oxidoreductase 1 and glutathione-S-transferase] and human AKR1C subfamily enzymes (AKR1C1, AKR1C2, and AKR1C3), AKR1B10 is transcriptionally regulated by the activation of nuclear factor-erythroid 2 p45-related factor 2 (Nrf2)-Kelch-like ECH-associated protein 1 (Keap1)-dependent signaling [37]. As the Nrf2-Keap1 signaling is activated in response to various stimuli including ROS [38], sulforaphane [39], and HNE [40], it is assumed that ROS and lipid peroxides formed during the MMC treatment are involved in the upregulation of AKR1B10 through the activation of this signaling. This assumption may be supported by recent findings that incubation of colorectal cancer cells with bortezomib, an inhibitor of proteasome that causes ROS generation [41], induces upregulation of AKR1B10 [42]. Lee *et al.* [43] reported that resistance of HT29 cell induction against MMC decreases the expression level of NAD(P)H:quinone oxidoreductase 1 that is transcriptionally activated by the Nrf2-Keap1 pathway. To reconcile the apparently contradictory expression patterns between AKR1B10 and NAD(P)H:quinone oxidoreductase 1, the detailed mechanism underlying transactivation of these enzymes has to be elucidated in future.

We provide evidence that the AKR1B10 inhibitor restores MMC sensitivity in HT29/MMC cells. The concentrations used in this study are comparable to those that are effective to inhibit intracellular metabolism by AKR1B10 using Hela cells [35]. This inhibitor potently inhibits both AKR1B10 and AKR1B1 [35], which exhibit reductase activities toward HNE and 4-oxo-2-nonenal [24,44]. However, AKR1B1 was not expressed in HT29 cells and in their MMC-resistant cells, supporting the selective inhibition of the catalytic activity of AKR1B10 by the inhibitor. Interestingly, the inhibitory effect was observed for 3 days as is evident by the progressive inhibition curves (Fig. 5a). This would result from the high affinity of the inhibitor to AKR1B10 ($K_i = 2$ nmol/l) [35] and/or its hardly metabolized feature in the cells. Therefore,

Fig. 5



Aldo–keto reductase (AKR) 1B10 inhibitor sensitizes HT29/mitomycin-c (MMC) cells to MMC toxicity. (a) Effect of the inhibitor on cell proliferation. HT29/MMC cells were suspended in the growth medium supplemented with 0.5 μmol/l MMC and seeded at a density of 2×10^4 cells/well in a 48-well multiplate. After the addition of 0 (open circle), 5 (closed circle), 10 (open square), or 20 μmol/l inhibitor (closed square) into the medium, the cells were cultured for 48 or 96 h, and the cell numbers were estimated. (b) Effect of the inhibitor on caspase-3 activation. HT29 cells and HT29/MMC cells were treated for 24 h with 10 μmol/l MMC. HT29/MMC cells were also pretreated for 2 h with the AKR1B10 inhibitor before the MMC treatment. The caspase-3 activity is expressed as the fold increase in the activity ratio of the treated cells to the untreated HT29 cells. *Significant difference from HT29 cells treated with MMC alone, $P<0.05$. #Significant difference from HT29/MMC cells treated with MMC alone, $P<0.05$.

AKR1B10 inhibitors might be used as adjuvant therapy for cancer chemotherapeutic drug resistance, in which AKR1B10 is overexpressed.

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