Topical application of valrubicin has a beneficial effect on developing skin tumors

Stine M. Andersen, Cecilia Rosada, Frederik Dagnaes-Hansen¹, Ina G. Laugesen, Elisabeth de Darkó², Tomas N. Dam³ and Karin Stenderup⁴

Department of Dermatology, Research Center S, Aarhus University Hospital, P.P. Oerumsgade 11, building 15, 8000 Aarhus C, Denmark, ¹Institute for Medical Microbiology and Immunology, University of Aarhus, Aarhus, Denmark and ²Valderm ApS, 2800 Lyngby, Denmark and ³Department of Dermatology, Roskilde Hospital, 4000 Roskilde, Denmark

¹To whom correspondence should be addressed.
Tel: +0045 89 49 19 07; Fax: +0045 89 49 18 50;
Email: karin_stenderup@email.dk

Valrubicin is a second generation anthracycline characterized by an excellent safety profile presenting no skin toxicity or necrosis upon contact. In its current liquid formulation (Valstar®; Indevus Pharmaceuticals, Lexington, MA), it is approved solely for the treatment of bladder cancer. Recently, valrubicin was incorporated in a cream formulation rendering this drug available for topical application. The cytostatic property of valrubicin can, thus, be employed for treating hyperproliferative skin diseases as was recently described for psoriasis. In the present study, the effect of topical application of valrubicin was investigated in skin tumor development; we hypothesized that valrubicin may be employed in treating actinic keratosis, a hyperproliferative skin condition that may transform into malignancy. A two-stage chemical mouse skin carcinogenesis model that represents the multistage etiology of human skin cancer—from developing papillomas to squamous cell carcinoma (SCC) was used. Moreover, two human skin SCC cell lines: DJM-1 and HSC-1 were cultured, to further investigate the effect of valrubicin in vitro. Cell viability was assessed by adenosine triphosphate presence, proliferation as proliferative cell nuclear antigen expression and apoptosis as cytokeratin 18 cleavage, caspase activation, poly-adenosine diphosphate-ribose-polymerase cleavage and bax and bcl-2 regulation. Valrubicin significantly inhibited tumor formation in the mouse skin carcinogenesis model and significantly decreased cell viability of the cultured human skin SCC cells. In both mouse skin and SCC cells, proliferation was significantly decreased. Apoptosis was significantly increased in SCC cells but unchanged in the treated mouse skin at study completion. This study demonstrated that topical application of valrubicin has a beneficial effect in treating developing skin tumors.

Introduction

Anthracyclines are excellent cytostatic drugs and very effective antineoplastic agents, however, their use as topical agents has not been possible due to their induction of contact toxicity and necrosis; in fact, severe tissue damage upon accidental leakage of anthracyclines into subcutaneous tissue has been observed during intravenous therapy (1–3). In the pursuit to improve the safety profile of these effective drugs, the second generation anthracycline: valrubicin (N-trifluorooracetyladriamycin-14-valerate) was developed by modifying doxorubicin (Adriamycin®; Pharmacia S.p.A, Milan, Italy) by two side chain substitutions producing a molecule characterized by an improved safety profile and the lack of skin toxicity, thus permitting topical use (1,4). A cytostatic treatment for topical use is therefore available to potentially treat hyperproliferative skin diseases such as actinic keratosis (AK), squamous cell carcinomas (SCCs) and psoriasis. In fact, valrubicin in a cream formulation was recently shown to induce resolution of psoriasis upon topical treatment (5).

The aim of the present study was to investigate the effect of topical valrubicin treatment on skin tumor development. AK is a potential premalignant condition of the skin presenting thick, scaly or crusty patches of hyperproliferative keratinocytes, which may progress into SCC (6), a non-melanoma skin cancer currently experiencing an increasing incidence rate (7). Treatment of AK and SCC often includes photodynamic therapy, excision, cryotherapy and/or topical treatment with immune-modulating or cytostatic drugs (8).

The available topical treatments, however, are associated with local skin reactions such as ulcerations, rash, pruritus and discomfort that may extend beyond the application site (8); consequently, an unmet need for improved topical treatments exists. Of note, adverse skin reactions were not observed in topical valrubicin-treated human psoriasis skin or surrounding mouse skin in the psoriasis xenograft transplantation mouse model (5).

To investigate the effect of topical application of valrubicin on non-melanoma skin tumor development in vivo, we used the recognized two-stage chemical mouse skin carcinogenesis model where the mouse skin is exposed to a cancer promoter and then repeatedly subjected to a proliferating agent (9,10). Valrubicin in the cream formulation was applied once daily after the appearance of tumor formation and was able to reduce the number and size of the tumors, without induction of visible adverse skin reactions. Moreover, we assessed the effect of valrubicin on tumor cell viability, proliferation and induction of apoptosis in vitro in two established human skin SCC cell lines: DJM-1 (11) and HSC-1 (12). Valrubicin was shown dose dependently to decrease cell viability that, in part, may be due to the observed decrease in proliferation and induction of apoptosis in the two cell lines.

This study demonstrated a beneficial effect of valrubicin in preventing skin tumor development, an effect shown to be related to decreased cell proliferation and increased apoptosis.

Materials and methods

Mice

Thirty-nine female BALB/c mice, 6–8 weeks old, (Taconic M & B, Silkeborg, Denmark) were housed under standard conditions with controlled temperature and humidity and 12 h light–dark alternating cycles. The mice received a commercial diet and water ad libitum. The well-being of the animals was controlled daily. Animal studies were carried out with approval of the Danish Experimental Animal Inspectorate.

Two-stage chemical mouse skin carcinogenesis model

The mice were shaved on the dorsal side and treated with a single application of tumor initiator 7,12-dimethylbenz[a]anthracene (DMBA; 25 μg in 100 μl acetone; Sigma–Aldrich, St Louis, MO) on day 1. After 1 week, 12-O-tetradecanoylphorbol 13-acetate (TPA; 4 μg in 100 μl acetone; Sigma–Aldrich) was applied three times weekly for a total of 22 weeks. After 6 weeks, visible tumors had developed, and the mice were divided into three treatment groups, representing equal numbers of tumors. Group 1 received valrubicin cream (1% formulation) topical treatment once daily 5 days weekly (n = 13), group 2 received vehicle cream topical treatment once daily 5 days weekly (n = 13) and group 3 was left untreated (n = 13). Valrubicin and vehicle cream formulations were generously provided by Valderm ApS (Lyngby, Denmark). Tumors were defined as papillomas elevated >1 mm, >1 mm in diameter and present for at least 2 weeks. Tumor diameters were measured with a Vernier caliper and the tumor volume (V) calculated as V = 4/3 × π × d₁ × d₂ × d₃, where d₁, d₂ and d₃ are the three cross-sectional diameters (13,14). To measure potential systemic uptake of valrubicin, retro-orbital blood samples were collected after 4 days of treatment and on the day of killing. The isolated plasma samples were stored at –80°C until measurement.

Cell cultures

Two human skin SCC cell lines: DJM-1 (generously provided by Yasou Kitajima, Gifu University, Gifu, Japan) (11) and HSC-1 (Health Science Institute, Japan) (12) were cultured, to further investigate the effect of valrubicin in vitro. Cell viability was assessed by adenosine triphosphate presence, proliferation as proliferative cell nuclear antigen expression and apoptosis as cytokeratin 18 cleavage, caspase activation, poly-adenosine diphosphate-ribose-polymerase cleavage and bax and bcl-2 regulation. Valrubicin significantly inhibited tumor formation in the mouse skin carcinogenesis model and significantly decreased cell viability of the cultured human skin SCC cells. In both mouse skin and SCC cells, proliferation was significantly decreased. Apoptosis was significantly increased in SCC cells but unchanged in the treated mouse skin at study completion. This study demonstrated that topical application of valrubicin has a beneficial effect in treating developing skin tumors.
Research Resources Bank, Osaka, Japan) (12) and HaCaT, a spontaneously transformed normal keratinocyte cell line originally established by N.E. Fusenig (15) were employed. All cell lines were grown in Dulbecco’s Modified Eagle Medium (Invitrogen, Carlsbad, CA), containing 1% penicillin, streptomycin and gentamycin (Bie & Berntsen, Aabyhøj, Denmark), at standard conditions: 37°C, 5% CO₂ and 95% humidity. Medium for the DIM-1 cell line contained additional 10% fetal bovine serum (Invitrogen), 0.4 µg/ml hydrocortisone (H0396; Sigma–Aldrich), 20 ng/ml epidermal growth factor (13247-051; Invitrogen) and 84 ng/ml cholera toxin (C8052; Sigma–Aldrich). Medium for HSC-1 cell line contained additional 20% fetal bovine serum (Invitrogen) and medium for HaCaT cell line contained additional 10% fetal bovine serum (Invitrogen).

Primary normal human epidermal keratinocytes (NHEKs) were isolated from three healthy donors undergoing skin reductive surgery as described previously (16). NHEK were grown in standard conditions in keratinocyte serum-free medium (Invitrogen) containing the supplements for keratinocyte growth (epidermal growth factor and pituitary extract: Invitrogen) and 5 µg/ml gentamicin.

Cell viability in vitro

The effect of valrubicin on skin SCC cell viability was measured upon exposure of DIM-1 and HSC-1 cell lines to valrubicin (0.05, 0.10, 0.50 and 1.00 µg/ml), AD41 (N-trifluoroacetyladriamycin), the principal active metabolite of valrubicin, 0.50, 1.00, 5.00 and 10.00 µg/ml) or doxorubicin (0.05, 0.10, 0.50 and 1.00 µg/ml) for 24 and 48 h. Cell viability was assessed by the CellTiter-Glo® Viability Assay Kit (Promega, Madison, WI) and expressed as percentage compared with vehicle-treated cultures. Cells were seeded in a concentration of 2000 cells per well (6500 cells/cm²) in Petri dishes (10 cm Ø) and cultured until at least 80% confluence, before stimulation for 2, 6 and 24 h, at these concentrations, decreased cell viability of skin SCC cells was still not detectable after 24 h. To isolate cellular proteins, cells were washed twice in ice-cold phosphate-buffered saline and snap frozen in liquid nitrogen. Cells were lysed in ice-cold lysis buffer (50 mM Tris–HCl, pH 6.8, 10 mM dithiotreitol, 10 mM β-glycerophosphate, 10 mM sodium fluoride, 0.1 mM sodium orthovanadate, 10% glycerol, 2.5% sodium dodecyl sulfate and 1 mM phenylmethylsulfonyl fluoride and Complete®–EDTA-free protease inhibitor cocktail (Roche Diagnostics) and stored at −80°C until use.

Statistics

Student’s t-test was employed to determine differences in mean numbers of tumors, percentage of mice with tumors, percentage of cell viability, PCNA protein levels, percentage of apoptotic cells and the caspase 3 activity. P values <0.05 were considered significant.

Results

Topical valrubicin treatment has a beneficial effect on tumor development in the two-stage chemical mouse skin carcinogenesis model

A two-stage chemical mouse skin carcinogenesis model was used to investigate the effect of topical application of valrubicin on the development of skin tumors. Upon induction and promotion of the tumors by DMBA and TPA exposure, mice were divided into three groups and subjected to once daily topical application of either valrubicin or vehicle cream, whereas the last group was left untreated. TPA was applied throughout the study and mice were exposed to treatment for 16 weeks. Tumors were counted once weekly and the mean number of tumors in each treatment group is shown (Figure 1a). Tumor formation was maximal after 4 weeks of treatment for the vehicle and the untreated group with approximately five and four tumors in each group in average, respectively. In the valrubicin-treated groups, tumors were not observed until week 7 of treatment where approximately one tumor in average per mouse was observed. Posttreatment, the mean number of tumors in the valrubicin-treated group was significantly lower than in both the vehicle and the untreated group. No difference in the mean number of tumors was found between the vehicle and the untreated group.

At killing, the percentage of mice with tumors in the valrubicin-treated group was significantly lower than in the vehicle and the untreated group (Figure 1b). The valrubicin-treated group had a total of five tumors, whereas the vehicle and the untreated group had 30 and 37 tumors, respectively. The tumor size was measured with a Vernier caliper and the tumor volume (V) calculated; besides decreasing the number of tumors, valrubicin was also found to decrease the tumor volume (Figure 1c). Representative mice from the valrubicin (Figure 1d) and the vehicle cream-treated groups (Figure 1e) are shown.

The well-being of the animals was not affected by either valrubicin or vehicle cream treatment, as indicated by the recorded weight gain and general well-being of each mouse throughout the study (data not shown); noteworthy, no skin adverse reactions to valrubicin in the treated animals (Figure 1d) was observed. As systemic absorption of valrubicin might be a concern, due to skin penetration or oral uptake resulting from the grooming activities of the mice, blood samples were collected after 4 days of treatment start and at killing. Valrubicin was not detected in any blood samples, however, AD41, its main active metabolite, and doxorubicin, as metabolic product,
were found in 9 and 4 of 13 mice in the range of 1.0–21.0 ng/ml and 5.8–13.3 ng/ml at the end of study, respectively.

Valrubicin decreases cell viability of human skin SCC cells in vitro

Valrubicin was demonstrated to have a beneficial effect on developing skin tumors in mouse skin, and to investigate the effect of valrubicin on human material, we investigated the effect on cultured human skin SCC cells. In this assay, AD41, the principal active metabolite, was included to assess whether it may contribute to the effects observed by valrubicin. AD41 has previously been demonstrated to localize in the nucleus where it intercalates with DNA thus affecting replication (19), whereas valrubicin is localized in the cell cytoplasm. Both valrubicin and AD41 were able to inhibit cell viability after 24 and 48 h in a dose-dependent manner. Valrubicin showed a significant inhibition of cell viability of both DJM-1 and HSC-1 cells already after 24 h stimulation at the highest dose (1.00 μg/ml) and after 48 h at the dose of 0.10 μg/ml (Figure 2a and d). AD41 also inhibited cell viability of both cell lines at a 5- to 10-fold higher dose as compared with valrubicin (Figure 2b and e). Doxorubicin was found to be most effective in inhibiting cell viability (Figure 2c and f). For comparison, valrubicin and AD41 has previously been shown to decrease cell viability of both HaCaT and NHEK cells (5).

Valrubicin decreases proliferation potential in vivo and in vitro

Decreased tumor growth in vivo and decreased cellular viability in SCC cells may in part be caused by a decrease in cellular proliferation. To investigate whether the effect seen in vivo on tumor formation may be ascribed to an inhibition of keratinocyte proliferation, the nuclear protein fraction was isolated from treated skin of valrubicin-treated (n = 11) and vehicle (n = 12)-treated mice, and the expression levels of the proliferation marker PCNA were measured. It was found that topical treatment with valrubicin cream decreased the PCNA expression as compared with vehicle treatment (P = 0.058, Figure 3a). This indicates that valrubicin may explicate its beneficial effect on preventing tumor formation, in part, by decreasing the proliferation potential of mouse skin cells in vivo. To further investigate the antiproliferative effect of valrubicin on developing human skin tumors, the effect of valrubicin in human keratinocytes in both malignant and premalignant stages was evaluated. For this purpose, the human skin SCC cell lines DJM-1 and HSC-1, HaCaT and NHEK

Fig. 1. Topical valrubicin treatment has a beneficial effect on tumor development in the two-stage chemical mouse skin carcinogenesis model. Mouse tumor development was initiated with DMBA and promoted with TPA. After initial tumors were observed, mice were divided into three treatment groups and subjected to 16 weeks of treatment: group 1 received daily topical treatment with valrubicin cream (n = 13), group 2 received vehicle cream (n = 13) and group 3 was left untreated (n = 13). Tumors were counted once weekly and expressed as mean number of tumors per treatment group (a). The percentage of mice with tumors, in each group, was calculated once weekly (b). * indicates significant difference at killing between both vehicle and valrubicin and untreated and valrubicin (P < 0.05). In each group, tumor size was measured and the volume calculated (c). Four representative mice showing the effect of valrubicin (d) and vehicle cream (e) treatment are shown.
cells were grown *in vitro* and exposed to valrubicin. Doxorubicin was included as positive reference. In all cell types, valrubicin was shown to decrease the expression level of PCNA (Figure 3b–e). In HSC-1, HaCaT and NHEK cells, the decrease was significant after 2 h of stimulation, whereas in DJM-1 cells, the decrease was not significant until 6 h of stimulation.

**Valrubicin induces apoptosis**

Decreased cell viability can result from either a reduction in cell proliferation and/or an induction of apoptosis. Thus, the effect of valrubicin in inducing apoptosis was also investigated. *In vitro*, the human skin SCC cell lines DJM-1 and HSC-1 and NHEK cells were investigated and stimulated for 1 h with valrubicin at a concentration corresponding to 20% inhibition of cell viability at 24 h; doxorubicin was included as positive treatment control. HaCaT cells were omitted in this investigation, as increased apoptosis in this cell line induced by valrubicin has previously been demonstrated (5). Positive staining for the cleavage product of cytokeratin 18, indicative of apoptosis, was demonstrated after exposure to valrubicin and doxorubicin in all three cell types. DJM-1 cells exposed to doxorubicin showed a significant 5-fold increase in apoptotic cell number (25%) compared with control cells (5%). Apoptosis induction by valrubicin was not determined (Figure 4a). HSC-1 cells showed a significant increase in apoptotic cell number upon exposure to valrubicin (7%) and doxorubicin (4%) compared with their respective controls (no apoptotic cells detected) (Figure 4b). NHEK cells showed as well a significant increase in apoptotic cell number compared with controls (6 and 3% upon exposure to valrubicin and doxorubicin, respectively) (Figure 4c). A representative staining upon valrubicin (Figure 4d) and control (Figure 4e) stimulation are shown.

To further investigate valrubicin’s mode of action in inducing apoptosis, we investigated the activation of several apoptotic caspases. *In vitro*, the human skin SCC cell lines DJM-1 and HSC-1 cells were stimulated with valrubicin and AD41 at concentrations where decreased cell viability of skin SCC cells was still not detectable after 24 h; doxorubicin was included as positive treatment control. After 24 and 48 h exposure, caspases 3 and 7 were significantly activated in DJM-1 cells by both valrubicin and AD41 (Figure 5a). Valrubicin and AD41 only slightly increased the activity of caspases 3 and 7 in HSC-1 cells, and the effect was seen only after 48 h (Figure 5b). No activation of caspases 8 or 9—upstream caspases—was observed (data not shown).

A downstream effect of valrubicin’s activation of caspases 3 and 7, in the event of apoptosis, is the cleavage of PARP, a protein that binds specifically at DNA strand breaks. *In vivo*, total and cleaved PARP expression levels were measured in mouse skin treated with valrubicin (*n* = 11) and vehicle (*n* = 12), however, no expression of cleaved PARP was detected upon topical treatment with either valrubicin or vehicle cream (Figure 6a). *In vitro*, the human skin SCC cell lines DJM-1 and HSC-1, HaCaT and NHEK cells were stimulated with valrubicin at a concentration where decreased cell viability of skin SCC cells was still not detectable after 24 h. Cleaved PARP was detected in DJM-1, HSC-1 and HaCaT cells indicating induction of apoptosis (Figure 6b), however, no cleaved PARP was detected in NHEK cells (Figure 6b).

In addition to PARP, the effect of valrubicin on the expression levels of the pro-apoptotic protein bax and the anti-apoptotic protein bcl-2 was as well investigated both *in vivo* and *in vitro*. *In vivo*, it was found that topical treatment with valrubicin cream upregulated the expression level of bax, however, the expression level of bcl-2 was not affected (data not shown). *In vitro*, the expression levels of bax and

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**Fig. 2.** Valrubicin decreases cell viability of human skin SCC cells *in vitro*. The human skin SCC cell lines DJM-1 and HSC-1 were cultured in the presence of valrubicin (a and d), AD41 (b and e) and doxorubicin (c and f). All three drugs dose dependently inhibited cell viability of both cell types. AD41 inhibited proliferation at a 5- to 10-fold higher concentrations as compared with valrubicin. Results shown are representative of four independent experiments. * indicates significant difference between drug and vehicle treatment (*P* < 0.05).
bcl-2 were observed to be regulated in HSC-1 and NHEK cells, but not in DJM-1 and HaCaT cells (data not shown).

Discussion

Valrubicin is an anthracycline with recognized cytostatic characteristics and antineoplastic effects similar to its parental drug, doxorubicin (20). Nonetheless, valrubicin is superior to other anthracyclines since it exhibits a favorable safety profile in regards to contact toxicity and cardiotoxicity upon systemic exposure (1,21). The modifications made to the doxorubicin molecule include a valerate moiety addition and an N-trifluoroacetyl substitution. These modifications increase the lipophilicity of the molecule and seem to play a role in the improved skin and cellular absorption. Also, in contrast to doxorubicin, valrubicin demonstrates binding to cytoplasmatic protein, a way valrubicin seems to explicate its effect (5,22). Valrubicin has been shown to inhibit growth of leukemia cells (23) and melanoma cells (24) and has proven efficient in systemic treatment of SCC of the head and neck (25). Currently, valrubicin is approved for the treatment of bladder cancer, where the therapeutic potential is achieved by contact to the cells of the bladder wall (1,26,27). Until now, however, it has not been possible to use valrubicin for other topical applications in the available liquid formulation.

The incorporation of valrubicin in a cream formulation suggests future use of this drug in new clinical settings and prompts investigation of the effect of valrubicin in hyperproliferative skin diseases. Recently, topical application of valrubicin was shown to ameliorate psoriasis, a disease characterized by hyperproliferative keratinocytes (5).

In the present study, the effect of valrubicin on the premalignant and malignant conditions AK and SCC was addressed. Whether AK transforms into SCC or not is still under debate (6); some consider AK as a distinct disease (28) and others consider it as merely a variant form of SCC (29,30). Nevertheless, both AK and SCC are hyperproliferative conditions that may benefit from valrubicin treatment. To study an antineoplastic treatment addressing tumor development and progression, the two-stage chemical carcinogenesis mouse model was found suitable (9,10). In this model, the malignant transformation is initiated by exposure of the mouse skin to the carcinogen DMBA and promoted by TPA. Upon repeated applications of TPA, the DMBA-modified cells hyperproliferate and produce papillomas that may spontaneously transform into SCC (31), a scenario comparable with the transformation of human AK into SCC.

A histological examination of the largest tumors showed protrusion of the basal membrane, indicative of malignant tumor development (data not shown). In order to assess the nature of the induced tumors, TPA was withdrawn from a group of mice (n = 8) when presenting maximal tumor appearance (four to five tumors in average per mouse). During the following 8 weeks, some of the previously observed tumors spontaneously resolved, indicating that not all tumors in this model represented malignant tumors (data not shown). Valrubicin in the cream formulation was applied for 16 weeks after skin tumor appearance and found to significantly inhibit tumor development both in tumor number and size, suggesting an effect on both premalignant and malignant growths.
Interestingly, valrubicin and TPA have both been demonstrated to bind to protein kinase C (PKC): TPA as an activator and valrubicin as a competitive inhibitor of this serine–threonine phosphokinase (9,32). It could therefore be speculated that the observed effect of valrubicin on tumor development was merely a consequence of blocking TPA activation of PKC, as opposed to a direct inhibition of tumor development. This, however, seems unlikely as it has been shown that TPA binding to the phospholipids of the membrane bilayer, preceding PKC activation, hinders the ability of valrubicin to inhibit TPA action (32); moreover, in this study, TPA was applied 30 min prior to valrubicin treatment, and TPA has been shown to explicate its effect within 10 min after application in vitro (33). Nevertheless, a partial inhibition of TPA activation by valrubicin cannot be ruled out. Also, it cannot be excluded that TPA and valrubicin explicate their effects through distinct routes possibly owing to a potential different affinity for the various PKC isoforms (34,35), of which at least five are expressed in keratinocytes (36–38). Which PKC isoforms are targeted by valrubicin is still unknown and requires further research.

In the two-stage chemical carcinogenesis mouse model, TPA is applied in a solution of acetone, which gives rise to perturbed skin that may facilitate penetration and drug absorption. Therefore, systemic uptake of valrubicin was assessed by measuring the presence of

![Fig. 4](https://academic.oup.com/carcin/article-abstract/31/8/1483/2477414)

**Fig. 4.** Valrubicin induces apoptosis via cleavage of cytokeratin 18 in vitro. Induction of apoptosis by valrubicin was evaluated by staining for the cleavage product of cytokeratin 18. The human skin SCC cell lines DJM-1 and HSC-1 and NHEK cells were stimulated with valrubicin and doxorubicin for 1 h, and positive stained cells (brown precipitate) were counted. Percentage of positive cells is shown for DJM-1 (a), HSC-1 (b) and NHEK (c) cells. Results shown are representative of three independent experiments, and NHEK is representative of three individual donors. * indicates significant difference between drug and vehicle treatment ($P < 0.05$). A representative staining upon valrubicin (d) and vehicle (e) stimulation are shown.

![Fig. 5](https://academic.oup.com/carcin/article-abstract/31/8/1483/2477414)

**Fig. 5.** Valrubicin induces apoptosis via caspases 3 and 7 activation in human skin SCC cells in vitro. Valrubicin’s mode of action in inducing apoptosis was evaluated by measuring the activity of caspases 3 and 7 in vitro. The skin SCC cell lines DJM-1 and HSC-1 were cultured in the presence of valrubicin, AD41 and doxorubicin. Caspases 3 and 7 activity was measured after 24 and 48 h. In DJM-1 cells, caspases 3 and 7 were activated by all three drugs (a). In HSC-1 cells, caspases 3 and 7 were activated after 24 h by doxorubicin, and after 48 h, only slightly by valrubicin and AD41 (b). Results shown are representative of three independent experiments. * indicates significant difference between drug and vehicle treatment ($P < 0.05$).
Valrubicin has a beneficial effect on skin tumors

The in vivo study showed a significant effect of valrubicin on tumor development. A decreased cell viability, *in vitro*, of the human skin SCC cell lines was as well shown confirming the cytotoxic property of valrubicin (1,21,26,40). The primary valrubicin metabolite AD41 that binds to DNA was also included in the *in vitro* study and observed to decrease cell viability although at a concentration 5- to 10-fold higher than valrubicin. This has also previously been shown for HaCaT and NHEK cells (5) suggesting that AD41 is not solely responsible for the effect of valrubicin on cell viability.

As decreased tumor growth *in vivo* and decreased cellular viability in SCC cells may, in part, be caused by a decrease in cellular proliferation, proliferation was assessed by measuring the expression level of PCNA. Valrubicin was shown to reduce PCNA expression *in vitro*, as well as *in vivo* in the hyperproliferative cells of the human skin SCC type, exposed to the drug in the soluble form. The effect *in vitro* was compared with HaCaT cells and primary keratinocytes that also showed reduced PCNA expression upon stimulation.

Decreased cell viability may also be a consequence of increased apoptosis and therefore the effect of valrubicin on apoptosis in human skin SCC cells was investigated. Cleavage of cytokeratin 18 is an early event in apoptosis and it was employed to identify an increase in the number of apoptotic cells in valrubicin-treated cell cultures. Valrubicin was demonstrated to induce apoptosis in the investigated cell lines, a result also obtained previously in HaCaT cells (5). In further investigation of valrubicin’s mode of action in inducing apoptosis, an increased activity of the apoptotic markers caspases 3 and 7 upon stimulation with valrubicin was found in both human skin SCC cell lines. Similar to valrubicin, AD41 also increased the activity of caspases 3 and 7, although at a concentration 2-fold higher than valrubicin.

A downstream effect of increased activity of caspases 3 and 7 is the cleavage of PARP. *In vivo*, no cleavage of PARP was detected indicating no increase in apoptosis at the time of skin retrieval. It may therefore be speculated that increased apoptosis took place only in the early phases of treatment and that a level of steady state was achieved after 16 weeks of treatment. To clarify this matter, additional studies on mouse skin *in vivo* and *in vitro* are needed. *In vitro*, cleaved PARP was detected in both human skin SCC cell lines as well as in the hyperproliferative HaCaT cell line supporting the hypothesis of valrubicin-induced apoptosis.

An investigation of regulation of the pro- and anti-apoptotic markers bax and bcl-2, as disequilibrium between these two markers is associated to induction of apoptosis (14,41,42), supported the observation that valrubicin induced apoptosis in HSC-1 cells. No regulation, however, was seen in DJM-1 and HaCaT cell lines. The lack of regulation in these cell lines could be ascribed to a defect in p53 expression as shown for HaCaT cells that express a mutated p53 (43).

Valrubicin’s ability to induce DNA damage and apoptosis is only limited described in the literature. However, its way of action may be suggested based on data published on the valrubicin analog AD198 (44). This analog possesses an N-benzyl group where valrubicin possesses a trifluoroacetyl group but is otherwise identical in the chemical structure; like valrubicin, it is more lipophilic and exerts a cytotoxic effect on cancer cells (45). Also, it possesses a 14 valerate side chain that mimics diacylglycerol, the natural substrate of PKC, and binding to the C1 domain of PKC delta and epsilon has been limited described in the literature. However, its way of action may be speculated based on data published on the valrubicin analog AD198 (44). This analog possesses an N-benzyl group where valrubicin possesses a trifluoroacetyl group but is otherwise identical in the chemical structure; like valrubicin, it is more lipophilic and exerts a cytotoxic effect on cancer cells (45). Also, it possesses a 14 valerate side chain that mimics diacylglycerol, the natural substrate of PKC, and binding to the C1 domain of PKC delta and epsilon has been shown (35,44). In addition, induction of apoptosis was shown as a downstream event of AD198 binding to PKC delta (46,47). Due to these molecular similarities, it may be speculated that valrubicin may induce apoptosis by binding to PKC in a similar manner as AD198.

Knowing that valrubicin mimics the di-acyl-glycerol molecule in its valerate side chain, it would be of interest to investigate the role of valrubicin in PKC regulation and downstream signaling pathways because PKC has been involved in both cancer promotion and proliferation.

In conclusion, we have demonstrated that valrubicin in a topical cream formulation has an antagonistic effect on tumor development. Moreover, valrubicin was shown to reduce cell viability in hyperproliferative human skin cancer cells, in part, due to decreased proliferation but also induced apoptosis. Valrubicin’s mode of action is still not fully understood, and further studies are needed in order to entirely appreciate the potential of this drug. Nevertheless, we propose an outstanding possibility to use this anthracycline for topical application in hyperproliferative skin diseases where surgery or other invasive approaches are unadvisable.
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References
22. Kalra,N. et al. (1982) Metabolic activation of N-acylanthracyclines pre-