Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical Pharmacology 65 (2003) 603–610
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

Structure activity analysis of the pro-apoptotic, antitumor effect of nitrostyrene adducts and related compounds

Sylvia Kaap[a,b], Iris Quentin[a], Dereje Tamiru[b], Mohammed Shaheen[b], Kurt Eger[b], Hans Jürgen Steinfelder[a,*]

[a]*Institute of Pharmacology & Toxicology, University of Göttingen, Robert-Koch-Str. 40, D-37075 Göttingen, Germany*
[b]*Institute of Pharmacy, University of Leipzig, Liebigstr. 18, D-04103 Leipzig, Germany*

Received 16 October 2002; accepted 21 November 2002

Abstract

In the present study, we outlined the part of the molecule mediating the prominent pro-apoptotic effect of the Michael adduct of ascorbic acid with *p*-chloro-nitrostyrene, a new synthetic phosphatase inhibitor. The nitrostyrene (NS) moiety was identified as the structure essential for apoptosis induction. NS and its ascorbic acid adducts displayed LC_{50} values of 10–25 μM with no significant reduction of potency in okadaic acid resistant cells overexpressing the MDR1 P-glycoprotein. Induction of apoptosis by NS derivatives and the protein phosphatase 2A inhibitor cantharidic acid was proven by the analysis of caspase-3 activation and subsequent fragmentation of DNA. Further structure activity analysis revealed the necessity of the nitro group at the β-position of the side chain. The pro-apoptotic potential of adducts of NS with pyrimidine- or pyridine-derivatives varied between NS and a progressive reduction in potency up to a nearly complete loss of cytotoxicity. Substitutions at the benzene core of NS suggested a prominent enhancement of toxicity only by substitutions at the 2- or 3-position. Heterocyclic aromatics can substitute for the benzene ring of NS albeit with a 2–3-fold reduced potency. In conclusion, nitrostyrene was identified as the core structure mediating the pro-apoptotic effect of a new synthetic phosphatase inhibitor. Further studies defined a nitrovinyl side chain attached to an aromatic ring as the pharmacophore structure of a new group of pro-apoptotic agents. These observations present the basis for the development of a new group of anticancer drugs.

Keywords: Nitrostyrene derivatives; Drug-induced apoptosis; Phosphatase inhibitors; Caspase activation; DNA fragmentation; Multidrug-resistance

1. Introduction

Protein phosphatase inhibitors have been shown in various reports to be cytotoxic and display an antitumor potential [1,2] which apparently is the consequence of apoptosis induction in animal and human tumor cell lines [3–5]. These inhibitors include the shellfish toxins okadaic acid and calyculin A and the blister beetle toxin cantharidin or some of its derivatives [6,7]. All these drugs are fairly selective inhibitors of Ser/Thr phosphatases 1 and 2A [8–11]. With the exception of calyculin A they are also at least 10-fold more potent inhibitors of PP2A than of PP1. Fostriecin is an experimental anticancer drug [12,13] which as phosphatase inhibitor displays a much higher selectivity for PP2A than any of the other agents [14]. A problem in clinical trials with fostriecin was its limited availability [15] which might now be solved since its complete synthesis has been reported recently [16]. The limited availability of natural inhibitors urged for the investigation of potential synthetic inhibitors of Ser/Thr-phosphatases. Such studies were successful with Michael adducts of ascorbic acid analogues with α,β-unsaturated carbonyl compounds, especially with acrolein [17]. These

* Corresponding author. Tel.: +49-551-395777; fax: +49-551-399652.
E-mail address: hsteinfe@med.uni-goettingen.de (H.J. Steinfelder).

Abbreviations: CA, cantharidic acid; PP, protein phosphatase; MDR, multidrug-resistance; MBA, methylene blue assay; AEBSF, 4-(2-aminoethyl)benzenesulfonyl fluoride; DEVD-AFC, DEVD-7-amino-4-trifluoromethylcoumarin; NS, *trans*-β-nitrostyrene; NVF, nitrovinylfuran; NVI, nitrovinylindole; DNS, dinitrostyrenes; 3-NS, 3-nitrostyrene; CiA, *trans*-cinnamic acid; HCiA, hydrocinnamic acid; Cl-NS, 4-chloro-nitrostyrene; NS-Asc, ascorbic acid adduct of nitrostyrene; MeO, 4-methoxy; TriMeO, 3,4,5-trimethoxy; DAP, 3-(2-nitro-1-phenyl-ethyl)-2,6-diamino-pyridine; TAP, 5-(2-nitro-1-phenyl-ethyl)-2,4,6-triamino-pyrimidine; DAHP, 5-(2-nitro-1-phenylethyl)-2,6-diamino-pyrimidin-4-ol; P-AHP, 5-(2-nitro-1-phenylethyl)-2-pyridin-2-yl-6-amino-pyrimidin-4-ol.

0006-2952/02/$ – see front matter © 2002 Elsevier Science Inc. All rights reserved.
doi:10.1016/S0006-2952(02)01618-0

ascorbic acid adducts which as spiro compounds possess a spiroacetal moiety comparable to those present in the natural compounds okadaic acid, tautomycin and calyculin A could be shown to be fairly selective inhibitors of PP1 [17]. The therapeutic antitumor options of Ser/Thr phosphatase inhibitors, especially PP2A inhibitors, motivated us to test three of these synthetic phosphatase inhibitors for a potential pro-apoptotic effect [18]. Herein we observed that the two most potent PP1 inhibitors, ascorbic acid adducts with acrolein, had no effect on PP2A activity and no pro-apoptotic potential in the micromolar range while the Michael adduct of ascorbic acid with 4-chloro-nitrostyrene was a potent PP2A inhibitor and also a potent inducer of apoptosis at micromolar concentrations.

Our present study is based on these observations. Here we investigated which part of the molecule of this new agent is necessary for the pro-apoptotic effect and which further modifications might either enhance or reduce this effect. First, the nitrostyrene moiety was conclusively identified as the essential pro-apoptotic core structure. Then, we studied substitutions at the NS benzene ring and substituted the NS core by related heterocyclic aromatic structures, e.g. in nitrovinylfuran or in nitrovinylindole, to compare the pro-apoptotic potency of these derivatives with that of NS. Finally, we tested whether Michael adduct formation of NS with compounds other than ascorbic acid affected the cytotoxicity of the NS core.

2. Materials and methods

2.1. Materials

Trans-β-nitrostyrene, 3-nitrostyrene and all three dinitrostyrenes were purchased from Lancaster. *Trans*-cinnamic acid and hydrocinnamic acid were from Sigma-Aldrich. The caspase-3 substrate DEVD-AFC was obtained from Bachem.

2.2. Synthesis of nitrostyrene derivatives and related agents

4-Chloro-, 4-methoxy-, 3,4,5-trimethoxy-*trans*-β-nitrostyrene, nitrovinylindole and nitrovinylfuran were synthesized *via* Henry condensation reaction [19] of nitromethane with the respective corresponding aldehydes. For NVF potassium hydroxide was used as catalyst, for all other agents ethylene diammonium diacetate was the catalyst. The reactions were performed under sonification for 3 hr at 22°. The synthesis of the ascorbic acid adducts is described in [18]. The dodecanoic acid-4-(2-nitroethenyl)phenyl ester of NS was synthesized from 4-hydroxy-*trans*-β-nitrostyrene and dodecanoic acid dissolved in dichloromethane containing 4-pyrrolidin-1-yl-pyridine as catalyst. After addition of *N*,*N*′-dicyclohexylcarbodiimide the mixture was stirred for 18 hr at room temperature and the adduct was purified by column chromatography.

The adducts of nitrostyrene: 3-(2-nitro-1-phenyl-ethyl)-2,6-diamino-pyridine-NS, 5-(2-nitro-1-phenyl-ethyl)-2,4,6-triamino-pyrimidine-NS, 5-(2-nitro-1-phenylethyl)-2,6-diamino-pyrimidin-4-ol and 5-(2-nitro-1-phenylethyl)-2-pyridin-2-yl-6-amino-pyrimidin-4-ol were generated as described in [20] by Michael addition of the respective substituted heterocycles with nitrostyrene in an ethyl acetate water (1:1) mixture.

2.3. Cell culture

Rat pituitary GH_3 tumor cells were cultured in DMEM medium supplemented with 8% fetal calf serum at 37° in a humidified atmosphere of 95% air and 5% CO_2.

2.4. Viability testing—methylene blue assay

The day before treatment 15,000 cells per well were plated in the inner 60 wells of 96-well plates. Treatment with the respective agents was done in triplicate in complete DMEM medium (8% fetal calf serum) for 24 hr. Thereafter the medium was aspirated and 100 μL of fixative added (10% formaldehyde in 0.9% NaCl (v/v)) for at least 30 min at room temperature. The fixative was decanted and cells were stained with 100 μL 1% methylene blue in 0.01 M sodium-borate buffer (w/v; pH 8.5) for another 30 min [21]. After decanting the dye solution the wells were washed four times with 0.01 M sodium-borate buffer (pH 8.5). To elute the dye from the cells 100 μL 50% ethanol in 0.1 M HCl (v/v) were added to each well and the plates incubated for 10 min at room temperature. After shaking the plates, the dye content was determined by measuring absorption at 650 nm in a microplate reader (Molecular Dynamics). The OD of untreated cells was set to 100% and the values of the treated samples referred to this control value.

2.5. Analysis of DNA fragmentation

In order to study apoptotic fragmentation of DNA 1 million cells were plated per well of a 6-well plate and treated 24 hr later with the respective agent for another 24 hr. DNA fragments were isolated according to the column isolation protocol described in [22]. After neutral lysis of the recovered cells with SDS, proteins and genomic DNA were precipitated with a CsCl solution and the DNA fragments in the supernatant isolated by the use of GFX columns (Pharmacia). DNA was separated by agarose gel electrophoresis on a 1.6% NuSieve gel (Biozyme). Ten microliters of sample were mixed with 3 μL loading buffer containing orange G and loaded on the gels (Gibco Minigel 6 cm × 8 cm). Gels were run for about 35 min at 90 V, stained with ethidiumbromide and documented with the Biodoc system (Biometra) after transillumination at 312 nm.

2.6. Fluorometric assay of caspase-3 like activity

For the measurements of caspase-3 like activity 1 million cells were plated per well of a 6-well plate and treatment started after 24 hr. After the indicated periods of time cells were scraped off the plates while still being in the medium. Cells and medium were transferred to 2 mL vials. Cells were separated from the medium by centrifugation at 500 *g* for 10 min at 4°. The supernatants were sucked off and cells lysed on ice for 15 min in 125 μL of buffer (pH 7.4) consisting of 10 mM HEPES (w/v), 0.1% NP-40 (w/v), 0.01 mM Digitonin and 1 mM AEBSF. After centrifugation at 14,000 *g* for 10 min at 4° the supernatant was transferred to a new vial, frozen in liquid nitrogen and stored until further use at −20°.

After thawing the samples on ice 100 μL were pipetted into a well of a 96-well plate. To each sample were added 150 μL assay buffer (lysis buffer plus 4 mM NaEDTA and 15 mM DTT). The buffer contained the substrate DEVD-AFC (final concentration: 25 μM). The enzymatic reaction was run at 37°. The liberated amount of AFC was repeatedly measured in a fluorometer (Canberra Packard, $E_x = 425$ nm; $E_m = 530$ nm). From the linear part of the increase in fluorescence the enzymatic activity per mg protein was calculated and the activation by the respective treatments is presented relative to the activity of the untreated controls. In parallel the protein content was measured in a 10 μL sample with the biscinchoninic acid reaction (Pierce).

2.7. Data analysis and presentation

Data for individual agents are given as percentage of viability while setting the viability in the absence of agents as 100%. Graphs were designed and curves were fitted with SigmaPlot (SPSS Science). Values presented are means ± SE (N = 3). The LC_{50} values calculated from the graphs represent the concentration reducing viability by 50%.

3. Results

Fig. 1 gives an overview of the agents used in the present study to gain insights in the structural requirements for the strong pro-apoptotic effect of nitrostyrene derivatives. First, we confirmed in another tumor cell line our previous results obtained in HIT hamster B-cells [18], in which we described a new group of pro-apoptotic agents also inhibiting PP2A. In the present study we used rat pituitary GH_3 tumor cells to initially evaluate the viability loss induced by the Michael adduct of ascorbic acid with 4-chloro-nitrostyrene (Fig. 1A), the most potent substance in the previous study. In addition to the parental rat pituitary GH_3 cells we included a GH_3 subclone being at least 10-fold less sensitive to the pro-apoptotic effect of the phosphatase inhibitor okadaic acid. The resistant subclone (GH_3-OA100) is overexpressing the P-glycoprotein mediating the MDR1 multidrug-resistance phenotype and displays a comparable 10-fold lower sensitivity towards the anti-cancer drugs doxorubicin and actinomycin D [23]. Both cell lines were affected to the same extent by the Cl-NS derivative with an LC_{50} of about 25 μM (Fig. 2). The nitrostyrene adduct of ascorbic acid lacking the chloro-substitution appeared to be equally effective. Interestingly, NS itself was the most potent cytotoxic agent ($LC_{50} \sim 15$ μM). None of these agents displayed a loss in effectiveness as a consequence of the MDR1 phenotype

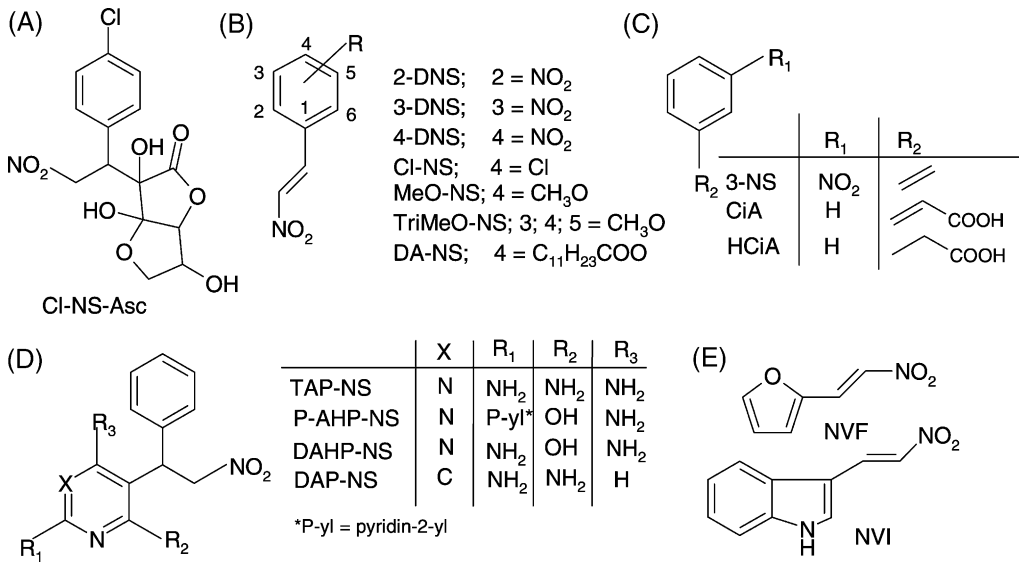

Fig. 1. Overview of tested agents (A) Michael adduct of ascorbic acid with *p*-chloro-nitrostyrene, a new synthetic phosphatase inhibitor [18], (B) ring substituted nitrostyrenes, (C) agents used to evaluate the role of the nitro group at the β-position (3-NS; cinnamic acid; hydrocinnamic acid), (D) pyrimidine and pyridine derivatives, (E) related aromatic heterocycles (NVF: nitrovinylfurane; NVI: nitrovinylindole).

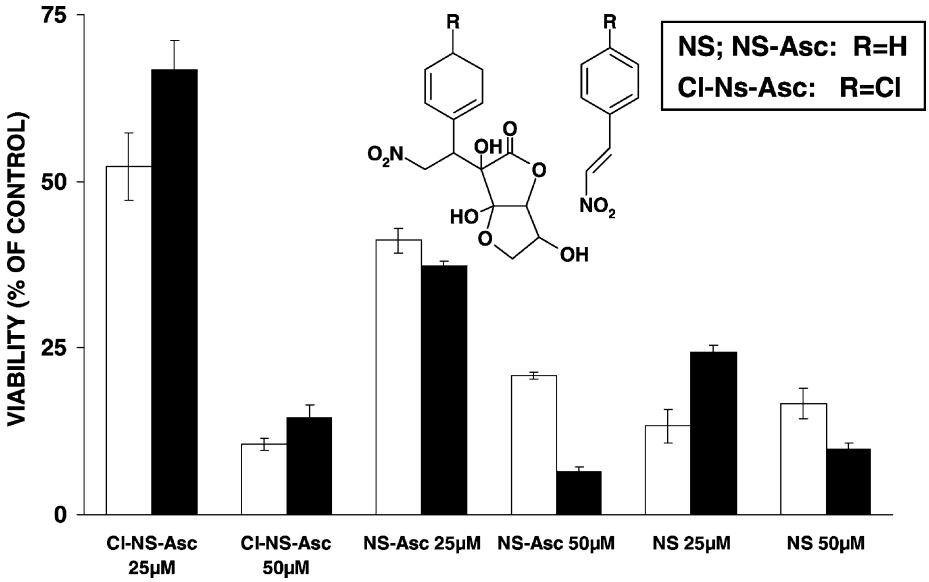

Fig. 2. Effect of Cl-NS-Asc, NS-Asc and NS on viability of parental GH_3 rat pituitary tumors cells (open columns) and GH_3-OA100 cells (filled columns), a subclone that is resistant to 100 nM okadaic acid. This line is at least 10-fold less sensitive to okadaic acid, doxorubicin and actinomycin D than the parental cells.

in the GH_3-OA100 cells. Thus, the nitrostyrene moiety of the new agent apparently was sufficient to promote cell death in these tumor cells and its effect was not hampered by the MDR1 phenotype.

Next, we confirmed that NS induced cell death occurred *via* apoptosis. As a marker of apoptosis we studied the induction of DNA fragmentation by NS, its 4-chloro-substituted derivative and the respective Michael adducts of ascorbic acid in parental GH_3 cells (Fig. 3). While both chloro-derivatives resulted in a distinct DNA ladder at 25 μM (Fig. 3A), NS itself as well as its ascorbic acid adduct (Fig. 3B) appeared to induce DNA fragmentation even at 10 μM. With all treatments the most prominent ladder was observed at 25 μM. When compared to their respective adducts both nitrostyrenes displayed ladders at 25 μM that were most prominent at the bands representing lower molecular weight DNA fragments. This observation suggests that apoptosis was induced by the nitrostyrenes themselves and progression in this case was more rapid than with the respective adducts of ascorbic acid.

Apoptosis specific DNA fragmentation is the result of the preceding activation of caspases, especially downstream effector caspases like caspase-3. Thus, the time-dependent activation of caspase-3 was tested in a fluorometric assay using the substrate DEVD-AFC. The effect of NS and its ascorbic acid adduct was compared to that of the phosphatase inhibitor cantharidic acid at the concentration of 25 μM. All three agents resulted in an approximately 40-fold increase of caspase-3 activation after 18 hr (Fig. 4). After 15 hr NS and its ascorbic acid adduct had already reached a comparable level while activity in cantharidic acid treated cells was still about 25% below that observed after 18 hr. This observation suggested a faster progression of apoptosis induced by NS and its derivative compared to cantharidic acid (see also Fig. 8B).

Ascorbic acid itself revealed no toxicity up to 1 mM (data not shown). Taken together, these data had definitely identified nitrostyrene as the core structure required for the pro-apoptotic activity of the ascorbic acid adducts. Therefore, all further experiments were intended to refer the

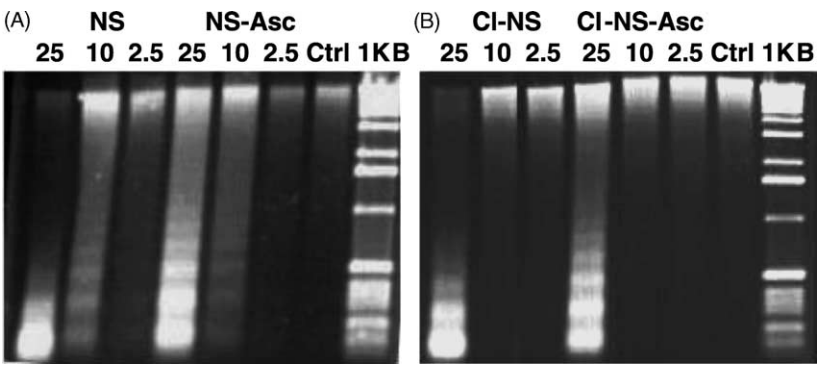

Fig. 3. DNA fragmentation induced by treatment of GH_3 cells with (A) NS-Asc or NS or (B) Cl-NS and Cl-NS-Asc.

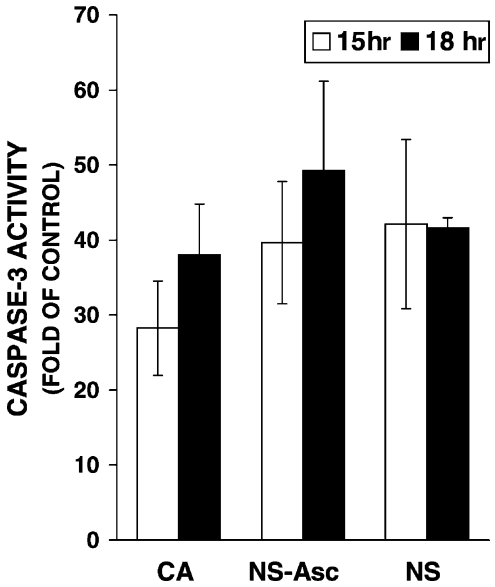

Fig. 4. Activation of DEVDase (caspase-3 like) activity by NS-Asc, NS or cantharidic acid (CA) after 15 and 18 hr.

effects observed with related agents to those achieved by NS itself. Our intention was to further elucidate structural modifications that might either enhance or decrease the pro-apoptotic activity of the NS core.

In the following structure activity analysis we used viability tests to evaluate the consequences of chemical modifications on the potency of NS. First, we tested whether the formation of adducts with aromatic heterocycles (Fig. 1D) instead of ascorbic acid affects the cytotoxic potency of the NS core (Fig. 5). The adduct with 2,4,6-triamino-pyrimidine was as potent ($LC_{50} \sim 15$ μM) and as effective as NS itself, the adduct with 2,4-diamino-6-hydroxy-pyrimidine was equally effective but 2-fold less potent ($LC_{50} \sim 30$ μM) while the adduct with 2-pyridinyl-4-amino-6-hydroxy-pyrimidine was distinctly less potent ($LC_{50} \sim 75$ μM). Finally, the adduct with 2,6-diamino-pyridine displayed only a remote cytotoxic effect (approximately 20% viability loss) at 100 μM.

Next, we compared NS with the related aromatic heterocyclic compounds nitrovinylfuran and nitrovinylindole. Both agents clearly displayed cytotoxic properties (Fig. 6) but were less potent than NS by 2–3-fold (NVI: $LC_{50} \sim 30$ μM; NVF: $LC_{50} \sim 45$ μM). Comparative analysis of NVF and its adducts with 2,4,6-triamino-pyrimidine, 2,4-diamino-6-hydroxy-pyrimidine and 2,6-diamino-pyridine revealed an order of potency (data not shown) resembling the one observed with the respective NS adducts.

Since all of the tested agents contained a nitro group at the β-position of the side chain we tested whether its dislocation to the 3-position of the benzene ring (3-NS) or a replacement by the bioisosteric carboxyl group at the β-position either in cinnamic or hydrocinnamic acid affects the pro-apoptotic property (Fig. 1C). All three agents were

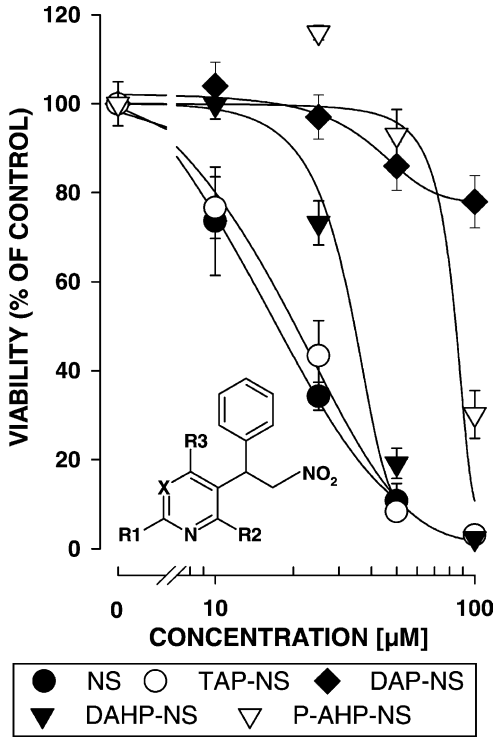

Fig. 5. Cytotoxic potency of pyrimidine and pyridine adducts of NS compared to that of NS itself. Cells were incubated for 24 hr with the indicated concentrations of NS and the respective adducts with 2,4,6-triamino-pyrimidine (TAP-NS [X = N; R1,2,3 = NH_2]), 2,4-diamino-6-hydroxy-pyrimidine (DAHP-NS [X = N; R1,3 = NH_2; R2 = OH]), 2-pyridinyl-4-amino-6-hydroxy-pyrimidine (P-AHP-NS [X = N; R1 = pyridin-2-yl; R2 = OH; R3 = NH_2]) and 2,6-diamino-pyridine DAP-NS (X = C; R1,2 = NH_2; R3 = H).

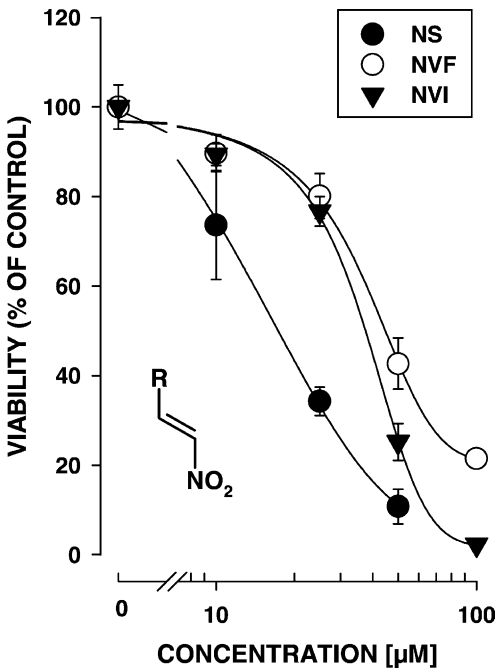

Fig. 6. Substitution of the benzene ring by the aromatic heterocycles furan and indole. Comparison of the 24 hr concentration-dependent cytotoxicity of nitrovinylfuran (NVF: R = 2-furyl) and nitrovinylindole (NVI: R = 3-indolyl) with that of nitrostyrene (NS: R = phenyl).

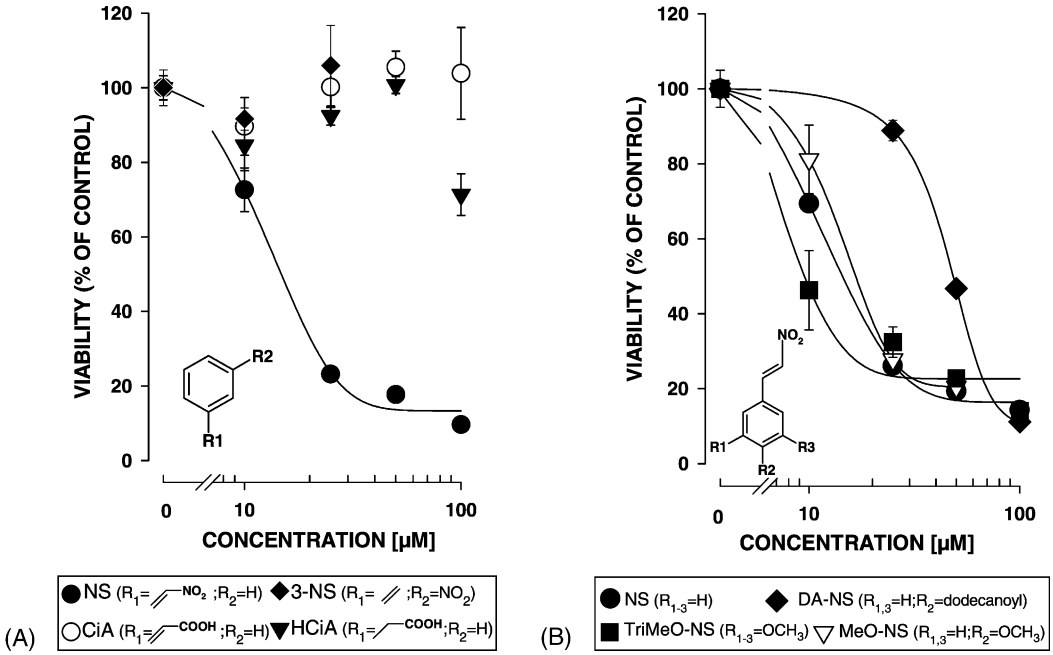

Fig. 7. Modifications in the NS core structure. (A) Role of the nitro group at the β-position. The nitro group was either transferred to the 3-position (3-NS) or substituted by a carboxy group (cinnamic and hydrocinnamic acid). (B) Effects of ring substitutions by methoxy groups or dodecanoic acid on NS toxicity.

without cytotoxic effect (Fig. 7A) up to 50 μM (3-NS) or even 100 μM (cinnamic or hydrocinnamic acid). Thus, apparently the nitro group at the β-position is an absolute requirement for the observed pro-apoptotic effect.

Thereafter, we investigated whether substitutions at the benzene ring might affect the pro-apoptotic potency of NS. Data with 4-halogenated NS suggested a comparable potency (F-NS, Cl-NS) (data not shown). In addition we tested substitutions at position 4 with a methoxy group or even larger substituents like dodecanoic acid (Fig. 1B). The potency of 4-MeO-NS was comparable to that of NS (LC_{50} ∼ 15–20 μM) while 3,4,5-tri-MeO-NS appeared to be more potent (LC_{50} ∼ 10 μM) suggesting substitutions at positions other than at the 4-position might have a greater impact on the pro-apoptotic potency (Fig. 7B). Finally, the introduction of a larger substitution at the 4-position like

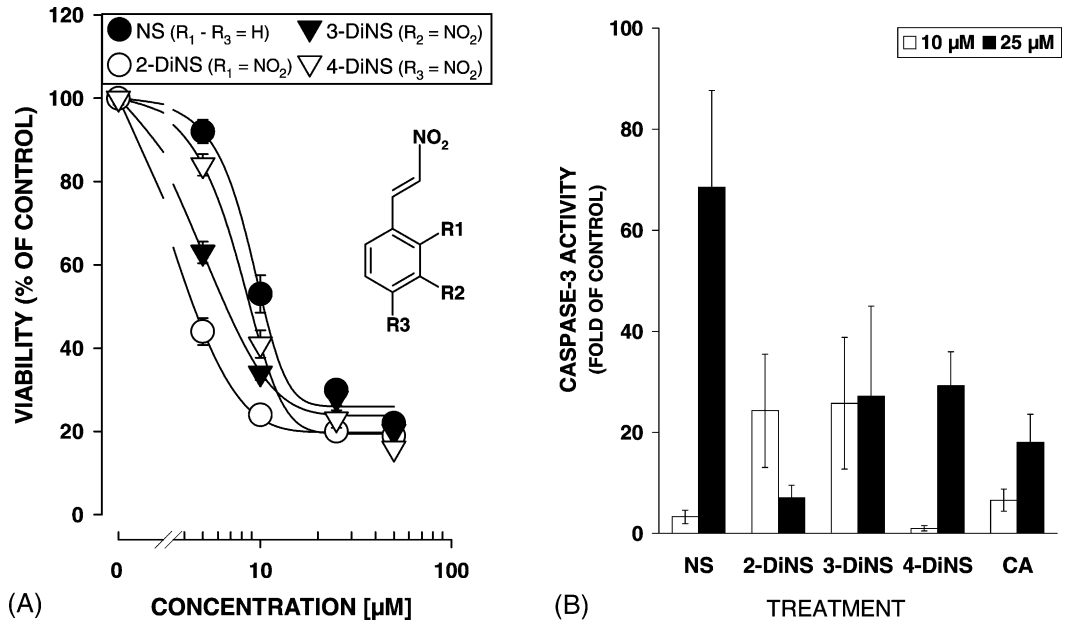

Fig. 8. Comparison of the pro-apoptotic effect of NS and 2-, 3- and 4-dinitrostyrene (2-,3-, 4-DNS). (A) Viability testing after a 24 hr treatment. (B) Caspase-3 activation after 6 hr treatment with NS, its DNS derivatives and the known PP2A inhibitor cantharidic acid. Agents were added at 10 μM (open columns) and 25 μM (filled columns).

the ester with dodecanoic acid reduced the pro-apoptotic potency by 3–4-fold ($LC_{50} \sim 50$ μM).

Since the nitro group appeared to be necessary and on the other hand substitutions at the benzene ring might increase the potency of NS we compared the potency of three different β-dinitrostyrenes (DNS) having an additional substitution of a nitro group at positions 2, 3 or 4 with NS (Fig. 8A). All dinitrostyrenes were at least as effective as the mother compound. While 4-DNS displayed only a marginally higher potency, 3-DNS was clearly more potent ($LC_{50} \sim 10$ μM) and 2-DNS was the most potent of all tested agents ($LC_{50} \sim 5$ μM). In order to support this observation we compared NS with the dinitrostyrenes in caspase-3 activation assays (Fig. 8B). Again the order of potency was 2-DNS $\geq$ 3-DNS $>$ 4-DNS $\geq$ NS. The two more potent dinitrostyrenes, 2- and 3-DNS, were strong caspase activators even after 6 hr at 10 μM, while NS and 4-DNS induced caspase-3 only at 25 μM. Cantharidic acid was again distinctly less effective at this time point supporting the hypothesis of a slower progression of apoptosis than with NS or the DNS. The observation that 2-DNS at 25 μM displayed a lower induction than at 10 μM appeared strange but can be interpreted as a result of a very fast progression of apoptosis at the higher concentration. Thus, with this treatment probably cells were detected which were already severely damaged and have lost activated caspase in the course of secondary necrosis with its characteristic disintegration of cell membranes.

4. Discussion

The search for new antitumor drugs has been empowered by the continuous progress in understanding the mechanisms underlying drug-induced apoptosis. In addition, mechanisms of resistance in tumors towards established chemotherapeutic drugs are better understood and can be taken into account when screening for agents that circumvent resistance by acting *via* different initiator mechanisms. Thus, these new agents might be effective in primarily resistant tumor cells. The inhibition of the activity of various protein phosphatases, especially PP1 and 2A, by natural toxins has been related to a potential antitumor activity of these drugs which is the consequence of apoptosis induction [1–8,12–14]. Generally these toxins are expensive and are also only available to a limited amount.

With the increasing insights in the therapeutic potentials of selective phosphatase inhibitors attempts to identify, generate and characterize new natural or synthetic phosphatase inhibitors have been intensified [24]. In addition, structural modifications have been performed in order to extent the potential of already known and characterized natural phosphatase inhibitors. Some recent papers have dealt with the aspect of using and further investigating cantharidin derivatives as anticancer drugs [25–27] and also generating new derivatives of cantharidin [28–32]. These agents retain the inhibitory activity of the mother compound towards Ser/Thr phosphatases and also display prominent pro-apoptotic potencies which might be useful in new anticancer regimens. Interestingly, some of these derivatives appear to display a selective cytostatic effect towards tumor subgroups, e.g. colon cancer cells [27,29] which are usually considered to be fairly insensitive to most commonly used anticancer drugs.

We have described easily available synthetic agents inhibiting PP2A activity which are also potent inducers of apoptosis. We identified the core structure for apoptosis induction by the most potent of these agents [18]. This agent was a Michael adduct of 4-chloro-nitrostyrene with ascorbic acid. Its NS moiety was proven to be essential for apoptosis induction.

Apoptosis by NS was shown by the time- and concentration-dependent increase of caspase-3 activity and subsequent DNA fragmentation. NS and its ascorbic acid adduct were effective in the same concentration range (10–25 μM) as cantharidic acid, a well known inhibitor of PP2A and inducer of apoptosis [6,7]. Interestingly, caspase activation data suggest a distinct faster progression of apoptosis by NS derivatives when compared to cantharidic acid.

Based on these observations a more detailed structure activity analysis was performed. Within the NS core structure the nitro group was required at the β-position and could not be replaced by a carboxyl group. The benzene ring on the other hand could be replaced by aromatic heterocycles like furan or indole. This modification retained the pro-apoptotic efficacy suggesting an important contribution of the nitro vinyl structure or the respective saturated nitro ethyl side chain attached to an aromatic ring system. Since such a replacement resulted in a reduction of the pro-apoptotic potency, nitrostyrene as the most potent of these agents appears the basic structure for further developments. Substitution at the benzene ring, especially at positions 2 and 3 by a second nitro group or positions 3,4,5 by methoxy groups enhanced the pro-apoptotic potency by about 2–3-fold. Thus, this initial structure activity analysis provided many details to be kept in mind when the nitrostyrene core is considered as the basis for the development of derivatives that might be useful in new anticancer regimens.

In summary, in the present paper we have characterized nitrostyrene as the structural core underlying the pro-apoptotic effect of a recently identified synthetic inhibitor of PP2A-like activity [18]. For this fast and extensive pro-apoptotic activity a nitro vinyl side chain attached to an aromatic ring could be identified as the underlying pharmacophore structure. Further studies should concentrate on pharmacokinetic aspects, e.g. water solubility, stability at neutral or slightly acid pH which is found in tumors, the form of drug application and finally tissue distribution. Pharmacodynamic aspects of further developments should concentrate on the aspects of specifically targeting the nitrostyrene core to tumor cells, reducing its toxicity to

healthy tissue and finally evaluating its efficacy in tumor lines resistant to standard anticancer drugs.

Acknowledgments

Thanks for synthesis of P-AHP-NS to A. Ludwig, Leipzig and of FNS to M. Klemm, Leipzig. This work was supported by grants from the Deutsche Forschungsgemeinschaft (STE 422/4-3) and the Faculty of Medicine, University of Göttingen, to H.J. Steinfelder and the Innovationskolleg Chemisches Signal und biologische Antwort and the Fonds der Chemischen Industrie to K. Eger.

References

[1] Tachibana K, Scheuer PJ, Tsukitani Y, Kikuchi H, Van Eugen D, Clardy J, Gopichand Y, Schmitz FJ. Okadaic acid, a cytotoxic polyether toxin from two marine sponges of the genus *Halichondria*. J Am Chem Soc 1981;103:2469–71.

[2] Kato Y, Fusetani N, Matsunaga S, Hashimoto K. Calyculins, potent antitumor metabolites from the marine sponge *Discodermia calyx*: biological activities. Drugs Exp Clin Res 1988;14:723–8.

[3] Boe R, Gjertsen BT, Vintermyr OK, Houge G, Lanotte M, Doskeland SO. The protein phosphatase inhibitor okadaic acid induces morphological changes typical of apoptosis in mammalian cells. Exp Cell Res 1991;195:237–46.

[4] Kiguchi K, Glesne D, Chubb CH, Fujiki H, Huberman E. Differential induction of apoptosis in human breast tumor cells by okadaic acid and related inhibitors of protein phosphatases 1 and 2A. Cell Growth Differ 1994;5:995–1004.

[5] von Zezschwitz C, Vorwerk H, Tergau F, Steinfelder HJ. Apoptosis induction by inhibitors of Ser/Thr phosphatases 1 and 2A is associated with transglutaminase activation in two different human epithelial tumor lines. FEBS Lett 1997;413:147–51.

[6] Morana SJ, Wolf CM, Li J, Reynolds JE, Brown MK, Eastman A. The involvement of protein phosphatases in the activation of ICE/CED-3 protease, intracellular acidification, DNA digestion, and apoptosis. J Biol Chem 1996;271:18263–71.

[7] Krautheim A, Brechlin P, Becker K, Winkler M, Steinfelder HJ. Hamster pancreatic beta cell lines with altered sensitivity towards apoptotic signalling by phosphatase inhibitors. Br J Pharmacol 2000;129:687–94.

[8] Ishihara H, Martin BL, Brautigan DL, Karaki H, Ozaki H, Kato Y, Fusetani N, Watabe S, Hashimoto K, Uemura D, et al. Calyculin A and okadaic acid: inhibitors of protein phosphatase activity. Biochem Biophys Res Commun 1989;159:871–7.

[9] Haystead TA, Sim AT, Carling D, Honnor RC, Tsukitani Y, Cohen P, Hardie DG. Effects of the tumor promoter okadaic acid on intracellular protein phosphorylation and metabolism. Nature 1989;337:78–81.

[10] Honkanen RE. Cantharidin, another natural toxin that inhibits the activity of serine/threonine protein phosphatases types 1 and 2A. FEBS Lett 1993;330:283–6.

[11] Honkanen RE, Codispoti BA, Tse K, Boynton AL. Characterization of natural toxins with inhibitory activity against serine/threonine protein phosphatases. Toxicon 1994;32:339–50.

[12] Hotz MA, Del Bino G, Lassota P, Traganos F, Darzynkiewicz Z. Cytostatic and cytotoxic effects of fostriecin on human promyelocytic HL-60 and lymphocytic MOLT-4 leukemic cells. Cancer Res 1992;52:1530–5.

[13] de Jong RS, de Vries EG, Mulder NH. Fostriecin: a review of the preclinical data. Anticancer Drugs 1997;8:413–8.

[14] Walsh AH, Cheng A, Honkanen RE. Fostriecin, an antitumor antibiotic with inhibitory activity against serine/threonine protein phosphatases types 1 (PP1) and 2A (PP2A), is highly selective for PP2A. FEBS Lett 1997;416:230–4.

[15] de Jong RS, Mulder NH, Uges DR, Sleijfer DT, Hoppener FJ, Groen HJ, Willemse PH, van der Graaf WT, de Vries EG. Phase I and pharmacokinetic study of the topoisomerase II catalytic inhibitor fostriecin. Br J Cancer 1999;79:882–7.

[16] Boger DL, Ichikawa S, Zhong W. Total synthesis of fostriecin (CI-920). J Am Chem Soc 2001;123:4161–7.

[17] Witt UG, Schultz JE, Dolker M, Eger K. Inhibition of protein phosphatases by Michael adducts of ascorbic acid analogues with alpha, beta-unsaturated carbonyl compounds. Bioorg Med Chem 2000;8:807–13.

[18] Fathi AR, Krautheim A, Kaap S, Eger K, Steinfelder HJ. Michael adducts of ascorbic acid as inhibitors of protein phosphatase 2A and inducers of apoptosis. Bioorg Med Chem Lett 2000;10:1605–8.

[19] Luzzio FA. The Henry reaction: recent examples. Tetrahedron 2001;57:915–45.

[20] Taylor EC, Lin B. Exploitation of a new route to fused pyrroles: synthesis of TNP-351, homo-MTA, and 5-arylpyrrolo[2,3-]pyrimidines. Tetrahedron Lett 1999;40:4027–30.

[21] Oliver MH, Harrison NK, Bishop JE, Cole PJ, Laurent GJ. A rapid and convenient assay for counting cells cultured in microwell plates: application for assessment of growth factors. J Cell Sci 1989;92:513–8.

[22] Steinfelder HJ, Quentin I, Ritz V. A fast and sensitive technique to study the kinetics and the concentration dependencies of DNA fragmentation during drug-induced apoptosis. J Pharmacol Toxicol Methods 2000;43:79–84.

[23] Ritz V, Marwitz J, Sieder S, Ziemann C, Hirsch-Ernst KI, Quentin I, Steinfelder HJ. Contribution of mdr1b-type P-glycoprotein to okadaic acid resistance in pituitary GH_3 cells. Naunyn-Schmiedeberg's Arch Pharmacol 1999;360:116–21.

[24] McCluskey A, Sim AT, Sakoff JA. Serine–threonine protein phosphatase inhibitors: development of potential therapeutic strategies. J Med Chem 2002;45:1151–75.

[25] Yang EB, Tang WY, Zhang K, Cheng LY, Mack PO. Norcantharidin inhibits growth of human HepG2 cell-transplanted tumor in nude mice and prolongs host survival. Cancer Lett 1997;117:93–8.

[26] Chen YN, Chen JC, Yin SC, Wang GS, Tsauer W, Hsu SF, Hsu SL. Effector mechanisms of norcantharidin-induced mitotic arrest and apoptosis in human hepatoma cells. Int J Cancer 2002;100:158–65.

[27] Peng F, Wei YQ, Tian L, Yang L, Zhao X, Lu Y, Mao YQ, Kan B, Lei S, Wang GS, Jiang Y, Wang QR, Luo F, Zou LQ, Liu JY. Induction of apoptosis by norcantharidin in human colorectal carcinoma cell lines: involvement of the CD95 receptor/ligand. J Cancer Res Clin Oncol 2002;128:223–30.

[28] Ho YP, To KK, Au-Yeung SC, Wang X, Lin G, Han X. Potential new antitumor agents from an innovative combination of demethylcantharidin, a modified traditional Chinese medicine, with a platinum moiety. J Med Chem 2001;44:2065–8.

[29] McCluskey A, Bowyer MC, Collins E, Sim ATR, Sakoff JA, Baldwin ML. Anhydride modified cantharidin analogues: synthesis, inhibition of protein phosphatases 1 and 2A and anticancer activity. Bioorg Med Chem Lett 2000;10:1687–90.

[30] Sakoff JA, Ackland SP, Baldwin ML, Keane MA, McCluskey A. Anticancer activity and protein phosphatase 1 and 2A inhibition of a new generation of cantharidin analogues. Invest New Drugs 2002;20:1–11.

[31] McCluskey A, Walkom C, Bowyer MC, Ackland SP, Gardiner E, Sakoff JA. Cantharimides: a new class of modified cantharidin analogues inhibiting protein phosphatases 1 and 2A. Bioorg Med Chem Lett 2001;11:2941–6.

[32] McCluskey A, Keane MA, Walkom CC, Bowyer MC, Sim AT, Young DJ, Sakoff JA. The first two cantharidin analogues displaying PP1 selectivity. Bioorg Med Chem Lett 2002;12:391–3.