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Additive Interaction of Cisplatin and Histone Deacetylase Inhibitors Combined Treatment in Rhabdomyosarcoma Cells – An Isobolographic Analysis

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Abstract. *Background/Aim: The aim of this study was to assess the anticancer effect and the type of pharmacologic drug–drug interaction of cisplatin (CDDP) and histone deacetylase inhibitors (HDIs) combined treatment on the rhabdomyosarcoma cell line. Materials and Methods: The antiproliferative actions of cisplatin and suberoylanilide hydroxamic acid (SAHA, vorinostat), as well as valproic acid (VPA) alone and in combination, were evaluated using the tetrazolium dye-based MTT cell proliferation assay and isobolographic analysis. Results: All tested compounds inhibited proliferation of rhabdomyosarcoma cancer cells in a dose-dependent manner. The combinations of CDDP with SAHA and CDDP with VPA produced additive interaction with type-I isobolographic analysis. Conclusion: When adding SAHA or VPA to CDDP therapy, one can expect additive anticancer effects in the rhabdomyosarcoma cell line.*

Rhabdomyosarcoma (RMS) is the most frequent childhood sarcoma of soft tissues (1). It originates from mesenchymal stem cells and can be found either as primary neoplasm or as a component of a heterogenous malignancy. Four histological subtypes of RMS are distinguished including alveolar rhabdomyosarcoma (ARMS), embryonal rhambdomyosarcoma (ERMS), pleomorphic rhamdomyosarcoma and sclerosing/spindle cell rhabdomyosarcoma (2). Unfortunately, patients with RMS have a poor prognosis caused by late diagnosis, metastasis and local recurrence (3). Multimodal therapy of RMS includes chemotherapy combined with surgery and radiotherapy. One of chemotherapeutics used in the treatment of RMS is cisplatin (CDDP). Platinum complexes crosslink with DNA strands, which ultimately triggers cells to die in a programmed way. Cisplatin (CDDP) treatment of cancers activate several molecular mechanisms that induce apoptosis including oxidative stress, induction of p53 signalling and cell-cycle arrest, down-regulation of proto-oncogenes, and anti-apoptotic protein (4). Unfortunately, cisplatin (CDDP) treatment display a number of side-effects that limit its clinical use (5). In order to overcome drug-resistance and reduce toxicity combination therapies of cisplatin with other cancer drugs (4) or natural compounds (6-8) constitute an alternative therapeutic approach.

New chemotherapeutics, which could be applied for RMS treatment, are histone deacetylases inhibitors recently introduced to clinical use for treatment of certain cancer types. HDIs are responsible for regulation of gene transcription through chromatin remodeling. They block histone deacetylation which allows to restore pathways silenced in cancer cells, leading to cell-cycle arrest, apoptosis or changes in cancer cells differentiation (9). HDIs exhibit anti-proliferative activities to various types of cancer cells both *in vitro* and *in vivo* (10). On the other hand they have relatively low toxicity against normal cells (11).

One of HDIs, which is used alone or in combination with other anticancer drugs, is vorinostat (SAHA) which was approved by the U.S.A. FDA in October 2006 for the treatment of refractory cutaneous T-cell lymphoma (12). Vorinostat has been proven to inhibit growth of lymphoma (13), acute myeloid leukemia (14), myelodysplastic syndrome (15), advanced melanoma (16) and advanced solid tumors (17). It was also found to suppress the growth and induce cell death of human RMS *in vitro* (18). Moreover, SAHA diminished embryonal rhabdomyosarcoma (ERMS) tumor growth and progression by inducing myogenic

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differentiation as well as reducing the self-renewal and migratory capacity of ERMS cells (19). SAHA also induced inhibition of cells proliferation and led to a significant radiosensitization of RMS tumor cell lines (20).

Valproic acid (VPA) is an approved drug and has been used in the treatment of epilepsy, manic-depressive disorders and migraines, as well as in cancer therapy, since its new molecular mechanism of action was discovered – the inhibition of histone deacetylases (21). VPA exhibits *in vitro* and *in vivo* antitumor activities against neuroblastoma (22), glioma (23), leukemia (24) and lymphomas (25). VPA was also found to have antitumor effects in solid tumors including breast cancer (26) gastric cancer (27) sarcoma (28) or non-small cell lung cancer (29) by inducing apoptosis, promoting cell cycle arrest, enhancing cell differentiation and tumorigenicity inhibition (10). VPA was shown to prevent formation of rhabdomyosarcoma tumors in Patched heterozygous mice model of RMS development (30).

In our study we aimed to test if the combination of SAHA or VPA with CDDP will have better therapeutic effect in TE671 rhabdomyosarcoma cells than the compounds applied alone, as well as to reveal the type of pharmacologic drug-drug interaction between CDDP and HDIs in RMS.

Materials and Methods

Cell culture. TE671, human rhabdomyosarcoma cell line was obtained from the European Collection of Cell Cultures (ECACC). Mycoplasma free cell culture was conducted in standard conditions (37˚C, in a humidified atmosphere with 5% CO_2) in DMEM/F12 Ham medium (Sigma – Aldrich, St. Louis, MO, USA) supplemented with 10% v/v fetal bovine serum and antibiotics penicillin (100 g/ml) and streptomycin (100 g/ml) (Sigma Aldrich).

Cells treatment. Cisplatin and VPA were purchased from Sigma Aldrich, whereas SAHA was from Cyman Chemical (Ann Arbor, MI, USA). Cisplatin and VPA were dissolved in phosphate buffered saline (PBS) with Ca^{2+} and Mg^{2+}, and SAHA was solubilized in dimethyl sulfoxide (DMSO). Cells at optimized concentrations of $1{,}0\times10^4$ cells/ml were incubated for 96 h with increasing concentrations of the VPA (17-830 μg/ml, equivalent to 0.1-5.0 mM), SAHA (0,026-2,6 μg/ml, equivalent to 0.0001-0,01 mM), or CDDP (0,01-15 μg/ml) to assess the IC_{50} concentration for each compound.

MTT assay. MTT assay was used to determine cell viability as described previously (31). The relative viability of the treated cells compared to that of the control cells was expressed as % of cells viability.

Isobolographic analysis. Isobolographic analysis was performed as described previously (26), with the exception that TE671 cell line was used. Isobolography is a statistical method allowing the precise characterization of pharmacodynamic interaction between drugs in both, preclinical and clinical studies (32). To start isobolographic analysis of interaction between CDDP and SAHA or VPA, we measured the percent inhibition of cell viability per increasing doses of CDDP, SAHA and VPA used alone in the rhabdomyosarcoma cell line. Subsequently, the dose-response effects for each investigated anti-cancer compound (*i.e.*, CDDP, SAHA, VPA) in the rhabdomyosarcoma cell line (TE671) were fitted with log-probit linear regression analysis as recommended by Litchfield and Wilcoxon (33). Log-probit method allowed to calculate median inhibitory concentrations (IC_{50}s) for CDDP, SAHA or VPA, when administered singly. The test for parallelism of dose-response effects for CDDP and SAHA or VPA, as described in more details in our previous studies (26, 34, 35) revealed that CDDP had its dose-response effect non-parallel to that of SAHA and VPA in the rhabdomyosarcoma cell line (TE671) measured by the MTT assay. The type of interactions between CDDP and SAHA or VPA in the cancer cell line TE671 was isobolographically analyzed according to the methodology described elsewhere (36). From the experimentally denoted IC_{50} values for the drugs administered alone, median additive inhibitory concentrations of the mixture of CDDP with SAHA or VPA at the fixed-ratio of 1:1 ($IC_{50\ add}$) – *i.e.*, concentrations of the mixture, which should theoretically inhibit cell viability in 50% were calculated as described earlier (34). Subsequently, the experimentally-derived $IC_{50\ mix}$ at the fixed-ratio of 1:1 was determined based on the concentration of the mixtures of CDDP with SAHA or VPA, inhibiting 50% of cell viability in the cancer cell line (TE671) measured *in vitro* by the MTT assay. The separate concentrations of CDDP and SAHA or VPA in the mixture were calculated from, the $IC_{50\ mix}$ values by multiplying this value by the respective proportions of particular drugs. Additional information concerning the isobolographic analysis has been published elsewhere (34, 36).

Statistical analysis. The data were analyzed using GraphPad Prism software with one-way ANOVA and Tukey *post hoc* tests. $p<0.05$ was considered to indicate a statistically significant difference. Results were presented as mean±standard error of the mean (SEM). Log-probit analysis was used to determine the experimentally-derived IC_{50} and $IC_{50\ mix}$ values for CDDP, SAHA and VPA, when the drugs were administered alone or in combination for the fixed-ratio of 1:1 (33). Difference between the experimentally-derived $IC_{50\ mix}$ values for the mixture of CDDP with SAHA or VPA and the theoretically additive $IC_{50\ add}$ values was statistically verified by using the unpaired Student's *t*-test, as presented elsewhere (36).

Results

HDI and CDDP elicit anti-cancer properties in rhabdomyosarcoma cell line. We have shown a decrease of rhabdomyosarcoma cancer cells proliferation in the dose-dependent manner after VPA, SAHA, or CDDP treatment (Figure 1). The IC_{50} values were the concentrations resulting in 50% cell growth inhibition by a 96-h exposition to active agents as compared with control (untreated cells). IC_{50} values for TE671 cell line treated with VPA, SAHA, CDDP was established as follows: 196.4 μg/ml for VPA, 0.052 μg/ml for SAHA and 0.591 μg/ml for CDDP (Table I).

Anti-proliferative effects of SAHA and VPA administered singly and in combination with CDDP to the TE671 cell line. The independent administration of CDDP, SAHA and VPA resulted in a clear-cut anti-proliferative effect in the

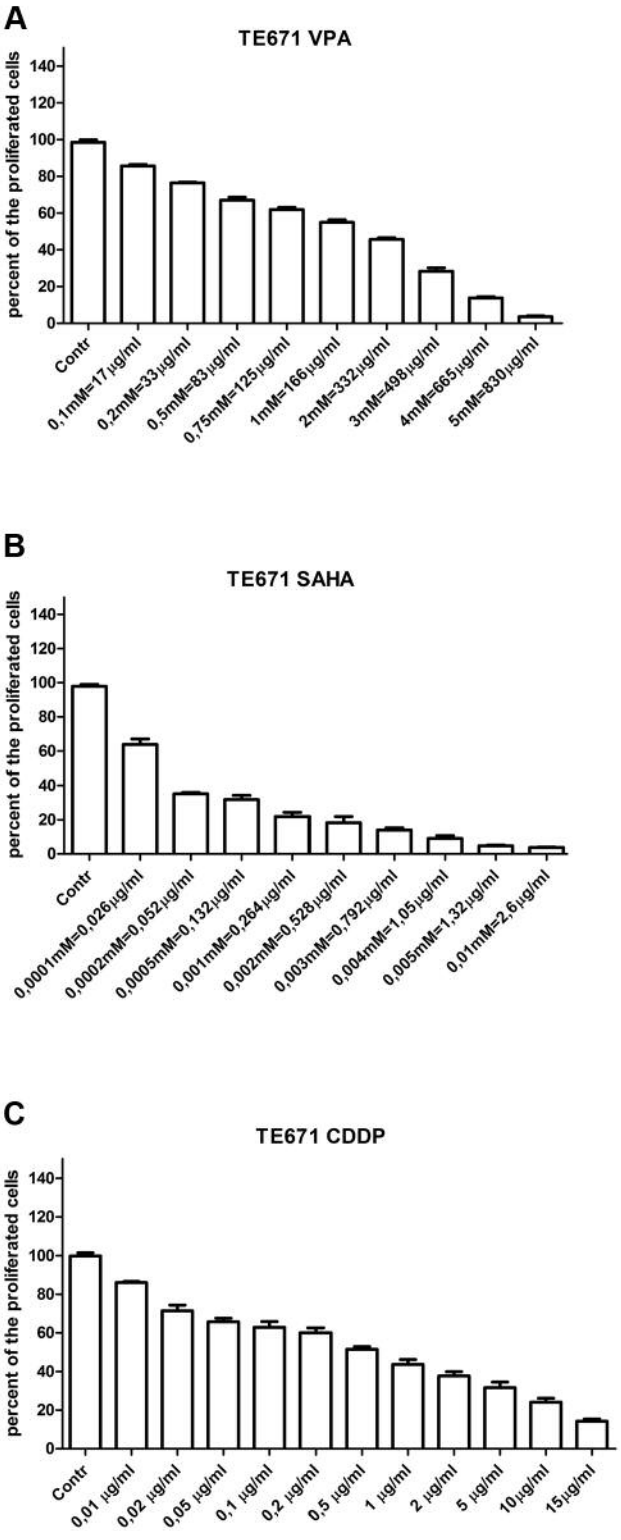

Figure 1. *Inhibition of proliferation in TE671 cancer cells with (A) valproic acid (VPA), (B) suberoylanilide hydroxamic acid (SAHA) and (C) cisplatin (CDDP), analyzed by an MTT assay. Data represent mean optical densities±S.E.M., (*p<0.05; **p<0.01; ***p<0.001 versus control; Student's test).*

Table I. *Anti-proliferative effects of CDDP, SAHA and VPA administered singly in the TE671 cancer cell line.*

Drug	IC_{50} (µg/ml)	*n*	CFP	*p/q*	Parallelism
CDDP	0.591±0.187	48	0.363 (q)	-	
SAHA	0.052±0.028	36	0.766 (p1)	2.110	not parallel
VPA	196.4±80.13	60	0.801 (p2)	2.206	not parallel

Results are presented as median inhibitory concentrations (IC_{50} values in µg/ml ±S.E.M.) of CDDP, SAHA and VPA administered alone with respect to their anti-proliferative effects in the rhabdomyosarcoma cell line (TE671) measured *in vitro* by the MTT assay. *n* – total number of items used at concentrations whose expected anti-proliferative effects ranged between 4 and 6 probits (16% and 84%); CFP – (*q* and *p*) curve-fitting parameters; *p/q* – ratio of *p* and *q* values. Test for parallelism between two dose-response effects (CDDP *vs*. SAHA and CDDP *vs*. VPA) was performed according to the procedure as described in details earlier (35).

rhabdomyosarcoma cell line TE671 (Figure 2A and B). Log-probit dose-response effects for CDDP, SAHA and VPA allowed for calculation of their IC_{50} values that amounted to 0.591±0.187 µg/ml, 0.052±0.028 µg/ml and 196.4±80.13 µg/ml, respectively (Table I). The test for parallelism of dose-response effects between CDDP and SAHA and CDDP and VPA confirmed that the log-probit lines of these compounds were non-parallel to one another (Table I, Figure 2A and B).

Isobolographic analysis shows additive interactions between HDI and CDDP in TE671 cells. The combinations of CDDP with SAHA and CDDP with VPA (both, at the fixed-ratio of 1:1) produced the definite anti-proliferative effects in the TE671 cell line. The experimentally determined $IC_{50\ mix}$ values for the two-drug mixture were 0.243±0.028 µg/ml for the combination of CDDP with SAHA (Table II, Figure 3A), and 127.7±21.26 µg/ml for the combination of CDDP with VPA (Table II, Figure 3B). With type I isobolographic analysis, no statistical difference was observed between the $IC_{50\ mix}$ and $IC_{50\ add}$ values with unpaired Student's *t*-test and thus, the analyzed interactions between CDDP and SAHA or VPA were additive (Table II).

Discussion

In the last years, little progress has been made in identifying new therapeutic agents and approaches to treat RMS (37). Multi-agent chemotherapy –mostly combined use of vincristine, dactinomycin, and cyclophosphamide (VAC)– has been the standard treatment for RMS for more than forty years. Introduction of other treatment systems like IVA (ifosfamide, vincristine and dactinomycin) or CEV (carboplatin, etoposide and vincristine), as well as IVE (ifosfamide, vincristine and

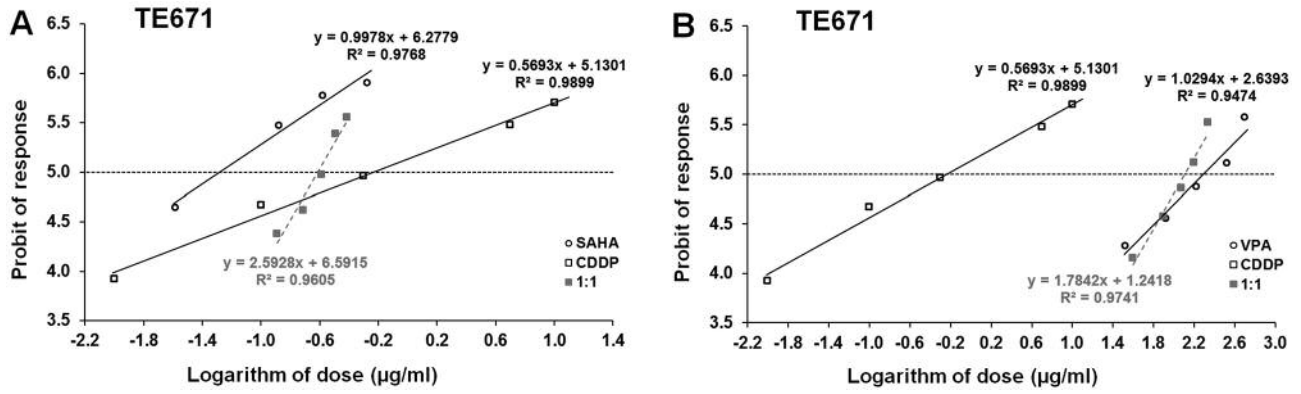

Figure 2. *Log-probit dose-response effects for cisplatin (CDDP) and suberoylanilide hydroxamic acid (SAHA) (A), and for cisplatin (CDDP) and valproic acid (VPA) (B), administered both, separately and in combination at the fixed-ratio of 1:1, displaying the anti-cancer effects of the drugs in the rhabdomyosarcoma cell line TE671 measured in vitro by MTT assay. Logarithm of doses of CDDP, SAHA and VPA, when administered singly and in combination at the fixed-ratio 1:1 are placed on the abscissa, whereas the anti-proliferative effects evoked by these drugs and transformed to probits of response are placed on the ordinate of the Cartesian coordinate system according to Litchfield and Wilcoxon (1949) (33). Dose-response effects are linearly related and are presented on each graph; where y – is the probit of response, and x – is the logarithm (to the base 10) of a drug dose, R^2 – coefficient of determination.*

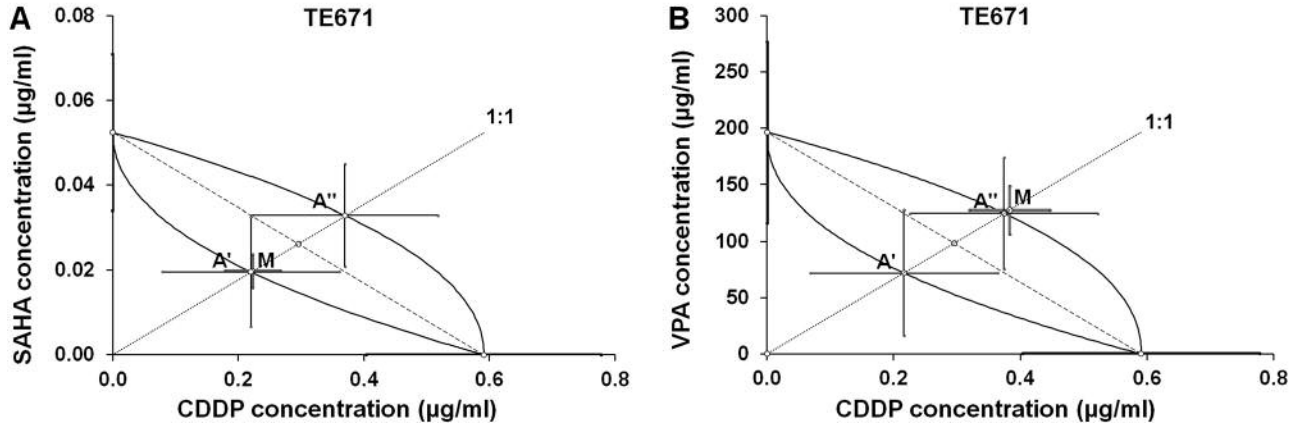

Figure 3. *Isobolograms illustrating additive interactions between cisplatin (CDDP) and suberoylanilide hydroxamic acid (SAHA) (A), and cisplatin (CDDP) and valproic acid (VPA) (B) in terms of their anti-cancer effects in the rhabdomyosarcoma cell line TE671 measured in vitro by the MTT assay. Median inhibitory concentrations (IC_{50}) for CDDP, SAHA and VPA are plotted graphically on the abscissa (CDDP) and ordinate (SAHA and VPA), respectively. The lower and upper isoboles of additivity are illustrated as curves connecting the IC_{50} values for CDDP and SAHA or VPA. The line starting from the point (0, 0) corresponds to the fixed-ratio of 1:1 for the combination of CDDP with SAHA or VPA. Points A' and A" depict the theoretically calculated $IC_{50\ add}$ values for both, lower and upper isoboles of additivity, whereas the point M corresponds to the experimentally-derived $IC_{50\ mix}$ value for the total dose of the mixture expressed as proportions of CDDP and SAHA or CDDP and VPA that produced a 50% anti-proliferative effect. For every IC_{50} value the S.E.M. values are presented as horizontal and vertical error bars.*

etoposide) did not improve survival or reduce the intensity of local therapy, but was associated with increased toxicity (38). Recent data suggest favorable response to platinum-based systemic chemotherapy for RMS treatment at different localizations in the body (39-42).

On the other hand, new generation of anticancer drugs –histone deacetylase inhibitors were introduced to the clinic for the treatment of some types of tumors (43). Pre-clinical studies showed potential of HDI use in the treatment of RMS. Second-generation histone deacetylase inhibitor JNJ-26481585 induced mitochondrial-dependent apoptosis and suppressed tumor growth *in vivo* in two pre-clinical RMS models (44), whereas expression of NAD^+ dependent histone deacetylases sirtuins 1 and 2 was crucial for the survival of

Table II. *Type I isobolographic analysis of interactions between CDDP and SAHA or VPA at the fixed-ratio combination of 1:1 in the TE671 cancer cell line.*

Combination	IC_{50} mix (μg/ml)	n mix	$^{\#}IC_{50\ add}$ (μg/ml)	*n* add	$^{+}IC_{50}$ add (μg/ml)	*n* add
CDDP+SAHA	0.243±0.028	120	0.240±0.154	80	0.403±0.160	80
CDDP+VPA	127.7±21.26	120	72.11±55.83	104	125.1±49.33	104

Results indicated median inhibitory concentrations (IC_{50} values in μg/ml ±S.E.M.) of two-drug mixtures. (CDDP+SAHA and CDDP+VPA), determined either experimentally ($IC_{50\ mix}$) or theoretically calculated as additive ($IC_{50\ add}$), inhibiting proliferation in 50% of tested cells in the cancer cell line (TE671) measured *in vitro* by the MTT assay. n_{mix} – total number of items used at those concentrations whose expected anti-proliferative effects ranged between 16% and 84% (*i.e.*, 4 and 6 probits) for the experimental mixtures; *n* add – total number of animals calculated for the additive mixture of the drugs examined ($n_{add}=n_{\_CDDP} + n_{\_SAHA\ -\ 4}$) or ($n_{add}=n_{\_CDDP} + n_{\_VPA\ -\ 4}$); $^{\#}$ – $IC_{50\ add}$ value calculated for the lower line of additivity; $^{+}$– $IC_{50\ add}$ value calculated for the upper line of additivity.

rhabdomyosarcoma cell, and pharmacological inhibition of sirtuins impaired the autophagy process and induced tumor cell death (45). Finally, SAHA inhibited growth of RMS cells (18) and significantly increased chemotherapeutic drug-induced apoptosis in both embryonal and alveolar RMS cell lines, including several anticancer agents that are used in the clinic for the treatment of RMS such as doxorubicin, etoposide, vincristine and cyclophosphamide (46).

In the present study, we analyzed efficacy of treatment and the type of pharmacological drug–drug interaction of two HDIs (SAHA or VPA) with cisplatin to assess potential application of combined treatment in TE671 rhabdomyosarcoma cells. To the best of our knowledge, such combinations had not been tested in RMS cells, but were shown to be very effective in other cancer cells types, including non-small cell lung cancer cells (47), oral-squamous cell carcinoma (48), as well as in platinum-resistant ovarian cancer cells (49). Our experiments revealed that the combination of CDDP with SAHA or CDDP with VPA showed additive interaction on the viability of TE-671 cells. This assessment was revealed by using advanced isobolographic analysis of drug-drug interaction. This very efficient method is seldom performed to establish pharmacological type of drug-drug interaction in cancer-related studies. Instead, usually simple correlations between tested compounds are demonstrated, where only limited number of random chosen doses are selected, without precise method of drug-type interaction analysis (50, 51).

By showing additive interaction of tested compounds in RMS cells, our results strongly suggest application of such drug combinations in other pre-clinical models, including animal xenografts. Additionally, VPA is a widely used anti-epileptic drug, with known pharmacokinetics and adverse effects (52), thereby no toxicity studies are necessary. Although direct cellular or molecular mechanism responsible for observed anti-cancer effect of HDIs and CDDP is not known, some genetic and epigenetic events related to histone acetylation and associated with RMS progression were recently discovered, confirming potential use of HDIs in RMS treatment. In ERMS, mutations in BCL-6 co-repressor (BCOR), which encodes a transcriptional repressor that interacts with histone deacetylases (HDACs) was confirmed (37, 53). It was also reported, that transcriptional suppression of the *Pax3–FOXO1* allele by HDAC inhibitor entinostat resulted in much more favorable prognosis in ARMS animal model (54). Finally, vironostat was recently discovered as an agent that inhibits RMS tumor growth *in vivo*, induces apoptosis and inhibits invasion of RD and Rh30 RMS cell lines through a novel, ROS-dependent molecular mechanism of action due to epigenetic repression of cMyc and subsequent downregulation of SP transcription factors independent of histone acetylation (55). All these data suggest that HDIs could be promising anticancer agents for RMS, especially when combined with other cytostatic drugs or treatment strategies, as it was demonstrated in other cancers types. Our previous study proved that combination therapy of SAHA or VPA with CDDP increased apoptosis and cell-cycle arrest in MCF7, T47D and MDA-MB-231 human breast cancer cell lines, enabling to use lower doses of both drugs to obtain augmented anticancer effect (34). Our recent results were consistent with previously reported. Moreover, they were in accordance to published data that revealed SAHA inhibited cell proliferation and intensified the anti-proliferative effects of CDDP both *in vitro* (head and neck cancer cells), and *in vivo* (decreased tumor metastasis in mouse xenograft models) (56). The mechanism responsible for the combining treatment that increase CDDP sensitivity in cancer cells could be related to amplification of the accessibility of DNA to CDDP and transcriptional regulators by the epigenetic changes mediated by HDIs (57).

In conclusion, our study using isobolographic method of pharmacological drug-drug interaction analysis proved that combined medication of HDIs with CDDP could be used in the treatment of rhabdomyosarcoma cancer cells to improve their antitumor effects and decrease their doses compared to those administered separately.

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