Diaminothiazoles evade multidrug resistance in cancer cells and xenograft tumour models and develop transient specific resistance: understanding the basis of broad-spectrum versus specific resistance

Smreti Vasudevan, Sannu Ann Thomas, Krishnankutty C.Sivakumar1, Reena J.Komalam2, Keerthi V.Sreerekha2, Kallikat N.Rajasekharan2 and Suparna Sengupta*

Division of Cancer Research and 1Distributed Information Sub-Centre, Rajiv Gandhi Centre for Biotechnology, Trivandrum, India and 2Department of Chemistry, University of Kerala, Trivandrum, India

To whom correspondence should be addressed. Tel: +91 471 2529475; Fax: +91 471 2348096; Email: ssengupta@rgcb.res.in

Abstract

Acquired drug resistance poses a challenge in cancer therapy. Drug efflux is the most common mechanism of resistance displayed by hydrophobic drugs beyond a certain size. However, target specific changes and imbalance between the pro- and anti-apoptotic proteins are also found quite often in many tumours. A number of small antimitotic agents show high potential for multidrug resistant tumours, mainly because they are able to evade the efflux pumps. However, these compounds are also likely to suffer from resistance upon prolonged treatment. Thus, it is important to find out agents that are sensitive to resistant tumours and to know the resistance mechanisms against small molecules so that proper combinations can be planned. In this report, we have studied the efficiency of diaminothiazoles, a novel class of tubulin targeting potential anticancer compounds of small size, in multidrug resistant cancer. Studies in model cell lines raised against taxol and the lead diaminothiazole, DAT1 [4-amino-5-benzoyl-2-(4-methoxy phenyl amino) thiazole], and the xenograft tumours derived from them, show that diaminothiazoles are highly promising against multidrug resistant cancers. They were able to overcome the expression of efflux protein MDR1 and certain tubulin isotypes, could sensitize improper apoptotic machinery and ablated checkpoint proteins Bub1 and Mad2. Further, we have found that the resistance against microtubule binding compounds with higher size is broad-spectrum and emerges due to multiple factors including overexpression of transmembrane pumps. However, resistance against small molecules is transient, specific and is contributed by target specific changes and variations in apoptotic factors.

Introduction

Drug resistance is a major concern in cancer chemotherapy rendering tumour resurgence and treatment failure. Cellular drug efflux is a mechanism of resistance for which drugs effective against all targets suffer (1). Further, in many cancers, the balance between pro- and anti-apoptotic proteins is lost leading to apoptosis resistance against different antitumour agents (2). The efficacy of microtubule binding drugs, which take a major role in cancer treatment, is also hampered severely due to acquired drug resistance. These compounds primarily disrupt microtubule dynamics thereby causing a mitotic block which is followed by apoptosis (3). Microtubule binding drugs normally bind to the β subunit of tubulin preventing either polymerisation or depolymerisation of microtubules. Mechanisms of resistance against microtubule binding agents in cancer cells are intricate...
and could stem from cellular drug efflux by the plasma membrane associated ABC (adenosine triphosphate binding cassette) transporter proteins, deficient apoptotic signalling, differential expression/mutation in the tubulin isotype composition and alteration of spindle checkpoint proteins (4, 5).

Hydrophobic drugs like taxanes and vinca alkaloids are good substrates of the major efflux pumps (MDR1, MRP) and thus their expression by cancer cells leads to active extrusion of such drugs (6–8). They are also less active in tumours that express certain isotypes of tubulin due to ineffective drug-target interactions (9). βIII and βIII are the isotypes that are mostly implicated in resistance (10–12). Cancer cells overexpressing anti-apoptotic proteins or where pro-apoptotic proteins like Bax are inactivated also show resistance to taxol and vinca alkaloids (13, 14). Moreover, deficiency or alterations in spindle checkpoint proteins like MAD1, MAD2, Bub1, BubR1 lead to lack of activation of spindle checkpoint thereby causing improper mitotic block and poor response against antimitic agents (5, 15). Compounds that are capable of binding to the isotypes expressed in resistant cancer cells and active in cells overexpressing P-glycoprotein efflux pump or cells with compromised apoptotic proteins or spindle checkpoint response would have an added advantage in cancer chemotherapy.

Antimitotic compounds like Combretastatin A4, E7010, epothilone are able to kill tumour cells resistant to taxol or vinca alkaloids and thus, an analogue of epothilone B (ixabepilone) has been approved in the clinic, whereas combretastatin, 2-methoxy estradiol and E7010 are in clinical trials (16–19). Generally, compounds with a molecular weight lower than 330 daltons are poor substrates of P-glycoprotein efflux pump and thus are capable of eluding the pump (20). However, small molecules may also face resistance problems as is observed in the cases of combretastatin A4, E7010 and podophyllotoxin (21–23). The resistance pattern exhibited by cancer cells against small molecules may differ and the mechanism of resistance developed is not fully understood.

Diaminothiazoles are a novel class of antimitotic and antiangiogenic compounds (24–26). They are capable of inducing apoptosis by both intrinsic and extrinsic pathways and in cells where the mitochondrial pathway is blocked (27). Lead diaminothiazole DAT1 [4-amino-5-benzoyl-2-(4-methoxy phenyl amino) thiazole] has been shown to bind to the classical colchicine site of tubulin and depolymerise microtubules reversibly (24, 28). In the present study, efficacy of diaminothiazoles in resistant cells has been tested by modelling and experimental studies. We found that diaminothiazoles are effective in multidrug resistant cells and validated this finding in xenograft tumour models. In addition, using model resistant cell lines against taxol and DAT1, we have compared the resistance mechanisms developed in cancer cells against large and small tubulin binding compounds.

### Materials and methods

#### Materials

Dulbecco’s modified Eagle’s medium and fetal bovine serum were purchased from GIBCO. 3-[4,5-Dimethylthiazole-2-yl]-2,5-diphenyl tetrazolium bromide, 4′,6-diamidino-2-phenylindole (DAPI) and all other fine chemicals including the drugs Paclitaxel (Taxol®), Colchicine, Vinblastine, 5-fluorouracil, Doxorubicin, Combretastatin A4, Podophyllotoxin and Epothilone B were from Sigma, St Louis, MO. Antibodies against βII and βIV and IV tubulin isotypes were from Biogenex, USA; P-glycoprotein, MAD2 and Bub1 from Abcam; Bcl-2, Bcl-xl, Bax from Santa Cruz, pBcl-2 and cleaved Caspase-3 were from Cell Signaling. Anti-mouse and anti-rabbit horse radish peroxidase were procured from Sigma. ECL Plus western blotting detection system was from Amersham, UK.

#### Cell lines

Epithelial derived colon carcinoma cell line HCT116, uterine sarcoma cell line MES-SA along with its multidrug resistant variant MES-SA/Dx5 were purchased from ATCC, and breast adenocarcinoma line MCF7 was obtained from National Cancer Institute, USA who authenticated the cell lines by short tandem repeat analysis (intraspecies). Frozen stocks of cells from the reference stock were made within passage three and stored in liquid nitrogen. For experiments, cells were never used beyond 3 months after revival.

#### Synthesis of the compounds

DAT1 and its analogues DAT22, DAT34 and DAT7 were synthesized by modification of a solid phase synthesis (29) (details to be described elsewhere) in more than 95% purity.

#### Cell culture and generation of resistant cell lines

HCT116 and MCF7 cells were cultured under standard conditions as described previously (27). Taxol resistant variants of HCT116 cell line (HCT116/TaxR) and MCF7 cell line (MCF7/TaxR) were established by exposing HCT116 and MCF7 cells, respectively, to a step wise increasing concentration of taxol (Paclitaxel) in Dulbecco’s modified Eagle’s medium. After each dose of drug increment, the surviving colonies were allowed to repopulate until the HCT116 and MCF7 cells could tolerate 75 and 10nM of taxol, respectively. Cells were subcultured at this stage and frozen for future studies. These taxol resistant variants were maintained in taxol free medium. Likewise, cells resistant to the lead diaminothiazole DAT1 (HCT116/DAT1R and MCF7/DAT1R) were raised from HCT116 and MCF7 cells up to 1 μM DAT1 concentration but these cells were maintained in DAT1 containing media. However, experiments to study the effects of compounds on these cells were done in drug free media.

#### Cytotoxicity assay

Cytotoxicity studies for the compounds in different cell lines were conducted by 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide assay (30). The index for resistance was calculated using formula: resistance index (RI) = (IC of the compound in the resistant subline)/IC of the compound in parental cell line).

#### Animal studies

Breeding pairs of Nonobese Diabetic/Severe Combined Immunodeficiency (NOD/SCID) mice were obtained from ‘Jacksons Laboratories,’ USA. They were housed and inbred at the Small Animal House Facility of the institute under pathogen free, isothermal conditions. All the animal experiments were conducted as per the approved guidelines of Institute Animal Ethics Committee which is under the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Govt. of India. HCT116/TaxR cells (1.5 million) were suspended in 0.2ml of phosphate buffer saline and injected subcutaneously into the flank region of 6–8 week old, female SCID mice to form xenograft tumours. In about 25 days, when the tumour attained appreciable size (60–120mm³), the mice were randomly divided into three groups (n = 10/group). DAT1 was dissolved in the vehicle cremophor-ethanol (1:1), diluted in phosphate-buffered saline and administered on thrice weekly basis, intraperitoneally at doses of 10 and 20 mg/kg body weight to the first and second group, respectively. The third group composed of control animals which received only the vehicle control. The caliper measurement of the tumour and the body weight of the animal were recorded weekly. Tumour volume was calculated as (length × width × height)/2. The animals were checked for any signs of distress. After 12 doses, the animals were killed and the tumour tissue was harvested, immediately snap frozen, homogenised and protein lysates were made...
using RIPA lysis buffer. Mean tumour growth inhibition was calculated as [1 - (mean treatment group volume/mean control group volume)] × 100. A small part of tumour tissue was minced under aseptic conditions and seeded in Dulbecco’s modified Eagle’s medium to initiate primary cells.

Similarly, HCT116 (1 million) and HCT116/DAT1R (1.5 million) cells were injected to form tumours. After 28 days, tumour tissue was dissected and protein lysates were made.

**DAPI (4′, 6-Diamidino-2-Phenylindole) staining for studying apoptosis**

Cells were seeded on cover slips and respective drugs were added at 70% confluency. After 18h, cells were fixed in methanol, rehydrated in phosphate-buffered saline and stained with DAPI (1 µg/ml). Mounted coverslips were visualized using fluorescence microscope (Olympus IX73).

**Western blotting**

Western blotting was performed as described earlier (27). Equal amounts of protein were immunooblotted and probed with specific primary antibodies for different proteins for 16h at 4°C followed by anti-mouse/anti-rabbit horseradish peroxidase conjugated secondary antibody for 1h at room temperature. The proteins were visualised using enhanced chemiluminescence detection system. β-actin was used as loading control.

**Statistical analysis**

For western blots, densitometry was conducted using BD Quantity One software (Bio-Rad). Data were normalised to loading control before statistical analysis. Expression of wild-type HCT116/control cells was taken as one and fold change was calculated for the resistant cells/treatments. Results were depicted as mean ± standard deviation. For animal studies results are shown as mean ± standard error of mean. Statistical analysis was conducted using Prism Graph Pad software. P values were estimated using Student’s unpaired t-test. Value of P < 0.05 was considered to be statistically significant.

**Results**

**Docking of diaminothiazole analogues with β-glycoprotein and β- tubulin isotype homology models**

Overexpression of protein efflux pump is one of the major resistance mechanisms acquired by tumour cells. A preliminary docking study of diaminothiazoles with β-glycoprotein was performed using the web server developed by Bikadi et al (31). Docking energies of 14 diaminothiazole compounds (Supplementary Figure S1, available at Carcinogenesis Online) with β-glycoprotein have been tabulated in Supplementary Table S2, available at Carcinogenesis Online. Docking energy for most of the diaminothiazole analogues were found to be higher compared with taxol and vinblastine, which are known substrates of β-glycoprotein. Lower binding affinity of diaminothiazoles with β-glycoprotein indicates that diaminothiazoles could be poor substrates of the pump and would possibly overcome β-glycoprotein mediated efflux mechanisms in resistant tumour cells.

Specific β- tubulin isotypes have been found to be differentially expressed in normal epithelial cells and patient tumours treated with antimitotic agents (32). Based on the C-terminal amino acid differences (33), homology models of the various β- tubulin isotypes for colchicine binding sites were constructed.

Diaminothiazole analogues were docked to these isotype models. All the compounds docked flexibly to αβII, αβII/V isotypes which are implicated in antimitotic drug resistance (Supplementary Table S3, available at Carcinogenesis Online). Docking of diaminothiazoles with αβIV tubulin isotype could not be studied due to non-availability of data for amino acid substitution at the colchicine binding site. Docking studies indicated that diaminothiazoles could be effective in drug resistant cells expressing varied β-tubulin isotypes.

**Cytotoxicity studies of anticancer agents in multidrug resistant cell lines**

To verify the in silico results and to determine the potency and degree of resistance suffered by cells against various diaminothiazole analogues, model multidrug resistant sublines—HCT116/TaxR and MCF7/TaxR were developed from parental cell line HCT116 and MCF7; which could grow in media containing 75 nM (6 times IC50) and 10 nM (2 times IC50) taxol, respectively. Cytotoxicity studies were performed in both parental and resistant sublines for taxol and other clinically used anticancer agents along with diaminothiazoles. As shown in Table 1, HCT116/TaxR cells were resistant to taxol by about 93 times as compared with wild-type HCT116 cells, and they were cross resistant to vinblastine (32-fold), doxorubicin (7.5-fold) and colchicine (3-fold). But there was no significant change in the sensitivity profile for DNA binding agent 5-fluorouracil. Combretastatin A4, Podophyllotoxin and Epothilone B displayed comparatively lower IC50 values in HCT116/TaxR cells than in parental HCT116 cells. Three potent diaminothiazole analogues—DAT1, DAT22 and DAT34 were also found to be cytotoxic in HCT116/TaxR cells with similar efficacy as in the parental HCT116 cells (Table 1). The MCF7/TaxR cells, which were developed for a shorter time span of exposure to taxol, were resistant to taxol by 34 times, displayed cross resistance towards vinblastine (7 times) and colchicine (2 times). Diaminothiazole DAT1 was also active in MCF7/TaxR cells (Table 2). Similarly DAT1 was found to be cytotoxic in the resistant uterine sarcoma MES-SA/Dx5 cells, which has been reported previously to show multidrug resistant phenotype by overexpressing P-glycoprotein (34), when compared with other antimitotic agents like taxol (RI 1126) and vinblastine (RI 357 6) (Table 2). Thus, cytotoxicity studies indicated that diaminothiazoles were efficacious in multidrug resistant cancer cells.

It has been observed that small molecules also face the problem of resistance upon prolonged treatment (35). However, limited information is available about the mechanism of resistance developed by cells against small molecules. The mechanisms are likely to differ from those against large molecules as small molecules are normally exempted from transmembrane pumps. Thus, we became interested to study whether prolonged treatment with diaminothiazoles could induce resistance mechanisms in cancer cells and the resistance mechanisms evolved therein. Using the lead diaminothiazole DAT1 on HCT116 cells and MCF7 cells, we generated resistant sublines, HCT116/DAT1R, HCT116/DAT1Rυ, and MCF7/DAT1R, by stepwise selection with the compound DAT1.

**Cytotoxicity studies in DAT1 resistant sublines**

To determine the resistance status of DAT1 resistant cells, cytotoxicity measurements were performed. HCT116/DAT1R cells showed 40 times increase in IC50 value for DAT1 (Table 1). These cells showed slight cross resistance to antimitotic compounds colchicine, podophyllotoxin, combretastatin A4 and more active diaminothiazole analogues DAT22, DAT34 and a higher cross resistance to DAT7 (20 times), an analogue which had similar IC50 value to DAT1 in the parental HCT116 cells (Table 1). Other anticancer agents, both large molecules (molecular weight > 500) such as taxol, vinblastine, doxorubicin and small molecules (molecular weight < 500) such as 5-fluorouracil and epothilone B, exhibited similar or better potency in HCT116/DAT1R cells (Table 1). Cytotoxicity studies in an intermediate line grown up to 500 nM DAT1 concentration (HCT116/DAT1Rυ) showed 11
Cytotoxicity of diaminothiazole analogues and some anticancer agents in wild-type colon cancer cells, resistant sublines and in primary cultures from resistant tumours

<table>
<thead>
<tr>
<th>Compound (molecular weight)</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; (µM) (RI)</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; (µM) (RI)</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; (µM) (RI)</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; (µM) (RI)</th>
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<tbody>
<tr>
<td>DAT1 (325.42)</td>
<td>0.02 (1.23)</td>
<td>0.01 (1.13)</td>
<td>0.03 (1.11)</td>
<td>0.00 (0.69)</td>
</tr>
<tr>
<td>DAT7 (355)</td>
<td>0.02 (1.23)</td>
<td>0.03 (1.13)</td>
<td>0.04 (1.11)</td>
<td>0.00 (0.69)</td>
</tr>
<tr>
<td>DAT22 (385.48)</td>
<td>0.04 (1.13)</td>
<td>0.03 (1.13)</td>
<td>0.05 (1.00)</td>
<td>0.00 (0.69)</td>
</tr>
<tr>
<td>Colchicine (414)</td>
<td>0.05 (1.13)</td>
<td>0.03 (1.13)</td>
<td>0.06 (1.00)</td>
<td>0.00 (0.69)</td>
</tr>
<tr>
<td>Podophyllotoxin (414)</td>
<td>0.06 (1.13)</td>
<td>0.03 (1.13)</td>
<td>0.07 (1.00)</td>
<td>0.00 (0.69)</td>
</tr>
<tr>
<td>Epothilone B (507)</td>
<td>0.07 (1.13)</td>
<td>0.03 (1.13)</td>
<td>0.08 (1.00)</td>
<td>0.00 (0.69)</td>
</tr>
<tr>
<td>Taxol (853.91)</td>
<td>0.25 (93.33)</td>
<td>0.25 (93.33)</td>
<td>0.26 (93.33)</td>
<td>0.25 (93.33)</td>
</tr>
<tr>
<td>5-Fluorouracil (130)</td>
<td>0.3 (1.5)</td>
<td>0.3 (1.5)</td>
<td>0.3 (1.5)</td>
<td>0.3 (1.5)</td>
</tr>
<tr>
<td>Doxorubicin (579.98)</td>
<td>0.3 (1.5)</td>
<td>0.3 (1.5)</td>
<td>0.3 (1.5)</td>
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<tr>
<td>Podophyllotoxin (414)</td>
<td>0.3 (1.5)</td>
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<td>Epothilone B (507)</td>
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Values represent for cells isolated from one mouse out of three tested. Standard deviation could not be calculated for DAT1 for HCT116/DAT1R primary cells as the values reduced drastically in second or third passage.

Mechanism of cell death in the resistant cell line HCT116/TaxR

To check whether the resistant cells preserve their diaminothiazole induced cell death mechanism as in the drug sensitive HCT116 cells (22), we checked for apoptosis in the lead diaminothiazole DAT1 treated cells. Nuclear condensation was measured by labelling the cells with DNA binding agent DAPI. Both HCT116 and HCT116/TaxR cells treated with DAT1 (0.5 µM) showed condensed chromosomes at the periphery of nuclear membrane and fragmented nuclear bodies indicating apoptosis (Figure 1C). Although 20 nM taxol treatment could bring apoptosis in parental HCT116 cells, a very high concentration of taxol (1 µM) was required to bring similar effect in the HCT116/TaxR cells (Figure 1C). Upon treatment with 0.5 µM DAT1, 53% of HCT116 cells and 49% of HCT116/TaxR cells showed condensed chromatin when stained with DAPI (Figure 1D).

Cleaved caspase-3 fragments were also observed in the resistant HCT116/TaxR cells upon 1 µM taxol and 0.5 µM DAT1 treatments (Figure 1E). Cleavage of executionary caspase-3 to its 17/19 kDa active fragments indicated that a caspase dependent cascade was induced followed by apoptosis in HCT116/TaxR cells upon DAT1 treatment.

Further, a cell cycle analysis by flow cytometry detected 58% cells in mitosis after 18 h of 0.5 µM DAT1 treatment which was followed by a sub-G<sub>1</sub> peak of 45.1% after 48 h (Supplementary Figure S4A, available at Carcinogenesis Online). About 20 nM taxol was insufficient to induce significant mitotic arrest in these cells (Supplementary Figure S4B, available at Carcinogenesis Online) even though in parental HCT116 cells, it was able to induce times resistance to DAT1 and showed lesser degree of cross resistance against the other diaminothiazole analogues DAT7, DAT22 and colchicine (Table 1).

The MCF7/DAT1R cells displayed 23 times resistance against the selecting agent DAT1. These cells also showed cross resistance to DAT1 analogues and colchicine but were sensitive to taxol and vinblastine (Table 2).

In vivo antitumour activity of DAT1 in drug resistant xenograft tumour models

To determine whether DAT1 would be effective against xenograft tumours bearing multidrug resistant cells, taxol resistant HCT116/TaxR cells were subcutaneously injected in SCID/NOD mice. After tumour induction, DAT1 was administered in two concentrations—10 and 20 mg/kg body weight, on thrice weekly basis and tumour volume was calculated every week. As shown in Figure 1A and B, after 28 days (12 treatment doses) the vehicle control group had larger tumour volume (mean tumour volume = 1215 mm<sup>3</sup>) than the groups treated with 20 and 10 mg/kg body weight (mean tumour volume 438 and 578 mm<sup>3</sup>, respectively). Mean tumour growth inhibition under this condition was 64% and 52% for the treatment groups 20 and 10 mg/kg body weight, respectively. No significant body weight decrease was observed in the treatment groups. Further to check whether the drug resistant phenotype persisted in the xenograft tumour models, tumour tissue was excised after 28 days, made into single cells and cultured. 3-(4,5-Dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide assay conducted with these cells indicated that in the taxol resistant tumour cells, the multidrug resistant phenomenon persisted, whereas in the cells derived from DAT1 resistant tumour, the resistance against DAT1 reduced drastically and was completely lost after subsequent passaging (Table 1).
67.5% mitotic arrest after 16h (Supplementary Figure S4C, available at Carcinogenesis Online). The concentrations of DAT1 and taxol used were 1.6 times of their IC\textsubscript{50} in HCT116 cells. Thus, DAT1 retained its capacity to induce mitotic block and apoptosis in multidrug resistant cells.

**Characterisation of resistant cell lines**

It was observed that taxol resistant cells were stable in drug free medium whereas cells resistant against DAT1 lost their resistance status after subsequent passaging, so they had to be periodically maintained in DAT1 containing media to maintain selective pressure of the compound. To know the mechanism of action of dianimothiozoles in multidrug resistant cells and to compare with those in DAT1 resistant cell lines, we further characterised the variant sublines.

**Expression of P-glycoprotein in resistant cells and xenograft tumour**

Reverse transcriptase PCR showed that HCT116/TaxR cells overexpressed MDR1 messenger RNA when compared with parental HCT116 cells (Supplementary Figure S5, available at Carcinogenesis Online). Western blotting also showed that the multidrug resistant variants HCT116/TaxR and MCF7/TaxR expressed the 170kDa P-glycoprotein, whereas no detectable P-glycoprotein was observed in the parental cells (Figure 2A and B). However, the DAT1 resistant variants–HCT116/DAT1R\_\textsubscript{HCT}, HCT116/DAT1R and MCF7/DAT1R did not express P-glycoprotein (Figure 2A and B) implying that the resistance displayed by these cells was not arising from the overexpression of P-glycoprotein efflux pump. Western blotting with tumour lysates also showed that the xenograft tumours bearing HCT116/TaxR cells overexpressed P-glycoprotein, which was not observed in the tumour bearing HCT116 or HCT116/DAT1R cells (Figure 2C).

**Efficacy of DAT1 in cells with ectopically expressed P-glycoprotein**

In a further attempt to confirm whether the expression of P-glycoprotein efflux pump deters the cytotoxic action of DAT1 and to eliminate the potential confounding factors associated with the taxol resistance, we transfected HCT116 cell line with the pHaMDRwt transfected HCT116 cells (Supplementary Figure S6B and C, available at Carcinogenesis Online). However, in the pHaMDRwt transfected HCT116 cell line, 20nM taxol treatment could not induce significant apoptosis even though in HCT116 and HCT116 pcdNA transfected cells it induced apoptosis effectively (Supplementary Figure S6B and C, available at Carcinogenesis Online). This showed that overexpression of P-glycoprotein in the pHaMDR1 transfected HCT116 cells protected cells against the cytotoxic action of taxol, whereas DAT1 could still act successfully.

**Altered β-tubulin isotype composition in resistant cells**

Next, we determined the βIII, βIII and IV tubulin isotype expression in the drug sensitive and its resistant variants by western blotting (Figure 2D and E). As reported, the isotype profile varied with cell type. βIII isotype expression was significantly increased in the multidrug resistant variant HCT116/TaxR compared with parental HCT116 cells. βIII expression also moderately increased. However, βIV isotype was downregulated in these cells. In contrast, the expression of βII and IV isotypes were found to be significantly downregulated in DAT1 resistant HCT116/DAT1R cells, whereas there was no change in the βIII tubulin level. However, both MCF7/TaxR and MCF7/DAT1R cells raised from breast cancer line MCF7, which had very low levels of intrinsic βII, βIII and IV tubulin isotypes, displayed a significant increase in all these isotypes (Figure 2D and E).

**Expression of apoptotic proteins in wild-type and resistant cells**

The Bcl-2 family proteins are important regulators of apoptosis. An increase in the ratio of anti-apoptotic/pro-apoptotic proteins denotes that the cell has become resistant to apoptosis. The intrinsic levels of Bcl-2, Bcl-xl and Bax were checked in HCT16, MCF7 and their taxol and DAT1 resistant sublines (Figure 3A left and right panels). Compared with parental HCT116 and MCF7 cells, the ratio of Bcl-2/Bax was significantly increased in both taxol resistant and DAT1 resistant cells. The anti-apoptotic protein Bcl-xl did not have much influence in the acquired resistance against taxol and DAT1 except in HCT116/DAT1R cells, where it showed significant increase in the Bcl-xl/Bax ratio (Figure 3C).

In the parental HCT116 cells, there was a downregulation of Bcl-2 expression upon both taxol and DAT1 treatments (Figure 3B, left panel) resulting in a drastic change in the Bcl-2/Bax ratios (Figure 3C). In the taxol resistant HCT116/TaxR cells, taxol at 20nM concentration could not discernibly alter the expressions of these pro- and anti-apoptotic proteins. A higher drug concentration of Taxol (1 μM) was required for Bcl-2 downregulation and phosphorylation (Figure 3B, right panel). However, similar
(0.5 µM) DAT1 concentration could downregulate anti-apoptotic protein Bcl-2 and could upregulate the expression of pBcl-2 in the taxol resistant cells. No significant change was observed in the Bcl-xl/Bax ratio by either taxol or DAT1 treatment in both drug sensitive and resistant sublines.

Expression of checkpoint proteins in the resistant cells

Faithful segregation of the sister chromatids during mitosis is governed by various spindle checkpoint proteins. Deregulation in the expression of these proteins is associated with chromosomal instability leading to aneuploidy and resistance to spindle poisons like taxol (37). We compared the expression of proteins MAD2 and Bub1 in the resistant lines (Figure 4A and B). MAD2 expression was found to be downregulated in both taxol resistant colon cancer line HCT116/TaxR and breast cancer line MCF7/TaxR cells (0.48 and 0.68 times, respectively when compared with the wild-type cells) (Figure 4B). Bub1 expression, however, was found to be suppressed in HCT116/TaxR but not in MCF7/TaxR. No significant change in the expression
of MAD2 and Bub1 checkpoint proteins were recorded in both the DAT1 resistant lines HCT116/DAT1R and MCF7/DAT1R. In parental HCT116 cells, upregulation of MAD2 and Bub1 happened within 6 and 12 h, respectively by both DAT1 (0.5 μM) and taxol (20 nM) (Figure 4C). At these time points, 0.5 μM DAT1 could upregulate these proteins in HCT116/TaxR cells also, whereas a much increased concentration of Taxol (1 μM) was required to upregulate these proteins thus sensitizing the checkpoint and nullify the effect of resistance. To gain an insight at cellular level, we checked for the expression of protein Bub1 in both taxol sensitive and resistant cells by immunocytochemistry. In metaphase cells, Bub1 remains attached to the kinetochore to sense any unattached or improperly attached microtubule with the kinetochore allowing it to get corrected after which the cell passes to anaphase. In untreated HCT116 cells at metaphase, Bub1 was found to be localized in small amount at the kinetochore. Upon treatment of both taxol and DAT1, when distorted and broken spindles were induced, the kinetochore expression of Bub1 increased to a large extent to reciprocate the improperly attached microtubules to the kinetochores (Figure 4C). At these time points, 0.5 μM DAT1 could upregulate these proteins in HCT116/TaxR cells also, whereas a much increased concentration of Taxol (1 μM) was required to upregulate these proteins thus sensitizing the checkpoint and nullify the effect of resistance. To gain an insight at cellular level, we checked for the expression of protein Bub1 in both taxol sensitive and resistant cells by immunocytochemistry. In metaphase cells, Bub1 remains attached to the kinetochore to sense any unattached or improperly attached microtubule with the kinetochore allowing it to get corrected after which the cell passes to anaphase. In untreated HCT116 cells at metaphase, Bub1 was found to be localized in small amount at the kinetochore. Upon treatment of both taxol and DAT1, when distorted and broken spindles were induced, the kinetochore expression of Bub1 increased to a large extent to reciprocate the improperly attached microtubules to the kinetochores (Supplementary Figure S7, available at Carcinogenesis Online, upper panels). In the resistant variant HCT116/TaxR cell metaphase, the expression of Bub1 in the kinetochore decreased further indicating inefficient spindle checkpoint. However, 0.5 μM DAT1 was highly efficient to enhance Bub1 expression in the kinetochore of these cells also, whereas 1 μM taxol was needed to increase its expression (Supplementary Figure S7, available at Carcinogenesis Online, lower panels).

**Discussion**

Many antimitotic compounds, due to their antiproliferative action, are the foci of cancer treatment. Target of most of the successful antimitotic compounds so far is tubulin. Taxoids, vinca alkaloids, an epothilone analogue Ixabepilone, are being used in the clinics whereas a good number of tubulin binding compounds are in clinical trials (4). Treatment with taxoids and vinca alkaloids suffer severely by acquired resistance and the mechanisms of resistance have been studied in detail. Highly potent small molecules, like Epothilone B, E7010, Combretastatin A4 and 2-methoxy estradiol, show promise on resistant tumours against taxoids and vinca alkaloids, mainly because they are able to evade the transmembrane pumps (16,22,38,39). However, these compounds also evoke resistance in cancer cells upon prolonged treatment as has been our experience with cisplatin (40,41) tamoxifen (42), 5-Fluouracil (43). Thus, it is important to find out agents that are effective against resistant tumours as well as to know the resistance mechanisms against small molecules so that proper combinations can be planned. However, resistance mechanisms against small antimitotic compounds...
have not been explored except a few limited studies (21–23). In this report, we have found that diaminothiazoles, a novel class of potential anticancer compounds of small size, were highly effective in multidrug resistant cancer cell lines and in resistant tumour models. We have also analysed and compared the resistance mechanisms of model cell lines resistant against taxol showing multidrug resistant phenotype and a lead diaminothiazole DAT1 resistant sublines. The resistance profile of colon carcinoma cell line raised against taxol varied for agents with varying size. HCT116/TaxR cells displayed maximum RI against taxol and vinblastine followed by doxorubicin and colchicine. These cells did not exhibit resistance against diaminothiazoles and 5-fluorouracil. Combretastatin A4, podophyllotoxin and epothilone B displayed better activity in this resistant subline. A limited study with another taxol resistant breast cancer cell line MCF7/TaxR also showed similar trend. This is in accordance with the overexpression of P-glycoprotein in these cell lines (Figure 2A–C) which are known to efflux a wide range of hydrophobic compounds ranging from 330 to 4000 daltons (20). Epothilone, however, despite being a compound with medium size (Molecular weight 507), showed better activity in taxol resistant cells.

On the other hand, the DAT1 resistant sublines HCT116/ DAT1R and MCF7/DAT1R developed maximum resistance against the selecting agent DAT1 and moderately towards some of its own analogues. However, these cells did not exhibit cross resistance against most compounds tested. Lack of multidrug resistance is in accordance with the fact that these cells did not express P-glycoprotein. The HCT116/DAT1R cells showed low resistance against other colchicine site binders. The support of these results also came from tumour studies where primary cells isolated from tumour tissues of HCT116/TaxR showed multidrug resistance but those from tumour tissues of HCT116/DAT1R showed loss of resistance even against diaminothiazoles (Table 1). The same phenomenon was reported earlier by Yoshimatsu et al. (22), who found that resistant murine leukemia cells developed against E7010, showed slight resistance against other colchicine site binders. This indicates that the resistance emerging in cancer cells from diaminothiazoles can be easily treated with many other effective anticancer drugs.

The lead compound DAT1 could induce mitotic arrest, followed by apoptosis in HCT116/TaxR cells. Both taxol and DAT1 resistant HCT116 and MCF7 variants showed an increase in the Bcl-2/Bax ratio which contributed to their resistance. DAT1 in its normal effective concentration of 0.5 µM could bring down this ratio in both HCT116 and HCT116/TaxR cells (Figure 3B and C) and thus was able to induce apoptosis effectively. An earlier study also showed that DAT1 was able to induce apoptosis in
colon cancer cells where the anti-apoptotic proteins Bcl-2 and Bcl-xl were ectopically expressed or the pro-apoptotic protein Bax was knocked out (27). Higher concentration of taxol (1 µM) was required to bring down Bcl-2/Bax ratio and to induce apoptosis in these HCT116/TaxR cells.

Tubulin isotype composition has been implicated in antimicrotubule drug resistance. These isotypes vary basically within their 15–20 C-terminal amino acids which are presumed to be binding sites for the various microtubule associated proteins (44). Thus, tubulin isotype composition influences the drug binding parameters. βII and βIVb tubulin isotypes are constitutively expressed, whereas other isotypes are mostly tissue specific (45). Increased levels of βII (10) and βIII tubulin are associated with taxane resistance in lung, breast, ovarian and gastric cancer (12). βII tubulin isotype has been reported to be present in the cell nuclei of many cancers like stomach, colon, pancreas, liver, bone, lung, breast and prostate (46). βIII isotype is found to be upregulated in many metastatic and aggressive tumours (47). βIV tubulin isotype has been found to be differentially regulated in resistant cells (48). Finding drugs that would bind and work against these isotypes will thus aid to widen the therapeutic window of treatment (33). Here, we found by both in silico and wet lab studies that diaminothiazoles worked with similar efficacies in multidrug resistant HCT116/TaxR cells.

Tubulin isotype expression profile was found in resistant colon and breast cancer cells. In colon cancer, DAT1 displayed lower levels of βII, III and IV tubulin isotypes, whereas in breast cancer cells, DAT1 resistance was accompanied with higher expression of these isotypes. Earlier a study that dealt with an acquired resistance against Combretastatin A4 had indicated a reduction in the βIII and IV isotypes (21), although the data could not be confirmed due to the absence of a loading control in the western blot where the expression levels were compared.

Low checkpoint protein expression like Mad1, Mad2, Bub1 and BubR1 corresponds to lack of spindle checkpoint activation. Recent reports have correlated cells with spindle checkpoint

Figure 4. Expression of cell cycle checkpoint proteins in parental and resistant variants. About 20 µg of total cellular protein was loaded for western blotting to check the expression of MAD2 and Bub1 using antibodies as specified in Methods section. Blots are representative of at least two independent experiments. Data have been normalised to the control actin expression. The graph quantitates the fold change in expression of MAD2 and Bub1 proteins from the western blot. ** indicates that the fold change in expression of the checkpoint proteins is significant at P < 0.01 when compared with parental cells. (A) Untreated cells to show the intrinsic protein levels. (B) MAD2 and Bub1 expression in wild-type and resistant cells upon DAT1/Taxol treatment for indicated time points.
defects to resistance against antimitotic drugs like taxol (5,15). Complementary to this, acquired resistance has been correlated with deficiency in the mitotic checkpoint protein expression (49). In this study, we found that taxol resistant cells showed low Mad2 and Bub1 expression. The lead dianionothezole DAT1 could restore these proteins in HCT116/TaxR cells in a concentration that could enhance these protein concentrations in the taxol sensitive HCT116 cells also, whereas a 50 times higher concentration of taxol was required to restore these expressions in the resistant cells. In DAT1 resistant breast and colon cancer cells, we did not find significant change in the expression of these checkpoint proteins.

Thus, using taxol resistant and DAT1 resistant HCT116 and MCF7 sublines, we have found that the resistance against microtubule binding compounds with higher size is multifactorial and stable whereas resistance against microtubule binding compounds with small size are short-lived, specific and is contributed by target specific changes and variations in apoptotic factors. A corollary to this finding is that cancer cells acquiring resistance against large molecules are likely to be multidrug resistant, whereas resistant cells against small molecules would be treatable by many efficacious compounds against that tumour. It is known that methylation and acetylation play a role in P-glycoprotein expression levels (50). Future studies to detect the epigenetic changes that define each resistance mechanism and their relation with drug structure would throw light on the basis of difference between different classes of drugs of varying size. We have also found from this study that dianionothezoles are highly promising against multidrug resistant cancers both in vitro and in vivo.

Supplementary material
Supplementary Figures and Tables S1–S23 can be found at http://carcin.oxfordjournals.org/

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