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Michael Adducts of Ascorbic Acid as Inhibitors of Protein Phosphatase 2A and Inducers of Apoptosis

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Abstract—Michael adducts of ascorbic acid with α,β-unsaturated carbonyl compounds have been shown to be potent inhibitors of protein phosphatase 1 (PP1) without affecting cell viability at the respective concentrations. Here we were able to show that higher concentrations can partially inhibit PP2A activity and concomitantly induce apoptotic cell death. A nitrostyrene adduct of ascorbic acid proved to be a more potent and effective inhibitor of PP2A as well as a stronger inducer of apoptosis. These adducts only slightly lost their cytotoxic potential in multidrug resistant cells that were 10-fold less sensitive to apoptosis induction by okadaic acid and vinblastine.

Introduction

Signal transduction by reversible phosphorylation of proteins depends on the function of specific ser/thr phosphatases (PP). These enzymes have been divided into four major categories depending on their substrate specificity and the dependency of their activity on divalent cations.[1] Two subtypes, PP1 and PP2A, are not influenced by divalent cations. Their role for signal transduction has been extensively studied with natural toxins such as okadaic acid (OA) and calyculin A which are selective and potent inhibitors.[2] As early as 1991 it was shown that the prolonged inhibition of these two enzymes resulted in the induction of apoptotic cell death in various tumour cells.[3] Terpenoids such as thyrsiferyl-24-acetate and cantharidin are more selective inhibitors of PP2A but are still powerful inducers of apoptosis.[4–6] Such cytostatic properties might be used therapeutically in the treatment of tumours. Testing for anti-tumour activity was already done with norcantharidin and the antibiotic fostriecin which are both preferential inhibitors of PP2A.[7,8] The role of this phosphatase subtype for the determination of cellular survival was supported by the observation that the knockout of the PP2Aα gene resulted in intrauterine death of the affected animals.[9] In this context we investigated the potency of Michael adducts of ascorbic acid for a potential inhibitory effect on PP2A and the associated cytotoxic and pro-apoptotic properties. Experiments were performed with HIT cells, a hamster insulin secreting cell line that responded with apoptosis to treatment with the phosphatase inhibitors OA[10] and cantharidic acid (CA).[11] Interestingly, these cells can be induced to loose their sensitivity for OA.[11] This resistance was achieved by the overexpression of the P-glycoprotein mediating drug extrusion which results in the multidrug resistance (MDR) phenotype compromising the efficiency of many cytostatic drugs.[12] Resistant cells remained completely responsive to death signalling by the terpenoid CA.[11]

Synthesis of Michael adducts of ascorbic acid

The reaction of acrolein with the Michael donor 6-*O*-palmitoyl-L-ascorbic acid resulted in the spiro-compound **1** when the reaction was carried out in isopropanol and in the pyranosyl compound **2** when methanol was used as solvent.[13] The synthesis of adduct **3** was achieved with L-ascorbic acid as donor for a Michael reaction with *p*-chloro-nitrostyrene.[14]

Cell culture, viability assays and isolation of OA-resistant cells[11]

HIT cells were cultured in RPMI 1640 medium supplemented with 10% foetal calf serum at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. Viability was assessed by measuring the conversion of the tetrazolium salt MTT by mitochondrial dehydrogenases. The magenta-coloured formazan product was quantified at 550 nm

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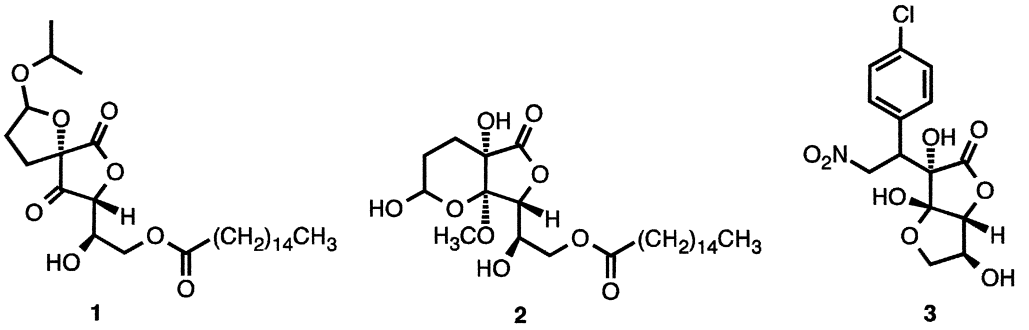

Figure 1. Structures of acrolein (**1**,**2**) and *p*-chloro-nitrostyrene (**3**) Michael adducts of 6-*O*-palmitoyl-L-ascorbic or L-ascorbic acid, respectively.

against a background of 650 nm in a microplate reader (Molecular Devices, USA). OA-resistant sublines were isolated by selecting HIT cells surviving the starting concentration of 10 nM of OA and subsequently raising the concentration up to 100 nM. The respective subclones were labeled HITxxR where the numbers refer to the concentrations of OA constantly present during cultivation.

Measurement of PP2A activity

The dephosphorylation of casein, a fairly specific substrate of PP2A,[15] by an extract from HIT cells was determined with a modified colorimetric assay as described.[11] Inorganic liberated phosphate was measured by the formation of a coloured complex between acidified phosphomolybdate and malachite green at 650 nm in a microplate reader.[16]

Caspase 3 activation and DNA fragmentation analysis

To measure the activation of caspase 3 as a consequence of apoptosis induction, untreated and treated cells were lysed by repeated freeze thawing. Caspase activity was quantified by measuring the colour development resulting from the cleavage of the specific tetrapeptide substrate DEVD-pNA[17] (25 μM) at 405 nm.[11]

Fragmentation of genomic DNA as the result of a caspase activated endonuclease was studied in a modification of described procedures and solutions.[18] The isolation of DNA fragments was achieved with GFX miniprep columns supplied by Pharmacia (Freiburg, Germany). DNA was separated on a 1.6% Nusieve agarose gel and visualized after staining with ethidiumbromide on a transilluminator at 312 nm and documented by video analysis (Biometra, Goettingen, Germany).

Results and Discussion

Acrolein adducts with palmitoyl ascorbic acid (**1**,**2** — Fig. 1) have been shown to be potent inhibitors of PP1 with IC_{50} values of 3 μM and 11 μM, respectively.[13] Here, these agents were tested for an inhibitory effect on casein dephosphorylation by a HIT cell extract (Fig. 2A). Casein is a substrate of PP2A but not of PP1.[15] To evaluate the

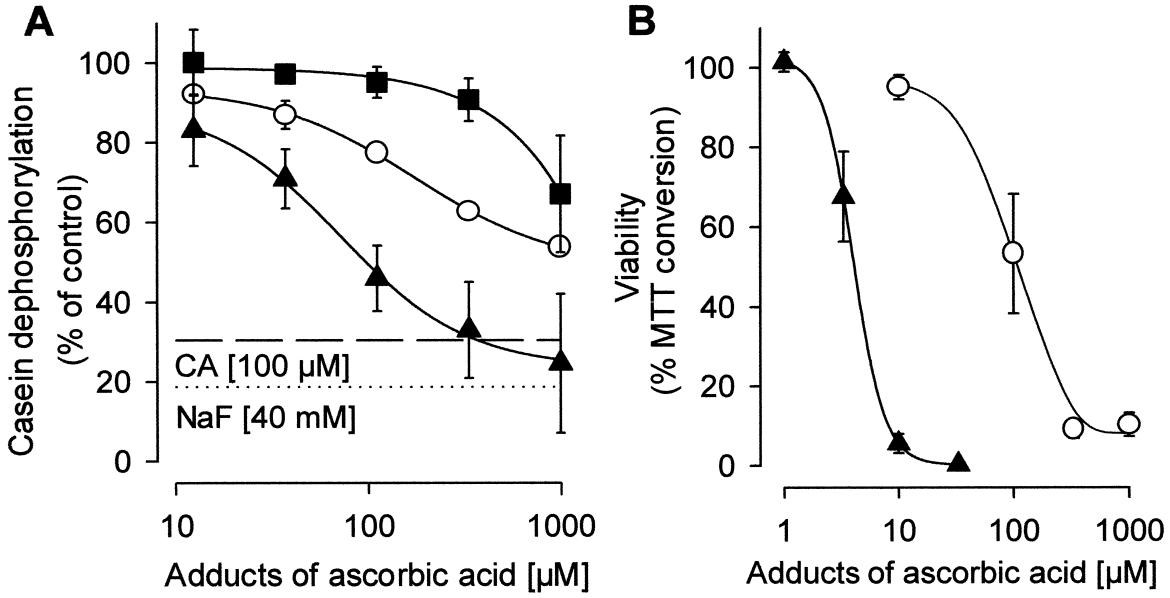

Figure 2. (A) Effect of adducts **1** (filled squares), **2** (open circles) and **3** (filled triangles) on casein dephosphorylation by HIT cell extracts. Inhibitory efficacy was compared to that observed with CA (100 μM broken line) and NaF (40 mM dotted line). $N = 3–7 \pm$ SEM; (B) Cytotoxicity of **2** (open circles) and **3** (filled triangles). Viability loss after 48 h of treatment was measured by MTT conversion and expressed relative to controls. $N = 4 \pm$ SEM.

general characteristics of this assay system, inhibition by maximally effective concentrations of the nonspecific phosphatase inhibitor NaF and the PP2A specific inhibitor cantharidic acid are included in the figure. The pyranosyl adduct **2** inhibited dephosphorylation activity up to 40% at concentrations exceeding 100 μM while the spiro compound **1** displayed only some inhibition at 1000 μM. The efficacy of **2** was about 45–50% of the maximal inhibition observed with CA (100 μM — broken line) or 225 nM OA (data not shown). The IC_{50} was about 175 μM. In addition, an adduct of L-ascorbic acid with *p*-chloro-nitrostyrene was also tested for its inhibitory effect on PP2A activity. This adduct **3** inhibited casein dephosphorylation more potently and effectively than agent **2**. The maximal inhibition was comparable to that observed with CA. The IC_{50} was about 70 μM. Because of this capacity to inhibit PP2A, adducts **2** and **3** were studied for their cytotoxic potential. Both agents were able to induce a complete viability loss albeit the LC_{50} values differed significantly (Fig. 2B) between 5.6 μM for **3** and 100 μM for **2**. Thus, as a death inducer adduct **3** was about 15–20-fold more potent than adduct **2**.

In order to test if this viability loss was the consequence of apoptosis induction, both agents were compared with CA (10 μM) for characteristic markers of apoptosis, e.g., activation of the apoptosis specific protease caspase 3 (Fig. 3A) and the subsequent activation of a caspase activated endonuclease that is responsible for DNA fragmentation (Fig. 3B). Caspase 3 activation was measured by cleavage of the tetrapeptide substrate DEVD-pNA. After 12 h 10 μM CA and agent **3** at concentrations of 6.25 μM and 12.5 μM induced caspase activation which further increased after 24 h. Agent **2** (100 μM) significantly raised caspase activity only after 24 h. Thus, agent **3** appeared to be comparable in its time and concentration response to CA as an inducer of apoptosis and was again at least 10-fold more potent than agent **2**. The similarities of **3** with CA were confirmed with data on DNA fragmentation (Fig. 3B) where agent **3** after 12 and 24 h appeared to be even more potent as an inducer of DNA cleavage than 10 μM CA. While 12.5 μM of **3** produced the most pronounced fragmentation rate after 12 h, 6.25 μM of **3** and 10 μM of CA reached their maximum after 24 h. At 12.5 μM of **3** no more small fragments could be detected after 24 h. This is most likely the consequence of progression of apoptosis into a state where membranes become leaky and the small fragments enter the culture medium and are lost in the isolation procedure.

In order to test for a loss of this response in drug-resistant tumour cells, the cytotoxic effect of both adducts was tested in OA-resistant cells selected by culturing these cells on the indicated nanomolar concentrations of OA. The multidrug resistance (MDR) phenotype of the HIT100R is the consequence of a *P*-glycoprotein mediated reduced accumulation of cytostatic drugs[11] that is observed with many tumour populations.[12] These cells are about 8–10 times less sensitive to OA and vinblastine than the parental cells.[11] In viability assays, the LC_{50} values of agent **2** (Fig. 4B) were raised from about 100 μM to 126 μM in HIT100R cells. Adduct **3**, the more potent inducer of apoptosis displayed a slight, less than 2 fold loss of sensitivity with LC_{50} values of about 5.6 μM in parental and 7.9 μM in HIT100R cells (Fig. 4B).

In conclusion, adducts of ascorbic acid with α,β-unsaturated carbonyl compounds such as acrolein or nitrostyrene which interact with PP1 also affect PP2A activity. The inhibition observed with high micromolar concentrations of the acrolein adducts **1**,**2** was only part of that observed with the natural toxins OA and CA. On the other hand, the *p*-chloro-nitrostyrene adduct **3** inhibited PP2A activity as effectively as the natural toxins. A cytotoxic effect as a consequence of apoptosis induction could also be detected with adducts **2** and **3**. Since these agents displayed only a negligible loss of the apoptotic response in OA-resistant cells displaying the MDR-phenotype, they might offer the potential for use as cytostatic drugs in multidrug resistant tumour cells. This conclusion appeared to be especially true for the nitrostyrene adduct **3** which had a LC_{50} value of less than 10 μM.

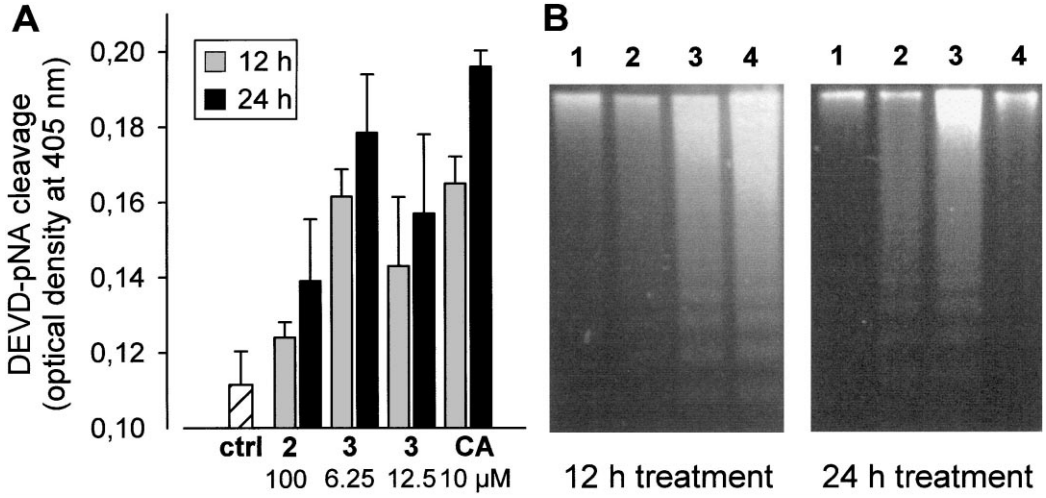

Figure 3. (A) Caspase 3 activation in HIT cells following 12 and 24 h treatment with the respective compounds. $N = 6 \pm$ SEM; (B) Comparison of DNA fragmentation in cells treated with CA and **3** for 12 or 24 h. 1 = control; 2 = 10 μM CA; 3 = 6.25 μM **3**; 4 = 12.5 μM **3**.

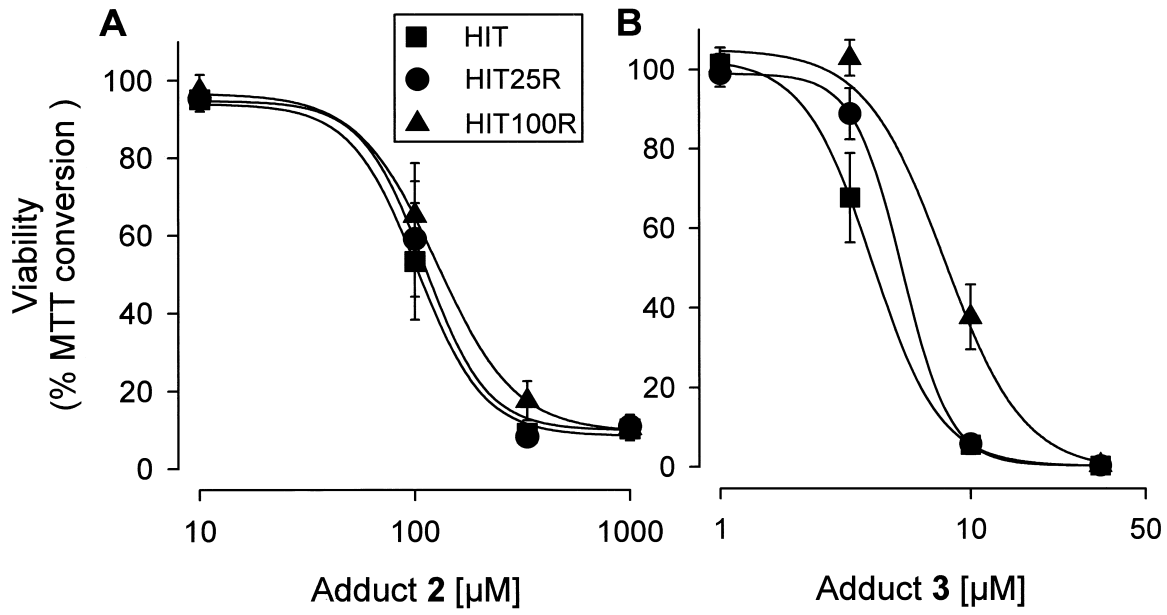

Figure 4. Cytotoxicity of adducts **2** and **3** in OA-resistant HIT cells expressing different states of multidrug (MDR) resistance. $N = 4 \pm$ SEM.

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