Original Article

Comparison of DNA demethylating and histone deacetylase inhibitors hydralazine-valproate versus vorinostat-decitabine in cutaneous T-cell lymphoma in HUT78 cells

Alejandro Schcolnik-Cabrera1*, Guadalupe Domínguez-Gómez1*, Alfonso Dueñas-González2

1Division of Basic Research, Instituto Nacional De Cancerología, México; 2Unidad De Investigacion Biomédica En Cancer, Instituto De Investigaciones Biomédicas UNAM/Instituto Nacional De Cancerología, México. *Equal contributors.

Received April 30, 2018; Accepted May 31, 2018; Epub June 5, 2018; Published June 15, 2018

Abstract: Purpose: Cutaneous T-cell lymphoma (CTCL) is an uncommon extranodal non-Hodgkin T-cell lymphoma that originates from mature T lymphocytes homed at the skin. Epigenetic alterations observed in CTCL are not limited to overexpression of Histone Deacetylases but also to DNA hypermethylation. The known synergy between Histone deacetylase inhibitors (HDACi) and DNA methyltransferases inhibitors (DNMTi) suggests that combining these agent classes could be effective for CTCL. Methods: In this study, the combinations of the HDACi and DNMTi hydralazine/valproate (HV) and vorinostat/decitabine (VD) were compared in regard to viability inhibition, clonogenicity, pharmacological interaction and cell cycle effects in the CTCL cell line Hut78. In addition, the effect of these combinations was evaluated in normal peripheral blood mononuclear cells. Results: The results show that each of the DNMTi and HDACi exerts growth inhibition, mostly by inducing apoptosis as shown in the cell cycle distribution. However, in the combination of HV the interaction is more synergic and also it inhibits the clonogenic capacity of cells over time. Additionally, the HV combination seems to affect in a minor degree the viability of peripheral blood mononuclear cells. Conclusions: The results of this study and the preclinical and clinical evidence on the efficacy of combining HDACi with DNMTi strongly suggest that more studies are needed with this drug class combination in CTCL, particularly with the hydralazine-valproate scheme, which is safe, and these drugs are widely available and administered by oral route.

Keywords: CTCL, HDACi, DNMTi, hydralazine, valproate, vorinostat, decitabine, synergy

Introduction

Cutaneous T-cell lymphoma (CTCL) is an uncommon extranodal non-Hodgkin T-cell lymphoma that originates from mature T lymphocytes homed at the skin, and represents the majority of all primary cutaneous lymphomas. Among them, mycosis fungoides (MF) accounts for almost 50% of all primary cutaneous lymphomas. This lymphoma is characterized by having a chronic and relapsing course, and since there are no curative modalities with the possible exception of patients with minimal patch and plaque lesions confined to the skin, the treatment, which includes external beam radiation, phototherapy, chemotherapy and biological agents, is used sequentially for the patients [1]. Most patients with limited disease burden, in general, show long-term survival and are commonly treated with corticosteroids, topical nitrogen mustard, phototherapy and other forms of systemic treatments. Systemic approaches such as bexarotene, denileukin diftitox, alemtuzumab, lenalidomide, Toll-like receptor agonists, pralatrexate, bortezomib, vorinostat, romidepsin, as well as cytotoxic chemotherapy, are frequently employed in patients with advanced and erythrodermic MF [2, 3].

CTCL overexpresses several histone deacetylases (HDACs), which include HDACs 1, 2, and 6, and this disease is highly sensitive to HDAC inhibitors (HDACi) [4, 5]. However, the epigenetic alterations observed in CTCL are not limited to overexpression of HDACs but also to DNA hypermethylation [6]. On this regard, van Doorn et al. by using differential methylation hybridization, reported on 28 CTCL samples, 35 CpG
(cytosine-guanine) islands hypermethylated in at least 4 of these 28 samples, plus 6 of 8 genes hypermethylated and analyzed with a candidate-gene approach. Of note, treatment with the DNA methyltransferase inhibitor (DNMTi) decitabine led to demethylation and re-expression of BCL7a and PTPRG, in the CTCL cell line MyLa [7].

This observation, and the known synergy between HDACi and DNMTi [8, 9], suggests that combining these agent classes could be effective for CTCL. In fact, preclinical studies combining these two agent classes have been undertaken, with promising results in lymphoma [10, 11]. However, in our knowledge, only the drug combination of romidepsin with azacitidine has been preclinically tested in CTCL [12]. The fact that the HDACi valproate in combination with the DNMTi hydralazine has shown promising efficacy in patients with CTCL, and because they are well tolerated, led us to compare this pharmacological combination against the one HDACi vorinostat and the DNMTi decitabinein the Hut78 CTCL cancer cell line. Our results demonstrate that hydralazine and valproate exert more synergism than decitabine and vorinostat against CTCL cells with similar cell cycle effects, but a higher reduction in clonogenic capacity of treated cells over time.

Materials and methods

Cell line

The CTCL cell line Hut78 was obtained from the ATCC (Manassas, VA, USA). Hut78 cell line and healthy leukocytes recovered from buffy coats were cultured at 37°C in a humidified atmosphere containing 5% CO₂ in complete medium, composed of RPMI-1640 medium supplemented with 10% fetal bovine serum and 1% antibiotic-antimycotic solution (all from Invitrogen Life Technologies, Carlsbad, CA, USA).

Healthy leukocytes

Buffy coats were kindly obtained from the blood bank of the Instituto Nacional de Cancerología (Mexico) for leukocyte recovery. Briefly,uffy coats contained in heparinized Terumo bags (Terumo BCT, Lakewood, CO, USA) were treated at 1:1 ratio with ACK lysing buffer (Life Technologies), mixed for 5 min in a platform mixer and then centrifuged at 250 G during 20 min to lyse the erythrocytic content. This process was repeated 3 times until a free-erythrocyte white pellet-containing leukocyte was obtained. Cells were then resuspended in RPMI-1640 complete medium and used for viability assay experiments.

Viability curves in the Hut78 cell line

Hut78 cells were seeded into 12-well microtiter plates (Corning) at a density of 5×10⁴ cells/well into 1 mL complete medium. The following day, cells were treated for a 72 hour-period with either hydralazine (Sigma, St. Louis, MO, USA), magnesium valproate (Psicofarma, México), vorinostat (Sigma, St. Louis, MO, USA), or decitabine (Sigma, St. Louis, MO, USA) at escalating doses. All the compounds but vorinostat were resuspended in distilled water, and the latter was resuspended in absolute ethanol (Sigma, St. Louis, MO, USA). Fresh complete medium containing each drug was changed every 24 hours by recovering all the content of each well, with posterior centrifugation (5 min at 120 G), decantation of the supernatant and resuspension of the cell pellet in the medium-containing drug. After 72 hours of treatment, pellets were recovered and resuspended in 1 mL RPMI-1640 medium to assess cell viability by trypan blue exclusion assay. Briefly, resuspended cells were gently mixed at 1:1 ratio with trypan blue stain solution (Life Technologies) and then cell viability was evaluated with a TC10™ Automated Cell Counter (Bio-Rad). The cytotoxic effect of each treatment was expressed as a percentage of cell viability relative to control cells treated with the vehicle of each compound at the highest evaluated dose. The data of the dose-response curve at different concentrations per drug was plotted in the SigmaPlot software 10.0. The percentage of growth inhibition was calculated, and IC₀⁻₁₅₀ values (the concentration of each drug that achieves 20%-50% growth inhibition) were graphically obtained from the survival curves.

Pharmacological interactions

Increasing doses of hydralazine (ICₓ₀, ICₓ₀, ICₓ₀, and ICₓ₀) were combined with their respective increasing doses of valproic acid (ICₓ₀, ICₓ₀, ICₓ₀, and ICₓ₀). At the same time, increasing doses of vorinostat (ICₓ₀, ICₓ₀, ICₓ₀, and ICₓ₀) were combined with their respective increasing doses of decitabine (ICₓ₀, ICₓ₀, ICₓ₀, and ICₓ₀). The resulting mixes (HV ICₓ₀, HV ICₓ₀, HV ICₓ₀, HV ICₓ₀, HV ICₓ₀, for hydralazine and valproate, and DV ICₓ₀, DV ICₓ₀, DV ICₓ₀, and DV ICₓ₀, for vorinostat and decitabine) were employed for viability curve
In vitro comparison of hydralazine valproate versus vorinostat decitabine in CTCL

Table 1. Combinatory index values, recommended symbols and descriptions for determining synergism, antagonism or addition using the Chou-Talalay formula

<table>
<thead>
<tr>
<th>Range of CI</th>
<th>Symbol</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;0.1</td>
<td>++++</td>
<td>Very strong synergism</td>
</tr>
<tr>
<td>0.1-0.3</td>
<td>+++</td>
<td>Strong synergism</td>
</tr>
<tr>
<td>0.3-0.7</td>
<td>+++</td>
<td>Synergism</td>
</tr>
<tr>
<td>0.7-0.85</td>
<td>++</td>
<td>Moderate synergism</td>
</tr>
<tr>
<td>0.85-0.9</td>
<td>+</td>
<td>Slight synergism</td>
</tr>
<tr>
<td>0.9-1.1</td>
<td>±</td>
<td>Nearly additive</td>
</tr>
<tr>
<td>1.1-1.2</td>
<td>-</td>
<td>Slight antagonism</td>
</tr>
<tr>
<td>1.2-1.45</td>
<td>--</td>
<td>Moderate antagonism</td>
</tr>
<tr>
<td>1.45-3.3</td>
<td>---</td>
<td>Antagonism</td>
</tr>
<tr>
<td>3.3-10</td>
<td>----</td>
<td>Strong antagonism</td>
</tr>
<tr>
<td>&gt;10</td>
<td>------</td>
<td>Very strong antagonism</td>
</tr>
</tbody>
</table>

assays. Cells were seeded into 12-well microtiter plates at 5x10^4 cells/well with 1 mL of complete medium, and then treated for a 72 hour-period with the different combinations of the ICs of HV and VD. After 72 hours, pellets were recovered and resuspended in 1 mL RPMI-1640 medium to assess cell viability by trypan blue exclusion assay.

Synergism evaluation

HV and VD interactions were determined using the combination index (CI) method from the mathematical formula of Chou and Talalay (CalcuSyn software, Biosoft). The CI is a numerical representation of the pharmacological interaction, which takes into account the dose-response curve of each drug and the growth inhibition curve of the drug combination to determine synergism, addition or inhibition. This model uses the formula f_i / f_u = [D/D_m], where f_i is the fraction of cells inhibited, f_u = 1-f_i, the unaffected fraction, D is the concentration of the drug, D_u is the potency of the drug, and m is the shape of the dose-effect curve. Table 1 shows the interpretation of CI values with the corresponding pharmacological interaction.

Cell cycle assays

After performing combinatory viability assays with the most synergistic doses, cells were stained with propidium iodide (Sigma) for 1 h and analyzed for DNA content on the flow cytometer BD FACS Canto™ II (BD Biosciences). Debris and aggregates were gated out during data acquisition, and 20,000 gated events were collected for each sample. Cell cycle analyses were performed employing the ModFit LT software (Verity Software House). Results are expressed in percentage of cells for each cell phase.

Clonogenic capacity assays

After performing combinatory viability assays with the most synergistic doses, remaining cells were seeded into 25 cm² cell culture flasks (Corning) at a density of 2x10^3 cells/flask into 5 mL complete medium. The whole medium was changed every 48 hours with a fresh complete medium. Cells were let growing during 2 weeks and then viability was measured by trypan blue exclusion assay.

Viability evaluation in healthy leukocytes

The most synergistic doses of each drug combination were employed during a 72 h-period in healthy leukocytes to evaluate viability. Healthy leukocytes were seeded into 12-well microtiter plates at a density of 5x10^4 cells/well into 1 mL complete medium. The following day, cells were treated for a 72 hour-period with either HV or VD at the most synergic doses, and the medium with the respective drug mixture was changed every 24 hours as with the Hut78 cell line. After 72 hours of treatment, cellular viability was measured by trypan blue exclusion assay.

Statistical analysis

Three independent experiments were performed and data was expressed as means ± SD. Data were statistically analyzed by using GraphPad Prism V6 software (GraphPad Software Inc., La Jolla, CA, USA). Significant differences were determined using one-way analysis of variance (ANOVA) followed by Tukey correction to determine significant difference between each test group against its respective control. A P value of <0.05 was considered statistically significant.

Results

Individual DNMTi and HDACi agents diminish in a dose-dependent manner cellular viability in the Hut78 lymphoma cell line

To demonstrate that the epigenetic agents, either individual DNMTi or HDACi decrease the
In vitro comparison of hydralazine valproate versus vorinostat decitabine in CTCL

Figure 1. Dose-response curves of hydralazine, valproate, vorinostat and decitabine individually in the Hut78 cell line. Hydralazine (A), valproate (B), vorinostat (C) and decitabine (D) were employed as single drugs at increasing doses, and after 72 h of treatment cellular viability was evaluated. Each concentration was compared against its respective control. ***: P<0.001.

Figure 2. Dose-response curves of the pharmacological combinations of hydralazine-valproate and vorinostat-decitabine in the Hut78 cell line. Hydralazine-valproate (A) and vorinostat-decitabine (B) were employed at increasing IC doses and after 72 h of treatment, cellular viability was evaluated. Each concentration was compared against its respective control. ***: P<0.001. The drug µM concentrations corresponding to each IC (20, 30, 40 and 50 respectively) for each agent are the following: Hydralazine 1.7, 6.2, 14.97 and 29.5. Magnesium Valproate: 140, 220, 330, 450. Vorinostat: 0.52, 0.83, 1.16, 1.48, and Decitabine: 0.3, 0.34, 0.39 and 0.49.
In vitro comparison of hydralazine valproate versus vorinostat decitabine in CTCL cellular viability of the CTCL cell line Hut78, cells were treated with increasing doses of hydralazine, valproate, vorinostat or decitabine. As shown in Figure 1, hydralazine shows its inhibitory effects starting at 2.5 µM in a dose-dependent manner. These effects were statistically significant. The inhibition with valproate was observed since 0.125 mM and was also dose-dependent. At doses higher than 3mM, viability was 0% (data not shown). Both vorinostat and decitabine also inhibited viability in a dose-dependent manner. For vorinostat, significant inhibition started at 0.5 µM and the highest effect was seen at 2 µM. The highest effect

Figure 3. Cellular effect of the pharmacological combinations of hydralazine-valproate (A) and vorinostat-decitabine (B) on the Hut78 cell line. 4X pictures taken at 24, 48 and 72 h after starting each regimen of the IC drug combinations of hydralazine-valproate or vorinostat-decitabine.
In vitro comparison of hydralazine valproate versus vorinostat decitabine in CTCL

The corresponding IC\(_{50}\) were 29.95, 450, 1.48 and 0.49 µM for hydralazine, valproate, vorinostat, and decitabine, respectively. Next, we treated the cells either with the combination of hydralazine and valproate (HV) or with the combination of vorinostat and decitabine (VD), using the IC values given by SigmaPlot. Figure 2 shows the combined effect and doses for the HV and VD combinations. For HV there was observed a dose-dependent effect on viability among the four ICs, being higher at IC\(_{50}\) doses, whereas for VD the IC\(_{20}\) and IC\(_{40}\) combinations induced the same reduction, which was more pronounced at IC\(_{40}\) and IC\(_{50}\) doses. A representative figure of the cell culture effect for both combinations at different IC combination over time is shown in Figure 3A and 3B.

With these viability percentages, the pharmacological interaction was determined using the CompuSyn software. CompuSyn generates the combination index (CI) for each drug combination, and a CI value below 1 indicates synergy. As shown in Figure 4 and Table 2, for HV, IC\(_{50}\) was clearly synergic with a CI of 0.548, whereas for the combination of VD only the IC\(_{20}\) scheme showed a borderline synergy, in the range of a nearly additive combination. All the others CIs for VD were clearly above 1, indicative of an antagonistic relationship.

Figure 4. Pharmacological interaction between the drug combinations. Hydralazine-valproate schemes (A) show antagonism between the IC\(_{20}\) and IC\(_{30}\) doses, with a CI above 3. In contrast, the IC\(_{40}\) doses generate a CI of 0.999, demonstrating a nearly additive interaction. The IC\(_{50}\) doses induce a synergistic interaction, with a CI of 0.548. On the other hand, vorinostat-decitabine combinations (B) tend to exert a nearly additive interaction within all the ICs evaluated. The only CI value below 1 is the resulting of IC\(_{20}\) interactions, with a CI of 0.957 (nearly additive). The IC\(_{30}\) scheme induces a slight antagonism interaction with a CI value of 1.19, and the IC\(_{40}\) and IC\(_{50}\) combinations generate a nearly additive effect with a CI value of 1.074 and 1.069, respectively.

of decitabine was observed at 1 µM, but inhibition started at 0.25 µM.

The combinations of hydralazine-valproate and of vorinostat-decitabine work in a synergistic way to reduce cellular viability

In order to determine the pharmacological interaction between these agents, the IC\(_{20}\), IC\(_{30}\), IC\(_{40}\) and IC\(_{50}\) doses for each of the agents were determined using the SigmaPlot software.

From this point on, we used the most synergic doses of each drug mixture, named HV IC\(_{50}\) and VD IC\(_{20}\) for further experiments. As a way to compare doses with both drug combinations, we employed the conditions HV IC\(_{20}\) and HV IC\(_{40}\), VD IC\(_{20}\) and VD IC\(_{50}\). Since vorinostat is resuspended in pure ethanol, we generated two controls for VD combinations, one for the VD IC\(_{20}\) and
In vitro comparison of hydralazine valproate versus vorinostat decitabine in CTCL

<table>
<thead>
<tr>
<th>Drug combination</th>
<th>( f_a )</th>
<th>CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>HV IC(_{20})</td>
<td>0.146746</td>
<td>3.408</td>
</tr>
<tr>
<td>HV IC(_{50})</td>
<td>0.241617</td>
<td>3.329</td>
</tr>
<tr>
<td>HV IC(_{90})</td>
<td>0.531805</td>
<td>0.999</td>
</tr>
<tr>
<td>HV IC(_{50})</td>
<td>0.726923</td>
<td>0.548</td>
</tr>
<tr>
<td>VD IC(_{20})</td>
<td>0.507458</td>
<td>0.957</td>
</tr>
<tr>
<td>VD IC(_{50})</td>
<td>0.525263</td>
<td>1.192</td>
</tr>
<tr>
<td>VD IC(_{50})</td>
<td>0.671353</td>
<td>1.074</td>
</tr>
<tr>
<td>VD IC(_{50})</td>
<td>0.76435</td>
<td>1.069</td>
</tr>
</tbody>
</table>

Drug interactions were evaluated with the CalcuSyn software by comparing the individual drug and the drug combinations effects on cellular viability. CI values >1 are indicative of antagonistic relationships, while <1 are synergistic. CI values close to 1 are summative. HV: hydralazine + valproate; VD: vorinostat + decitabine; \( f_a \): fraction of affected cells, or percentage of reduction in cellular viability as compared with controls; CI: combinatorial index.

the other for the VD IC\(_{50}\) schemes. Therefore, we used the same volume of ethanol required for vorinostat at IC\(_{50}\) dose for its control, while employing the same volume of ethanol required for vorinostat at IC\(_{50}\) dose for its control. Regarding HV combinations, because both hydralazine and valproate are resuspended in distilled water, we used only one control with the same volume of water as the employed in the HV IC\(_{50}\) dose for comparison.

**Hydralazine-valproate and vorinostat-decitabine combinations significantly promote cell death as demonstrated by flow cytometry**

To further investigate the effect of these treatments in cell cycle, flow cytometry assays with propidium iodide were performed with IC\(_{20}\) and IC\(_{50}\) doses of the HV and VD schemes. For both combinations at IC\(_{20}\) of doses, there were no statistically significant differences in cell cycle distribution. However, when treated with IC\(_{90}\) doses, both couple of drugs significantly increased the percentage of cell death, in a very similar manner (Figure 5).

**The hydralazine-valproate IC\(_{50}\) drug scheme promotes a maintained reduction in the clonogenic capacity of treated cells over time**

To determine the cellular effects of these interactions upon the clonogenic capacity of treated cells, clonogenic assays with cellular viability evaluation were performed for both combinations with the IC\(_{20}\) and IC\(_{50}\) doses. As shown in Figure 6, a small effect on cellular viability was observed for the IC\(_{20}\) doses for HV, but it was clearly more marked and statistically significant when IC\(_{50}\) doses were employed. On the other hand, no viability reduction was observed with either IC\(_{20}\) or IC\(_{50}\) doses for VD.

**Healthy PMNCs are resistant to both hydralazine-valproate and vorinostat-decitabine combinations**

Finally, to determine which of these combinations would affect more to normal cells, freshly isolated peripheral blood mononuclear cells (PBMNCs) were treated with both HV and VD combinations with either IC\(_{20}\) or IC\(_{50}\) doses, as shown in Figure 7. No differences in cellular viability were seen with any of the drug schemes at IC\(_{20}\) concentrations, as compared with the respective controls. However, there was observed a higher decrease in cellular viability with both treatments at IC\(_{50}\) doses, being slightly more marked with the combination of VD, although none of them demonstrated a statistically significant difference.

**Discussion**

In this *in vitro* study in which the antitumor effects of different epigenetic agents were evaluated in the Hut78 CTCL cancer cell line, the results show that each of the DNMTi and HDACi exerts growth inhibition, mostly by inducing apoptosis as shown in the cell cycle distribution. However, in the combination of HV the interaction is more synergic and also it inhibits the clonogenic capacity of cells over time. Additionally, the HV combination seems to affect in a minor degree the viability of peripheral blood mononuclear cells.

The therapy of CTCL is challenging since even with the use of HDACi as single agents the response rates are below 40%. Beyond the clinical study with hydralazine and valproate in CTCL [13], there are yet no clinical head-to-head comparisons of different HDACi, nor clinical studies of any HDACi combined with any DNMTi for CTCL. However, preclinical studies in a model of CTCL demonstrate that the HDACi romidepsin and the DNMTi azacitidine are synergic in their epigenetic modulatory effects and apoptosis [12]. Likewise, but in a model of dif-
In vitro comparison of hydralazine valproate versus vorinostat decitabine in CTCL

In fuse large B-cell lymphoma (DLBCL), the combination of panobinostat with decitabine also results in synergic growth inhibition and apoptosis [10]. The results here reported on the higher synergy demonstrated by the pharmacological interaction with hydralazine and valproate support the results of a recently reported phase II study with these drugs in untreated and pre-treated CTCL, yielding responses above 70% [14]. Thus, the findings of this study, and the preclinical studies combining these two agent classes, strongly suggest that additional clinical studies with combined epigenetic therapy are highly needed, which promises to increase the efficacy of CTCL treatment.

There are a number of preclinical studies testing the combination of different HDACi with DNMTi, and all of them show that the antitumor effects are increased and that the combination potentiates the expression of candidate genes. Decitabine and vorinostat induce apoptosis in myeloid leukemia cells, accompanied by survivin downregulation [15]. On the other hand, in estrogen receptor (ER) negative breast cancer cells, decitabine and trichostatin A increase up to 300-400 fold the expression of the ER gene [16]. In ovarian cancer, decitabine and vorinostat show G2/M arrest and apoptosis in cancer cell lines, while in xenografts they induce the expression of

Figure 5. Effects of the IC$_{50}$ and IC$_{90}$ doses of HV and VD combinations on the cell cycle of the Hut78 cell line. There was observed a statistically significant increase in the percentage of cells in the subG0 peak, indicative of apoptosis, with both drug combinations at IC$_{50}$ doses. The VD combination at IC$_{50}$ also showed an increase in the G2-M phase.

Figure 6. Effects of the combinations at IC$_{20}$ and IC$_{50}$ doses of HV and VD combinations cellular viability after clonogenic assays. There was a statistically significant decrease in clonogenicity only in the combination of HV at IC$_{50}$. 

Decitabine and vorinostat induce apoptosis in myeloid leukemia cells, accompanied by survivin downregulation [15]. On the other hand, in estrogen receptor (ER) negative breast cancer cells, decitabine and trichostatin A increase up to 300-400 fold the expression of the ER gene [16]. In ovarian cancer, decitabine and vorinostat show G2/M arrest and apoptosis in cancer cell lines, while in xenografts they induce the expression of
In vitro comparison of hydralazine valproate versus vorinostat decitabine in CTCL

imprinted tumor suppressor genes [17]. Antitumor effects are also shown by combining decitabine with vorinostat in the pancreatic cancer cell line MiaPaCa2, both in vitro and in vivo [18]. At least eight studies in lung cancer have shown that combining these two agent classes is able to increase apoptosis by diverse mechanisms [19-25]. Similar results have been reported in esophageal cancer [26], endometrial carcinoma [27], Ewing sarcoma [28], and colon cancer cells [29]. Thus, there is a strong preclinical support for the use of dual epigenetic therapy.

Clinical studies combining either azacitidine or decitabine with vorinostat have yielded conflicting results. Craddock et al. [30] reported that azacitidine and vorinostat did not increase the efficacy in acute myeloid leukemia, as well as neither the combination of vorinostat with either lenalidomide or azacitidine in high-risk myelodysplastic syndrome [31]. In relapsed or refractory acute lymphoblastic leukemia (ALL), decitabine and vorinostat followed by re-induction chemotherapy were tolerable and it demonstrated clinical benefits in relapsed patients with ALL. Methylation differences were identified between responders and non-responders, indicating interpatient variation, which could impact in clinical outcome [32]. Likewise, double epigenetic modulation of gemcitabine/busulfan/melphalan with azacitidine/vorinostat is feasible and highly active in patients with refractory/poor-risk relapsed lymphomas. Authors conclude that this regimen with epigenetic modulation warrants further study [33]. Finally, a phase I study by Stathis et al. [34] found that the combination of decitabine with vorinostat is tolerable on both concurrent and sequential schedules in previously treated patients with advanced solid tumors or non-Hodgkin’s lymphoma, recommending for phase II studies a regimen of sequential decitabine at 10 mg/m²/day on days 1 to 5, and vorinostat at 200 mg twice a day on days 6 to 12. This schedule demonstrated activity with prolonged disease stabilization in different tumor types. Taken together, these clinical data with our results on the synergistic interaction between these two agent classes, strongly suggest that further exploration of these combinations should be pursued. Interestingly, as observed in this study while VD exhibits almost none synergy, hydralazine and valproate were highly synergistic at the doses of IC₅₀ and only this combination was able to reduce the clonogenicity of Hut78 cells.

CTCL can be considered in general an indolent disease with years of survival. Therefore, the main goal of the therapy is to improve skin lesions and to avoid pruritus, without affecting the quality of life by the side effects. Our results showed a minor effect of the combination of hydralazine valproate upon PBMCs, indicating interpatient variation, which could impact in clinical outcome [32]. Likewise, double epigenetic modulation of gemcitabine/busulfan/melphalan with azacitidine/vorinostat is feasible and highly active in patients with refractory/poor-risk relapsed lymphomas. Authors conclude that this regimen with epigenetic modulation warrants further study [33]. Finally, a phase I study by Stathis et al. [34] found that the combination of decitabine with vorinostat is tolerable on both concurrent and sequential schedules in previously treated patients with advanced solid tumors or non-Hodgkin’s lymphoma, recommending for phase II studies a regimen of sequential decitabine at 10 mg/m²/day on days 1 to 5, and vorinostat at 200 mg twice a day on days 6 to 12. This schedule demonstrated activity with prolonged disease stabilization in different tumor types. Taken together, these clinical data with our results on the synergistic interaction between these two agent classes, strongly suggest that further exploration of these combinations should be pursued. Interestingly, as observed in this study while VD exhibits almost none synergy, hydralazine and valproate were highly synergistic at the doses of IC₅₀ and only this combination was able to reduce the clonogenicity of Hut78 cells.

![Figure 7. Effects of the drug combinations at IC₂₀ and IC₅₀ doses in the viability of PBMCs. There were no statistically significant differences in cellular viability between both combinations, though a slightly lower viability was observed for the IC₅₀ dose of VD.](image-url)
advanced solid tumors, and even in healthy volunteers [13, 14, 35, 36].

Some limitations of this study are the lack of mechanistic investigation on the molecular basis for the differential synergism between these two agent classes; that a single cell line was investigated, and that the patterns of global gene expression and DNA methylation need to be investigated. Nevertheless, a recent study by Qu et al., shows that the clinical response of CTCL to HDACi strongly associates with a concurrent gain in chromatin accessibility [37], and it is well-known that, both HDACi and DNMTi, cooperate in inducing locus-specific and global chromatin decompaction [38, 39]. In addition, the same work by Qu et al., [37] found that the role of the host immune system may be equally important in CTCL. It is known that azacitidine causes an interferon response in cancer [40]. Interestingly, in breast cancer, the combination of hydralazine and valproate induces overexpression of IFN-response pathway genes and 8-fold up-regulation in MHC class I-A and -B molecules [41].

In conclusion, the results of this study performed on a single CTCT line and the preclinical and clinical evidence on the efficacy of combining HDACi with DNMTi suggest that more preclinical and clinical studies are needed with this drug class combination in CTCL, particularly with the hydralazine-valproate scheme, since hydralazine and valproate are safe and widely available drugs administered by oral route.

Acknowledgements

Alejandro Schcolnik-Cabrera is a student belonging to the Plan de Estudios Combinadosen Medicina (PECEM), UNAM.

Disclosure of conflict of interest

None.

Address correspondence to: Alfonso Dueñas-González, Division of Biomedical Research in Cancer, Instituto De Investigaciones Biomédicas UNAM/Instituto Nacional De Cancerología, México. Tel: +52 55 13532386; +52 55 54853034; E-mail: alfonso_duenasg@yahoo.com

References


In vitro comparison of hydralazine valproate versus vorinostat decitabine in CTCL


In vitro comparison of hydralazine valproate versus vorinostat decitabine in CTCL


