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SHORT COMMUNICATION

Dihydrodiol dehydrogenase in drug resistance and sensitivity of human carcinomas

Bih Fang Pan · J. Arly Nelson

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Abstract We previously reported (UroOncology 1:165, 2001) cross-resistance and collateral-sensitivity to 2-chlorodeoxyadenosine (CldAdo) and fludarabine (FaraA), respectively, in a human renal cell carcinoma selected for resistance to 2′-deoxytubercidin (Caki-dTub). Insofar that these drugs generally demonstrate cross resistance rather than collateral sensitivity, we further examined the bases for this phenomenon. Both CldAdo and FaraA induce apoptosis, as the triphosphates, via binding to Apaf-1. In the presence of cytochrome *c*, this binding leads to activation of procaspase 9 to active caspase 9 that induces apoptosis through its activation of caspase 3. CldAdo and FaraA induced caspase 3 activities in wild type and Caki-dTub cell lines in a dose-dependent manner that paralleled the cross-resistance (CldAdo, 200-fold) or collateral sensitivity (FaraA, 20-fold) with regard to cell viability. The activation of caspase 3 was inhibited by the caspase 9 inhibitor, Z-LEHD-FMK, suggesting that both drugs act via the same pathway. By differential display and direct enzyme analysis, dihydrodiol dehydrogenase (DDH) was observed to be profoundly underexpressed in the Caki-dTub compared to wild-type Caki-1 cells. Stable transfection of the Caki-dTub cells with a vector encoding the enzyme led to partial reversal of the resistance to CldAdo. Resistance to cisplatin has recently been ascribed to overexpression of DDH in a human ovarian carcinoma cell line (Deng et al. in J Biol Chem 227:15035, 2002). It is tempting to speculate a mutation in the Apaf-1 nucleotide binding site that reduces (CldAdo) or increases (FaraA) toxicity in the Caki-dTub cells; however, the recent finding by others in a human ovarian carcinoma cell line suggests that DDH expression mediates the cross-resistance and perhaps, collateral-sensitivity.

Keywords Dihydrodiol dehydrogenase · Cross-resistance · 2-Chlorodeoxyadenosine · Apoptosis · Fludarabine · Caspase 3

Introduction

In pharmacology, drug "resistance" is a relative term, implying drug sensitivity in some reference tissue or tumor. Collateral-sensitivity is a term used to describe the phenomenon of increased drug sensitivity in a tumor that is otherwise resistant to another agent. Although this phenomenon has been repeatedly observed in the experimental setting, to our knowledge it has not been clinically exploited. The biochemical basis for resistance to cisplatin in a human ovarian cancer cell line has recently been reported to be due to overexpression of the enzyme, dihydrodiol dehydrogenase (DDH) [1]. This enzyme catalyzes the reversible

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B. F. Pan (✉)
Department of Molecular Pathology, Unit 951,
The University of Texas M. D. Anderson Cancer Center,
1515 Holcombe Boulevard, P.O. Box 301429,
Houston, TX 77230-1429, USA
e-mail: bpan@mdanderson.org

J. A. Nelson
Division of Pediatrics,
The University of Texas M. D. Anderson Cancer Center,
1515 Holcombe Boulevard,
Houston, TX 77230-1429, USA

oxidation of cis-diols to orthoquinones, and it has been studied extensively for its role in the metabolism of polycyclic aromatic hydrocarbons as carcinogens [2]. Although the mechanism(s) by which DDH confers drug resistance (or perhaps, collateral sensitivity) is not known, its overexpression has been unequivocally established to mediate the resistance to cisplatin in the human ovarian carcinoma cells referred to above [1]. Interestingly, this ovarian carcinoma selected for resistance to cisplatin also demonstrates collateral sensitivity toward the mitotic spindle poisons, vincristine and vinblastine [3]. Nevertheless, DDH transfected parental cells showed resistance to cisplatin, but not sensitive to paclitaxel and vincristine [4]. Herein, we describe an analogous phenomenon in the human renal cell carcinoma selected for resistance to a deoxyribonucleoside, i.e., Caki-dTub cell which is cross-resistant to 2-chlorodeoxyadenosine (CldAdo) and collateral-sensitive to fludarabine (FaraA) [5].

Materials and methods

Cell culture and selection for resistance to dTub

Caki-1 cells were obtained from the American Type Culture Collection (Rockville, MD). A dTub-resistant cell line (Caki-dTub) was established by gradually increasing the concentration of dTub from 1 to 100 μM during a 1-year period [5]. The Caki-dTub cells were then maintained in McCoy's 5 A medium containing 100 μM dTub. Cell viability was determined colorimetrically using the MTT assay [6] following a 6-day exposure to drugs in McCoy's 5 A medium.

Measurement of caspase 3 activation

The caspase 3 activity kit purchased from Oncogene Research Products (Boston, MA) was used as reported [7]. Cells were treated with CldAdo or FaraA for 1 day in the presence or absence of 20 μM caspase inhibitor, i.e., Z-DEVD-FMK or Z-LEHD-FMK for caspase 3 or 9, respectively. Cleavage of the substrate, DEVD conjugated with 7-amino-4-trifluoromethyl coumarin, was measured by a fluorescent microplate reader (Fluoroskan Ascent FL, Labsystems, Inc., Franklin, MA). Emission was monitored at 510 nm using an excitation wavelength of 390 nm.

Differential expression of DDH

Total RNA obtained from Caki-1 and Caki-dTub cells was used to prepare cDNAs using the enhanced differential display technique of Linskens et al. [8]. The primers for performing differential display were obtained from Genosys (The Woodlands, TX). Using the 5′-GCG, CAA,GCT,TTT,TTT,TTT,TTM,G-3′ (M = A, G, or C) 3′-primer and 5′-CGG,GAA,GCT,TTG,CAG,TGT, GTG,A-3′ 5′-primer revealed a notably underexpressed gene from Caki-dTub cells that was extracted from the electrophoresis gel and reamplified by PCR. The PCR product was cloned into the PCR 2.1 vector (Invitrogen, Carlsbad, CA) and sequenced. The product was found to be identical to DDH isoform I [9]. DDH enzymatic activity [10] was determined by the increase in absorbance at 340 nm upon incubating whole cell homogenates (0.25 mg protein/reaction) with 1.5 mM 1-acenaphthenol, 0.25 mM β-NADP, 50 mM glycine/NaOH buffer at pH 9.0 at room temperature.

Transfection of Caki-dTub cells with a vector encoding DDH

A pcDNA3 eukaryotic expression vector containing the full-length human DDH isoform I insert was generously provided to us by Dr. Trevor Penning of the University of Pennsylvania. FuGENE 6 Transfection Reagent from Roche Molecular Biochemicals (Indianapolis, IN) was used for transfections of no vector, pcDNA3 vector, and pcDNA3-DDH I into Caki-dTub Cells. Stable transfectants were selected by G418 resistance using standard procedures. All negative control cells without vector transfection were killed, which indicated that the positive clones were survived due to transfection.

Results

Drug resistance and sensitivity of Caki-dTub cells

We reported earlier [5] the multidrug resistant and collateral sensitive phenotype of Caki-dTub cells. The resistance to CldAdo is not due to failure of the cells to acquire CldAdo from the medium, metabolize it to nucleotides, or to incorporate the analog into DNA. That is, the Caki-dTub cells metabolized CldAdo as well as the Caki-1 cells, indicating normal activity of the activating enzyme, deoxycytidine kinase. It was also established that cytochrome c release was the same in Caki-dTub and Caki-1 cells upon treatment of both cell types with CldAdo. Thus, we hypothesize that the resistance is due to a "down-stream" defect in the induction of apoptosis by CldAdo in the Caki-dTub cells. Indeed, the Caki-dTub cells are resistant to

induction of caspase 3 compared to the Caki-1 wild type cells (Fig. 1a). Further, the dose-response relationship essentially parallels that of drug-induced toxicity (upper portion versus lower portion of Fig. 1a). In a similar manner, the collateral sensitivity of Caki-dTub cells to FaraA is reflected in an increased ability of the drug to induce caspase 3 in this as compared to the wild type cells (Fig. 1b). Again, the enhanced sensitivity to caspase 3 activation parallels that of reduction in cell viability. It is likely that both drugs induce caspase 3 activation via caspase 9 insofar that the caspase 9 inhibitor prevented the enhancement by each agent (Fig. 2). Further confirmation that the caspase 3 activation occurred is provided by inhibition of its activity using the caspase 3 inhibitor as described in the legend to Fig. 2.

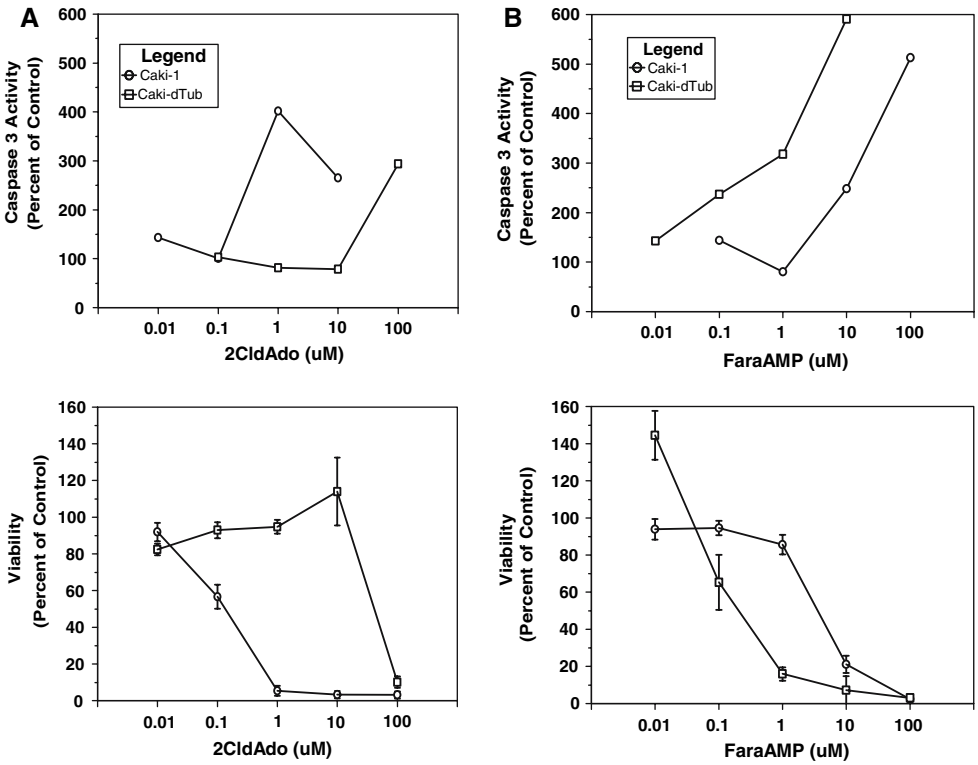

Fig. 1 **a** The resistance of Caki-dTub cells to CldAdo correlates with the lack of activation of caspase 3. **b** The collateral-sensitivity of Caki-dTub cells to FaraA correlates with the activation of caspase 3. The *upper figures* illustrate the activation of caspase 3 as a function of CldAdo or FaraA dose, and the *lower figures* show the corresponding reduction in cell viability. Caspase 3 activity was measured after a 1-day exposure to CldAdo using the fluorescent substrate, DEVD conjugated to 7-amino-4-trifluoromethyl coumarin. Cell viability was determined by MTT assay after a 6-day exposure to CldAdo or FaraA. (*Open circle*), Caki-1; (*open rectangle*), Caki-dTub

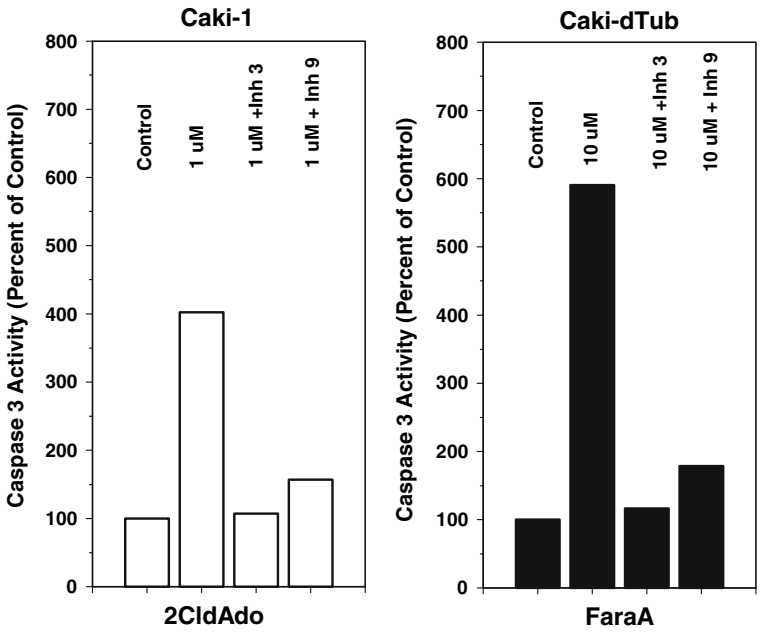

Fig. 2 The caspase 3 inhibitor, Z-DEVD-FMK, and the caspase 9 inhibitor, Z-LEHD-FMK, prevent the activation of caspase 3 by CldAdo or FaraA. Caspase 3 activity was determined as described in the legend to Fig. 1 after a 1-day exposure to the drugs in the presence or absence of the caspase 9 or caspase 3 inhibitors (50 μM)

DDH expression

As discussed in the Materials and methods section, DDH was found to be greatly underexpressed in the Caki-dTub cells by differential display and sequencing of the electrophoretic band expressed in Caki-1 cells (Fig. 3a). The underexpression of the enzyme was confirmed by direct enzyme analysis (Fig. 3b). Transfection of the Caki-dTub cells with a vector encoding the DDH enzyme partially reversed the resistance to Cld-Ado (Fig. 4). We were unable to study the collateral sensitivity toward FaraA in the transfectants since the vector alone (pcDNA3 without insert) conferred resistance to the drug (data not shown). Apparently, the enzyme, aminoglycoside phosphotransferase, responsible for G418 (aminoglycoside) resistance also confers resistance to FaraA.

Discussion

DDH expression and drug resistance

A role for DDH overexpression in drug resistance was first reported in a human colon carcinoma (HT29 cells) selected for resistance to ethacrynic acid [11]. The inducible expression of this enzyme has been observed [10], and promoter elements have been identified [12]. The potential importance of DDH expression to clinical outcome was first suggested in a study of 381 non-small cell lung cancer patients [13]. In this study, the 5-year survival rate for "high" expressers was about 20% compared to approximately 80% for "low" expressers. The authors were led to investigate DDH after identifying its differential expression in patient cancer and noncancerous tissues. The patients were studied between 1986 until 1999 and they were treated with 5-fluorouracil, cisplatin, adriamycin and/or etoposide. The drug resistance as a function of DDH expression was confirmed in vitro (Dr. K-C Chow, personal communication). These observations assumed additional significance upon the report by Deng et al. [1] that DDH overexpression is responsible for the resistance of a human ovarian carcinoma cell line to cisplatin [3]. Specifically, by microarray cDNA expression analysis, these authors identified differential expression of five genes including DDH; however, upon transfection of the wild-type cells, only DDH was found to confer resistance to cisplatin. The cisplatin resistant cell line also demonstrates collateral sensitivity toward vinca alkaloids [3] and taxol. It is of potential clinical significance to note that taxol and cisplatin are currently the mainstay drugs for the treatment of ovarian carcinoma, a common and problematic disease [14]. Up-regulation of DDH in human ovarian carcinoma cell lines resistant to cisplatin has been independently confirmed [15].

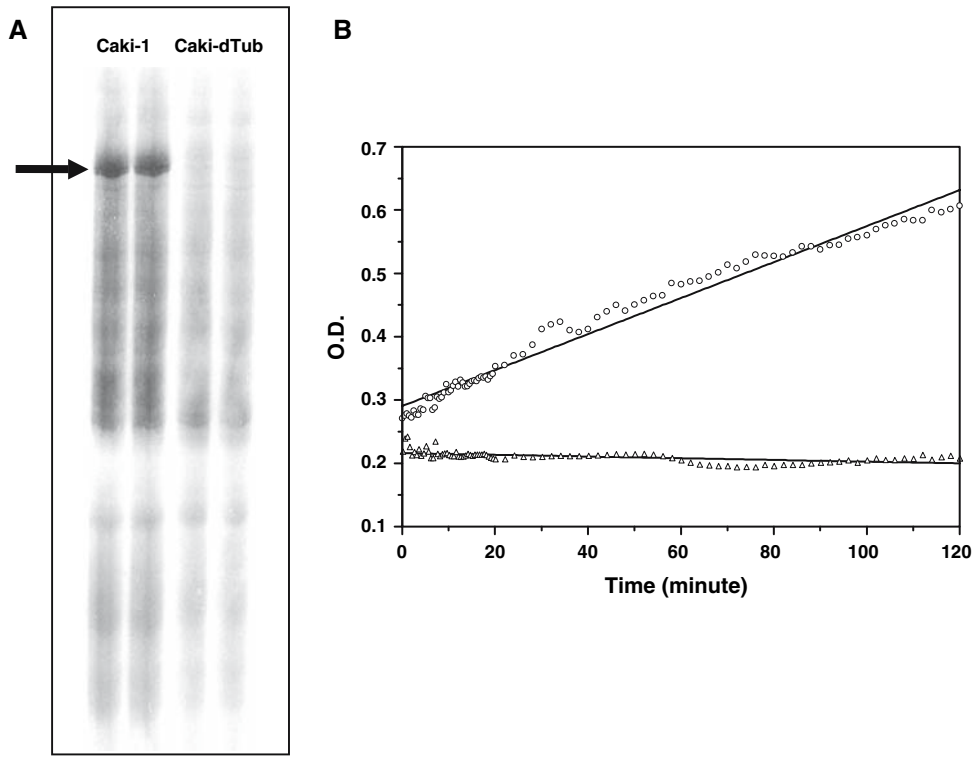

Fig. 3 **a** DDH is underexpressed in Caki-dTub cells by differential display. The *arrowed band* in Caki-1 sample was cut, extracted, reamplified by PCR and cloned into PCR 2.1 vector. DDH isoform 1 was identified by sequence analysis. **b** DDH activity is reduced in Caki-dTub cells. The enzyme was measured in whole cell homogenates using 1-acenaphthenol as substrate. The formation of NADPH was measured by the increase in absorbance at 340 nm. The enzyme activity in Caki-1 cells as determined during the first 20 min incubation is 2.39 nmol/min/mg protein. *Open circle* Caki-1; *open triangle* Caki-dTub

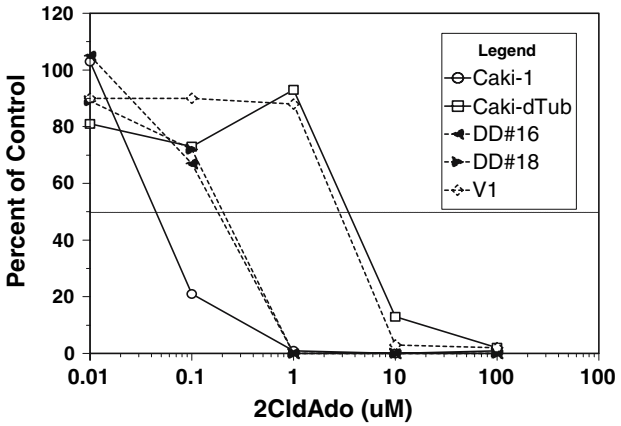

Fig. 4 Transfection of Caki-dTub cells with a vector encoding DDH partially reverses the resistance to CldAdo. Caki-dTub cells were treated with a pcDNA3 eukaryotic expression vector containing the full-length sequence for DDH isoform I. Stably transfected cells were selected for G418 resistance. Cell viability was determined by MTT assay as described in Materials and methods. *Open circle* Caki-1; *open square* Caki-dTub; *open diamond* clone 1 of pcDNA3 vector transfected Caki-dTub cell; *filled left triangle* clone 16 of DDH 1 transfected Caki-dTub cell; *filled right triangle* clone 18 of DDH 1 transfected Caki-dTub cell

Herein we report, using differential display and direct enzyme analysis, the underexpression of DDH in a human renal carcinoma selected for resistance to the deoxyribonucleoside analog, dTub. The Caki-dTub cells exhibit marked cross-resistance to other analogs including CldAdo (200-fold), arabinosyl cytosine and gemcitabine (20-fold). On the other hand, the Caki-dTub cells are some 20-fold collaterally sensitive to FaraA and troxacitabine [5]. The resistance to CldAdo may be a result of underexpression of DDH since transfection of the Caki-dTub cells with a vector expressing DDH partially reversed the resistance (Fig. 4). On a clinical note, CldAdo and FaraA are the drugs of choice for treating CLL [16], i.e., if similar observations could be extended to this disease, it may be possible to exploit the collateral sensitivity in selected patients.

Caspase 3 activation in the multidrug resistance and collateral sensitivity of Caki-dTub cells

Both CldAdo and FaraA induce apoptosis via activation of caspases [17, 18]. Specifically, they bind as their deoxyribonucleoside triphosphates to Apaf-1. In the presence of cytochrome c, this leads to activation of caspase 9 that subsequently activates caspase 3. As shown herein, the activation of caspase 3 correlates in a dose-dependent manner with the reduction in viability in the wild type and Caki-dTub cells. Namely, the concentration of CldAdo required to enhance caspase 3 in the Caki-dTub cells is about 200-fold that needed for wild-type Caki-1 cells whereas the reverse is true for FaraA, i.e., less drug is required to activate caspase 3 or to reduce cell viability in the Caki-dTub compared to Caki-1 cells (Fig. 1). Thus, it is tempting to speculate that a mutation has occurred in the Apaf-1 nucleotide binding site of Caki-dTub compared with Caki-1 cells such that there is reduced efficacy for CldAdo and enhanced efficacy for FaraA (as the triphosphates) in mediating caspase 9 activation.

Conclusions

Given the diverse structures of the drugs described above for which the toxic effects are apparently modified by DDH expression, it seems unlikely that this drug-metabolizing enzyme could deactivate/activate each of them, directly. Perhaps more likely, DDH plays a role in mediating the induction of caspases and/or apoptosis by these agents. DDHs are members of the aldo-keto reductase gene superfamily [2] known to catalyze the reversible oxidation of dihydrodiols to catechols that rapidly autoxidize to ortho-quinones. This pathway suppresses the formation of the ultimate carcinogenic species of polycyclic aromatic hydrocarbons (PAH anti- and syn-diol epoxides); however, the process of autooxidation yields reactive oxygen species [19]. In this manner, DDH activity can initiate processes leading to apoptosis. The enzyme, via catalysis of added substrate, has been shown capable of mediating the induction of cell death in human breast carcinoma MCF-7 cells [20], and overexpression of an aldose reductase activity has been shown to lead to a redox imbalance in pancreatic β-cells that induces apoptosis [21]. In summary, over- or underexpression of DDH can alter reactive oxygen species in a manner that may favor or prevent apoptosis, and sufficient evidence exists to warrant continued study of DDH as a predictor of drug response in human carcinomas of great clinical importance.

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