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Report

Treatment of drug-resistant human neuroblastoma cells with cyclodextrin inclusion complexes of aphidicolin

Martin Michaelis,[1] Jaroslav Cinatl,[2] Jens-Uwe Vogel,[1,2] Pavla Pouckova,[3] Pablo Hernáiz Driever[4] and Jindrich Cinatl[1]

[1]Institut für Medizinische Virologie, Zentrum der Hygiene, Klinikum der JW Goethe-Universität, Paul-Ehrlich-Strasse 40, 60596 Frankfurt am Main, Germany. [2]Abteilung für Hämatologie und Onkologie, Zentrum der Kinderheilkunde, Klinikum der JW Goethe-Universität, Theodor-Stern-Kai 7, 60590 Frankfurt am Main, Germany. [3]Institute of Biophysics, First Medical Faculty, Charles University, Americká 532, 252 29 Dobrichovice, Prague, Czech Republic. [4]Klinik für Pädiatrie mit Schwerpunkt Hämatologie und Onkologie, Charité, Campus Virchow-Klinikum, Augustenburger Platz 1, 13353 Berlin, Germany.

Treatment failure in most neuroblastoma (NB) patients is related to primary and/or acquired resistance to conventional chemotherapeutic agents. Aphidicolin (APH), a tetracyclic diterpene, exhibits specific cytotoxic action against NB cells. The purpose of this study was to compare antitumoral efficacy of APH in parental NB cell lines and cell subclones that exhibit drug resistance to vincristine (VCR), doxorubicin (DOX) and cisplatin. Due to poor solubility of APH in water, γ-cyclodextrin (γ-CD) inclusion complexes of APH were used for systemic treatment of xenotransplanted parental and VCR-resistant UKF-NB-3 tumours. APH and its γ-CD inclusion complexes inhibited growth of parental and drug-resistant NB cells at equimolar doses *in vitro*. Growth of VCR-sensitive and -resistant NB tumors was inhibited at equal doses in a dose-dependent fashion *in vivo*. These results indicate that the specific cytotoxic activity of APH against NB cells *in vitro* and *in vivo* is independent of cellular mechanisms facilitating drug resistance to conventional chemotherapeutic drugs. Hence, taking into account our previous findings that APH acts synergistically with VCR and DOX, APH might be an additive tool for the therapy of NB and is suitable for evaluation in clinical studies of NB treatment protocols. [© 2001 Lippincott Williams & Wilkins.]

Key words: Cisplatin, doxorubicin, mice, vincristine.

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Correspondence to J Cinatl, Institut für Medizinische Virologie, Zentrum der Hygiene, Klinikum der JW Goethe-Universität, Paul-Ehrlich-Strasse 40, 60596 Frankfurt am Main, Germany.
Tel: (+49) 69 6301-6409; Fax: (+49) 69 6301-4302;
E-mail: Cinatl@em.uni-frankfurt.de

Introduction

Neuroblastoma (NB) is defined by immature neuroblastic cells derived from neural crest cells and accounts for 8–10% of pediatric malignancies, being second after acute lymphoblastic leukemia. Risk factors include age over 1 year, disseminated disease at diagnosis, *MYCN* amplification and deletion of 1p36→ter. Combination of various treatment modalities including surgery, chemotherapy and irradiation has improved overall survival during the last two decades. Nevertheless, prognosis for high-risk patients remains dismal, despite increasing intensity of therapy.[1,2] Resistance of NB cells to chemotherapeutic agents such as vincristine (VCR), doxorubicin (DOX) and cisplatin is often present at diagnosis and increases during therapy. Mechanisms involved in this phenomenon include dysregulation of the apoptotic pathway and multidrug resistance gene expression. Therefore, new and specific anti-NB agents are needed that ideally have a greater therapeutic ratio and act independently of the aforementioned pathways.

The tetracyclic diterpene antibiotic aphidicolin (APH), produced by *Cephalosporium aphidicola*,[3] reversibly inhibits eukaryotic DNA polymerases α and δ, without affecting DNA polymerase β and γ, reverse transcriptase or prokaryotic DNA polymerase II.[4–6] APH specifically kills a number of different tumor cells, whereas the viability of other human malignant and non-malignant cells is not affected.[4,7–9] The mechanism of the specific action of APH towards some tumor cells remains unclear. As APH displays poor solubility in water, it cannot be administered directly *in vivo*.

Cyclodextrins (CD) are widely used to solubilize poor water-soluble substances. They are cyclic oligosaccharides consisting of six or more glucose molecules linked by α-(1,4)-glycosidic bonds that form inclusion complexes with lipophilic substances. CD are part of approved pharmaceutical products for drugs such as itraconazole and prostaglandin E_1.[10] The *in vivo* pharmacokinetics of the incorporated substances are, if there is any influence, only slightly influenced by the incorporation into CD complexes.[11] γ-CD consists of eight unsubstituted glucose molecules and has been employed to enable systemic administration of substances with a wider range of host molecules than the other smaller CD. However, its use was limited for a long time as it could not be produced at high yields and economic costs. With the development of methods to produce γ-CD economically it became of greater interest.[12,13]

We hypothesized that due to the peculiar anti-NB activity of APH, APH and the γ-CD inclusion complex of APH (APH-CD) exert equal cytotoxic effects on NB cell lines sensitive and resistant to VCR, DOX and cisplatin *in vitro*. Subsequently, the efficacy of APH-CD to treat established xenografted VCR-resistant and -sensitive NB tumors was investigated as VCR resistance is considered to be facilitated by multidrug resistance-associated protein (MRP), a gene that is overexpressed in *MYCN* amplified NB.

Materials and methods

Materials

All culture media and media supplements were purchased from Seromed (Berlin, Germany). APH, VCR, cisplatin and DOX were purchased from Sigma-Aldrich Chemie (Deisenhofen, Germany). γ-CD was obtained from AnalytiCon (Potsdam, Germany).

Preparation of APH-CD

Solutions were prepared freshly directly before the beginning of the experiments. APH and γ-CD were used in equal molecular concentrations. A suspension of APH was prepared in water under stirring. γ-CD was added to the suspension. Complex formed automatically during stirring within about 4 h, leading to a clear solution. Before usage solutions were filtered through sterile 0.22-μm filter units.

Cells

Human NB cell line IMR-32 was obtained from ATCC (Rockville, MD). The NB cell lines UKF-NB-2 and UKF-NB-3 were established from metastasis harvested in relapse in two of our patients with Evans stage 4 NB.[9,14] The cells were propagated in IMDM supplemented with 10% FBS, 100 IU/ml penicillin and 100 mg/ml streptomycin at 37°C in a humidified 5% CO_2 incubator.

Resistant cell lines were obtained as described before[15] by long-time incubation of tumor cells with increasing concentrations of VCR, DOX and cisplatin, respectively.

Cytotoxicity assay

Drug toxicity in NB cells was determined by measurement of the reduction in mitochondrial uptake of the dye MTT[16] carried out as described previously.[17,18] Briefly, cells were suspended in culture medium (4×10^5 cells/ml) and 50 μl aliquots were pipetted into 96-well microtiter plates. Medium (50 μl aliquots containing various concentrations of the test compounds) was then added. On day 4 of incubation the MTT solution (2 mg/ml) was added, the plates were incubated at 37°C for 4 h and cells lysed in PBS containing 20% (w/v) SDS and 50% (v/v) *N*,*N*-dimethylformamide adjusted to pH 4.5. The absorbance at 570 nm was determined for each well using a multiwell ELISA reader. After subtracting the background absorbance, results were expressed as percentage of control cultures in the absence of drug. The IC_{50} values were calculated. The results are mean of three experiments.

Animals

Female, 4- to 6-week-old outbred CD-1 strain athymic nude mice (nu/nu) (AnLab, Charles River, Czech Republic) weighing 18–20 g were kept under sterile conditions and obtained sterile nutrition and water

To establish xenotransplanted NB tumors 5×10^6 NB tumor cells were injected s.c. into the right flank of mice. Cells were administered together with Matrigel in a total volume of 0.2 ml. Tumor size was then measured using a calliper and calculated by the formula volume = (length × width^2)/2.

Treatment of mice with s.c. tumors

Treatment was started after tumors grew to a size of about 100 mm^3 (range 65–190 mm^3). Mice with VCR-sensitive (UKF-NB-3) and VCR-resistant (UKF-NB-3^rVCR[20]) tumors were treated in the same manner. Animals received 0.1 ml of drug solution i.p. twice a day. Total daily dose of APH (administered as γ-CD inclusion complex) in group A was 15 mg/kg body weight and in group B 7.5 mg/kg body weight. No

higher APH doses could be examined because of the limited solubility of the APH-CD complex in water. Group C received 0.5 mg/kg body weight VCR once a week in the first 2 weeks. As a control, group D was given γ-CD 7.5 mg/kg body weight/day and group E was treated with PBS only. Treatment was performed for 30 days. At day 10 and 20 one mouse of each group was sacrificed. Blood was sampled from the eye plexus of one mouse of each group and blood count was prepared to detect possible effects of APH treatment on the blood count.

After day 30 remaining mice were sacrificed and treated in the same manner. Tumor size was measured and body weight controlled every third day

Statistics

Tumor volumes, body weights and blood counts of treatment groups were compared for statistically significant differences using Jandel SigmaStat (version 2.0, Jandel, Erkrath, Germany).

Results

Resistant cells

The IC_{50} values for cisplatin, DOX, VCR, APH and APH-CD against established drug-resistant NB cell lines *in vitro* are shown in Table 1. IC_{50} concentrations of cisplatin-, DOX- and VCR-resistant NB cell lines for the corresponding drugs are markedly higher than the IC_{50} levels of parental NB cell lines. DOX and VCR resistance is associated with reciprocal chemoresistance for subclones of both parental UKF-NB 2 and UKF-NB 3 cells. Evidently, antitumoral action of APH and APH-CD *in vitro* was equally effective against drug-sensitive parental and subsequent drug-resistant NB subclones. Nevertheless, APH-CD was about 2-fold less effective than APH in all NB cell lines. The maximum APH concentration in the APH-CD inclusion complex that inhibited 50% of tumor cell growth was 440 ng/ml, i.e. 1.3 μM. γ-CD did not influence cell growth of the different cell types in concentrations up to 20 μM.

Treatment of s.c. tumors

Influence of treatment on tumor growth. The influence of the different treatment regimens on the tumor growth is shown in Table 2 and in Figure 1. APH, administered as APH-CD, inhibited tumor growth of VCR-sensitive tumors (UKF-NB-3) in a dose-dependent manner.

In the control group E, treated with PBS solution, the average tumor size at day 30 reached 808±185% relative to the tumor size at the beginning of treatment. In group A, receiving 15 mg/kg body weight APH, the average tumor size was 238±109% relative to tumor size at the beginning of treatment. In the group B, treated with 7.5 mg/kg body weight APH, average tumor volume reached 427±73%. In the group D, treated with 7.5 mg/kg body weight CD, the average tumor size in the treated mice was 625±205%. Mice of group C, that received VCR 0.5 mg/kg body weight once a week 2 times during

Table 2. Effect of treatment on growth of neuroblastoma tumours *in vivo*

Treatment	Tumor size at day 30 relative to tumor size at day 0 (%)	
	UKF-NB-3	UKF-NB-3^{r}VCR^{20}
Control	808±185	833±81
15 mg APH/kg	237±109	295±102
7.5 mg APH/kg	427±73	318±130
VCR	54±10	1019±340
CD	624±205	712±130

Table 1. IC_{50} values of parental and resistant neuroblastoma cell lines

Cells	IC_{50} (ng/ml)				
	Cisplatin	DOX	VCR	APH	Aphidicolin-CD
IMR-32	126±38	9.7±1.1	0.21±0.031	240±35	300±51
IMR-32^{r}CDDP^{500}	1560±216	13.5±2.0	0.38±0.056	280±29	410±58
UKF-NB-2	165±19	6.5±0.8	0.31±0.023	220±24	380±65
UKF-NB-2^{r}DOX^{100}	210±25	397±44	124±14	190±16	420±58
UKF-NB-2^{r}VCR^{20}	135±17	65±7.3	85±9.3	210±24	440±81
UKF-NB-3	142±15	12.5±1.9	0.15±0.01	150±29	310±59
UKF-NB-3^{r}DOX^{100}	115±13	855±96	45.6±5.5	110±19	260±45
UKF-NB-3^{r}VCR^{20}	220±27	185±23	51.3±7.2	180±25	330±62

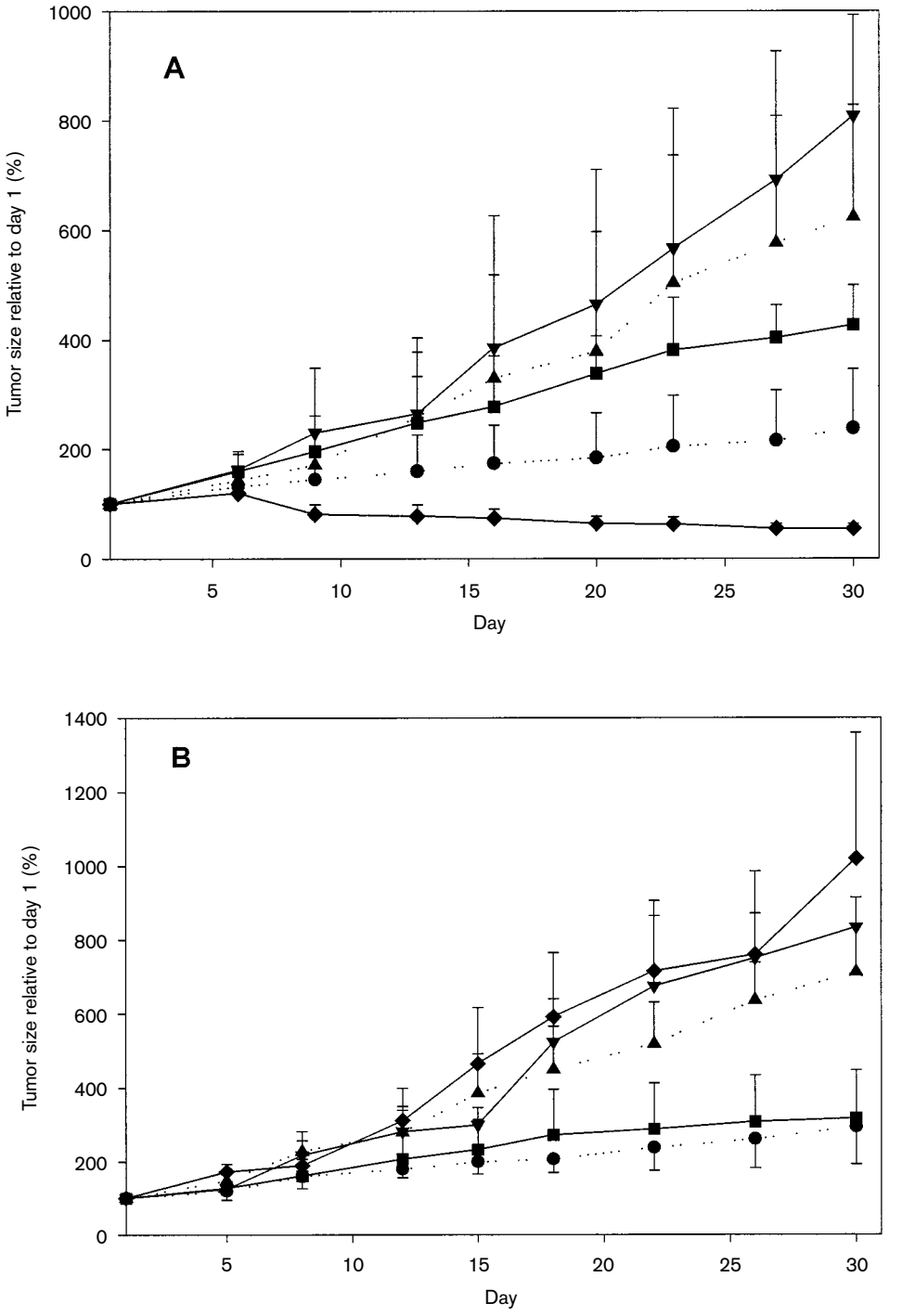

Figure 1. Influence of different treatment regimens on (A) UKF-NB-3 and (B) UKF-NB-3^{r}VCR^{20} tumors in mice: (a) APH (administered as γ-CD complex) 15 mg/kg/body weight/day (●), (b) APH (administered as γ-CD complex) 7.5 mg/kg/body weight/day (■), (c) VCR (◆), (d) γ-CD (▲) and (e) control (▼).

whole experiment, developed tumors with an average volume of $54 \pm 10\%$

One-way ANOVA indicated a significant difference between the mean values of the different treatment groups ($p < 0.001$). The groups were compared using all pairwise multiple comparison procedures (Tukey test). Significant differences ($p < 0.05$) were seen between the control group and groups treated with

VCR, APH 15 mg/kg body weight and APH 7.5 mg/kg body weight. No significant difference could be detected between the control group and the group receiving CD. No statistical difference was also seen between APH 15 mg/kg body weight and treatment with VCR or APH 7.5 mg/kg body weight, respectively. Nevertheless, VCR treatment was significantly more effective than treatment with APH 7.5 mg/kg body weight. VCR and APH 15 mg/kg body weight showed significantly higher tumor growth inhibition than treatment with CD, whereas CD and APH 7.5 mg/kg body weight did not influence tumor growth in a significantly different manner.

APH also inhibited tumor growth of VCR-resistant tumours (UKF-NB-3^{r}VCR^{20}) in a dose-dependent manner (Table 2 and Figure 1). In the control group E, treated with PBS solution, the average tumor size at day 30 of the three examined mice was $833 \pm 81\%$. In group A, receiving 15 mg/kg body weight APH, the average tumor size at day 30 was $295 \pm 102\%$, in group B (treated with 7.5 mg/kg body weight APH) it was $318 \pm 130\%$. Mice of group D (treated with 7.5 mg/kg body weight CD) developed tumors with a size of $712 \pm 130\%$. In group C, that received VCR 0.5 mg/kg body weight once a week 2 times during the whole experiment, the mice had tumors with an average volume of $1019.25 \pm 340\%$.

One-way ANOVA indicated a significant difference between the mean values of the different treatment groups ($p < 0.001$). The groups were compared using all pairwise multiple comparison procedures (Tukey test). Significant differences ($p < 0.05$) were detected between the control group and the groups treated with APH-CD. No significant difference was calculated between the control group and the groups receiving VCR and CD. APH treatment was significantly more antitumorally efficient than administration of VCR and CD, whereas no significant difference between the two APH concentrations was seen. VCR treatment was not significantly different from CD treatment.

Student's *t*-test was performed in order to compare growth inhibition of treatment regimens in VCR-sensitive and -resistant tumors. VCR-resistant and -sensitive tumors grew in the same manner (p=0.836). No significant difference could also be detected between tumor growth inhibition caused by APH-CD in sensitive and resistant tumors (APH 15 mg/kg body weight p=0.419, APH 7.5 mg/kg body weight p=0.221). Moreover, CD treatment did not influence tumor growth of resistant and sensitive UKF-NB-3 cells in a different manner (p=0.427). A significant difference between VCR-sensitive and -resistant NB tumors was only observed in the case of VCR treatment, indicating the high VCR resistance of the UKF-NB-3^{r}VCR^{20} cell line ($p < 0.001$).

Influence of treatment on body weight

The body weight at day 30 was expressed in percent relative to the body weight at the beginning of the experiment. One-way ANOVA was performed to detect significant changes in the differently treated groups. A statistically significant difference was only detected in the group of mice with VCR-sensitive tumors (p=0.049). The Tukey test only showed a significant difference ($p < 0.05$) between body weights of the control group and the VCR group. No other significant difference was detected. Within the group of mice with VCR-resistant tumors no statistically significant differences between the different treated groups could be detected (p=0.447).

Influence of treatment on blood counts

No significant differences between the different treatment groups could be detected (data not shown).

Discussion

The intention of these investigations was to examine whether APH exerts antineoplastic activity against NB cells that are resistant to the chemotherapeutic drugs VCR, DOX and cisplatin, that are commonly used in NB treatment protocols. APH and γ-CD inclusion complexes of APH reduced growth of parental drug-sensitive NB cells and drug-resistant NB subclones at equimolar doses *in vitro*. The IC_{50} levels of APH in NB cells sensitive and resistant to VCR, DOX or cisplatin did not differ significantly. APH-CD, which is used to overcome the poor solubility of APH in *in vivo* experiments, is also active against all used NB cell lines. However, the antitumoral action of APH-CD *in vitro* is about 2-fold lower than that of APH. This is most probably associated with some changes in the pharmacokinetics under *in vitro* conditions, due to the release of APH from its γ-CD inclusion complexes. The differences were not further investigated as CD are known to exhibit, if any, only a slight influence on drug pharmacokinetics *in vivo*.

Corroborating these findings, growth of vincristine-sensitive and -resistant human xenotransplanted NB tumors was inhibited by APH-CD treatment at equal doses. No signs of toxicity were observed in mice treated with APH-CD at antitumorally active concentrations. Our findings underline previous investigations that showed APH to be a promising

antineoplastic substance. It was already shown to induce cellular differentiation in leukemic[8,19] and rhabdomyosarcoma cells,[20] to reverse cisplatin resistance in ovarian cancer cells,[21] to enhance the antitumoral efficacy of platinum compounds and cytarabin,[22,23] to act synergistically with VCR and DOX *in vitro*,[24] and to enhance the effect of X-ray irradiation on melanoma cells.[25] These former studies showed that APH is suitable for use in combination with established therapy regimens that usually include chemotherapy and irradiation therapy.[1,2,26–28] In addition, our data demonstrate that APH offers potential for use against therapy-resistant tumor cells *in vitro* and *in vivo*. Therefore, it is a promising candidate for anticancer therapy with a remarkably low cytotoxicity against non-malignant cells.[4,7–9] In addition, the water-soluble APH derivative APH glycinate was already proven to be safe for use in humans in a phase I clinical trial.[29]

In summary, APH was able to kill normal and chemotherapy-resistant cells *in vitro*. *In vivo* APH-CD inhibited tumor growth of normal and VCR-sensitive cell lines significantly without any signs of toxicity. Therefore, APH-CD is worth being further investigated as an additional tool for antitumoral therapy and is suitable for evaluation in clinical studies of NB treatment protocols.

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