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Inhibition of Chemoresistance in Primary Tumor Cells by *Camellia sinensis non fermentatum* Extract Noviphenone (NPE®)

CHIARA GIORGI[1], URS E. GASSER[2], MARION E. LAFONT[1], HANS E. HOLZGANG[1] and VERENA FONTANA THALHAMMER[1,3]

[1]*Novelpharm AG, Schlieren, Switzerland;*
[2]*ClinResearch LTD., Aesch, Switzerland;*
[3]*Highperformance Scientific AG, Freienbach, Switzerland*

Abstract. *Background/Aim: Despite improvements in cancer therapy, life expectancy after tumor recurrence remains low. Relapsed cancer is characterized by drug resistance, often mediated through overexpression of multidrug resistance (MDR) genes. Camellia sinensis non fermentatum extract is known for its anticancer properties in several cancer cell lines and might improve cancer therapy outcome after tumor recurrence. Materials and Methods: Embryonal rhabdomyosarcoma cell lines, alveolar rhabdomyosarcoma cell lines and primary rhabdomyosarcoma MAST139 cells were used to test NPE® effects on cell viability in combination with chemotherapeutic agents. Cell viability was measured by the WST-1 assay and CV staining. Gene expression levels of chemotherapy-induced efflux pumps and their activity was assessed upon NPE® treatment by measuring doxorubicin retention through evaluation of the autofluorescence signal. Results: Administration of increasing doxorubicin concentrations triggered immediate adaptation to the drug, which was surprisingly overcome by the addition of NPE®. Investigating the mechanism of immediate adaptation, MDR1 gene overexpression was observed upon doxorubicin treatment. Although NPE® did not alter pump gene expression, it was able to reduce pump activity, thus allowing the chemotherapeutic agent to stay inside the cells to exert its full anticancer activity. Conclusion: NPE® might improve chemotherapeutic treatment by re-sensitizing relapsed tumors to anticancer drugs. Fighting MDR represents the key to overcome tumor relapse and improve the overall survival of cancer patients.*

Correspondence to: Chiara Giorgi, Novelpharm AG., Laettenstrasse 27, 8952 Schlieren, Switzerland. Tel: +41 434332160, Fax: +41 434332162, e-mail: c.giorgi@novelpharm.com

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Rhabdomyosarcoma is the most common soft tissue tumor of childhood with 4.5 cases per million children/adolescents per year (1, 2). It can be divided into two main subgroups with different outcomes reflecting distinct genetic backgrounds, alveolar and embryonal (3). Alveolar rhabdomyosarcoma (aRMS) is characterized by the tumor-specific chimeric transcription factor PAX3/7-FOXO1 (4, 5). The presence of the translocation makes aRMS tumor more aggressive than embryonal rhabdomyosarcoma (eRMS) and often displays resistance to conventional chemo- and radiotherapy, resulting in a 5-year survival rate of only 30% (2, 6). Indeed, multidrug resistance (MDR) is one of the principal mechanisms by which tumors become resistant to anticancer drugs and it represents an obstacle in a successful chemotherapy (7-11). Since MDR occurs against every effective drug, it is very important to understand and modulate signaling and proteins involved in MDR to improve chemotherapy (12, 13). Efflux pumps are transmembrane proteins belonging to the family of ATP-binding cassettes (ABC) involved in the active efflux of molecules from cells (8, 14). Hydrolysis of ATP confers the ABC pumps to actively transport molecules, in this case chemotherapeutics, to the outside of cells, thus reducing toxic effects. Also RMS MDR is associated with high expression of efflux pumps; indeed, biopsies from patients before and after chemotherapy showed a different expression pattern (15, 16). Thus, new and alternative solutions to overcome drug resistance and relapse both reflect a real medical need.

Camellia sinensis non fermentatum folium (green tea; GT) is known and used for its beneficial properties since ancient time (17) exhibiting distinctive pharmacological properties, such as anti-inflammatory (17-29), anti-oxidative (11, 18, 30-36), and anti-carcinogenic effects (37-42). For these characteristics, GT is widely studied in clinical assets for cancer prevention (43); specifically, our group investigated its role in skin protection upon radiotherapy in a previous study (44). Recently, research focused on the anti-carcinogenic

properties (45). Interestingly, it was shown that GT is capable of reducing efflux pump activity (ABCB1) and therefore improving chemotherapy (46). Moreover, Epigallocatechin gallate (EGCG), the major catechin of polyphenol content in GT, was described to be the substrate of ABCB1 pumps, therefore competing with chemotherapeutics for binding (46). EGCG in combination with chemotherapeutics was also reported to exert striking and promising synergistic effects on tumor growth in chemoresistant cell lines (47-49).

For these very reasons, we investigated the effect of Noviphenone (NPE®), a quantified GT extract produced by Novelpharm AG (Schlieren, Switzerland), on RMS cells in combination with chemotherapeutics.

Materials and Methods

Cell lines. eRMS and aRMS cell lines as well as primary cells from RMS were used in our studies. The aRMS cell line RH4 and the eRMS cell line RD were kindly provided by Peter Houghton (Greehey Children's Cancer Research Institute, San Antonio, TX, USA). The primary eRMS cells MAST111 and MAST139 have previously been described as SJRHB013758 (50). They were obtained from a patient at the time of tumor diagnosis (MAST111) and from the relapsed tumor (MAST139) after the patient had received chemotherapeutic treatment with doxorubicin and etoposide. Primary cells were cultured in Neurobasal Medium (Gibco, Reinach, Switzerland) supplemented with 100 U/ml penicillin/streptomycin (Thermo Fisher Scientific, Reinach, Switzerland), 2 mmol/l L-glutamine (Bioconcept AG, Allschwil, Switzerland), B27 supplement (Gibco), 20 ng/ml bFGF and 10 ng/ml EGF. Cell culture plates were coated with 0.2% gelatin. RH4, RD and PC3 were cultivated in DMEM medium, 10% fetal calf serum (FCS; Sigma-Aldrich, Buchs, Switzerland), 1% Penicillin/Streptomycin (Thermo Fisher Scientific), and 1% L-glutamine (Bioconcept). All cells were cultured under standardized conditions in 5% CO_2 at 37˚C.

Doxorubicin dose-response analysis in primary cells. Monotherapy: Primary diagnostic (MAST111) and relapsed (MAST139) tumor cells were used for doxorubicin dose-response analysis. MAST111 cells 12×10^3/well or MAST139 cells 6×10^3/well were plated without coating in 40 μl of Neurobasal Medium (Gibco) supplemented with B27 minus antioxidants (Life Technologies), 20 ng/ml bFGF, and 10 ng/ml EGF in a 384-well plate. After 24 h, doxorubicin (Selleck Chemicals, Munich, Germany) dissolved in DMSO was added at increasing concentrations ranging from 0-15 μM for MAST111 (two biological replicates in technical duplicates) and from 0-50 μM for MAST139 (five biological replicates in technical duplicates). Combination therapy: MAST139 cells 6×10^3/well were plated without coating in 20 μl Neurobasal Medium (Gibco) plus B27 minus antioxidants (Life Technologies), 20 ng/ml bFGF and 10 ng/ml EGF, in a 384-well plate. After 24 h, NPE® was added dissolved in 20 μl of Neurobasal Medium plus B27 minus antioxidants, 20 ng/ml bFGF, and 10 ng/ml EGF, reaching final concentrations of 0 μg/ml (control), 20 μg/ml, 40 μg/ml or 60 μg/ml in a volume of 40 μl medium/well. Subsequently, doxorubicin dissolved in DMSO was applied at increasing concentrations ranging from 0-50 μM to each NPE® concentration row (0 μg/ml, 20 μg/ml, 40 μg/ml or 60 μg/ml). Five biological replicates in technical triplicates were conducted.

Relative cell viability was measured 24 h after drug treatment using 5 μl WST-1 assay (Roche Diagnostics, Rotkreuz, Switzerland) dissolved 1:1 in medium without supplementation. As NPE® and doxorubicin are colored, the background was individually measured and subtracted for each concentration.

Dose-response curves were generated by plotting relative cell viability against the logarithm ($\log_{10}$) of doxorubicin concentrations. Non-linear regression curve fitting was performed to determine IC_{50} concentrations using GraphPad Prism software (Graph-Pad Software Inc).

Statistical analysis of drug response curves. We either compared IC_{50} values of doxorubicin monotherapy and doxorubicin/NPE® combinations or we recorded relative cell viability upon monotherapy and co-treatment at different concentrations of doxorubicin. Normality was examined by means of Kolmogorov-Smirnov test. Homoscedasticity was tested using Levene's test. Data was analyzed using one-way analysis of variance. Significance was set to $p<0.05$; all statistical analyses were performed using SPSS 22.0 for Windows (SPSS Inc., Chicago IL, USA) and Prism 6 for Mac OS X (Graph-Pad Software Inc.). Data are presented as mean value (95% confidence interval) for nonlinear regression curves or mean±standard deviation for actual data points.

Cell viability assay. RH4 or PC3 cells (1×10^4 per well) were plated in a 96-well plate in triplicates per condition. Treatment with 0.7 μM doxorubicin and 20/50 μg/ml NPE® was performed. Afterwards, medium was aspirated and cells were fixed with 4% paraformaldehyde (Thermo Scientific) for 15 min, stained with 0.01% Crystal Violet (CV; Thermo Scientific) and incubated at room temperature (RT) for 1 hr. Later, CV was removed and plate was left to dry overnight at RT. Then, 100 μl of methanol 100% were added for 1 h and absorbance was measured at ELISA reader at 570 nm. The experiment was performed 5 times in PC3 cells and 6 times in RH4.

Treatment, RNA extraction and Taqman (qRT-PCR). Cells (6×10^5 per well) were plated without coating and without antioxidants and treated for 24 h with 0.7 μM doxorubicin and/or 40 μg/ml NPE®. RNA was extracted using the Qiagen RNeasy Kit (Qiagen) and reverse-transcribed with oligo (dT) primers and Omniscript reverse transcriptase (Qiagen). qRT-PCR was performed under universal cycling conditions on an ABI 7900 instrument using TaqMan gene expression master mix (Thermo Scientific) and ABCB1 Taqman probe (Hs00184500_m1, Thermo Scientific) and data were analyzed with SDS 2.2 program. Cycle threshold (CT) values were normalized to GAPDH (GAPDH Taqman probe: Hs99999905_m1, Thermo Scientific). Relative expression levels were calculated using the ΔΔCT method based on experiments performed in triplicates. Graphics were generated using Prism 6 for Mac OS X (Graph-Pad Software Inc). The experiment was performed 5 times. Statistical analysis was performed on level of ΔΔCT values.

Pump assay. MAST139 cells (6×10^5/well) and RH4 cells (5×10^5 /well) were plated per condition in a 6-well plate. Cells were pre-treated for 24 h with 0.7 μM doxorubicin in order to induce pump ABCB1 overexpression. Cells were collected, counted and divided into several 1.5 ml tubes, according to the number of samples. Each tube contained 5×10^5 cells. Upon centrifugation, supernatant was collected and cells

were incubated for 1 h with 10 μM doxorubicin at 37 ˚C. Cells were centrifuged again and supernatant was discarded. Fresh medium was added to each sample and either NPE® (20, 30 ,40 and 100 μg/ml), Vitamin E 33 μM (VitE, D-α-Tocopherol polyethylene glycol 1000 succinate-TPGS; Sigma-Aldrich) and Vinblastine 22 nM (Merck KGaA, Darmstadt, Germany) were added and incubated for 1 h at 37˚C. Vinblastine and VitE TPGS were used as positive controls (51, 52). Upon incubation with the desired substance, cells were collected and lysed in 120 μl of lysis buffer (0.75 M HCl, 0.2% Triton X in isopropanol) for 20 min at 37˚C shaking. Finally, 100 μl of lysate was added to a black 96-black well plate and doxorubicin fluorescence was measured at ELISA reader: (Excitation: 460 nm; Emission: 610 nm). NPE® autofluorescence was measured and no fluorescence was detected (data not shown). The experiment was performed 3 times.

Results

Relapse primary tumor cells were more resistant to doxorubicin therapy than diagnostic primary tumor cells and NPE® improved doxorubicin treatment in relapsed tumor cells. Primary eRMS tumor cells were obtained from a patient at the time of cancer diagnosis and when the tumor relapsed after treatment with a chemotherapeutic combination of doxorubicin and etoposide. We performed cell viability assays in order to compare diagnostic and relapse cells regarding their sensitivity towards doxorubicin treatment (Figure 1A and 1B) and etoposide treatment (data not shown). In both cases the relapsed tumor cells were more resistant to chemotherapeutic treatment. The IC_{50} value of doxorubicin in the relapse cells was tremendously increased compared to the diagnostic cells (24-h treatment: 118 μM *vs.* 0.007 μM; $p<0.05$ Figure 1A).

While a non-linear regression curve is shown in Figure 1A, actual measuring points were plotted in Figure 1B allowing for the presentation of a very interesting progression of the curve. This curve shows an effect of doxorubicin on cell viability of relapsed tumor cells starting at a concentration of 0.224 μM (log: –0.65). The drug response is linear reaching a relative cell viability of 44% at a concentration of 1.36 μM (log: 0.134). Increasing the drug concentration from 1.36 μM (log: 0.134) to 8.24 μM (log: 0.916) leads to a sudden recovery of viable cells. Doxorubicin concentrations beyond 8.24 μM again induce a drop of relative cell viability. This progression indicates that doxorubicin treatment triggers adaptation of tumor cells within a certain concentration range during the first 24 h of treatment. Taken together, there is a long-time adaptation period comparing diagnostic to relapse cells as well as an immediate adaptation.

We further investigated possibilities to minimize the immediate adaption of primary relapsed tumor cells (MAST139) to doxorubicin treatment. Therefore, we repeated the 24 h cell viability assay in MAST139 cells and this time we included co-treatment of doxorubicin and NPE® using 0-60 μM doxorubicin and 0 μg/ml, 20 μg/ml, 40 μg/ml, and 60 μg/ml NPE®. Figure 1C shows a matrix of mean cell viability relative to 0 μg/ml NPE® and 0 μM doxorubicin upon combination of different doxorubicin and NPE® concentrations. More specifically, 20 μg/ml NPE® and 40 μg/ml NPE® were the appropriate concentrations to test for synergistic effects, whereas 60 μg/ml NPE® led to almost complete cell death with NPE® monotherapy. Interestingly, the combination of doxorubicin and NPE® was more effective than monotherapy of each compound as indicated by the red color of the heat map. For example, 40 μg/ml NPE® alone led to cell viability of 61% and 50 μM doxorubicin alone reduced cell viability to 63%, while the combination treatment resulted in only 12% cell viability. In the case of combination, it was sufficient to use 20 μg/ml NPE® and 0.408 μM or 15 μM doxorubicin to reach about 63% cell viability. Moreover, calculation of the combination index [(20 μg/ml NPE®)/(40 μg/ml NPE) + (0.408 μM or 15 μM doxorubicin)/(50 μM doxorubicin) <1] suggested synergism of NPE® and doxorubicin.

In Figure 1D, drug response curves of either doxorubicin monotherapy or combination therapy of doxorubicin and NPE® are demonstrated. In this case, the NPE® concentration rows (doxorubicin + 0 μg/ml NPE®, doxorubicin + 20 μg/ml NPE®, and doxorubicin + 40 μg/ml NPE®) were analyzed separately and then compared to each other. Importantly, NPE® was able to significantly lower the IC_{50} value of doxorubicin from 118 μM to 33 μM (20 μg/ml NPE®, $p=0.018$), or to 11 μM (40 μg/ml NPE®, $p=0.002$).

Analyzing actual measuring points, it became obvious that the reduction of the IC_{50} value was due to modulation of the previously observed immediate adaptation (Figure 1E). The addition of NPE® to doxorubicin treatment significantly attenuated the recovery of tumor cells at doxorubicin concentrations above 2.48 μM. At lower concentrations monotherapy and combination therapy had the same effect. Interestingly, also a mixture of antioxidants (DL-alpha-tocopherol-acetate, D-alpha-tocopherol, glutathione, catalase, superoxide-dismutase) that was applied to the medium (B27 with antioxidants) demonstrated this reduction of adaptation with significant effects at 27.4 μM ($p=0.046$; log: 1.44) and 50 μM ($p=0.016$; log: 1.70) of doxorubicin (Figure 1F). In summary, NPE®, as well as at least one of the ingredients of the antioxidant mixture were able to sensitize MAST139 primary tumor cells to doxorubicin by modulating immediate adaptation of cancer cells to drug treatment.

NPE® enhanced the toxic effect of doxorubicin on different cancer cell lines. The aRMS cell line RH4 and the prostate cancer cell line PC3 were used to investigate whether our results can be validated in different tumor types. Combination therapy and its consequent effect on cell viability was assessed by CV staining in different cell lines upon single and combination treatment of NPE® together with doxorubicin for

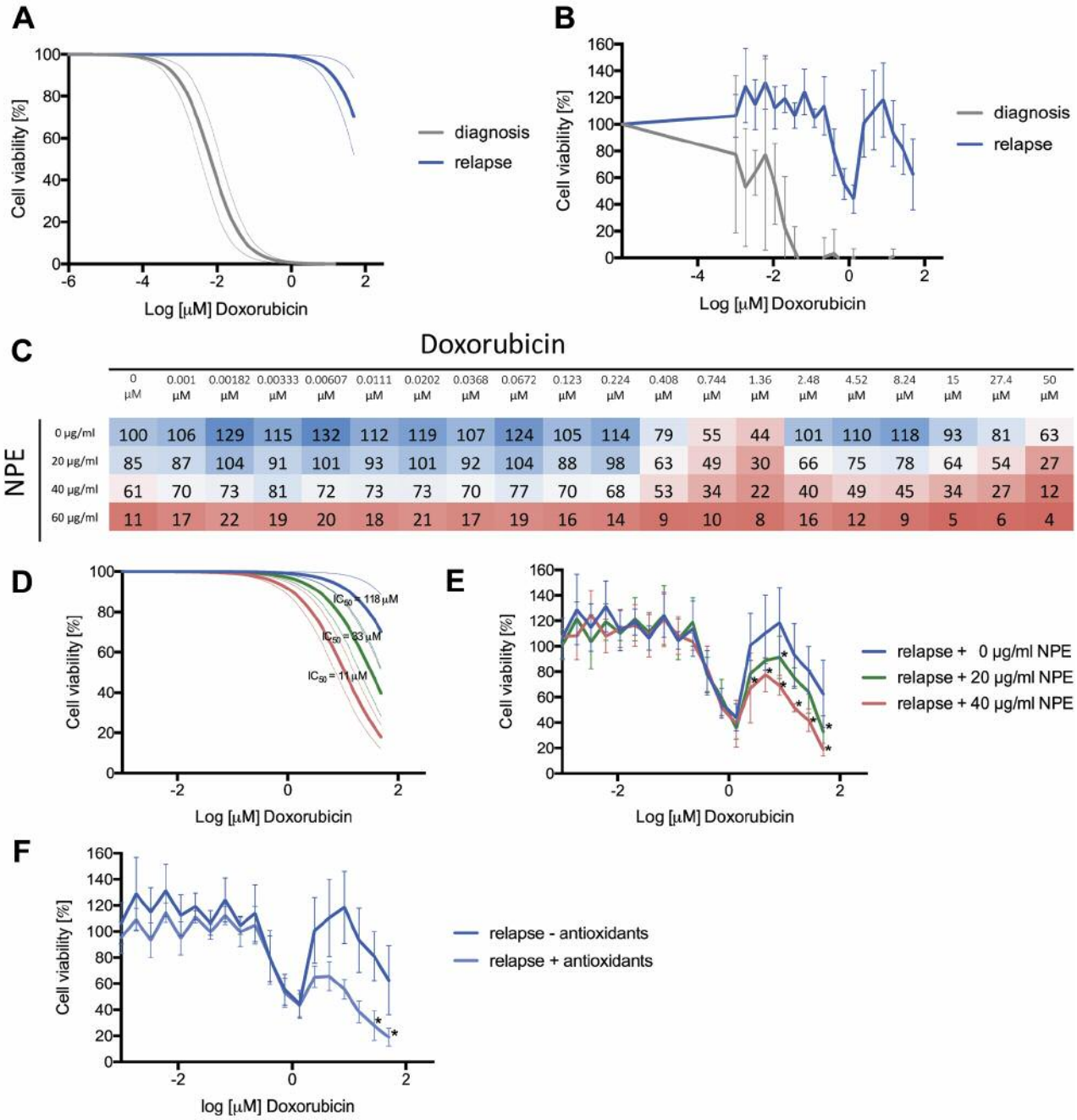

Figure 1. *Drug response curves of doxorubicin. Cells were treated with increasing concentrations of doxorubicin. The nonlinear regression curves (mean and 95% confidence interval indicated) of MAST111 (diagnosis; n=2) IC_{50}=0.007 μM and MAST139 cells (relapse; n=5) IC_{50}=118 μM. Differences were statistically significant (p< 0.05 according to unpaired t-test) (A). The mean and standard deviation (error bars) of MAST111 (diagnosis; n=2) and MAST139 cells (relapse; n=5) are shown in graph. Biological replicates were performed in technical duplicates (B). Mean cell viability of MAST139 cells was calculated upon treatment with different doxorubicin and NPE® concentrations (combined) relative to 0 μg/ml NPE® and 0 μM doxorubicin (n=5) (C). Cells were treated with increasing concentrations of doxorubicin, alone or in combination with NPE® at several concentrations, for 24 h. Graph shows nonlinear regression curve of cell viability of MAST139 cells (n=5) treated with Doxorubicin plus the indicated concentrations of NPE® relative to the control (MAST139 cells treated with 0 μM doxorubicin). Thin colored lined represent the corresponding 95% confidence interval. *p<0.05 (118 μM vs. 33 μM and 118 μM vs. 11 μM) significant according to repeated measures one-way analysis of variance (D). Means and standard deviations (error bars) of cell viability of MAST139 cells (n=5) of each NPE® concentration are also shown; *p<0.05 according to repeated measures one-way analysis of variance (E). Cells were incubated in the presence or absence of antioxidants, for 24 h. Graphs show mean and standard deviation (error bars) of cell viability of MAST139 cells in the presence (n=4) and absence (n=5) of antioxidants relative to 0 μM doxorubicin. *p<0.05 significant according to unpaired t-test (F). Biological replicates performed in technical triplicates.*

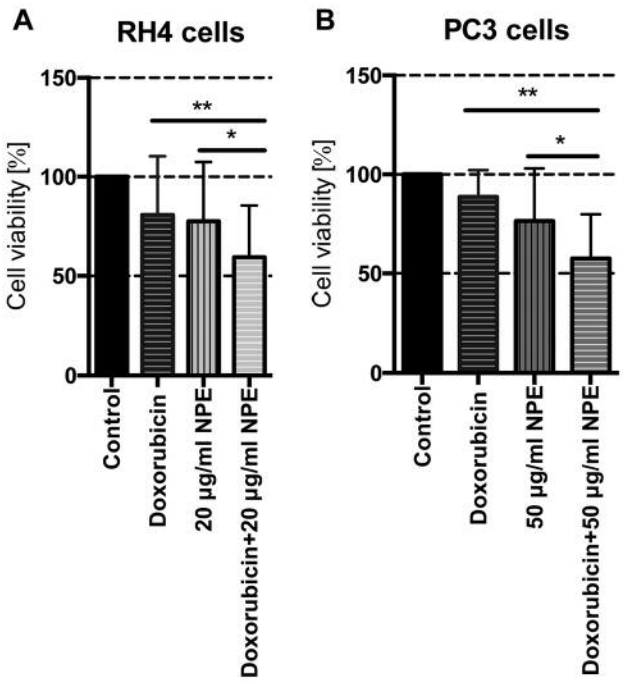

Figure 2. *Cell viability of cancer cell lines RH4 (A) and PC3 (B) upon treatment with doxorubicin and NPE® alone, or combined. *p<0.05 according to unpaired t-test.*

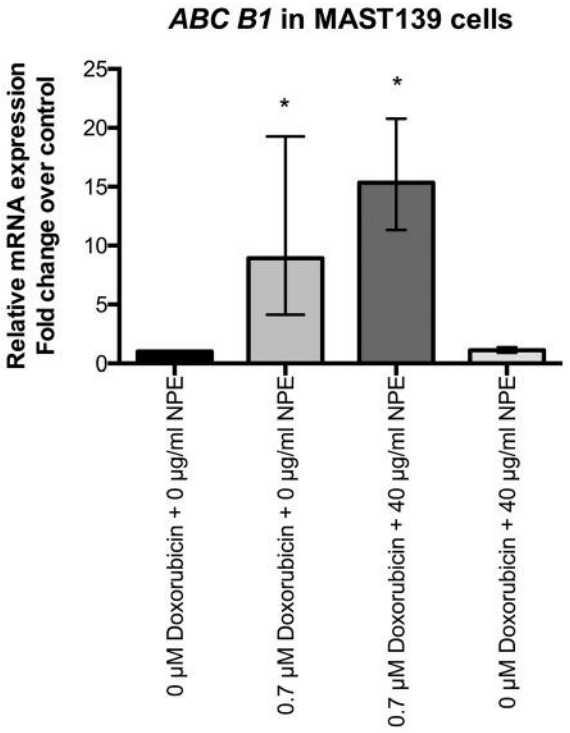

Figure 3. *Fold change of ABCB1 gene expression over control. Cells were treated with doxorubicin monotherapy, NPE® monotherapy or combination therapy for 24 h. Relative mRNA expression of ABCB1 was determined upon treatment in MAST139 cells. CT values relative to control treatment were measured by qRT-PCR and normalized to GAPDH expression. Columns show geometric mean of five independent experiments performed in triplicates; bars show 95% confidence interval; Statistical analysis was performed on level of ΔΔCT values. *p<0.05 according to Dunnett's multiple comparisons test.*

24 h. Interestingly, in RH4 cells (Figure 2A), a statistically significant reduction in cell viability was observed when doxorubicin treatment was applied in combination with 20 μg/ml NPE® (p=0.002 when comparing the combination to the single treatment; p<0.001 when comparing the combination to control). A similar significant effect was observed on PC3 cell viability after treatment with 50 μg/ml NPE® combined with doxorubicin (p=0.005 when comparing the combination to the single treatment) (Figure 2B).

Doxorubicin induced ABCB1 pump gene expression. The effects of doxorubicin on *ABCB1* pump gene expression were examined. Therefore, relative *ABCB1* expression was measured in the primary eRMS relapsed tumor cells MAST139 after incubation with 0.7 μM doxorubicin for 24 h. Gene expression analysis demonstrated a significant increase of *ABCB1* expression in MAST139 cells after treatment with doxorubicin (p=0.003) compared to the untreated control (Figure 3). In addition, induction of *ABCB1* expression by doxorubicin was also observed in our preliminary experiments in RH4 and RD cells (data not shown). Furthermore, it was tested whether NPE® could reverse the increase in *ABCB1* gene expression induced by doxorubicin, since it was hypothesized that this might be the basis for the possible sensitizing effect of NPE®; however, no down-regulation of pump gene expression level was observed.

NPE® inhibited the activity of ABCB1. When added to the medium, doxorubicin penetrates through the cell membrane, exerting its anticancer effect. Since it is an autofluorescent drug, it is possible to measure and quantify the amount retained, as an indication of pump activity. Thus, doxorubicin concentration will be higher in cells with inhibited pump activity. Cells were preincubated with 0.7 μM doxorubicin for 24 h to allow pump overexpression and later on, incubated for 1 h with 10 μM doxorubicin to induce drug incorporation.

Upon 1 h combination treatment of MAST139 cells with Vinblastine and 100 μg/ml of NPE®, a 40% increase in doxorubicin retention was observed (p=0.017) (Figure 4A). Unexpectedly, Vinblastine, NPE® or VitE TPGS alone did not produce any significant effect on primary cells, at least not after incubation for 1 h. However, in RH4 cells, already the lowest concentration of NPE® (20 μg/ml) significantly reduced efflux of doxorubicin (p<0.001) (Figure 4B). Similar results were achieved by the combinations of NPE® (30, 40, and 100 μg/ml) with VitE TPGS (p<0.001). Vinblastine

alone was less effective; however, its inhibitory function was improved by the combination with VitE TPGS and 100 μg/ml of NPE® ($p<0.001$).

Discussion

Cancer is the second leading cause of death and the 5-year survival rate of patients with a recurrent tumor is very low, often due to development of resistance to chemotherapeutic agents. Resistance can arise in different ways including enhanced expression of cellular transporters or DNA repair machinery, reduced drug uptake or down-regulation of drug targets, and modifications in apoptotic signaling, in detoxification processes, or in cell cycle regulation (7). For these reasons, we investigated new strategies to target chemoresistance by studying the effects of NPE®, a GT (*Camellia sinensis non fermentatum*) extract produced by Novelpharm AG, on RMS cell lines and primary cells, derived from diagnosis and relapse tumor, in combination with doxorubicin treatment. GT is currently under investigation for its beneficial effects, such as antioxidant, anti-inflammatory, antiviral, antibacterial and especially for its anticancer potential in different tumor types (36, 53-55).

For our experiments we used primary tumor samples. Comparing response to doxorubicin *in vitro* treatment of primary diagnostic and relapsed cells of a RMS tumor deriving from the same patient, a large difference was found in cell survival whereat relapsed cells were much less sensitive to drug treatment. The observed difference in IC_{50} values indicates a clear evolution of the relapsed tumor and development of resistance relative to the diagnostic biopsy, at least regarding its sensitivity towards doxorubicin, which most probably had occurred during chemotherapeutic treatment of the patient. This observation is in accordance with the evidence of *in vivo* treatment of recurrent tumors in the clinic (56). Interestingly, increasing doxorubicin concentrations in relapsed cells led to sudden recovery of viable cells suggesting not only a long-term adaptation acquired in the patient undergoing chemotherapy, but also an immediate adaptation to the drug *in vitro*.

Importantly, administration of NPE® to relapsed cells, and for validation to RH4 and PC3 cells, led to a reduction in cell viability, thus decreasing the IC_{50} value of doxorubicin and enhancing its anticancer effects by inhibiting the immediate adaptation response, but not the long-term adaptation. This fact might be interesting for clinical practice in order to inhibit resistance mechanisms occurring immediately during therapy. *In vivo* experiments remain to be conducted to further investigate this finding. However, several previous studies already used EGCG, the main catechin of GT, for successful *in vivo* treatment of xenograft tumors (57, 58). Inhibition of resistance was also obtained by treating MAST139 cells with doxorubicin in combination with an antioxidant mixture including Vitamin E, suggesting a similar mechanism and once more the benefit of combination therapies.

Furthermore, we investigated the mechanistic basis of resistance as well as the mechanism of inhibition by NPE®. One of the described resistance mechanisms for doxorubicin is mediated through overexpression of transmembrane proteins such as ABC transporters (59, 60). Our data demonstrated that a 24-h treatment with the chemotherapeutic agent doxorubicin, even at a very low concentration, significantly induced *ABCB1* expression in primary cells and cell lines, which might result in immediate adaptation and reduced drug efficacy due to enhanced drug efflux. These results are in accordance with other previous studies, which showed that the expression of *ABCC1* and *ABCB1* is strongly upregulated in RMS tissue samples of cancer patients after chemotherapy in comparison to the untreated biopsy samples (16). In addition, induction of *ABCB1* expression by doxorubicin has been demonstrated before (61). However, our results did not show any reduction of *ABCB1* at gene expression level by NPE®, suggesting an alternative mechanism that involves NPE®competing with chemotherapeutics as a substrate of the efflux pumps or acting on pump activity. Therefore, we performed doxorubicin retention assays to validate pump activity upon NPE® treatment. In addition, we used Vitamin E TPGS and Vinblastine, both known to inhibit the function of ABC pumps involved in MDR (62). Our results confirmed that NPE® has an inhibitory effect on pump activity, especially when combined with Vitamin E TPGS and/or Vinblastine in RH4 cells. Furthermore, it was demonstrated that combination of NPE® with Vinblastine resulted in a significant upregulation of doxorubicin inside MAST139 cells.

Interestingly, Vitamin E TPGS as well as EGCG have previously been shown to have an inhibitory effect on ABCB1 (63-68). Some of these publications indicated ABCB1 to be directly regulated by EGCG at gene expression level, whereas others observed regulation of pump activity like we did (68). However, besides inhibition of pump activity, NPE® might counteract immediate adaptation to doxorubicin at different levels, since we observed significant reduction of cell viability in MAST139 cells even when, in contrast to RH4 cells, the effect on pumps' activity with NPE® alone was not significant in these cells. Whether modulation of the experimental conditions could enhance the effect on pump activity in MAST139 cells remains to be tested.

Overall, we demonstrated that NPE® is capable of increasing doxorubicin retention by inhibiting the efflux pumps of the ABC family and therefore of inhibiting the resistance mechanism used by the cancer cells to survive chemotherapy.

In conclusion, NPE® could enhance the efficacy of chemotherapeutic agents by counteracting immediate adaptation, thus inhibiting drug resistance. NPE® might

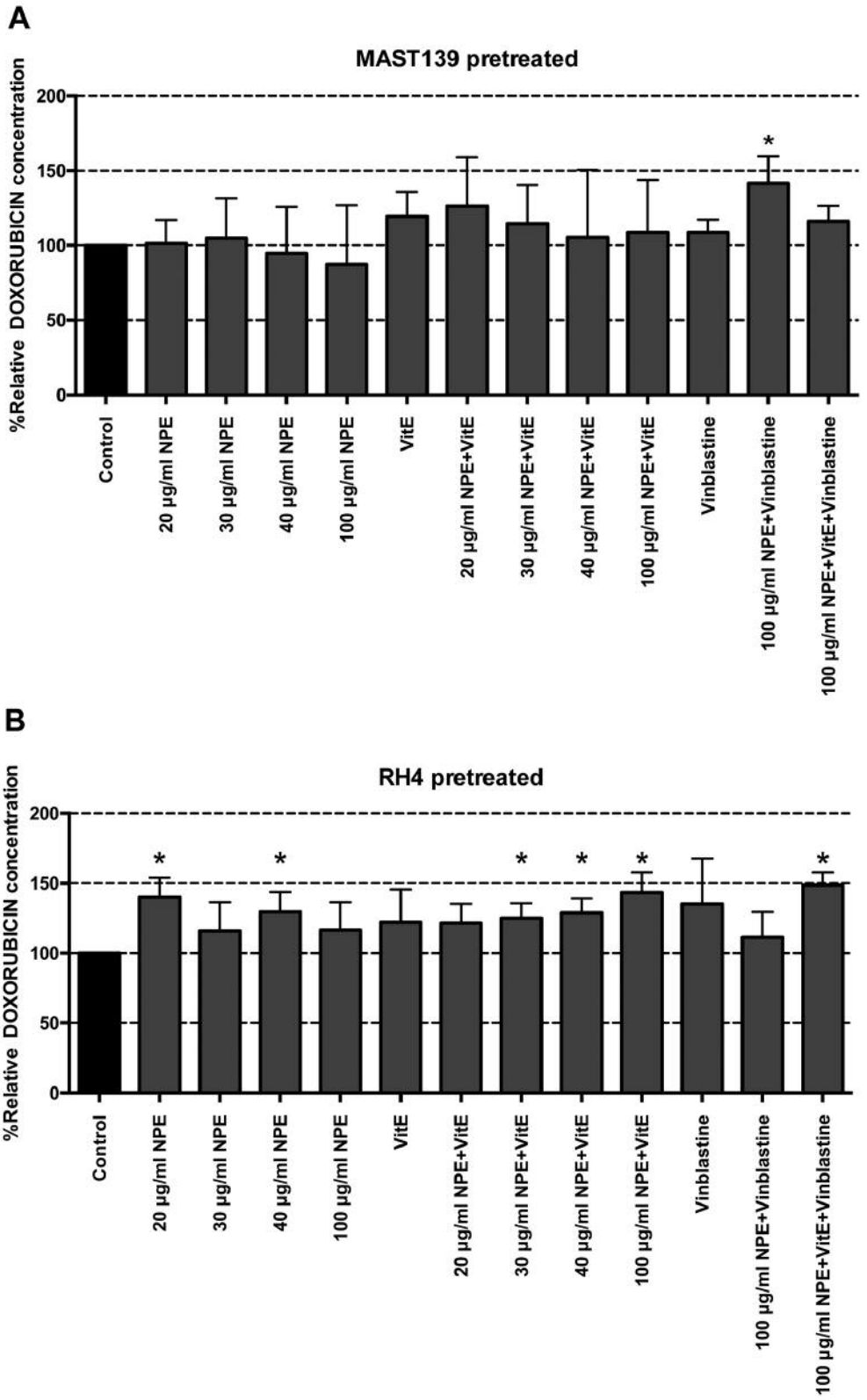

Figure 4. *Pump activity was measured by doxorubicin retention in MAST139 (A) and RH4 cells (B) treated with NPE®, Vitamin E (VitE), and Vinblastine alone or combined (NPE® plus VitE TPGS or NPE® plus Vinblastine). *p<0.05 according to unpaired t-test. Data were normalized to the untreated control and percentage of doxorubicin retention was correlated to a second internal control incubated with doxorubicin alone.*

therefore improve standard cancer therapy, which is of great interest, since single therapy is generally accepted not to be sufficient for a positive outcome. Combination treatment with natural products, with lower risk of developing adverse events, might be a reasonable approach to better manage cancer recurrence.

Authors' Contributions

V. Fontana Thalhammer and H. Holzgang initiated the project. C. Giorgi and V. Fontana Thalhammer planned and performed the experiments, analyzed the data, wrote and revised the article. Urs E. Gasser and Marion E. Lafont reviewed the manuscript.

Conflicts of Interest

There are no potential conflicts of interest.

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