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ORIGINAL ARTICLE

Protein kinase inhibitors emodin and dichloro-ribofuranosylbenzimidazole modulate the cellular accumulation and cytotoxicity of cisplatin in a schedule-dependent manner

Tetsuji Kurokawa · Guangan He · Zahid H. Siddik

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Abstract
Purpose Protein kinase inhibitors (PKI) have become prominent agents in cancer therapeutics. However, the specificity for target kinase inhibition can be poor and unwanted effects can emerge in combination regimens. The PKI emodin, for instance, can produce mixed results when combined with cisplatin, and we have sought a biochemical pharmacologic explanation for the negative cytotoxic effects.
Methods Human ovarian A2780 tumor cells were exposed to the PKI emodin or dichloro-ribofuranosylbenzimidazole (DRB) with cisplatin using several schedules, and cytotoxicity determined by a growth inhibition assay. Intracellular platinum levels and DNA adducts were estimated by flameless atomic absorption spectrophotometry.
Results When A2780 cells were exposed first to emodin or DRB and then to cisplatin alone, the cytotoxic effects of cisplatin were significantly enhanced, whereas simultaneous exposure did not enhance the cytotoxicity, but instead inhibited it in the case of DRB. The increase in activity of cisplatin in the sequenced schedule was not due to increases in intracellular levels of cisplatin or DNA adducts, whereas the cytotoxic inhibition was related to a significant fall in both intracellular platinum levels and DNA adducts, which were ascribed to inhibition in cisplatin uptake. Knockdown of hCtr1 (the human copper transporter 1) by siRNA abrogated this inhibition in cisplatin uptake.
Conclusion The results demonstrate that co-exposure of tumor cells to emodin or DRB with cisplatin inhibits platinum drug uptake by impacting the hCtr1 transporter and, thereby, reduce the cytotoxicity of cisplatin. Based on our findings, scheduling of the PKI and the cytotoxic agent should be a major consideration in the clinical design of combination regimens.

Keywords Cisplatin · Emodin · DRB · Drug transport · hCtr1 · Resistance

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Introduction

Protein kinases phosphorylate target proteins at serine, threonine or tyrosine residues, and, therefore, are critically involved in regulating every cellular process [1]. When specific protein kinases, such as those encoded by the HER-2/neu and Bcr/Abl genes, are deregulated, they promote tumor formation and its growth, and this has provided a rationale for developing inhibitors against these kinases as potential antitumor agents [2, 3]. The therapeutic approach usually involves the application of the inhibitor as a component of a combination regimen, and the successes of protein kinase inhibitors Herceptin and Gleevec in combination with cytotoxics (such as cisplatin, taxol, methotrexate and cytarabine) against HER-2/neu- and Bcr/Abl-positive tumors, respectively, have validated the approach, and have spurred further development of novel inhibitors in this field

[4–6]. It is noteworthy that some combinations, such as that involving the inhibitor Tarceva with cisplatin and gemcitabine [7], have not demonstrated any benefits, and the cause of such failures is not known.

Emodin (1,3,8-trihydroxy-6-methyl-anthraquinone), a biologically active natural compound extracted from the rhizomes of Rheum palmatum, is a protein kinase inhibitor that also targets the p185 kinase encoded by the HER-2/neu oncogene [8]. In preclinical studies, emodin strongly inhibits p185^{HER-2} and synergizes with cisplatin, doxorubicin and etoposide to overcome drug resistance and increase antitumor effects in HER-2/neu-overexpressing non-small cell lung tumor cells [9]. The inhibitor has also demonstrated an ability to preferentially repress transformation and growth of HER-2/neu-overexpressing breast tumor cells [5], and induce apoptosis in human hepatoma tumor cells [10]. In a separate study, however, emodin reduced the cytotoxic activity of cisplatin and ultraviolet radiation, in part by increasing the expression of ERCC1, a component of the nucleotide excision repair complex that repairs DNA lesions induced by cisplatin and UV [11]. Thus, under certain conditions, emodin protects cells from the apoptotic effects of antitumor agents.

The reason for emodin having contrasting effects on the cytotoxicity of antitumor agents is not clear, but is important to ascertain in order to fully exploit the potential of emodin or its derivatives as viable protein kinase inhibitors in cancer chemotherapy [12]. Since many such inhibitors are non-specific and inhibit phosphorylation of a number of proteins [3, 13], the possibility for antagonizing or nullifying their favorable effects by co-inhibiting other proteins cannot be discounted. In this respect, emodin, for instance, inhibits casein kinase 2 (CK2) more strongly than p185^{HER-2} (inhibitory concentration ID_{50}, 10 vs. 21 μM) [12–14], and this CK2 inhibition has the potential to modulate functions of membrane transporter proteins [15] that could attenuate the cytotoxicity of antitumor agents. Since such an effect may depend on the schedule used, we have examined discrete schedules to explore if this could explain both the increase and decrease in cytotoxicity of the combination comprising emodin and the antitumor agent. For this study, we selected cisplatin to combine with emodin in order to duplicate the combination that has been previously reported with mixed results. In addition to emodin, we selected a structurally different protein kinase inhibitor DRB (5,6-dichloro-1-β-D-ribofuranosyl-benzimidazole) to identify whether the effects are unique to emodin or are a consequence of a common mode of action of these inhibitors. In this respect, DRB also targets a variety of protein kinases, and overlaps with emodin in inhibiting several kinases, such as CK1 and CK2, and in inducing apoptosis in combination with cytotoxic agents [13, 16–19]. Our findings demonstrate that pre-exposure of human ovarian A2780 tumor cells to either emodin or DRB potentiates the cytotoxicity of cisplatin, whereas simultaneous exposure to cisplatin with emodin or DRB either negates this potentiation or inhibits antitumor effects. The failure to potentiate cisplatin potency in the simultaneous exposure protocol was due to reduced intracellular cisplatin accumulation.

Materials and methods

Chemicals

Cisplatin, obtained from Sigma–Aldrich (Milwaukee, WI, USA), was dissolved in normal saline, sterilized using 0.22-mm disc filters, and its concentration confirmed by flameless atomic absorption spectroscopy (FAAS) [20]. Emodin was also purchased from Sigma–Aldrich, with DRB acquired from Calbiochem (La Jolla, CA, USA). Both of these agents were dissolved in DMSO, and stock solutions stored as aliquots at −70°C. The MTT reagent was obtained from USB Corporation (Cleveland, OH, USA). siRNA duplexes (ID SASI_Hs01_00076329) targeting hCTR1 (the human copper transporter 1) and control siRNA (ID 4611) were purchased from Sigma-Genosys (The Woodlands, TX, USA) or Ambion (Austin, TX, USA).

Cytotoxicity studies

The ovarian A2780 tumor cell line, acquired from Dr. Thomas Hamilton, was used in these studies. It was originally established from a patient's biopsy prior to initiation of any chemotherapeutic regimen, and is sensitive to platinum-based drugs [21]. Tumor cells were grown at 37°C in RPMI 1640 medium containing 10% fetal calf serum and antibiotics (100 μg/ml streptomycin and 100 U/ml penicillin), and the effect of drugs on the growth of subconfluent cultures of A2780 cells was determined by the MTT assay using the 5-day continuous drug treatment protocol [22], which provides cytotoxicity data with platinum drugs that is comparable to those obtained with the trypan blue exclusion or the apoptotic Annexin V assay [23]. Briefly, A2780 cells (100 or 200/well) were plated into 96-well plates in 50-μl aliquots and allowed to adhere for at least 24 h at 37°C before initiating treatment with 50 μl of either the drug solution freshly diluted in complete media or the vehicle (saline or DMSO; the latter at a final cell exposure concentration of 0.1%). Cells were exposed to a single agent or to a combination of cisplatin and the protein kinase inhibitor according to one of four scheduling protocols (Fig. 1). Thus, the inhibitor was added to cells either (1) 24 h before (pre-exposure), washed and then simultaneously with cisplatin (Pre + Sim), (2) simultaneously with cisplatin (Sim), (3) as

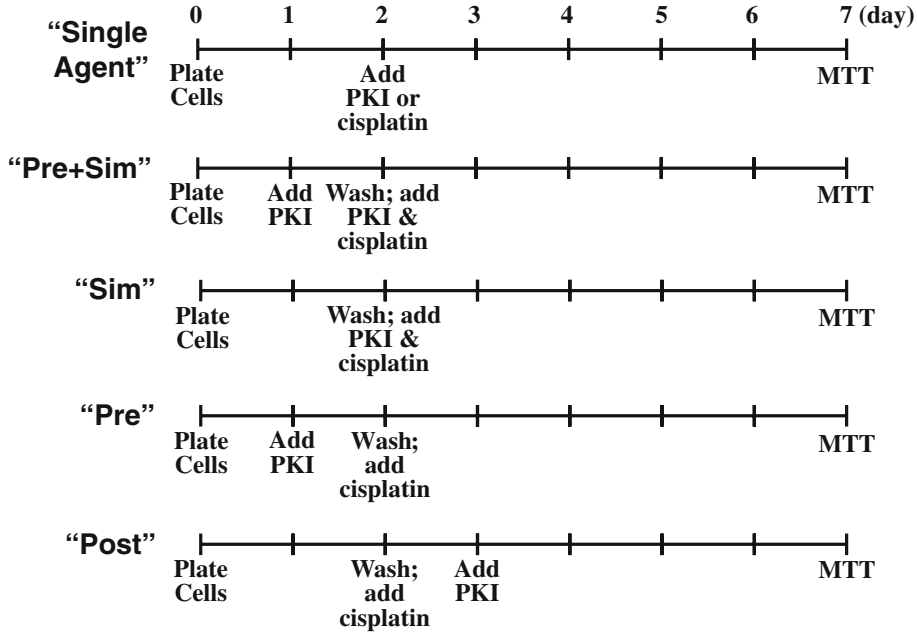

Fig. 1 Drug schedules of cisplatin with or without protein kinase inhibitors (*PKI*) emodin and DRB that were examined in the A2780 tumor model system. In each schedule, the growth inhibitory effects of the drugs were examined by the MTT assay 5 days after exposing cells to cisplatin

a 24 h pre-exposure before washing it out and then adding cisplatin only (Pre), or (4) 24 h after (post-exposure) cisplatin (Post). In each case, the cisplatin exposure time was 5 days. After this time, 50-µl aliquots of an MTT solution (3 mg/ml) were added to each well and plates were incubated for an additional 4 h at 37°C. The media was then removed and replaced with 100 µl of 100% DMSO to dissolve formazan crystals with agitation for 15 min on a shaker. The absorbance was measured at 570 nm using a multi-well scanning spectrophotometer (Molecular Devices, Sunnyvale, CA, USA). The IC_{10}, IC_{50}, and IC_{90} values were determined from a sigmoidal plot of log concentration against A_{570} reading as a percentage of control using the SigmaPlot software. Percent change in cisplatin activity was assessed by the equation [(IC_{50} in control cells − IC_{50} in cells treated with inhibitor) × 100/IC_{50} in control cells].

Intracellular platinum levels and DNA adducts

Evaluations of cellular platinum and DNA adduct levels were conducted in complete medium at 37°C, as described previously [24, 25]. In these biochemical pharmacologic studies, subconfluent cultures of A2780 cells (3–4 × 10^6) in 10-cm dishes were exposed for 4 h to freshly prepared cisplatin alone or simultaneously with either emodin or DRB. In pre-exposure studies, cells were incubated with emodin or DRB for 24 h, washed twice with PBS and then the biochemical pharmacology of cisplatin determined following a 4-h drug exposure. In efflux studies, subconfluent cells were exposed to cisplatin for 4 h, washed twice with PBS, and then incubated with only the protein kinase inhibitor or the vehicle. After 4 or 8 h of incubation, the cells were collected, washed twice with PBS, pelleted by microcentrifugation, resuspended in PBS, and separate aliquots removed to assess protein, cellular platinum and DNA adduct levels. For protein determination by the Lowry procedure, cell aliquots were first digested overnight in 2 M NaOH at 55°C. For DNA-platinum adduct levels, DNA was isolated from cell lysates using the QIAamp DNA Mini Kit (Qiagen, Valencia, CA, USA) using the manufacturer's instructions. Purity and concentration of extracted DNA was assessed by absorption at 260 and 280 nm. Platinum determination was undertaken by FAAS, with cell pellets first dissolved in hyamine hydroxide, as described previously [20].

Knockdown of hCtr1 expression

A2780 cells, grown in antibiotic-free medium for 24 h and then washed in OPTI-MEM I (Invitrogen, Carlsbad, CA, USA), were transfected for 5 h with 100 nM siRNA using LipofectAMINE 2000. After this time, the transfection mixture was removed, cells re-incubated in media without antibiotics for 19 h, and then assessed for knockdown of hCtr1 by the TaqMan Gene Expression real-time PCR assay (Applied Biosystems, Foster City, CA, USA; assay ID: Hs00741015_m1) using the manufacturer's protocol or exposed to cisplatin for 4 h either alone or in combination with the protein kinase inhibitor in antibiotic-free medium for assessment of biochemical pharmacology, as described above.

Statistics

Differences between groups were examined by Student's paired *t* test and were considered significant if $P < 0.05$.

Results

Sensitivity of tumor cells to cisplatin and protein kinase inhibitors

To establish basal sensitivity of ovarian A2780 tumor cells to the drugs of interest, and determine a suitable concentration of the protein kinase inhibitor for use in combination studies, sigmoidal dose-response cytotoxic curves were generated using the SigmaPlot program. The 4-parameter sigmoidal equation was used to provide the best fit to the data and generate good estimates for the IC_{10}, IC_{50} or IC_{90} drug concentrations that induce 10, 50 or 90% inhibition of cell growth, respectively. These values are presented in Table 1, from which the concentrations of 1 μM emodin and 2 μM DRB that approximate the minimally cytotoxic IC_{10} levels were selected for combination studies to assess the effect of protein kinase inhibitors on the cytotoxicity of cisplatin.

Modulation of cisplatin cytotoxicity by protein kinase inhibitors

The effect of emodin or DRB on cisplatin cytotoxicity was assessed by examining changes in the IC_{50} of the platinum drug, with an increase indicative of decreased cytotoxicity and, conversely, a decrease indicative of increased cytotoxicity. A2780 tumor cells were exposed to the combination treatment using four separate schedules. In the "Pre + Sim" protocol, when cells were first pretreated with the protein kinase inhibitor and then simultaneously with the platinum drug, no changes in the IC_{50} of cisplatin were observed (Table 2). However, when cells were exposed simultaneously to the two agents in the "Sim" protocol, emodin again had no effect on the IC_{50} (0.24 vs. 0.25 μM), whereas DRB significantly increased the IC_{50} of cisplatin by about 1.4-fold (0.24 vs. 0.34 μM). In contrast, in the "Pre" protocol, pre-exposure first to either inhibitor significantly decreased the IC_{50} of cisplatin by 1.4- to 1.8-fold (0.18 vs. 0.10 or 0.13 μM). To examine the potential of a post-exposure effect of the inhibitor on the cytotoxicity of cisplatin, the inhibitor was added 24 h after the platinum drug and growth inhibition examined on day 5. In this "Post" protocol, neither inhibitor had any significant effect on the IC_{50} of cisplatin.

Table 1 Activity of cisplatin, emodin and DRB against A2780 tumor cells

	Growth Inhibitory Concentration (μM)		
	IC_{10}	IC_{50}	IC_{90}
Cisplatin	0.095 ± 0.035	0.25 ± 0.05	0.68 ± 0.02
Emodin	0.96 ± 0.50	3.01 ± 0.52	7.58 ± 0.83
DRB	1.44 ± 0.13	3.52 ± 0.48	8.67 ± 1.62

Inhibitory concentrations (IC) suppressing cell growth by 10, 50 and 90% (IC_{10}, IC_{50} and IC_{90}, respectively) are shown as mean ± SEM of 3–5 independent experiments

Table 2 Schedule-dependent modulation of cytotoxic sensitivity of A2780 tumor cells to cisplatin by emodin and DRB

	IC_{50} of Cisplatin (μM)			
	"Pre + Sim"	"Sim"	"Pre"	"Post"
cisPt + Vehicle	0.25 ± 0.03	0.24 ± 0.02	0.18 ± 0.03	0.23 ± 0.05
cisPt + Emodin	0.23 ± 0.04	0.25 ± 0.02	0.10 ± 0.01*	0.20 ± 0.05
cisPt + DRB	0.24 ± 0.06	0.34 ± 0.04*	0.13 ± 0.02*	0.26 ± 0.04

The IC_{50} values represent cisplatin (cisPt) concentrations inducing 50% inhibition of cell growth in the presence or absence of emodin (1 μM) or DRB (2 μM) using the four schedules, and are shown as mean ± SEM of 3–5 independent experiments; * $P < 0.05$ versus "cisplatin + Vehicle"

To better compare the effects of protein kinase inhibitors on cisplatin cytotoxicity, the change in cisplatin activity in the four protocols was calculated, and the results with emodin and DRB presented in Fig. 2. The findings indicate that the only effect of emodin was a significant 45% increase in cisplatin activity in the "Pre" protocol. DRB significantly increased cisplatin activity in the same protocol by a similar margin. On the other hand, DRB also decreased the activity of cisplatin by about 40% in the "Sim" protocol. Thus, when the "Pre" and the "Sim" protocols were combined, the resultant "Pre + Sim" protocol with DRB produced a net effect on cisplatin activity that was not significantly different from cisplatin alone. The "Pre + Sim" protocol with emodin also had no effect, but this was surprising since the net effect of individual results obtained from the "Sim" and "Pre" protocols would be anticipated to show an increase in cisplatin activity.

Effect of protein kinase inhibitors on intracellular platinum and DNA adduct levels

Protein kinase inhibitors have a potential to modulate membrane transport proteins [15, 26], and any changes in cisplatin accumulation and resultant effect on DNA adduct levels can significantly influence cytotoxicity [22]. To examine the possibility that the modulation in cisplatin activity in the "Pre" or "Sim" protocol could be due to an alteration in the biochemical pharmacology of the platinum drug, A2780 cells were exposed to cisplatin for 4 h either

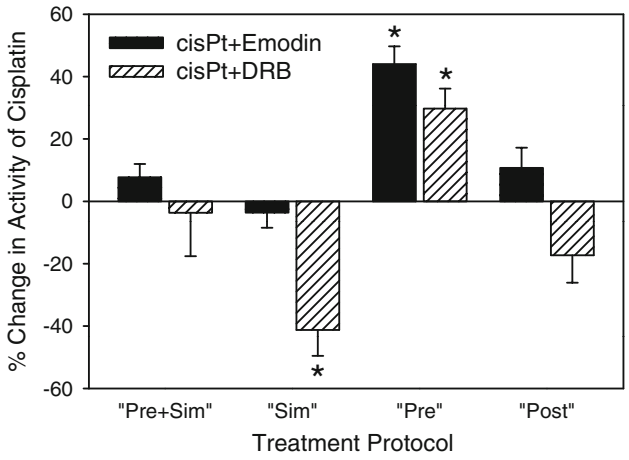

Fig. 2 Modulation of cisplatin cytotoxic activity by emodin or DRB. The results represent changes in IC_{50} as percent of controls (see "Materials and methods"), and show an increase or decrease in cisplatin (cisPt) cytotoxicity by emodin (1 μM) or DRB (2 μM) depending on the schedule employed. Results are presented as mean ± SEM of 3–5 independent experiments; $^*P < 0.05$ versus activity with cisplatin alone (represented by the horizontal line at zero)

after a 24-h pre-exposure to the inhibitor or in the simultaneous presence of the inhibitor (emodin or DRB), and intracellular levels of platinum and DNA adducts then assessed. The results demonstrate that pre-exposure of cells to the inhibitors had no effect on platinum levels (Fig. 3a) or DNA adducts (Fig. 3b) in A2780 tumor cells. Simultaneous presence of the inhibitor, on the other hand, significantly reduced intracellular platinum (Fig. 3c) and DNA adduct levels (Fig. 3d), which correlate with loss of cisplatin potency.

Effect of protein kinase inhibitor on efflux of cisplatin and DNA adduct repair

Reduced cellular accumulation of cisplatin in the presence of emodin or DRB may be due to reduced cisplatin uptake, its increased efflux or both. Since the activity of efflux transporters are known to be altered by protein kinase inhibitors [15, 26], the possible increase in efflux was first examined. In this specific study, A2780 tumor cells were allowed to accumulate cisplatin for 4 h, washed and then transferred to cisplatin-free media for assessment of platinum efflux in the absence or presence of emodin or DRB. The efflux of platinum from cells in absence of the inhibitor was evident by a time-dependent decline in its intracellular level over the 8-h period, with efflux approaching 35% by 4 h and 50% by 8 h (Fig. 4a). The efflux results were similar in the presence of emodin or DRB, and it was evident that reduced accumulation of cisplatin by the protein kinase

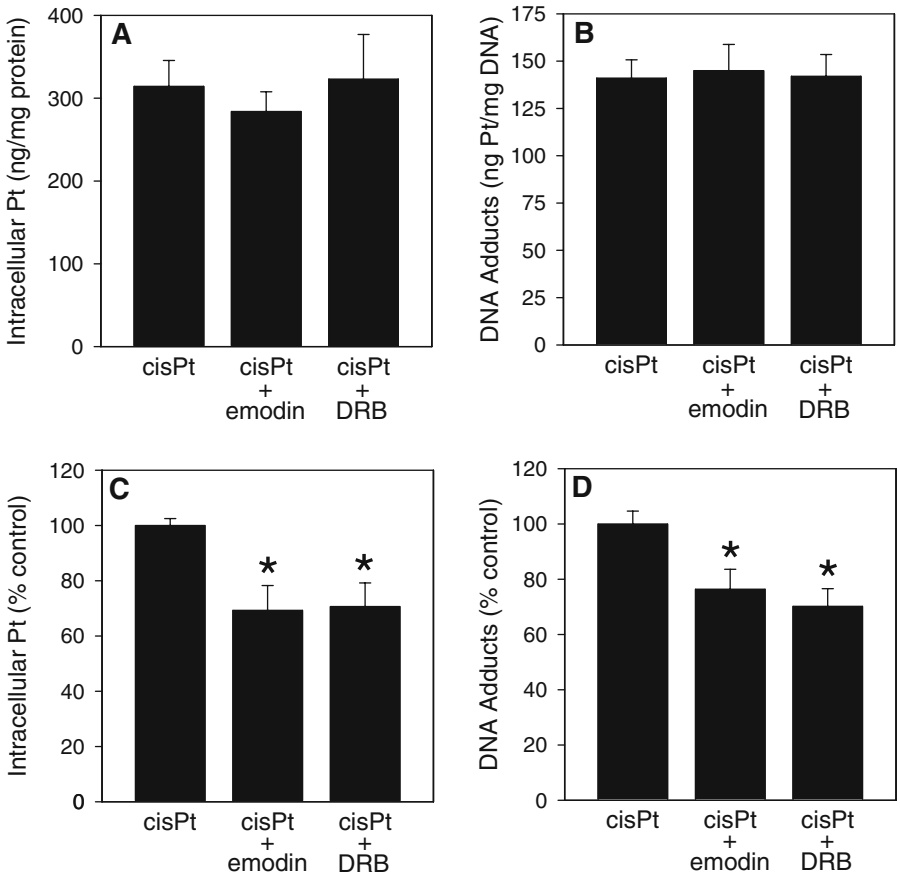

Fig. 3 Schedule-dependent modulation of intracellular platinum and DNA adduct levels by emodin or DRB. **a** Cells were pre-exposed to vehicle, emodin (1 μM) or DRB (2 μM) for 24 h, washed and then exposed to cisplatin (100 μM) for 4 h. Results are shown as platinum levels relative to intracellular protein in the sample. **b** Cells were treated as in **a** and the DNA extracted for the assessment of bound platinum (DNA adducts), which is expressed relative to DNA levels in the sample. **c**, **d** Platinum and DNA adduct levels were determined as in **a** and **b**, but following simultaneous exposure to emodin or DRB. Results are normalized to levels obtained with cisplatin alone. All values are presented as mean ± SEM of 3–5 independent experiments; $^*P < 0.05$ versus levels with cisplatin alone

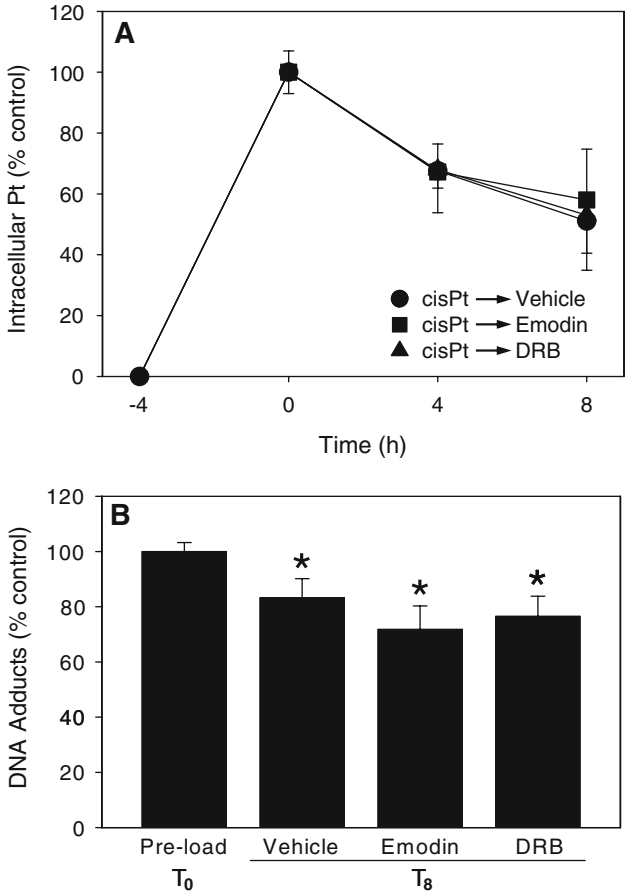

Fig. 4 The effect of emodin or DRB on the efflux of intracellular platinum and repair of DNA adducts. A2780 cells were exposed to cisplatin (cisPt; 100 μM) for 4 h to preload tumor cells, washed and then re-incubated for 4 or 8 h in the absence (vehicle control) or presence of either emodin (1 μM) or DRB (2 μM). **a** Intracellular platinum levels immediately after the 4-h preload (T_0) and during the 8-h efflux period. **b** DNA adducts present at T_0 and after an 8-h post-drug incubation period (T_8), with the difference ($T_0 - T_8$) indicative of DNA repair. Results are expressed as % of platinum or DNA adduct levels at T_0, and are presented as mean ± SEM of 3–5 independent experiments. *$P < 0.05$ versus T_0 levels with cisplatin alone

inhibitor was not due to increased efflux of the platinum drug.

Changes in DNA adducts were also examined at the 8-h post-cisplatin exposure time-point to assess DNA repair, particularly since emodin has been reported to induce ERCC1 and increase DNA repair [11]. The results in Fig. 4b indicate that the reduction in DNA adducts over the 8-h period, as an index of repair, was similar in cells whether exposed or not to the protein kinase inhibitor.

Role of hCtr1 transporter in the reduced accumulation of cisplatin by emodin or DRB

The results in Fig. 4a strongly suggest that inhibition in drug uptake is the underlying basis for reduced intracellular cisplatin levels affected by the protein kinase inhibitor. In this regard, hCtr1 is the major transporter for cisplatin uptake [27], and, therefore, we designed studies to examine if the transport function of hCtr1 is impacted by emodin or DRB. We reasoned that knockdown of hCtr1 by siRNA will prevent further effects of emodin on cisplatin accumulation if this transporter is causally involved. Since the levels of hCtr1 protein in A2780 cells are low and pose difficulty in detection by immunoblot analysis [27, 28], we assessed the effectiveness of siRNA in our A2780 model system by real-time PCR and a functional approach that monitored cisplatin uptake. The results demonstrate that siRNA oligos significantly decreased hCtr1 levels (Fig. 5a), cisplatin accumulation (Fig. 5b) and DNA adducts (Fig. 5c). The 30% reduction in uptake in our system was consistent with hCtr1 knockdown, and similar to that reported in A2780 cells following loss of this transporter through degradation [28] or in Ctr1-deleted yeast cells [29]. This confirmed the effectiveness of siRNA in our studies. Moreover, reduction in cisplatin uptake by knockdown of hCtr1 resulted in a corresponding reduction in DNA adducts.

We next examined the effect of protein kinase inhibitor on cisplatin uptake in A2780 cells pretreated with control or hCtr1 siRNA oligos. The significant reduction in platinum accumulation by emodin or DRB in control siRNA-treated cells was similar to that in hCtr1-knockdown cells that were not exposed to emodin or DRB (Fig. 5d). Furthermore, co-exposure of hCtr1-knockdown cells to cisplatin and either emodin or DRB did not further inhibit cisplatin uptake. Taken together, these data indicate that protein kinase inhibitors attenuate cisplatin uptake by impacting the hCtr1 transporter.

Discussion

The results of our study demonstrate that protein kinase inhibitors emodin and DRB modulate the sensitivity of cisplatin, and this was dependent on the schedule: notably, pre-exposure of tumor cells to the inhibitors potentiated the cytotoxicity of cisplatin, whereas simultaneous exposure with DRB inhibited the antitumor effects of the platinum drug. The reduced cytotoxicity was not due to enhanced efflux of cisplatin or increased DNA repair, but rather to its diminished uptake through a mechanism affecting the hCtr1 influx transmembrane transporter. Although emodin had similar effects to DRB on the biochemical pharmacology of cisplatin following simultaneous exposure, the cytotoxicity of cisplatin in this "Sim" schedule was surprisingly unchanged by emodin.

Protein kinases are often deregulated in cancers, and a number of inhibitors have been developed to inhibit phosphorylation of key proteins and improve cancer

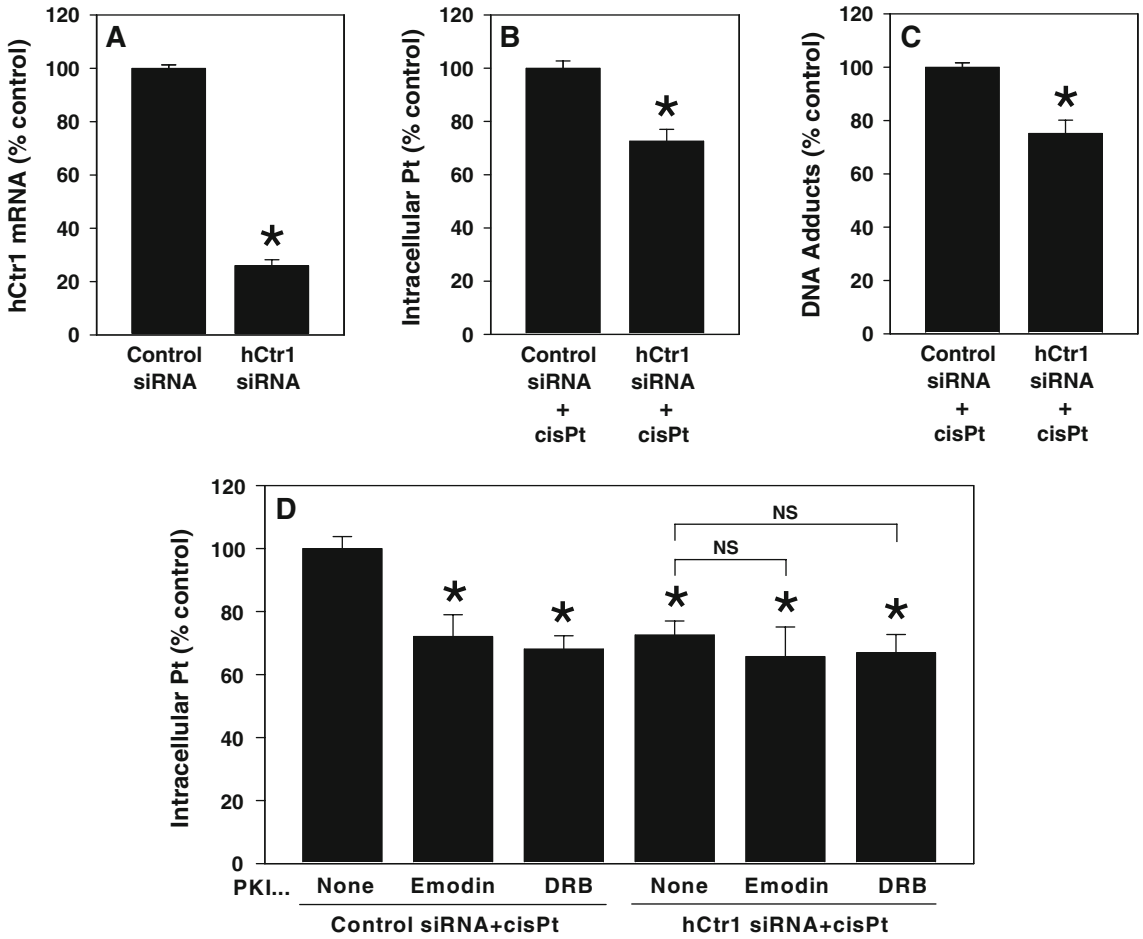

Fig. 5 The effect of hCtr1 knockdown on platinum accumulation and DNA adduct levels. A2780 cells in six-well plates were transfected with either control or hCtr1 siRNA oligos, and then exposed to cisplatin (cisPt; 100 μM) for 4 h in the absence (vehicle control) or presence of protein kinase inhibitor (PKI) emodin (1 μM) or DRB (2 μM). Cells were harvested and platinum and DNA adduct levels determined. The effect of siRNA is indicated by changes in **a** hCtr1 mRNA levels immediately prior to drug treatment, with expression normalized against the housekeeping gene GAPDH, **b** intracellular platinum levels in absence of emodin or DRB, **c** DNA adducts in absence of emodin or DRB, and **d** intracellular platinum levels in the presence of emodin or DRB. All results are shown as % of control siRNA treatment group in the absence of emodin or DRB, and are presented as mean ± SEM of 3–5 independent experiments; $*P < 0.05$ versus control siRNA/cisplatin in absence of PKI; *NS* not significant

management, usually in combination with cytotoxic agents [2, 3]. As a result of search for more effective inhibitors, emodin was identified as also having the potential to increase the cytotoxicity when simultaneously exposed with cisplatin, doxorubicin or etoposide against non-small cell lung cancer (NSCLC) cells overexpressing ectopic Her-2/neu [9]. In contrast, our studies demonstrate that simultaneous exposure of cells to emodin and cisplatin does not change the cytotoxicity of cisplatin. The reason for the discrepancy between the reported data and our results is not readily apparent, but one possibility is the use of relatively higher concentrations of cisplatin (50–75 μM) and emodin (30 μM) in the study with the NSCLC models. Although this high concentration of emodin can inhibit $p185^{Her\text{-}2}$ [12], it can also inhibit a number of other kinases [13] that may be favorable to cisplatin cytotoxicity in NSCLC cells.

For both emodin and DRB, pre-exposure of cells to the protein kinase inhibitors potentiated the cytotoxicity of cisplatin in our study; the exact mechanism of this potentiation is uncertain, but it is clear that the effect is not due to increased intracellular accumulation of the platinum drug or increased levels of DNA adducts. Since the increased cytotoxicity is not observed if exposure to the protein kinase inhibitor occurs 24 h after cisplatin, it follows that the potentiating mechanism of the inhibitor needs to be in effect at the time when cisplatin-induced DNA damage signals are transduced. If cytotoxic potentiation requires inhibition of one or more protein kinases, it is difficult to speculate which could be involved since the target specificity of such inhibitors is poor [3, 13, 14]. CK2 and $p185^{Her\text{-}2}$ kinases are sensitive targets of both emodin and DRB, but the concentration required to inhibit at least 50% activity in

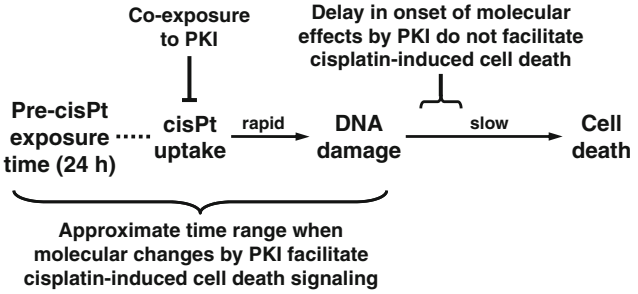

Fig. 6 Model demonstrating modulation of cisplatin cytotoxicity by emodin and DRB. The model indicates that DNA damage by cisplatin (cisPt) is rapid, but the signals leading to cell death proceed slowly. The effect of the PKI (protein kinase inhibitor) on cisplatin-mediated cell death is dependent on the schedule: co-exposure of the PKI with cisplatin immediately inhibits cisplatin uptake and decreases its cytotoxicity; intracellular molecular events of PKI initiated early (as, for instance, following pre-exposure in "Pre" schedule) potentiate cisplatin cytotoxicity; and molecular events of PKI initiated later (as, for instance, following post-exposure in "Post" schedule) do not impact cisplatin cytotoxicity. Treatment with PKI before and during cisplatin (that is, in the "Pre + Sim" protocol) both inhibit and potentiate cisplatin cytotoxicity, with no net change in cell death. The model suggests that in the "Sim" schedule, the favorable intracellular molecular effects of emodin must occur rapidly to neutralize its unfavorable effect from inhibiting cisplatin uptake, whereas the favorable molecular effects DRB must be slow in onset and cannot compensate for the unfavorable effect on cisplatin uptake, with the result that cytotoxicity is attenuated

a cell-free (10–15 μM) or cellular (20–40 μM) system [9, 12, 13, 18, 30–33] is substantially higher than those used in our study (emodin, 1 μM; DRB, 2 μM), and rules out these two kinases as causally involved. However, inhibition of other more sensitive kinases in potentiating cisplatin activity cannot be excluded, nor can the possibility that potentiation of cisplatin activity may not require inhibition of protein kinases.

In contrast to cytotoxic potentiation observed in the sequenced "Pre" schedule, the "Pre + Sim" protocol had no effect on cisplatin cytotoxicity, and the "Sim" protocol was either without effect, as with emodin, or inhibited the cytotoxicity, as with DRB. Although increased repair has the potential to explain some of the observations, we did not observe this in our studies. On the other hand, we did observe reduced accumulation of cisplatin and resultant lower DNA adduct levels in the co-presence of emodin or DRB. Reduced intracellular cisplatin was due to decreased uptake through a mechanism impacting hCtr1, the major transporter of cisplatin uptake [34]. However, a larger fraction of the uptake is by diffusion [35], and this likely explains why the protein kinase inhibitors or hCtr1 knockdown reduced uptake by only about 30%, which is a level consistent with the literature finding following degradation or deletion of this transporter [28, 29]. Reduced cisplatin uptake has also been reported with BAY-43-9006 [36], a Raf kinase inhibitor presently in clinical trials. This allows a pattern to emerge that although the protein kinase inhibitors emodin, DRB and BAY-43-9006 are structurally different and claimed to have distinct target specificity [12, 36, 37], they have a common ability to attenuate cisplatin uptake and its cytotoxic potency. This raises the possibility that other such inhibitors may also demonstrate similar effects.

Although hCtr1 is involved in the reduced uptake of cisplatin by emodin and DRB, the specific mechanism for this is not known. However, the fact that inhibition of cisplatin uptake by emodin or DRB is immediate following simultaneous exposure, and that washout of inhibitor in the "Pre" protocol has no effect on uptake suggests that inhibition requires a direct, but reversible interaction of the inhibitor with hCtr1, possibly through extracellular transmembrane segments. Such an interaction is likely not to require a consensus motif for ATP where the inhibitors could bind, since hCtr1 lacks this motif [38]. On the other hand, facilitated cisplatin uptake by hCtr1 involves the transporter either binding to the drug and internalizing by endocytosis or oligomerizing to form transmembrane channels for drug transport [34], and, therefore, it is possible that emodin may compete with cisplatin for the binding sites on hCtr1 responsible for transport of the platinum agent.

Based on the foregoing discussion, it is clear that reduced uptake of cisplatin and adduct formation by simultaneous combination with DRB is consistent with loss of cisplatin cytotoxicity. In contrast, emodin had a similar effect on the biochemical pharmacology of cisplatin but did not inhibit the cytotoxicity of the platinum drug in the simultaneous (Sim) schedule. The reason for this difference between emodin and DRB is not readily apparent, but an explanation may be advanced using the model shown in Fig. 6. The model takes into consideration two important but opposing effects of protein kinase inhibitors: First, they inhibit cisplatin uptake and cytotoxicity when used simultaneously; second, they augment cisplatin cytotoxicity when intracellular sensitizing events occur prior to signaling from cisplatin-induced DNA damage. In the "Pre" schedule, therefore, sensitization of cells to cisplatin is the only effect by the inhibitors and results in increased cytotoxicity. It follows then that in the "Pre + Sim" schedule, the observed cytotoxicity is a net effect of sensitization on the one hand and inhibition via reduced uptake on the other. In the "Post" schedule, the inhibitors are added to cell cultures after DNA damage signals following cisplatin are already transduced, which occurs rapidly, usually within 2 h after cisplatin treatment when induction of pro-apoptotic p53 is observed [39]. Thus, the cytotoxicity is unlikely to be influenced by the intracellular effect of inhibitors occurring later. These understandings provide a foundation on which to forward a plausible explanation for the contrasting cytotoxic results between emodin and DRB obtained in the "Sim" protocol. In this protocol, it

is likely that emodin not only reduces cisplatin uptake, but also increases sensitivity of cells to the low cisplatin concentration, so there is no net change in cytotoxicity. On the other hand, the reduced cisplatin accumulation affected by DRB is not compensated by an increase in sensitivity, with the result that the net effect is inhibition of cytotoxicity. Differential sensitization by emodin and DRB suggests that intracellular effects of emodin are rapid and occur in a timely manner to interact with cisplatin-induced signals, whereas those of DRB are slower relative to emodin and cisplatin. This indeed may be the case as emodin is reported to impact a variety of intracellular events from within seconds to 3 h [11, 30, 40, 41], whereas DRB takes 4–16 h [42–45], which is well after cisplatin initiates p53 induction [39]. This difference is further appreciated with the protein kinase target CK2, inhibition of which requires pretreatment of cells for 3 h with emodin [30], but 16 h with DRB [43]. The reason for the slower effects of DRB is not known, but appears to be consistent with the differential effects of emodin and DRB in modulating cisplatin cytotoxicity.

In conclusion, our results demonstrate that pre-exposure of ovarian A2780 tumor cells to emodin or DRB potentiates the cytotoxicity of cisplatin, whereas simultaneous exposure to cisplatin and the kinase inhibitor did not show this potentiation, due to inhibition in cisplatin uptake via an effect of the inhibitor on the hCtr1 influx transporter. These results are significant in that they demonstrate a strong schedule dependency for the combination of cisplatin with two structurally unrelated protein kinase inhibitors, and suggest the need to exercise preference for a sequential schedule in therapeutic applications, with the inhibitor given prior to the cytotoxic agent.

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