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

AGATA JARZĄB1, JAROGNIEW J. ŁUSZCZKI2, MAŁGORZATA GUZ1, EWELINA GUMBAREWICZ1, KRZYSZTOF POLBERG3 and ANDRZEJ STEPULAK1

1Department of Biochemistry and Molecular Biology, Medical University of Lublin, Lublin, Poland;
2Department of Pathophysiology, Medical University of Lublin, Lublin, Poland;
3Department of Otolaryngology, MSWiA Hospital, Lublin, Poland

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 rhabdomyosarcoma (ERMS), pleomorphic rhabdomyosarcoma 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
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% CO₂) 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 Ca²⁺ and Mg²⁺, and SAHA was solubilized in dimethyl sulfoxide (DMSO). Cells at optimized concentrations of 1.0x10⁴ 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 IC₅₀ 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 (IC₅₀) 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 IC₅₀ 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 (IC₅₀ 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 IC₅₀ 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 IC₅₀ 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 IC₅₀ and IC₅₀ 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 IC₅₀ mix values for the mixture of CDDP with SAHA or VPA and the theoretically additive IC₅₀ add values was statistically verified 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 IC₅₀ values were the concentrations resulting in 50% cell growth inhibition by a 96-h exposition to active agents as compared with control (untreated cells). IC₅₀ 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
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 showed 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 (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).

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

<table>
<thead>
<tr>
<th>Drug</th>
<th>IC_{50} (μg/ml)</th>
<th>n</th>
<th>CFP</th>
<th>p/q</th>
<th>Parallelism</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDDP</td>
<td>0.591±0.187</td>
<td>48</td>
<td>0.363 (q)</td>
<td>0.363 (q)</td>
<td>1.000 (p)</td>
</tr>
<tr>
<td>SAHA</td>
<td>0.052±0.028</td>
<td>36</td>
<td>0.766 (p1)</td>
<td>0.766 (p1)</td>
<td>2.110 (p)</td>
</tr>
<tr>
<td>VPA</td>
<td>196.4±80.13</td>
<td>60</td>
<td>0.801 (p2)</td>
<td>0.801 (p2)</td>
<td>2.206 (p)</td>
</tr>
</tbody>
</table>

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).

**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
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...
Although direct cellular or molecular mechanisms responsible for observed anti-cancer effect of HDIs and CDDP is not clear, thereby no toxicity studies are necessary. For RMS cells, our results strongly suggest application of such drug combinations in other pre-clinical models, including non-small cell lung cancer cells (47), oral-squamous cell carcinoma (48), and oral- tongue cancer cells), and particularly in platinum-resistant ovarian 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. Consequently, they were in accordance to published data that revealed SAHA inhibited cell proliferation and intensified 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 conclusion, our study using isobolographic method of drug–drug interaction analysis proved that the combination of CDDP with SAHA or VPA with CDDP, determined either experimentally (IC₅₀ mix) or theoretically calculated as additive (IC₅₀ 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 – a) or (n_add = n_CDDP + n_VPA – a); δ – IC₅₀ add value calculated for the lower line of additivity; *– IC₅₀ add value calculated for the upper line of additivity.

<table>
<thead>
<tr>
<th>Combination</th>
<th>IC₅₀ mix (μg/ml)</th>
<th>n mix</th>
<th>IC₅₀ add (μg/ml)</th>
<th>n add</th>
<th>IC₅₀ add (μg/ml)</th>
<th>n add</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDDP+SAHA</td>
<td>0.243±0.028</td>
<td>120</td>
<td>0.240±0.154</td>
<td>80</td>
<td>0.403±0.160</td>
<td>80</td>
</tr>
<tr>
<td>CDDP+VPA</td>
<td>127.7±21.26</td>
<td>120</td>
<td>72.11±55.83</td>
<td>104</td>
<td>125.1±49.33</td>
<td>104</td>
</tr>
</tbody>
</table>

Results indicated median inhibitory concentrations (IC₅₀ values in μg/ml ±S.E.M.) of two-drug mixtures, (CDDP+SAHA and CDDP+VPA), that the combination of CDDP with SAHA or CDDP with VPA showed additive interaction on the viability of TE671 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).
References


