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Report

Cytotoxicity of aphidicolin and its derivatives against neuroblastoma cells *in vitro*: synergism with doxorubicin and vincristine

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Disseminated neuroblastoma diseases are still indicated by a poor outcome despite treatment regimens including radiation therapy and high-dose chemotherapy with stem cell rescue. Therefore, new substances and treatment regimens are of interest. Aphidicolin (APH), a tetracyclic diterpene antibiotic produced by *Cephalosporium aphidicola*, has a specific toxicity for neuroblastoma cells. Furthermore, it was shown to enhance the effects of X-ray radiation and chemotherapy on malignant cells. To find new substances, 20 APH derivatives were tested for their anti-neuroblastoma efficacy *in vitro* in UKF-NB-2 cells. Five derivatives had antitumoral activity in neuroblastoma cells. A relationship between the structure and the antitumoral efficacy showed that the hydroxyl groups at C-3 and C-18 are essential for the antitumoral effects. Furthermore, antitumoral effects of APH in combination with doxorubicin and vincristine, both part of commonly used treatment regimens for disseminated neuroblastoma diseases, were tested in the neuroblastoma cell line UKF-NB-2. APH was found to act synergistically with vincristine and synergistically to additive with doxorubicin depending on the molecular ratio of the substances in combination. This may offer the chance to use APH and its derivatives as additional tools in the treatment of neuroblastomas. [© 2000 Lippincott Williams & Wilkins.]

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Introduction

Neuroblastoma (NB) represents the most common extracranial tumor of childhood. Between 8 and 10% of all cancer diseases in the childhood are caused by NB. The incidence is of about 8 cases in a million children up to an age of 15 years. Despite new treatment protocols the prognosis still depends on the kind of tumor. In patients with disseminated diseases the clinical outcome remains poor. Only about 15% of high-risk patients survive, even after intensive treatment including high-dose chemotherapy with stem cell rescue.[1,2] In addition, high-dose chemotherapy is associated with considerable toxic adverse effects. Therefore, new substances and treatment strategies for NB appear to be very desirable.

Aphidicolin (APH) is a tetracyclic diterpene antibiotic produced by *Cephalosporium aphidicola*[3] that inhibits eukaryotic DNA polymerases α and δ without interfering with the activities of DNA polymerase β and γ, reverse transcriptase or prokaryotic DNA polymerase II.[4–6] The binding to the DNA polymerases α and δ is reversible, and, therefore, it is commonly used as a synchronizing agent in experimental systems.[7] APH was shown to kill neuroblastoma cells *in vitro* without affecting the viability of most common human cell lines including human embryonic cell lines and different human tumor cell lines.[4,8–10] Its water-soluble derivative APH glycinate inhibited hu-

man neuroblastoma cell growth *in vivo*.[11] Cellular differentiation was influenced by APH in some leukemic[9,12] and a human rhabdomyosarcoma cell line.[13] The specific sensitivity of neuroblastoma cells towards APH remains unclear.

In a human ovary cancer cell line APH was able to reverse the cisplatin resistance and increased the sensitivity of cells obtained from primary ovarian tumors to different platinum compounds.[14,15] Moreover, the *in vitro* sensitivity of leukemic cells to ara-C became raised by APH treatment.[16]

Furthermore, APH was shown to enhance the effect of X-ray treatment on Cloudman melanoma cells.[17] As NBs are considered to be a radiosensitive tumors and the use of radiotherapy is indicated in the therapy of different types of NB,[1,2,18] this finding is very promising.

In this study 20 derivatives of APH were tested for their anti neuroblastoma activity in UKF-NB-2 cells. In order to examine the usefulness of APH in combination therapy with commonly used chemotherapeutics we tested the efficacy of APH together with doxorubicin (DOX) and vincristine (VCR) that are part of therapy regimens of disseminated neuroblastoma diseases[18–20] *in vitro*.

Materials and methods

Reagents

All culture media and media supplements were purchased from Seromed (Berlin, Germany). APH derivatives were obtained from AnalytiCon AG (Potsdam, Germany). APH, VCR and DOX were purchased by Sigma-Aldrich Chemie (Deisenhofen, Germany). The structure of APH can be seen from Figure 1. The structures of the derivatives of APH are shown in Table 1.

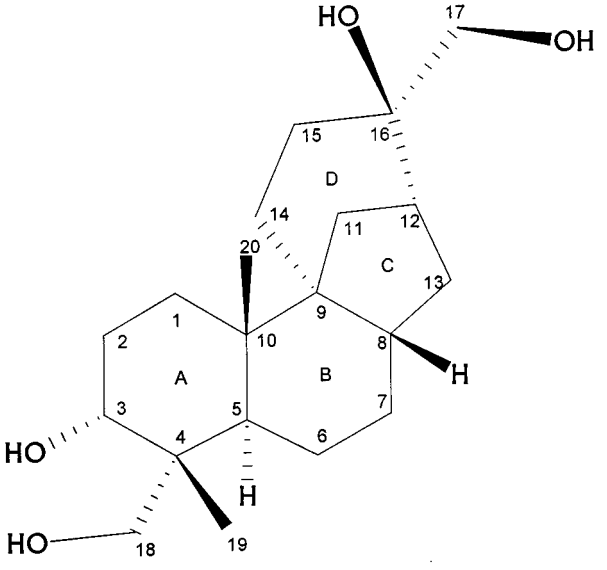
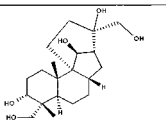
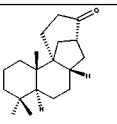
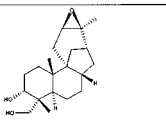
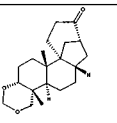
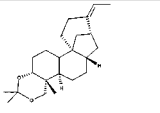
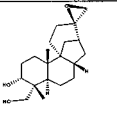
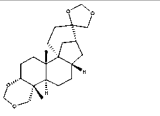
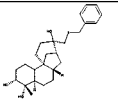
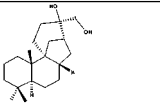
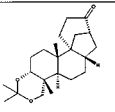
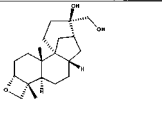
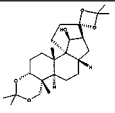
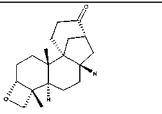
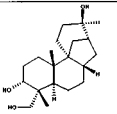
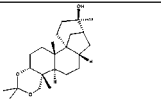
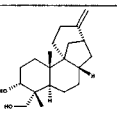
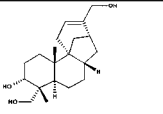
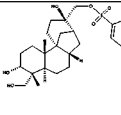
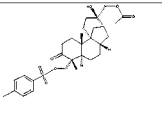
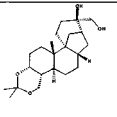


Figure 1. Molecular structure of APH.

Table 1. APH derivatives

K14249 MW: 354.49u		K14250 MW: 274.54u	
K14251 MW: 320.48u		K14252 MW: 318.46u	
K14253 MW: 344.54u		K14254 MW: 320.48u	
K14255 MW: 362.51u		K14256 MW: 430.65u	
K14257 MW: 306.49u		K14258 MW: 346.51u	
K14259 MW: 320.48u		K14260 MW: 420.59u	
K14261 MW: 288.43u		K14262 MW: 322.49u	
K14263 MW: 362.56u		K14264 MW: 304.48	
K14265 MW: 320.48		K14266 MW: 492.68	
K14267 MW: 532.70		K14268 MW: 364.53	

Cells

NB cell line UKF-NB-2 was established from metastasis harvested in relapse of our patients with Evans stage 4 neuroblastoma.[10,21] The cells were propagated in Iscove's modified Dulbecco's Medium (IMDM) supplemented with 10% fetal bovine serum (FBS), 100 IU/ml penicillin and 100 mg/ml streptomycin at 37°C in a humidified 5% CO_2 incubator.

MTT assay

The evaluation of the *in vitro* toxicity of the tested substances was performed using the MTT assay to determine the cell viability after the treatment.[22] Stock solutions of tested substances were diluted with MEM supplemented with 10% FBS starting with a 1:100 dilution and proceeding with 1:2 dilution steps. These dilutions were added to confluent cell layers in 96-well plates 4–5 days after seeding. Cells were incubated for 4 days at 37°C and 5% CO_2 atmosphere. After addition of 100 μl SDS solution (20% SDS in a 1:1 DMF/H_2O solution), plates were read on a multiwell scanning spectrophotometer at a wavelength of 620 nm and a reference wavelength of 690 nm. The extent of cytotoxicity is defined as the relative reduction of the optical density (OD) which correlates with the amount of viable cells in relation to cell control (=100%).

Combination studies

Combination studies were taken out in UKF-NB-2-cells. DOX, VCR and APH alone were used in different concentrations. Then combinations of APH with DOX and VCR, respectively, at three different molecular ratios were tested. To determine the effect of the tested drug the MTT assay was used. The IC_{25}, IC_{50} and IC_{90} were determined using CalcuSyn™ for Windows. The program was also used to calculate the combination index (CI) indicating whether the substances act in combination synergistically, additively or antagonistically. This calculation is based on the method of Chou and Talalay.[23] A $CI < 1$ indicates synergistic action, a $CI > 1$ indicates antagonistic action and a CI=1 indicates additive action.

Results

Cytotoxicity of APH and its derivatives

Antitumoral effects of APH and its derivatives were tested at concentrations ranging from 0.1 to 10 μM. APH inhibited growth of UKF-NB-2 cells at nanomolar concentrations with a IC_{50} value of 0.12 μM (Table 2). APH at a concentration of 0.5 μM killed all cells after 5 days of treatment. In the tested concentrations five of the tested APH derivatives showed an anti-neuroblastoma effect (Table 2). The derivatives K14254 and K14266 showed cytotoxic activities comparable to that of the parental compound with IC_{50} values of 0.11 and 0.13 μM, respectively. K14265, K14249 and K14251 were 5- to 20-fold less effective when compared with APH with IC_{50} values of 0.62, 0.71 and 2.0 μM, respectively.

Relationship between drug structure and antitumoral effects

A relationship between structure and antitumoral efficiency of tested derivatives was detected. The hydroxyl groups at C-3 and C-18 were found to be essential for the antitumoral effect. Derivatives without these hydroxyl groups showed no antitumoral effects. K14266, representing the tosylate ester of APH at hydroxyl group C-17, and K14254, having an intramolecular epoxide formed between the hydroxyl groups at C-16 and C-17, acted in comparable concentrations as APH. These two substances can be considered to represent prodrugs of APH. In K14249 the introduction of a hydroxyl group at C-11 leads to a 6-fold decrease of activity. In K14251 the hydroxyl group at C-17 is missing, and a epoxide group was introduced between C-15 and C-16 leading to a 20-fold decrease of activity. As the epoxide is supposed to be cleaved under *in vitro* conditions into two hydroxyl groups, the loss of antitumoral activity could be associated with the loss of hydroxyl group at C-17 and/or introduction of hydroxyl group at C-15. In K14265 the hydroxyl group at C-16 was eliminated leading to a double bond between C-15 and C-16. The loss of the hydroxyl group was associated with a 6-fold decrease of antitumor activity. The non-active derivatives K14262 and K14264 remained unchanged at C-3 and C-18 but were modified at C-16 and C-17, indicating

Table 2. Toxicity of APH derivatives toward NB cell line UKF-NB-2

Tested substance	IC_{50} (μM) in UKF-NB-3[a]
K14249	0.71
K14251	2.03
K14254	0.11
K14265	0.62
K14266	0.13
APH	0.12

[a]Values are mean of three experiments. Relative SD are <15%.

that severe modifications in this positions can also lead to total loss of activity.

Combination studies

To evaluate APH effects in combination with DOX or VCR various fixed-ratio concentrations of the drugs were tested. The dose–effect curves are shown for DOX in Figure 2 and for VCR in Figure 3. As calculated from the dose–effect curves the IC_{50} values for each compound in the combinations of APH with either DOX or VCR were markedly reduced in comparison to the values for the respective drugs alone (Table 3). When used as single agents the IC_{50} values for APH and VCR were 136 and 0.14 nM, respectively. When the two agents were combined at a molar ratio of 923:1, the IC_{50} values decreased to 38 nM for APH and 0.042 nM for VCR (Table 3). In the experiments with APH and DOX, the IC_{50} values for the single agents were 121 nM for APH and 3.5 nM for DOX. When the two agents were combined at a molar ratio of 58:1, 65.6 nM APH and 1.1 nM DOX inhibited 50% cell growth (Table 3).

The calculation of the combination index CI at the IC_{50} level showed that both combinations of APH and VCR (CI=0.58 for 923:1 molar ratio and 0.72 for 461.5:1 molar ratio, Table 4) as well as both combinations of APH and DOX (CI=0.86 both for 58:1 and 14.5:1 molar ratio) exert synergistic effects. APH and VCR also showed synergistic effects at the IC_{25} and IC_{90} levels while the combination with DOX was synergistic at the IC_{25} level and exhibited only additive effects at the IC_{90} level (CI=1.5 for 58:1 and 1.0 for 14.5:1 molar ratio; Table 4).

Discussion

In this study we present five APH derivatives effective against NB in nanomolar concentrations. The derivatives K14254 and K14266 represent substances with *in vitro* antitumor activity comparable to APH. At the drug concentrations under evaluation the derivatives showed no cytotoxicity against normal cells (data not shown).

A relationship between structure and antitumoral

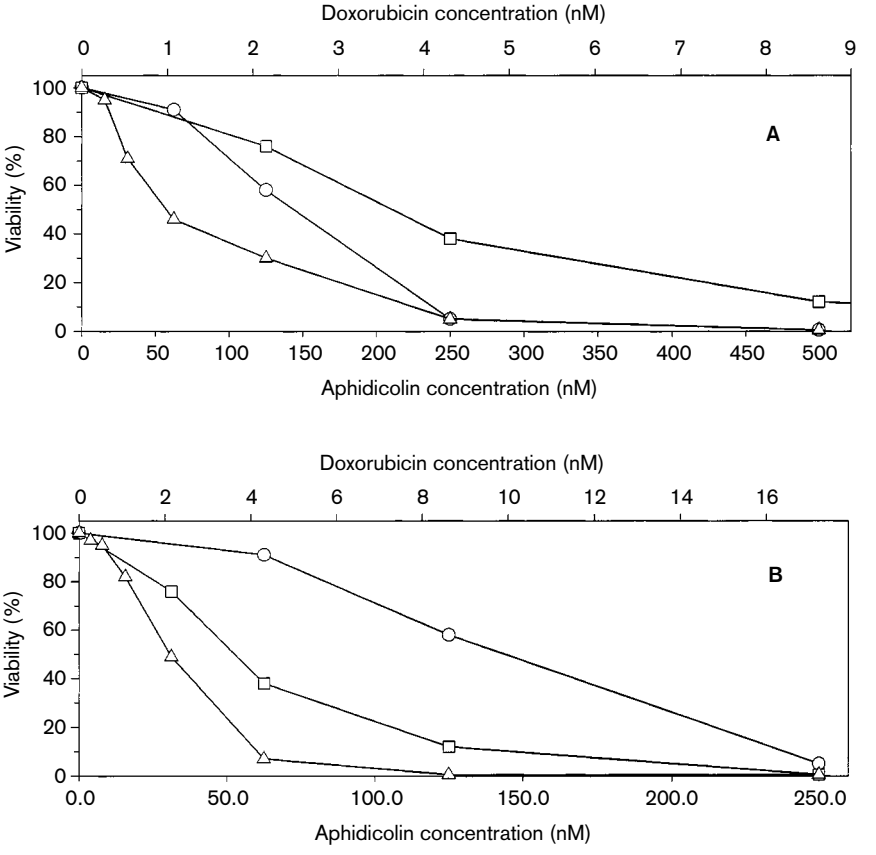


Figure 2. Effects of APH (○), DOX (□) and their combinations (△) on UKF-NB-2 cells. Molecular ratios of APH to DOX in the combinations are: (a) 58:1 and (b) 14.5:1.

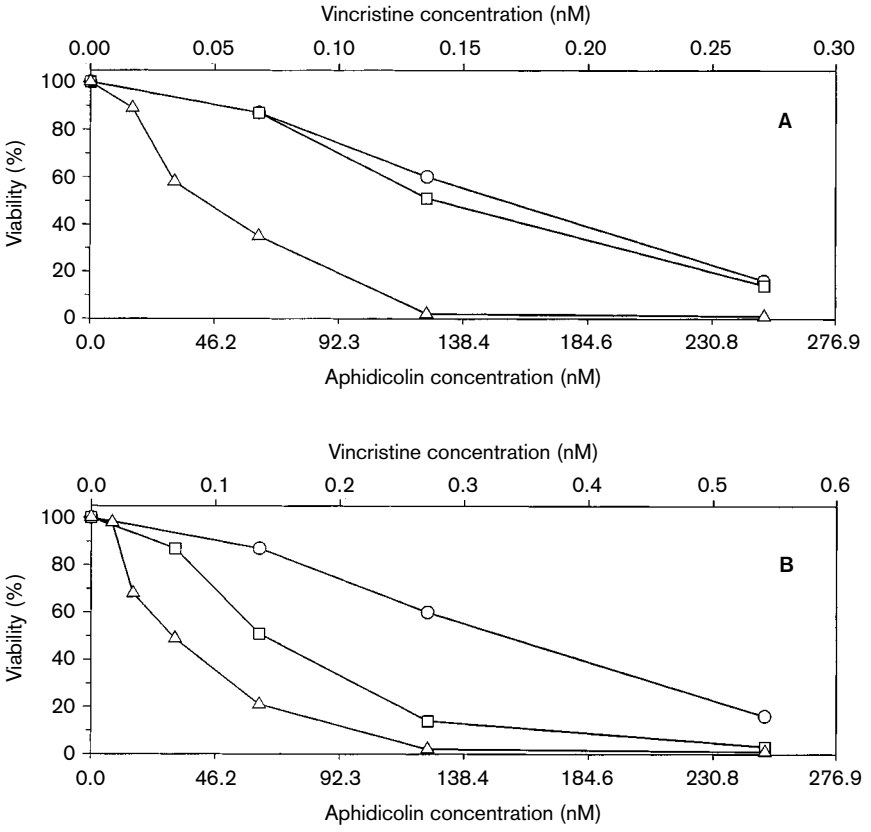


Figure 3. Effects of APH (○), VCR (□) and their combinations (△) on UKF-NB-2 cells. Molecular ratios of APH to VCR in the combinations are: (a) 923:1 and (b) 461.5:1.

Table 3. IC_{50} (nM) of APH, VCR, DOX and APH in fixed combinations

Drug combination (molecular ratio)	IC_{50} (nM)		IC_{50} of APH in combination (nM)		IC_{50} of VCR in combination (nM)		IC_{50} of DOX in combination (nM)	
APH	–	136	–	–	–	–	–	–
VCR	–	0.14	–	–	–	–	–	–
APH:VCR	–	–	–	–	–	–	–	–
	923 : 1	–	38.3	–	0.0415	–	–	–
	461.5 : 1	–	31.2	–	0.0677	–	–	–
	–	–	–	–	–	–	–	–
APH	–	122	–	–	–	–	–	–
DOX	–	3.5	–	–	–	–	–	–
APH:DOX	–	–	–	–	–	–	–	–
	58 : 1	–	65.6	–	–	–	1.13	–
	14.5 : 1	–	30.98	–	–	–	2.14	–

efficiency of the tested derivatives was detected. The elimination of hydroxyl groups at C-3 and C-18 was associated with a total loss of antitumor activity, whereas changes of the hydroxyl groups at C-16 or C-17 led to a decrease. The most effective derivatives K14266 and K14254 are considered to be prodrugs of APH. Although the chemical stability of the APH derivatives remained uninvestigated, the formation of a tosylate ester (K14266) and the formation of an intramolecular epoxide group (K14254) are likely to be hydrolyzed under *in vitro* conditions resulting in APH. This was underlined by their antitumoral activities comparable to APH. The other active compounds were clearly less effective indicating that

Table 4. Combination indices of APH in combination with VCR and DOX at fixed molecular ratios

Drug combination (molecular ratio)		Combination index at growth inhibition of		
		25%	50%	90%
APH:VCR	923 : 1	0.56	0.58	0.63
	461.5 : 1	0.69	0.72	0.79
APH:DOX	58 : 1	0.69	0.86	1.50
	14.5 : 1	0.79	0.86	1.00

changes in the molecular structure of APH led to a decreased affinity of the derivatives to their binding sites. No changes at all are possible at C-3 and C-18 without loosing anti-NB efficiency. Changes without total loss of activity were possible at C-16 and C-17 but were associated with a decrease of activity. Furthermore, major modifications in this positions also led to a total loss of activity as indicated by the non-active derivatives K14262 and K14264 that were unmodified at C-3 and C-18.

From our data no prognosis can be made concerning the relevance of our findings under *in vivo* conditions as no pharmacokinetic data so far is available. Nevertheless, in our study five promising APH derivatives which are worth being further investigated were found.

In the second part of our study we investigated the interaction of APH in combination with DOX and VCR, respectively, on neuroblastoma cell line UKF-NB-2. These two substances are commonly used in treatment regimens for disseminated neuroblastoma diseases.[18–20] We found APH to act synergistically *in vitro* with both of the substances. Since the outcome in high-risk neuroblastoma patients still remains unfavorable.[1,2] APH might be an additional tool in the therapy of disseminated neuroblastoma diseases. Furthermore, APH was shown to enhance the efficacy of X-ray irradiation in melanoma cells.[17] As radiation therapy in combination with chemotherapy is an essential element in the neuroblastoma therapy of high-risk patients,[1,2,18] the use of APH could improve the effects of the commonly used chemotherapeutics and radiotherapy.

Moreover, the potential of APH as an additional tool for the treatment of other kinds of cancer appears to be of great interest. Its ability to reverse cisplatin resistance and to increase the sensitivity of tumor cells to platinum compounds and ara-C was shown before.[14–16] These findings are underlined by our investigations showing APH to interact synergistically with DOX and VCR. Further research has to show if APH offers the possibility to improve the therapy of other kinds of cancer.

Additionally, APH was shown to be non-toxic for normal cells *in vitro*.[4,8–10] Growth inhibition that occured to normal cells at APH concentrations up to 10 μM was reversible after removal of APH.[4,8–10] *In vivo* testing of the water-soluble APH derivative APH glycinate in therapeutic concentrations was proved to be non-toxic in mice.[11] In phase I clinical trials of APH glycinate local toxicity at the injection site was dose limiting with a maximum tolerated dose of 4500 mg/m^2 in a 24 h continuous infusion study. This dose led to sustained plasma levels of 2.5–5 μM being significantly higher than those active against NB *in vitro*.[24] Therefore, the use of APH could result in a decrease of adverse effects leading to therapy regimes with less burden for the patients or the possibility to use higher doses to overcome even highly aggressive forms of NB.

Conclusions

In our study we found two new APH derivatives having a promising anti-neuroblastoma activity. A relationship between structure and antitumoral efficacy indicated the hydroxyl groups at C-3 and C-18 to be essential for the antitumoral effects. Moreover, we found APH to act synergistically with DOX and VCR in neuroblastoma cells. As disseminated neuroblastoma diseases are still marked by a poor outcome, new therapeutics are of great interest.

Therapy of high-risk neuroblastoma patients at present consists of radiation therapy and high-dose chemotherapy with stem cell rescue. As APH was shown to enhance the effect of X-rays in melanoma cells *in vitro*, it could be an additional tool in the therapy of neuroblastoma that increases the efficiency of both, radiotherapy and chemotherapy.

Moreover, further research has to show if APH is generally able to increase the effects of chemotherapeutics and radiotherapy in different tumors.

The therapeutical value of the two new antitumoral effective APH derivatives compared to APH will be evaluated in the future.

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