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ORIGINAL ARTICLE

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Serine/threonine protein phosphatase inhibition enhances the effect of thymidylate synthase inhibition

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Abstract *Purpose*: The serine/threonine protein phosphatases 1 (PP1) and 2A (PP2A) are key enzymes in regulating entry into the cell cycle, mitosis and apoptosis. Inhibition of PP1 and PP2A is associated with enhanced S-phase entry culminating in G_2/M arrest and apoptotic cell death. Thymidylate synthase (TS) is a key regulatory enzyme in DNA synthesis, inhibition of which is often a first-line treatment for colorectal carcinoma. In this study the effect of combining PP inhibition with TS inhibition in two colorectal cell lines was examined. *Methods*: Cantharidin and nolatrexed were used to inhibit PP and TS activity, respectively. The MTT cytotoxicity assay and cell cycle analysis were performed following single-drug treatment of HT29 and HCT116 colorectal cell lines. The median effect method was used to determine a combination index (CI), where drug antagonism was indicated by a CI > 1.1, additivity by a CI between 0.9 and 1.1, and synergism by a CI < 0.9. *Results*: Both cell lines were equally sensitive to cantharidin alone (GI_{50} values 5.4 and 7.3 μM), which induced a significant increase in the S-phase population of both cell lines within 6 h with a concomitant increase in DNA synthesis. This response culminated in G_2/M cell cycle arrest within 24 h and subsequent cell death. In response to nolatrexed alone, HT29 cells were more sensitive than HCT116 cells (GI_{50} 1.9 μM vs 9.8 μM), with G_1/S-phase cell cycle arrest occurring within 24 h in both cell lines. In HT29 cells, this was followed by cell death, whereas in HCT116 cells, a proportion of cells died following arrest but the predominant event was re-entry into the cell cycle. The simultaneous exposure of HT29 cells to the combination of nolatrexed and cantharidin in drug molar ratios of 1:1 and 1:2.5 for 72 h was synergistic producing composite CIs of 0.88 and 0.87, respectively. The sequence of nolatrexed followed by cantharidin 24 h later resulted in greater synergism (CI values of 0.75, 0.52, 0.55, 0.68 for molar ratios of 10:1, 1:1, 1:2.5, 1:10), whereas the reverse sequence was antagonistic, suggesting that the point of interaction is downstream of TS inhibition. In HCT116 cells only additive and antagonistic interactions were observed for any of the treatment combinations. The lack of synergism in these cells may be caused by the reduced sensitivity of these cells to nolatrexed as a single agent. *Conclusion*: The effect of TS inhibition can be enhanced by the inhibition of serine/threonine protein phosphatases.

Keywords PP1 · PP2A · TS inhibition · Combination analysis · Colon

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Introduction

The serine/threonine protein phosphatases 1 (PP1) and 2A (PP2A) (EC 3.1.3.16) are key enzymes in signal transduction mechanisms regulating entry into the cell cycle, mitosis, and apoptosis. PP1 and PP2A are often described as negative regulators of the cell cycle, inhibition of which results in enhanced movement through certain stages of the cell cycle. This enhanced movement following PP inhibition is mediated via the hyperphosphorylation of the retinoblastoma protein and its subsequent inability to bind with the E2F transcription factor, together with enhanced cyclin-dependent kinase activity by preventing the removal of inhibitory phosphorylations [17, 19, 32, 33]. Abnormal entry into the S phase of the cell cycle and checkpoint abrogation has been described for various PP inhibitors [4, 5, 6, 23, 26, 27, 28, 34].

PP1 and PP2A also control specific phases of mitosis including chromosome condensation, nuclear membrane disintegration, reorganization of cytoplasmic microtubules, spindle formation, chromatid separation, nuclear membrane reassembly and cytokinesis [5, 6, 10, 13, 26, 27, 28, 29, 34]. This role is exemplified in yeast and *Drosophila*, where gene mutations in PP1 and PP2A result in various mitotic defects including the inability to complete anaphase [17]. Similarly, PP inhibition by cantharidin, norcantharidin, fostriecin, okadaic acid, calyculin A, and microcystin also induces abnormalities in these mitotic events, culminating in cell cycle arrest and cell death [4, 5, 6, 23, 26, 27, 28, 34].

Apoptosis is also regulated by protein phosphorylation. A mitochondrial pool of PP2A regulates the phosphorylation status and activity of the protooncogene product bcl-2 [3, 7, 24]. PP2A inhibition induces bcl-2 hyperphosphorylation, inhibiting its activity and enhancing the apoptotic death pathway [2, 7]. Furthermore, antisense PP2A transfectants have reduced bcl-2 activity and are more susceptible to apoptosis [12].

Since serine/threonine PPs control key components of cell cycle progression as well as the death pathway, we have proposed that these enzymes are potentially novel targets for the treatment of malignancy [14, 15, 16, 17, 18]. Cantharidin is a small molecule PP1 and PP2A inhibitor [17] suitable for anticancer treatment. It is membrane-permeable, does not require intracellular activation, is not a substrate for p-glycoprotein, and does not cause myelosuppression. In cell lines cantharidin induces unique biphasic cell cycle alterations involving accelerated movement into the S phase of the cell cycle manifest by an increased S-phase population and enhanced DNA synthesis, culminating in G_2/M cell cycle arrest and apoptotic cell death [26]. Thus, in the present study we sought to determine the potential of combining PP inhibition with a conventional antimetabolite agent that mediates its effects during the S phase of the cell cycle. Other protein phosphatase inhibitors have previously been shown to enhance the effect of anticancer treatment, e.g. PP2A inhibition by okadaic acid and calyculin A have been shown to enhance ATRA-induced granulocyte differentiation [20] and to sensitize cells to radiation treatment [21], but the mechanism involved has not been clarified.

In the present study we explored the interaction of cantharidin with the thymidylate synthase (TS, EC 2.1.1.45) inhibitor, nolatrexed (Thymita, AG337; Zarix) (K_i 11 n*M*) [9, 30]. TS is a key regulatory enzyme in DNA synthesis and catalyses the conversion of dUMP to dTMP by methylation using the cosubstrate N5,N10-methylene tetrahydrofolate (CH_2-THF) as a methyl donor. This step is the only de novo source of dTMP, which is subsequently metabolized to dTTP exclusively for incorporation into DNA during synthesis and repair [11]. TS inhibition results in the depletion of dTTP as well as the accumulation of dUTP pools. The former induces an arrest in DNA synthesis while the latter stimulates a futile cycle of DNA uridine misincorporation and subsequent excision by uracil-DNA glycosylase (UDG).

TS inhibition plays a pivotal role in anticancer treatment and is the first-line treatment of many cancers particularly colorectal tumours [22]. The quinazoline TS inhibitor, nolatrexed, was specifically chosen in this study over more conventional agents since it is a small molecular noncompetitive direct and specific inhibitor of TS, with no requirement for active membrane transport or intracellular activation [9, 30]. We have previously shown that nolatrexed induces S-phase cell cycle arrest, biphasic mitochondrial alterations and subsequent apoptotic cell death in various leukaemia cell lines [25]. The lack of intermediate steps in the action of both cantharidin and nolatrexed enables the examination of the combined effects of these drugs without the confounding effect of interfering with the initial drug target interaction.

Methodology

Cell culture and stock solutions

Stock solutions were prepared as follows and stored at −20°C: cantharidin (Biomol, USA) 12.5 m*M* in dimethyl sulphoxide (DMSO); propidium iodide (Sigma, St. Louis, Mo.) 400 μg/ml in phosphate-buffered saline (PBS); RNase A (Sigma) 5 mg/ml in distilled water; and MTT (Sigma) 5 mg/ml in water. All cell lines were cultured at 37°C, under air containing 5% CO_2. HT29 cells (human colon carcinoma, p53 mutant) were maintained in DMEM (Trace Biosciences, Australia) supplemented with 10 m*M* sodium bicarbonate, and HCT116 (human colon adenocarcinoma, p53 wildtype) cells were maintained in McCoy's 5A (Sigma). All culture medium preparations were further supplemented with 10% foetal bovine serum, penicillin (100 IU/ml), streptomycin (100 μg/ml), and glutamine (4 m*M*). Cells were passaged every 3–7 days and all cell lines were routinely tested and found to be mycoplasma free.

In vitro growth inhibition assay

Cells in logarithmic growth were transferred to 96-well plates in 100 μl medium at a density of 2500–2800 cells per well. On day 1 (24 h after plating), 100 μl medium with or without the test agent was added to each well in triplicate. On day 4 at the end of the drug exposure period (72 h), the growth-inhibitory effects were evaluated using the MTT (3-[4,5-dimethylthiazol-2-yl] 2,5-diphenyl-tetrazolium bromide) cell growth assay and absorbance read at 540 nm. The GI_{50} is the drug concentration at which cell growth is inhibited by 50% of untreated control cells based on the difference between the optical density values on day 0 and those at the end of drug exposure.

Cell cycle analysis

Tumour cells in logarithmic growth were transferred to six-well plates at a density of 2×10^5–2.5×10^5 cells/well. On day 0 (24 h after plating), the cells were treated with or without the test agent. The cells were harvested 24 h after drug treatment and washed twice in PBS, fixed in 70% ethanol and stored overnight at −20°C. The cell pellet was incubated in 600 μl PBS containing propidium iodide (40 μg/ml) and RNase (200 μg/ml) for at least 30 min at room temperature. The samples (1.5×10^4 events) were analysed for fluorescence (FL2 detector, filter 575/30 nm bandpass) using a

FACScan (Becton Dickinson). Cell cycle distribution was assessed using Cell Quest software. Experiments were each performed on three to six separate occasions. Values are the means ± SEM.

DNA synthesis

DNA synthesis was assessed by administering [^{3}H]thymidine (1 μCi/ml, specific activity of 49 Ci/mmol; Amersham) to 1×10^6 cells during the final 30 min of a 2–6 h cantharidin exposure. At the end of the incubation period, the medium was removed, the cells were washed twice with PBS, then washed once with 10% TCA, followed by incubation with 10% TCA for 30 min at 4°C. The TCA-insoluble material was washed twice with ethanol, solubilized in 0.5 *M* NaOH, and then buffered to neutral pH. [^{3}H]Thymidine incorporation was determined by liquid scintillation spectrometry. The results are expressed as the means ± SEM of three independent experiments and are the fold increase in [^{3}H]thymidine uptake compared with untreated control.

Treatment schedule for combination analysis

The GI_{50} values obtained in response to nolatrexed and cantharidin as single agents were used to determine the optimal drug molar ratios (nolatrexed:cantharidin) for combination analysis. Since the response of each cell line to nolatrexed and cantharidin differed, various molar ratios were chosen including 10:1, 1:1, 1:2.5 and 1:10. A schedule of either simultaneous or sequential drug treatment was adopted. For simultaneous treatment the cells were plated as described above and 50 μl nolatrexed and 50 μl cantharidin were added on day 1 and the growth inhibition determined on day 4. For the sequential regimen 50 μl of drug was added on day 1 and 50 μl of the alternate drug was added on day 2. The control treatment for each regimen involved adding 50 μl of drug-free medium.

Combination assay

The combined drug effect was evaluated using the median effect principle [1]. The combination index (CI) was calculated using the formula: $CI=[(D)_1/(D_x)_1]+[(D)_2/(D_x)_2]+[(D)_1(D)_2/(D_x)_1(D_x)_2]$. Where $\alpha=1$ for mutually nonexclusive drugs and $\alpha=0$ for mutually exclusive drugs. $(D)_1$, $(D)_2$, and $(D)_1(D)_2$, are the doses of the separate drugs and their combination in a fixed ratio; and $(D_x)_1$, $(D_x)_2$, and $(D_x)_1(D_x)_2$ are the doses resulting in a growth inhibition of *x*%. These doses were calculated using the formula: $D=Dm[FA/(1-FA)]^{1/m}$ where Dm is the dose required to produce a 50% growth inhibition (GI_{50} values from the dose response curves), FA is the fraction affected (an FA value of 0.25 is growth inhibition of 25%), and m is the slope of the median-effect plot. Theoretically, a CI less than 1 is indicative of synergism, greater than 1 indicative of antagonism, and equal to 1 indicative of additivity. In our experience these limits are best set at $CI<0.9$ for synergism, and $CI>1.1$ for antagonism. A composite CI was calculated from the mean of the individual CIs at 0.5, 0.75, 0.9 and 0.95 FA.

Statistical analysis

The results were assessed by analysis of variance. Percentage data were arcsine-transformed before analysis. All values presented in the figures are means ± SEM of the nontransformed data. Data were analysed using Statistica (ver. 4.5; Statsoft, Tulsa, Okla.).

Results

Growth inhibition

In the MTT growth response assay HT29 cells were more sensitive to nolatrexed treatment after 72 h than HCT116 cells (GI_{50} 1.9 ± 0.4 μ*M* vs 9.8 ± 1.4 μ*M*; Fig. 1). Even at very high concentrations of nolatrexed (100 μ*M*), growth inhibition reached a maximum of 75% after 72 h. The response curves for cantharidin treatment of the cell lines produced similar GI_{50} values in HT29 (GI_{50} 5.4 ± 0.6 μ*M*), and HCT116 (GI_{50} 7.3 ± 0.6 μ*M*) cells. However, HT29 cells were actively killed by cantharidin (surviving fraction less than baseline) in contrast to HCT116 (surviving fraction approached baseline) in which cantharidin appeared to be only cytostatic. Cantharidin induced a steeper dose-response curve in both cell lines compared to nolatrexed. This is not surprising as antimetabolite drugs such as nolatrexed require the cells to be actively synthesizing DNA and therefore affect only a limited population of cells at any given time.

Cell cycle effect in response to nolatrexed

In order to further clarify the upstream effect of inhibiting TS and PP, temporal cell cycle changes were examined for each cell line in response to 5 μ*M* and 50 μ*M* nolatrexed (Table 1) and 25 μ*M* and 50 μ*M* cantharidin (Table 2). Since nolatrexed is a slow-acting antimetabolite, the cell cycle responses were examined over 120 h while cantharidin was limited to only 24 h. In HT29 cells, exposure to nolatrexed (5 μ*M*) for 24 h induced S-phase cell cycle arrest as shown by an increase in the S-phase population from 24% to 42%. This response continued for the next 72 h, together with a

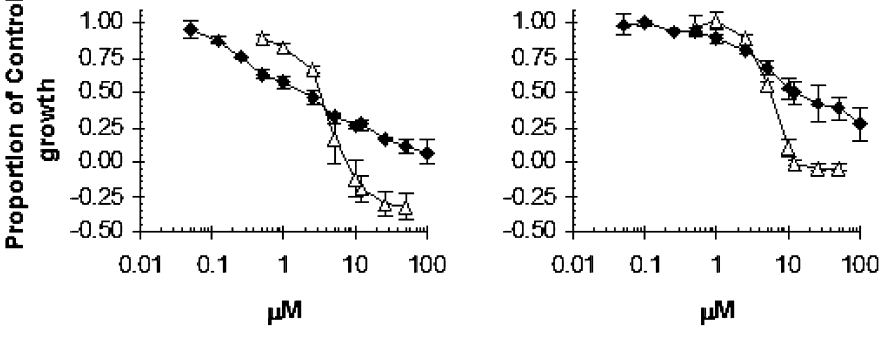

Fig. 1 Growth response curves of (*left*) HT29 and (*right*) HCT116 colorectal cell lines 72 h after continuous exposure to cantharidin (△) and nolatrexed (◆)

Table 1 Cell cycle distribution of HT29 and HCT116 colorectal cells following nolatrexed treatment. Values are means ± SEM

Cell line	Nolatrexed (μ*M*)	Exposure time (h)	Cell cycle distribution (% of total cells)			
			sub-G_1	G_1	S	G_2 + M
HT29	5	0	4.1 ± 0.4	38 ± 2.3	24 ± 1.1	34 ± 1.4
		24	6.3 ± 3.8	21 ± 6.2	42 ± 6.1*	30 ± 4.6
		48	10 ± 1.8*	14 ± 4.8*	52 ± 3.1***	24 ± 3.9
		72	22 ± 8.6	10 ± 1.9**	41 ± 4.2*	27 ± 3.8
		96	24 ± 7.1*	9 ± 1.3***	42 ± 3.5**	25 ± 5.0
		120	32 ± 9.6*	21 ± 10	27 ± 2.0	20 ± 7.2
	50	0	4.0 ± 0.3	39 ± 1.5	23 ± 0.8	34 ± 1.3
		24	4.0 ± 0.6	31 ± 5.9	34 ± 5.7	31 ± 4.0
		48	7.4 ± 2.3	31 ± 8.6	38 ± 6.6	24 ± 2.8
		72	22 ± 7.8	25 ± 5.8	34 ± 3.0*	19 ± 4.7*
		96	34 ± 14	20 ± 6.0*	31 ± 4.2	15 ± 7.6
		120	32 ± 7.7*	19 ± 9.2	34 ± 3.9*	15 ± 4.4*
HCT116	5	0	3.6 ± 0.9	41 ± 0.6	24 ± 2.6	32 ± 1.7
		24	5.4 ± 1.3	26 ± 2.5**	40 ± 4.6*	29 ± 3.4
		48	13 ± 2.5*	13 ± 1.6***	16 ± 2.2	58 ± 3.1**
		72	15 ± 3.3*	24 ± 0.6***	18 ± 1.7	43 ± 4.0*
		96	19 ± 3.4**	20 ± 1.8***	15 ± 1.5*	46 ± 3.4*
		120	18 ± 3.1**	21 ± 2.2**	16 ± 0.5*	46 ± 3.5*
	50	0	3.6 ± 0.5	42 ± 0.6	24 ± 2.4	31 ± 1.6
		24	4.4 ± 1.2	31 ± 4.1	43 ± 2.6**	22 ± 5.5
		48	20 ± 6.0*	21 ± 4.4*	41 ± 3.5*	17 ± 2.9*
		72	34 ± 5.6**	14 ± 4.1**	28 ± 1.1	24 ± 4.6
		96	40 ± 4.8***	22 ± 2.3**	24 ± 2.7	15 ± 2.7**
		120	33 ± 3.7***	18 ± 1.4***	25 ± 0.7	24 ± 2.2

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs control (zero dose of nolatrexed)

progressive decline in the G_1 population from 38% to 9%, and an increase in the proportion of dying cells from 4% to 24%. After 120 h of treatment S-phase arrest was no longer apparent and the predominant feature was cell death with 32% of the population presenting degraded DNA. A higher concentration of nolatrexed (50 μ*M*) also induced S-phase arrest with a population increase from 23% to 34%, however, it did not reach statistical significance until after 72 h of treatment. With this concentration of nolatrexed, the S-phase population was restricted to a narrow band close to the G_1/S phase boundary, making it more difficult to resolve the cell populations.

In HCT116 cells, exposure to nolatrexed (5 μ*M*) for 24 h also induced S-phase arrest with the cell population significantly increasing during this phase from 24% to 40%. In contrast to HT29 cells, by 48 h cells had progressed through to the G_2/M phase indicative of the cells reinitiating cell cycle progression. Although cell death accompanied these temporal changes the magnitude of the death response (sub-G_1 population) in HCT116 cells was less than that observed for HT29 cells. These cell cycle changes paralleled the reduced sensitivity of this cell line to TS inhibition (Fig. 1). A higher concentration of nolatrexed (50 μ*M*) also induced S-phase arrest in HCT116 cells within 24 h. This response was maintained for the next 24 h after which cell death was the predominating feature. The reinitiation of cell cycle progression observed at the lower concentration of nolatrexed was absent at the higher concentration.

Cell cycle effects in response to cantharidin

In HT29 and HCT116 cells cantharidin (25 μ*M*) induced a significant accumulation of cells in the S phase of the

Table 2 Cell cycle distribution of HT29 and HCT116 colorectal cell lines following cantharidin treatment. Values are means ± SEM

Cell line	Cantharidin (μ*M*)	Exposure time (h)	Cell cycle distribution (% of total cells)			
			Sub-G_1	G_1	S	G_2 + M
HT29	25	0	2 ± 0.5	44 ± 2	17 ± 1	36 ± 2
		6	1 ± 0.1	30 ± 6	32 ± 2**	39 ± 7
		24	7 ± 1*	11 ± 1**	12 ± 1	70 ± 2**
	50	0	2 ± 0.5	44 ± 2	17 ± 1	36 ± 2
		6	1 ± 0.1	34 ± 7	30 ± 2**	37 ± 8
		24	12 ± 2**	15 ± 4**	12 ± 2	60 ± 5*
HCT116	25	0	2 ± 0.4	43 ± 2	17 ± 1	38 ± 2
		6	6 ± 1	33 ± 1**	32 ± 2**	30 ± 3
		24	6 ± 0.3	26 ± 4	17 ± 3	53 ± 7*
	50	0	2 ± 0.4	43 ± 2	17 ± 1	38 ± 2
		6	5 ± 1	36 ± 2*	27 ± 2*	32 ± 3
		24	19 ± 2*	23 ± 3	11 ± 3	49 ± 4**

*$P < 0.05$, **$P < 0.01$ vs control (zero dose of cantharidin)

Table 3 DNA synthesis in HT29 and HCT116 colorectal cells after exposure to cantharidin

Cantharidin (μ*M*)	Exposure time (h)	^{3}H-Thymidine incorporation[a]	
		HT29	HCT116
25	2	3.5 ± 0.3	4.1 ± 1.2
	4	3.9 ± 1.2	4.5 ± 1.5
	6	2.5 ± 0.1	4.6 ± 0.6
50	2	3.7 ± 0.6	3.8 ± 1.2
	4	3.3 ± 0.7	4.2 ± 1.5
	6	2.3 ± 0.5	3.9 ± 0.8

[a]^{3}H-Thymidine incorporation into DNA is expressed as the fold increase compared with untreated control cells

cell cycle within 6 h. Within 24 h this response culminated in progression through the G_2/M phase and subsequent cell death. A similar response was observed at the higher concentration of 50 μ*M*. Cell cycle alterations induced by cantharidin were induced considerably earlier following drug treatment than those observed with nolatrexed.

Effect of cantharidin on DNA synthesis

In order to clarify the mechanism by which cantharidin induced the accumulation of cells in S phase, the effect of cantharidin on the rate of DNA synthesis after 2–6 h of drug exposure was examined (Table 3). HT29 and HCT116 cells showed a significant 2.5- to 4.6-fold increase in the rate of DNA synthesis in response to cantharidin when compared to untreated cycling cells. Thus, the accumulation of cells in the S phase of the cell cycle observed by cell cycle analysis appeared to be a consequence of accelerated movement into S phase rather than the result of cell cycle arrest. The increased incorporation of thymidine could be due to checkpoint abrogation leading to a greater proportion of cells undergoing DNA synthesis and/or an enhancement in the rate of DNA synthesis.

Combination analysis of thymidylate synthase and protein phosphatase inhibition

Although the cell cycle analysis characterized the upstream effect of inhibiting the target enzymes, the MTT assay was exploited to determine the long-term downstream cell growth inhibition when these drugs were used in combination. Combination analysis of nolatrexed and cantharidin was performed (Table 4) at molar ratios of 10:1, 1:1, 1:2.5, 1:10 with either a simultaneous or sequential treatment schedule. The simultaneous combination of nolatrexed and cantharidin induced a synergistic interaction in HT29 cells at molar ratios of 1:1 (CI 0.88) and 1:2.5 (CI 0.87). These molar ratios were equivalent to the GI_{50} ratio of the single agents for these cells. The simultaneous regimen in HCT116 cells showed additivity or antagonism (Fig. 2). Since cantharidin had previously been shown to mediate its effects considerably faster than nolatrexed, it was decided to administer the agents in a sequential fashion with nolatrexed on day 1 followed by cantharidin 24 h later. As a result, an even greater synergistic interaction was observed in the HT29 cells. Again the molar ratio with the greatest synergistic interaction was 1:1 (CI 0.52). The reverse sequential treatment was antagonistic in both cell lines.

In order to more clearly assess the magnitude of the synergistic response observed in the HT29 cell line, the growth inhibition at GI_{50}, GI_{90} and GI_{100} was determined for each drug alone and sequential treatment (nolatrexed followed by cantharidin) at the molar ratio of 1:1 (Table 5). This analysis showed that total growth inhibition was achieved (GI_{100}) in HT29 cells by administering 7.8 μ*M* nolatrexed on day 1 followed by

Table 4 Combination analysis in HT29 and HCT116 colorectal cells in response to various molar ratios of nolatrexed and cantharidin administered using either a simultaneous or sequential treatment schedule

Cell line	Molar ratio (nolatrexed:cantharidin)	Composite combination index[a]		
		Simultaneous[b]	Sequential[b, c]	
		Nolatrexed and cantharidin	Nolatrexed followed by cantharidin	Cantharidin followed by nolatrexed
HT29	10:1	1.74 ± 0.50	**0.75 ± 0.1**	16 ± 13
	1:1	**0.88 ± 0.22**	**0.52 ± 0.09**	2.89 ± 1.06
	1:2.5	**0.87 ± 0.22**	**0.55 ± 0.07**	1.38 ± 0.12
	1:10	0.96 ± 0.15	**0.68 ± 0.12**	1.16 ± 0.09
HCT116	10:1	1.79 ± 0.46	2.34 ± 1.26	2.74 ± 0.57
	1:1	1.06 ± 0.07	1.15 ± 0.11	1.74 ± 0.43
	1:2.5	1.05 ± 0.02	1.14 ± 0.05	1.22 ± 0.17
	1:10	0.94 ± 0.12	1.09 ± 0.04	0.89 ± 0.14

[a]Calculated as the mean CI at FA 0.5, 0.75. 0.9, and 0.95. A value of < 0.9 is indicative of a synergistic interaction (indicated in bold), a value between 0.9 and 1.1 indicates an additive interaction, a value > 1.1 is antagonistic
[b]The total incubation time was 72 h for both the sequential and simultaneous treatments
[c]The sequential schedule involved treating the cells with the first agent then administering the second agent 24 h later

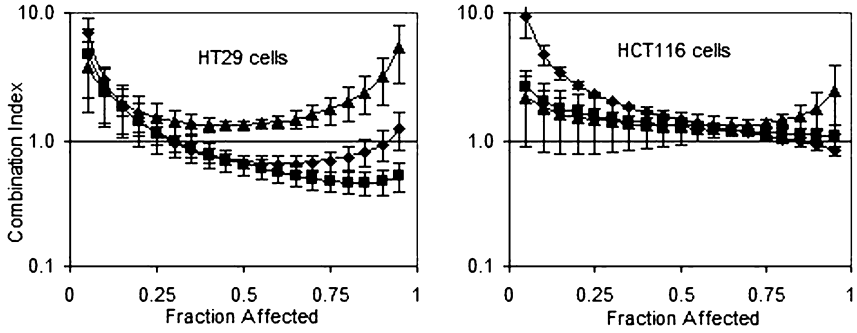

Fig. 2 Combination index curves for a 1:1 molar ratio of nolatrexed and cantharidin in HT29 and HCT116 cells. Treatment schedules were simultaneous (◆), nolatrexed followed by cantharidin (■), and cantharidin followed by nolatrexed (▲). Data points are the means of three to four independent experiments

Table 5 Growth inhibition in HT29 cells with nolatrexed and cantharidin either alone or in combination (nolatrexed followed by cantharidin 24 h later at a molar ratio of 1:1)

Treatment schedule		Growth inhibition day 4[a]	
Day 1	Day 2	Percent inhibition	Drug concentration (μM)
Nolatrexed	Medium	50	1.8 ± 0.2
Medium	Cantharidin	50	4.6 ± 0.4
Nolatrexed	Cantharidin	50	1.7 ± 0.5
Nolatrexed	Medium	90	> 50
Medium	Cantharidin	90	17 ± 3.5
Nolatrexed	Cantharidin	90	7.4 ± 0.9
Nolatrexed	Medium	100	> 50
Medium	Cantharidin	100	> 50
Nolatrexed	Cantharidin	100	15.7 ± 5.0

[a]Total drug concentrations required to induce 50, 90 and 100% growth inhibition (GI_{50}, GI_{90} and GI_{100}, respectively) when analysed on day 4 of the treatment schedule (72 h after treatment commenced)

7.8 μM cantharidin on day 2 (total molar concentration of 15.7 μM). In comparison, neither nolatrexed nor cantharidin alone at 50 μM could induce this level of growth inhibition.

Discussion

Using the colorectal cell lines HT29 and HCT116 we showed that nolatrexed, a specific TS inhibitor, induced G_1/S phase cell cycle arrest, a hallmark of TS inhibition. However, the effect of TS inhibition differed: in HT29 cells this arrest was closely followed by the induction of cell death, whereas in HCT116 cells, the predominant event following arrest was the re-entry of a large proportion of the cells into the cell cycle. This difference was more evident at low concentration (5 μM) than high concentration (Table 1). This process may explain the reduced sensitivity of HCT116 cells to nolatrexed as reflected by the larger GI_{50} value (9.8 μM) compared with that for HT29 cells (1.9 μM) (Fig. 1). Such a resistance to TS inhibition has been observed in cells that overexpress dUTPase, a pyrophosphatase responsible for maintaining low intracellular levels of dUTP [31], in which TS inhibition induces G_1/S phase cell cycle arrest and reduces cytotoxicity compared to wildtype cells. These studies indicate that the cytotoxic effect of TS inhibition results in the depletion of dTTP and G_1/S phase arrest, while the accumulation of dUTP pools and misincorporation of dUTP into DNA is cytotoxic. Cantharidin, a specific inhibitor of serine/threonine PP1 and PP2A, accelerated the entry of HT29 and HCT116 cells into the S phase of the cell cycle (Tables 2 and 3) culminating in G_2/M cell cycle arrest and cell death. Such a response has been reported for other PP inhibitors [4, 23]. As a result of these unique cell cycle events we decided to investigate whether a drug such as cantharidin would enhance the effect of TS inhibition as a consequence of increasing the proportion of cells in the S phase of the cell cycle.

This study showed that the simultaneous combination of nolatrexed and cantharidin induced cytotoxic synergism in HT29 cells, whereby the combination caused cytotoxicity greater than expected from simple addition (Tables 4 and 5). The optimum drug molar ratio for this combination was 1:1, which is comparable to the GI_{50} ratio. Greater synergism was observed when cantharidin was administered 24 h after nolatrexed. The induction of a synergistic interaction was clearly schedule-dependent as the administration of cantharidin followed by nolatrexed was not synergistic but antagonistic. This result was unexpected, as we anticipated greater cytotoxicity as a consequence of a greater proportion of cells in S phase at the time of nolatrexed treatment. Instead, the combination of nolatrexed followed by cantharidin was the most synergistic suggesting that an event downstream of TS inhibition was the point of interaction. In this regard, PP1 and PP2A are known to regulate key events of the apoptotic cell death pathway including p53 and bcl-2 phosphorylation. Inhibition of PP2A with okadaic acid and norcantharidin or the use of antisense PP2A is known to reduce bcl-2 activity and enhance apoptosis [2, 8, 12].

In HCT116 cells a synergistic interaction was not observed using any schedule of cantharidin and nolatrexed. HCT116 cells were fivefold less sensitive to nolatrexed treatment than HT29 cells and this was paralleled by flow cytometry studies which showed a greater tendency to cell cycle arrest followed by resumption, especially at lower concentrations. Theses studies suggest that either there is no direct link between

TS inhibition and PP inhibition in this cell line, or the two processes are mutually exclusive. Again it is possible that PP inhibition may affect components of the cell death pathway downstream of TS inhibition. This hypothesis is the subject of further investigations.

This study suggests that PP inhibition is a novel mechanism whereby the cytotoxicity of conventional chemotherapy may be enhanced. Despite the apparent pleiotropic action of protein phosphatases in human tissue, these findings support the contention that PP2A offers is a valid clinical target for the treatment of malignancy [17]. The clinical use of PP inhibitors in the fight against malignancy is in its infancy. Fostriecin, a potent PP2A inhibitor (IC_{50} 3.4 n*M*) found in *Streptomyces pulveraceus*, has entered a number of phase I clinical trials, but its oxidative instability has prevented its further development in oncology. Cantharidin and its demethylated analogue norcantharidin are currently used in China for the treatment of cancer, but their true potential in mainstream oncology is yet to be fully assessed. The possible mechanisms of interaction of PP inhibition and TS inhibition, some of which have been explored here, remain the object of ongoing investigation.

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References

1. Ackland SP, Kimbell R (1998) Antifolates in combination therapy. In: Jackman AL (ed) Antifolate drugs in cancer therapy. Humana Press, Totowa, p 365
2. Chen Y-H, Chen J-C, Yin S-C, Wang G-S, Tsauer W, Hsu S-F, Hsu S-L (2002) Effector mechanisms of norcantharidin-induced mitotic arrest and apoptosis in human hepatoma cell lines. Int J Cancer 100:158
3. Deng X, Ito T, Carr B, Mumby M, May WS (1998) Reversible phosphorylation of Bcl-2 following interleukin 3 or bryostatin 1 is mediated by direct interaction with protein phosphatase 2A. J Biol Chem 273:34157
4. Ghosh S, Schroeter D, Paweletz N (1996) Okadaic acid overrides the S-phase checkpoint and accelerates progression of G2-phase to induce premature mitosis in HeLa cells. Exp Cell Res 227:165
5. Giese G, Weigers W, Kubbies M, Scherbarth A, Traub P (1995) Okadaic acid co-induces vimentin expression and cell cycle arrest in MPC-11 mouse plasmacytoma cells. J Cell Phys 163:145
6. Gurland G, Gundersen GG (1993) Protein phosphatase inhibitors induce the selective breakdown of stable microtubules in fibroblasts and epithelial cells. Proc Natl Acad Sci U S A 90:8827
7. Haldar S, Jena N, Croce CM (1995) Inactivation of bcl-2 by phosphorylation. Proc Natl Acad Sci USA 92:4507
8. Haldar S, Basu A, Croce CM (1998) Serine 70 is one of the critical sites for drug-induced Bcl-2 phosphorylation in cancer cells. Cancer Res 58:1609
9. Hughes A, Calvert AH (1999) Preclinical and clinical studies with the novel thymidylate synthase inhibitor nolatrexed dihydrochloride (Thymitaq^{TM}, AG337). In: Jackman AL (ed) Antifolate drugs in cancer therapy. Humana Press, Totowa, p 229
10. Inada H, Togashi H, Nakamura Y, Kaibuchi K, Nagata K, Inagaki M (1999) Balance between activities of rho kinase and type 1 protein phosphatase modulates turnover of phosphorylation and dynamics of desmin/vimentin filaments. J Biol Chem 274:34932
11. Jackman AL, Calvert AH (1995) Folate-based thymidylate synthase inhibitors as anticancer drugs. Ann Oncol 6:871
12. Kang HS, Choi IP (2001) Protein phosphatase 2A modulates the proliferation of human multiple myeloma cells via regulation of the production of reactive oxygen intermediates and anti-apoptotic factors. Cell Immunol 213:34
13. Liao H, Li Y, Brautigans D, Gundersen GG (1998) Protein phosphatase 1 is targeted to microtubules by the microtubule-associated protein tau. J Biol Chem 273:21901
14. McCluskey A, Sakoff JA (2001) Small molecule inhibitors of serine/threonine protein phosphatases. Mini Rev Med Chem 1:43
15. McCluskey A, Bowyer MC, Collins E, Sim ATR, Sakoff JA, Baldwin ML (2000) Anhydride modified cantharidin analogues: synthesis, anti-cancer activity and selective inhibition of protein phosphatase 1 and 2A. Bioorg Med Chem Lett 10:1687
16. McCluskey A, Ackland SP, Gardiner E, Walkom CC, Sakoff JA (2001) The inhibition of protein phosphatases 1 and 2A: a new target for rational anti-cancer drug design? Anticancer Drug Design 16:291
17. McCluskey A, Sim ATR, Sakoff JA (2002) Inhibition of protein phosphatases as a therapeutic target. J Med Chem 45:1151
18. McCluskey A, Ackland SP, Bowyer MC, Baldwin ML, Garner J, Walkom CC, Sakoff JA (2003) Cantharidin analogues: synthesis and evaluation of growth inhibition in a panel of selected tumour cell lines. Bioorg Chem 31:66
19. Morana SJ, Wolf CM, Li JF, Reynolds JE, Brown MK, Eastman A (1996) The involvement of protein phosphatases in the activation of ICE/CED-3 protease, intracellular acidification, DNA digestion, and apoptosis. J Biol Chem 271:18263
20. Morita K, Nishikawa M, Kobayashi K, Deguchi K, Ito M, Nakano T, Shima H, Nagao M, Kuno T, Tanaka C, Shirakawa S (1992) Augmentation of retinoic acid-induced granulocytic differentiation in HL-60 leukemic cells by serine/threonine protein phosphatase inhibitors. FEBS Lett 314:340
21. Nakamura K, Antoku S (1994) Enhancement of X-ray cell killing in cultured mammalian cells by the protein phosphatase inhibitor calyculin A. Cancer Res 54:2088
22. Peters GJ, Ackland SP (1996) New antimetabolites in preclinical and clinical development. Exp Opin Invest Drugs 5:637
23. Roberge M, Tudan C, Hung SM, Harder KW, Jirik FR, Anderson H (1994) Antitumor drug fostriecin inhibits the mitotic entry checkpoint and protein phosphatases 1 and 2A. Cancer Res 54:6115
24. Ruvolo PP, Deng XM, Ito T, Carr BK, May WS (1999) Ceramide induces Bcl-2 dephosphorylation via a mechanism involving mitochondrial PP2A. J Biol Chem 274:20296
25. Sakoff JA, Ackland SP (2000) Thymidylate synthase inhibition induces S-phase arrest, biphasic mitochondrial alterations and caspase dependent apoptosis in leukaemia cells. Cancer Chemother Pharmacol 46:477
26. Sakoff JA, Ackland SP, Baldwin ML, Keane MA, McCluskey A (2002) Anticancer activity and protein phosphatase 1 and 2A inhibition of a new generation of cantharidin analogues. Invest New Drugs 21:1
27. Sassoon I, Severin FF, Andrews PD, Taba M, Kaplan KB, Ashford AJ, Stark MJR, Sorger PK, Hyman AA (1999) Regulation of *Saccharomyces cerevisiae* kinetochores by the type 1 phosphatase Glc7p. Genes Dev 13:545
28. Schwartz DA, Schultz RM (1991) Stimulatory effect of okadaic acid, an inhibitor of protein phosphatases, on nuclear envelope breakdown and protein phosphorylation in mouse oocytes and one cell embryos. Dev Biol 145:119
29. Thompson LJ, Bollen M, Fields AP (1997) Identification of protein phosphatase 1 as a mitotic lamin phosphatase. J Biol Chem 272:29693

30. Webber S, Bartlett CA, Boritzki TJ, Hillard JA, Howland EF, Johnson AL, Kosa M, Margosiak SA, Morse CA, Shetty BV (1996) AG337, a novel lipophilic thymidylate synthase inhibitor: in vitro and in vivo preclinical studies. Cancer Chemother Pharmacol 37:509
31. Webley SD, Welsh SJ, Jackman AL, Aherne GW (2001) The ability to accumulate deoxyuridine triphosphate and cellular response to thymidylate synthase (TS) inhibition. Br J Cancer 85:446
32. Wera S, Hemmings BA (1995) Serine/threonine protein phosphatases. Biochem J 311:17
33. Wolf CM, Morana SJ, Eastman A (1997) Zinc inhibits apoptosis upstream of ICE/CED-9 proteases rather than at the level of an endonuclease. Cell Death Differ 4:125
34. Yatsunami J, Fujiki H, Suganuma M, Yoshizawa S, Eriksson JE, Olsen MO, Goldman RD (1991) Vimentin is hyperphosphorylated in primary human fibroblasts treated with okadaic acid. Biochem Biophys Res Commun 177:1165