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Letter to the Editor

In classical Hodgkin lymphoma the combination of the CCR5 antagonist maraviroc with trabectedin synergizes, enhances DNA damage and decreases 3D tumor-stroma heterospheroid viability.

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Running title: Maraviroc enhances trabectedin activity in HL

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Authors’ contributions
N.C. and C.B. generated and interpreted data; D.A. and N.C. drafted the manuscript; D.A. supervised the study. All authors reviewed, revised, and approved the final manuscript.

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Supplementary data
Supplemental Figure S1. CCR5 expression and CCL5 secretion by HRS cells; Supplemental Figure S2. Maraviroc enhanced trabectedin cytotoxicity; Supplemental Figure S3. Maraviroc alone and in combination with trabectedin decreased cell viability of 3D heterospheroids formed by HRS cells and cHL-MSCs
Several preclinical and clinical studies demonstrated a key role of the CCL5/CCR5 axis in cancer, providing the rationale for clinical trials with the CCR5 antagonist Maraviroc. Indeed, maraviroc, approved by FDA as antiretroviral for the treatment of HIV infections, has been repurposed as a potential therapeutic option for cancer treatment since it demonstrated to inhibit tumor growth, metastasis formation, and the building of a tumor promoting microenvironment (TME). In breast cancer, CCR5+ tumor cells are characterized by increased DNA repair gene levels and maraviroc enhanced both the cell killing and the DNA damage mediated by the DNA damaging agent doxorubicin.

Tumor cells from classical Hodgkin lymphoma (cHL) (the Hodgkin and Reed Sternberg cells, HRS) secrete CCL5, express a functional CCR5 receptor, and patients with high CCL5 levels have poor prognosis. CCR5 is activated in both autocrine and paracrine manners by CCR5-ligands secreted by HRS cells, monocytes and cHL-Mesenchymal stromal cells (MSCs). Maraviroc decreases HRS cell growth in vitro and in tumor xenografts, synergizes with doxorubicin and brentuximab vedotin and decreases the self aggregation of HRS cells, monocytes and MSCs in three-dimensional (3D) heterospheroids, used as an in vitro model to study the TME.

Trabectedin covalently binds DNA, blocking transcription and interfering with DNA repair, and modifies the TME by reducing selectively tumor-associated macrophages. Its anticancer activities have been demonstrated in solid tumors and in preclinical models of hematologic malignancies. In cHL, trabectedin induces a potent in vitro antitumor activity, decreases cytokine levels and viability of heterospheroids formed by HRS cells and MSCs, inhibits tumor xenograft growth, monocyte infiltration and angiogenesis.

Considering that HRS cells express a functional CCR5 receptor and maraviroc synergizes with doxorubicin and affects TME interactions, our hypothesis is that this CCR5 antagonist, by
promoting DNA damage and disrupting the cross-talk between tumor cells and MSCs in 3D-heterospheroids could potentiate the cytotoxic effects of trabectedin.

Here we found that maraviroc in combination with trabectedin: exerted synergistic effects; enhanced DNA double strand breaks (DSBs); cooperated to decrease the viability of 3D tumor-stroma-heterospheroids.

In this study we used a panel of four authenticated chL-derived cell lines L-1236, L-428, KM-H2, and HDLM-2 (DSMZ, Germany), and chL-MSCs obtained from frozen lymph nodes and generated as previously described. Drug combinations effects were evaluated calculating the combination index (CI) using the CalcuSyn software (Biosoft, Ferguson, MO, USA) (Chou-Talalay method). CI values <0.9 indicate synergy, the lower the value the stronger the synergism. The phosphorylation of the H2AX histone (γ-H2AX) and γ-H2AX-foci formation were used to study the lethal double-strand DNA lesions. The combination of maraviroc (Sigma-Aldrich) with trabectedin (PharmaMar) was also tested in 3D heterospheroids. Heterospheroids were generated by co-culturing HRS cells and chL-MSCs (1.0 x 10^4/mL of each cell type) in RPMI-1640 medium containing 2% FCS, using plates coated with 20 mg/mL poly-HEMA (Sigma) to prevent cell adhesion. Statistical analysis was carried out using GraphPad Prism version 6.0 software (GraphPad, La Jolla, USA). Student’s t test was used to compare two groups and One-way ANOVA followed by Dunnett’s test to compare each of a number of treatments with a single control. A P-value <0.05 was considered significant.

First, we performed drug-combination studies with maraviroc and trabectedin using CCR5+ chL-derived cell lines (1236, L-428, KM-H2, HDLM-2). Maraviroc reduced in a dose-dependent manner the CCR5-CCL5 mediated autocrine growth of HRS cells (Figure 1A), without an apparent correlation with CCR5 expression (online Supplemental Figure S1A and S1B) nor CCL5 secreted
levels (online Supplemental Figure S1C). The combination of maraviroc with trabectedin resulted in a strong synergism (i.e. interaction between two or more drugs that determines greater effect than the sum of the individual effects of each drug) in KM-H2 (CI < 0.3) and in HDLM-2 cells (CI < 0.3), and a moderate synergism in L-1236 cells (CI ranging from 0.47 to 0.63) (Figure 1B). In L-428 cells, we observed synergy only at low drug concentrations (CI ranging from 0.57 to 0.98) (Figure 1B). Genetic lesions in members of the NF-κB and JAK/STAT pathways or TP53 alterations could be a possible explanation for the lower synergistic effects in L-428 cells.  

Then, we evaluated DNA fragmentation in HRS cells treated with maraviroc, trabectedin alone, or in combination. The phosphorylation of the histone H2AX (γ-H2AX) is one of the early events associated with the detection and processing of DNA DSBs and the formation of γ-H2AX-foci, arising when the cell identifies DNA lesions. For this purpose, we analyzed γ-H2AX induction and γ-H2AX-foci formation after short time drug incubation. Western blot assay showed that treatment of HRS cells with maraviroc alone did not or slightly induce DNA fragmentation. On the other hand, trabectedin induced γ-H2AX that was deeply enhanced by the combination with maraviroc (Figure 2A and 2B). To further validate that maraviroc in combination with trabectedin enhanced DNA damage, we evaluated the formation of nuclear γ-H2AX foci by confocal microscopy. Consistently with western blot assay (Figure 2A and 2B), after treatment with maraviroc only rare γ-H2AX foci were detected, whereas the combination with maraviroc enhanced the formation of γ-H2AX-foci by trabectedin (Figure 2C), confirming that the CCR5-antagonist in combination with trabectedin further promoted DNA fragmentation (Figure 2).

The high cytotoxic effects of drug combination respect to the slight activity of single treatments were confirmed by phase contrast photographs and by Annexin-V/7AAD staining (online Supplemental Figure S2).
However, even if maraviroc alone induced a very modest or no DNA fragmentation in HRS cells respect to breast cancer cells,\textsuperscript{4} we cannot exclude a possible role of CCR5 in enhancing DNA repair also in cHL. Alternatively, maraviroc could increase trabectedin concentration in HRS cells by competing for the drug transporters P-glycoprotein, substrate for both drugs.\textsuperscript{12}

In cHL, few HRS cells are surrounded by a protective and immunosuppressive TME, capable to decrease drug activity and to counteract the immune control of tumor growth.\textsuperscript{13} Heterospheroids represent a 3D model in which different cell types are cultured under non-adherent conditions. This \textit{in vitro} model, developed to mimic the cross-talk of HRS cells with the TME, was recently used to study the antitumoral activity of trabectedin alone,\textsuperscript{9} and of maraviroc in combination with doxorubicin.\textsuperscript{5}

cHL-MSCs can exert protective effects against anticancer drugs by their direct contact with tumor cells\textsuperscript{13,14} or by secreting tumor-promoting molecules, including CCL5.\textsuperscript{5} Since maraviroc reduces the self assembling of HRS cells with cHL-MSCs,\textsuperscript{5} it could cooperate with trabectedin not only by enhancing DNA damage in CCR5+ cells but also by counteracting the protective effects of the cross-talk HRS/cHL-MSCs. First, we evaluated the efficacy of the combination maraviroc-trabectedin in formed heterospheroids (direct contact). We cultured HRS cells (L-1236 or HDLM-2 cells) with cHL-MSCs under non-adherent conditions. After 24h (time necessary to obtain cell aggregation as heterospheroids), we added maraviroc and different concentrations of trabectedin\textsuperscript{9} (online Supplemental Figure S3). After 3 (online Supplemental Figure S3A and S3B) and 6 days (online Supplemental Figure S3C and S3D) of treatment, we evaluated drug effects. Both maraviroc and trabectedin decreased in a dose-dependent manner heterospheroid cell viability and their combination further increased cytotoxicity. These effects were more evident after 6 days of treatment in HDLM-2 and in particular in L-1236 cells (Online Supplemental Figure S3).
To demonstrate that the direct contact with cHL-MSCs can protect against the effects exerted by maraviroc, trabectedin and their combination, HRS cells and cHL-MSCs were cultured under non-adherent conditions and drugs were added before (at T=0) and after (at T=24h) their spontaneous aggregation in heterospheroids (see Figure 3A). After 6 days cell viability of heterospheroids formed by L-1236/cHL-MSCs (Figure 3B) and by HDLM-2/cHL-MSCs (Figure 3C) was evaluated. Maraviroc and trabectedin alone reduced cell viability of heterospheroids (Figure 3B and 3C) and their combination was significantly more efficacious than single treatments. Cytotoxicity was more evident when drugs were added before cell aggregation (heterospheroid formation, T=0) (black histograms), suggesting a drug protecting role of HL-MSCs and a cooperation between the two cell types, including the increased secretion and expression of prosurvival factors. Representative phase contrast photographs demonstrating the increased cytotoxic activity of the combination trabectedin-maraviroc in heterospheroids are shown in Figure 3D.

Taken together our results suggest that maraviroc can enhance trabectedin activity by reducing the tumor-promoting effects of the direct contact of HRS cells with cHL-MSCs and by inhibiting autocrine and paracrine effects induced by CCL5 secreted by HRS cells and tumor-educated cHL-MSCs.

In conclusion, maraviroc synergized with trabectedin, enhanced trabectedin induced DNA DSBs and decreased the protective effects of cHL-MSCs in heterospheroids. Therefore, this study offers an additional preclinical rationale for the use of maraviroc as a new therapeutic option to affect tumor microenvironmental interactions, to enhance the cytotoxic activity of DNA damaging agents and by decreasing their doses to reduce adverse side effects. These findings provides insights for future research to investigate the possibility to potentiate the
antitumoral activity of DNA damaging agents, including gamma radiation, currently used for the
treatment of cHL.¹⁵
References


Legend to figures

**Figure 1. Maraviroc synergized with trabectedin in HRS cells.** L-1236, L-428, KM-H2 and HDLM-2 cells (2.0×10^5 cells/mL), were exposed to increasing concentrations of maraviroc, trabectedin and the combination. (A) After 72h cell viability was evaluated by trypan blue dye exclusion. Results (percentage of control) are mean and SD of three independent experiments each run in triplicate. (B) Synergy was determined using CalcuSyn software (CI, combination index). Values of bar charts are mean CI values and SD of three experiments each run in triplicate. MVC, maraviroc; TB, trabectedin.

**Figure 2. Maraviroc enhanced DNA damage induced by trabectedin.** HRS cells were treated with maraviroc (100 µM), trabectedin (500 pM) and the combination for 24h. (A) Western blot for -H2AX and α-tubulin protein expression in cHL cell lines. Membrane strips were incubated with mouse anti-phospho-Histone H2A.X (Ser139)(clone JBW301)(Millipore) and mouse anti-α-Tubulin antibody clone B-5-1-2 (Sigma Aldrich) and revealed with Donkey anti-Mouse IgG (H+L chain) A90-137P (Bethyl Laboratories). Images were acquired using a ChemiDoc XRS system (Bio-Rad). Data are representative of four experiments. (B) Bar charts showing densitometric analysis of γ-H2AX expression normalized to α-tubulin as loading control. Protein quantification was performed by imagej software. Values are means and SD of four experiments. * P<0.05, treatments vs. control, One-way ANOVA followed by Dunnett’s test. ° P<0.05, trabectedin vs. trabectedin and maraviroc in combination, Student’s t test. (C) Immunofluorescence images (confocal microscopy) of γ-H2AX foci after drug treatment. HRS cells adherent to coverslips, were fixed, permeabilized and incubated with anti-phospho-Histone H2A.X (Ser139)(clone JBW301)(Millipore), followed by Alexa Fluor-488 anti-mouse secondary antibody (Thermo Scientific). Images were acquired with a
Leica TCS SP8 Confocal system (Leica Microsystems Heidelberg, Mannheim, Germany), using the Leica Confocal Software (LCS). MVC, maraviroc; TB, trabectedin.

Figure 3. Maraviroc cooperated with trabectedin to decrease cell viability in 3D heterospheroids formed by HRS cells and cHL-MSCs. (A) Schedule of HRS and cHL-MSCs treatment. (B) L-1236, or (C) HDLM-2 cells were cultured in non-adherent conditions with cHL-MSCs (1.0 × 10⁴/mL of each cell type) in 24 well plates and treated with trabectedin (360 pM), maraviroc (100 µM) alone or in combination. Drugs were added immediately (add. T= 0) and after heterospheroid formation (add. T=24h). After 6 days, cell viability was evaluated using the PrestoBlue Cell Viability Reagent (Invitrogen). Values are mean and SD of three experiments. *P<0.05 for T=0 vs. T=24h, Student’s t test. * P<0.05 treatments vs. medium, One-way ANOVA followed by Dunnett’s test. (D) Representative phase contrast micrographs of heterospheroids cultured with trabectedin, maraviroc and in combination (T=0). MVC, maraviroc; TB, trabectedin. add., added.
A

Drug addition (non-adherent conditions)

HRS cells (○) + cHL-MSCs (●) → Heterospheroids

B

L-1236/cHL-MSCs

C

HDLM-2/cHL-MSCs

D

medium  maraviroc (100 μM)  trabectedin (360 pM)  trabectedin + maraviroc

L-1236 + cHL-MSCs

HDLM-2 + cHL-MSCs

3 dimensional-heterospheroids
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Supplemental Figure S1. CCR5 expression and CCL5 secretion by HRS cells. (A) CCR5 expression was analyzed using the monoclonal mouse anti-CCR5 mAb (clone 45531; R&D Systems) followed by the FITC-conjugated goat anti-mouse IgG (Jackson Immuno Research). Assay results were detected by flow cytometry on a BD FACS Canto II flow cytometer. Data were analyzed using BD FACSDiva v.8.0.1 software (BD Biosciences, Milano, Italy). (B) Percentage of CCR5 positive cells (flow cytometry) (C) cHL cells were seeded at 2.0 × 10^5/ml in RPMI-1640 plus 10% FCS, and medium collected after 72 h. CCL5 was quantified using commercially available ELISA kit (Immunological Sciences). Three biological replicates tested in duplicate (n=6) and results are expressed as mean and SD.
Supplemental Figure S2. Maraviroc enhanced trabectedin cytotoxicity.

HRS cells were treated with maraviroc, trabectedin, or their combination. (A) Phase contrast microscopy showing the cytotoxic effects of the drugs after 24h treatment. (B) Representative cytofluorimetric dot blots of the cells double stained with Annexin-V-FITC (Thermo Fisher Scientific) and 7AAD (BD Pharmingen), and analyzed by flow cytometry after 48h treatment.

MVC, maraviroc; TB, trabectedin.
Supplemental Figure S3. Maraviroc alone and in combination with trabectedin decreased cell viability of 3D heterospheroids formed by HRS cells and chL-MSCs. (A, C) HDLM-2 and (B, D) L-1236 were cultured under non-adherent conditions with chL-MSCs to form heterospheroids (24h). Then cells were cultured with trabectedin (0-360 pM), maraviroc (100 µM) alone or in combination. After 3 and 6 days, cell viability was evaluated with Presto Blue assay. Values are mean and SD of three experiments. MVC, maraviroc; TB, trabectedin.