Histone deacetylase inhibition enhances the lymphomacidal activity of the anti-CD22 monoclonal antibody HB22.7

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ABSTRACT

HB22.7, an anti-CD22 monoclonal antibody has shown consistent preclinical activity against non-Hodgkin lymphoma (NHL). Histone deacetylase inhibitors (HDACi) have demonstrated efficacy in lymphoma and can modulate cell surface receptor expression. To augment the lymphomacidal activity of HB22.7 we examined the combination of AR42 (an HDACi) and HB22.7 in vitro and in vivo. The combination resulted in 10-fold increased potency in 6 NHL cell lines when compared to either drug alone. Both drugs reduced tumor progression in xenografts, but the combination was significantly more efficacious and resulted in regression of established tumors, without toxicity. AR42 inhibited HB22.7-mediated CD22 internalization, suggesting that increased efficacy could be due to higher availability of CD22. Overall, the synergistic effects of HB22.7 and AR42 on in vitro cytotoxicity and in vivo anti-tumor activity make this combination an attractive option for further pre-clinical and clinical evaluation.

1. Introduction

Non-Hodgkin lymphoma (NHL) is the sixth most common cause of cancer-related deaths in the United States, with over 60,000 new cases diagnosed in 2010 and over 332,000 people currently living with this disease [1–3]. Despite expanding therapeutic options, the majority of patients will eventually succumb to the disease. The fastest growing segment of NHL patients is the elderly and many of these patients tolerate chemotherapy poorly and this limits efficacy. Therefore, less toxic yet more effective treatments are needed. Monoclonal antibodies (mAb), such as rituximab, are currently the mainstay of treatment for recurrent and previously untreated NHL because of their specificity, safety, and efficacy. Given the success of mAb-based therapeutics, new antibodies are being tested as single agents or in combination with currently available agents.

CD22 is a cell-surface glyco-phospho-protein expressed by most normal and malignant B cells [4,5], and it is therefore of interest for the development of targeted therapies. CD22 is a member of the immunoglobulin superfamily as well as the recently described siglec (defined as a sialic-acid-containing ligand binding family) [6]. CD22 has been proposed to mediate its physiologic role in B-cell function in vivo through ligand-dependent and ligand-independent mechanisms [7]. Engagement of CD22 through ligand binding, crosslinking, or B-cell antigen receptor (BCR) co-activation results in phosphorylation of its cytoplasmic domain. This phosphorylation ultimately leads to modulation of BCR signaling resulting in inhibition of B-cell survival/proliferation [8–11] and pro-apoptotic effects in neoplastic B-cells [12].

HB22.7 is a ligand-blocking antibody that prevents interaction of CD22 with its sialic-acid-containing ligand(s). CD22-ligand binding mediates B cell survival and this explains why the CD22 ligand blocking mAb, HB22.7, has in vivo lymphomacidal activity. Moreover, stable surface expression of CD22 is dependent on ligand binding [7]; therefore, ligand-blocking antibodies cause rapid internalization of the CD22-antibody complex, making them ideal as naked antibodies-based therapeutics or as vehicles for CD22-targeted drug carriers. Non-blocking anti-CD22 mAbs, on the other hand, have demonstrated very limited pre-clinical efficacy [13].

As single agents, mAbs are effective, but tumoricidal activity is enhanced when used in conjunction with chemotherapeutic drugs. A substantial amount of research now indicates a role for...
deregulated epigenetic mechanisms in cancer, both in the development of tumors and their acquisition of chemoresistance [14–17]. As a result, chromatin-modifying enzymes such as histone methyltransferases and demethylases [14], histone acetyltransferases (HAT), and histone deacetylases (HDAC) [15–17] are promising therapeutic targets. Histone deacetylases catalyze the removal of negatively charged acetyl groups from lysine residues in the core histones, which results in a more compact and transcriptionally repressed chromatin structure. HDAC inhibition results in hyperacetylated, transcriptionally active chromatin. It is generally accepted that the anti-cancer activity of HDAC inhibitors (HDACi) is based on enhanced exposure of DNA to damaging agents, increased expression of tumor suppressor genes, increased expression of genes involved in DNA-damage recognition, and triggering of apoptotic cascades. AR42 is a phenylbutyrate-derived pan-HDAC that acts on Class 1 and Class 2 HDACs and exerts cytotoxic effects in tumor cells via multiple pathways [18,19]. It has activity in vestibular schwannomas [18,20,21], neoplastic B-cells, ovarian cancer [22,23], prostate adenocarcinoma [24] and T-cell lymphoma [25]. In combination with immunotherapeutics, HDACis and rituximab (α-CD20 mAb) have synergistic cytotoxic activity on NHL cells [26–28].

Here, we report the increased treatment efficacy with the combination of HDACi (AR42) and the HB22.7 anti-CD22 mAb. This report is the first to examine this combination, and highlights its potential clinical significance for improving B-cell NHL patient outcomes.

2. Materials and methods

2.1. Reagents

AR42 (OSU-HDAC42, 5-N-hydroxy-4-(3-methyl-2-phenylbutanamido) benzamide) was from Selleckchem. Anti-CD22 mAb HB22.7 was prepared as described previously [6]. All other chemicals were from Sigma-Aldrich.

2.2. Cell culture

The Burkitt’s B-cell lymphoma cell lines CA46, Raji, Ramos, Daudi, DG75, and NAMALWA were purchased from the American Type Culture Collection (ATCC; Manassas, VA). Cells were cultured in ATCC-formulated RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 100U/ml penicillin G and 100 µg/ml streptomycin at 37 °C using a humidified 5% CO2 incubator.

2.3. In vitro cytotoxicity

Cells (5 x 10⁶ per sample, 100 µl) were plated in triplicate in 96-well plates. Cells were treated with HB22.7 (1.2 µg/ml) and/or AR42 at indicated concentrations; untreated control cells received media only. After a 12, 24, 48, or 72-h incubation, cell viability was assessed using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega) according to the manufacturer’s instructions. Cell viability was calculated as a percent of the untreated control; the mean ± standard deviation (SD) of 3 independent experiments (each in triplicate) is reported.

2.4. Flow cytometry

For CD22 surface staining, cells were incubated with HB22.7 antibody (25 µg in 100 µl PBS/0.05% BSA) for 1 h at 4 °C, washed 3 times in the same buffer and incubated with PE-Cy5 secondary antibody at 1:100 for 30 minutes and washed as above. Data (10,000 events/sample) was acquired using a FACScan instrument (Becton Dickinson) and analyzed using Flow software (Treestar, Inc.).

Internalization of CD22 was directly assessed by measuring CD22 levels in Raji cells treated with AR42 and/or HB22.7 at 37 °C (where internalization occurs) and 4 °C (where internalization does not occur or is minimal). Internalized CD22 was calculated as the residual signal after incubation at the permissive temperature (37 °C) relative to the “total” signal at the non-permissive temperature (4 °C).

2.5. Clonogenic survival assay

Raji cells (1 x 10⁶/well) were plated in 0.5 ml of 0.3% agarose in phenol red-free RPMI 1640 supplemented with 10% FBS, on a 0.6% agarose base (in the same medium) in 24-well plates. Cells were treated with HB22.7 (0.4 µg/ml) and/or AR42 (0.25 µM) for one week. Negative controls were incubated with media only. Colonies were fixed (10:1 methanol:acetic acid), stained with 1% crystal violet in methanol, and counted. Colonies > 5 mm were counted and the fraction of surviving cells was calculated.

2.6. In vivo studies

Female athymic nude mice, 6–8 week old, were obtained from Harlan (Indianapolis, IN) and maintained in micro-isolation cages under pathogen-free conditions in the UC Davis animal facility. Mice were allowed to acclimatize for at least 4 days prior to an experiment. Mice were housed in a temperature-controlled room (20–22 °C) with a 12-h light–dark cycle and relative humidity of 45–55%. Mice were monitored for signs of pain and discomfort throughout the study; all procedures were conducted under approved protocols according to federal and institutional guidelines. Three days before tumor cell implantation mice received 400 rad/s of whole body radiation. To establish tumors, 5 x 10⁶ Raji cells were resuspended in PBS and subcutaneously implanted on to the flank of each mouse. Treatments were performed by intraperitoneal (i.p.) injections of HB22.7 (1.4 mg/mouse/dose), AR42 (10 mg/kg) or both, administered weekly for 6 weeks. Tumor size was measured 3 times/week and volume was calculated as d1 x d2 x d3 x 0.52. Weight was recorded 3 times/week. Blood was collected by tail nick once a week during treatment. At the end of the study, mice were euthanized and tumors were dissected and weighed.

2.7. Statistical analysis

In vitro cytotoxicity data was analyzed by a two-tailed, unpaired Student’s t-test. To obtain IC50 values, the dose–response data was fitted to a dose–response (inhibition) curve. Results for soft agar colony formation assays and resected tumor weight were analyzed by t-test. All statistical analysis, curve fitting and IC50 calculations were performed using GraphPad Prism software (San Diego, CA). A p value of <0.05 was considered significant. For pharmacological interactions, the effect of the drugs alone or in combination were analyzed for additive or synergistic effect using the Compusyn software [29].

3. Results

3.1. Cytotoxic effects of AR42 and HB22.7 on NHL cell lines

To determine the cytotoxicity of AR42 and HB22.7 alone or in combination, Raji cells were treated with the indicated concentrations of either drug or both for up to 72 h. As expected, substantial cell death was observed in a concentration-dependent manner for each drug individually. Treatment of Raji cells with a combination of AR42 and HB22.7 resulted in increased cell death, Fig. 1. After 24 h, the combination showed statistically significant greater cytotoxicity when compared to either drug alone, and this continued over a 72-h time course. To quantitatively analyze the cytotoxic potential of AR42 in combination with HB22.7, we determined IC50s by measuring cytotoxicity of AR42, HB22.7 or the combination over a range of concentrations, and extended the analysis to six NHL cell lines: CA46, Daudi, DG75, NAMALWA, Raji and Ramos

<table>
<thead>
<tr>
<th>NHL cells</th>
<th>IC50 (95% CI)</th>
<th>AR42 (µM)</th>
<th>HB22.7 (µg/ml)</th>
<th>AR42 (µM) + HB22.7</th>
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<tr>
<td>CA46</td>
<td>0.67 (0.26–1.70)</td>
<td>9.11 (3.50–23.7)</td>
<td>0.03 (0.01–0.10)</td>
<td></td>
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<tr>
<td>Daudi</td>
<td>0.72 (0.29–1.70)</td>
<td>7.96 (3.60–17.6)</td>
<td>0.06 (0.01–0.47)</td>
<td></td>
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<tr>
<td>DG75</td>
<td>0.59 (0.27–1.20)</td>
<td>10.31 (8.21–58.6)</td>
<td>0.03 (0.02–0.05)</td>
<td></td>
</tr>
<tr>
<td>NAMALWA</td>
<td>0.41 (0.20–0.80)</td>
<td>10.9 (5.22–21.6)</td>
<td>0.03 (0.01–0.09)</td>
<td></td>
</tr>
<tr>
<td>Raji</td>
<td>0.45 (0.08–2.51)</td>
<td>5.57 (3.14–18.2)</td>
<td>0.03 (0.01–0.10)</td>
<td></td>
</tr>
<tr>
<td>Ramos</td>
<td>0.49 (0.25–0.90)</td>
<td>8.00 (3.36–19.0)</td>
<td>0.03 (0.01–0.12)</td>
<td></td>
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</table>
Fig. 1. Combination of AR42 with HB22.7 results in greater cytotoxicity in NHL cells in vitro. (A) Time course of proliferation of Raji cells treated with AR42 (1.0 μM), HB22.7 (1.2 μg/ml) or both. The combined treatment resulted in a greater and earlier decrease in cell counts when compared to either drug alone or the untreated control. (B) Dose-inhibition curves for AR42, HB22.7 and AR42 plus HB22.7 (1.2 μg/ml) in six NHL cell lines. Addition of HB22.7 increased the cytotoxic efficacy of AR42 10-fold. IC50s are presented in Table 1.

All cell lines showed similar degrees of cytotoxicity to both agents. IC50s for AR42 ranged from 0.41 μM (NAMALWA) to 0.72 μM (Daudi), while IC50s for HB22.7 were between 5.57 μg/ml (Raji) and 10.0 μg/ml (NAMALWA). IC50s for each drug in each cell line are presented in Table 1. In all cases, the combination of the drugs was more cytotoxic than either drug alone. Indeed, IC50s for AR42 when combined with HB22.7 were in the range of 0.03 μM to 0.06 μM, indicating a 5 to 10 fold increase in potency (Table 1).

Fig. 2. AR42 inhibits HB22.7-mediated internalization of CD22 in Raji cells. (A) Raji cells were pretreated with AR42 (1 μM) and/or HB22.7 (1.2 μg/ml) for 12, 24, 48 and 72 h; surface CD22 was assessed as mean fluorescence intensity (MFI) by flow cytometry. (B) At 24-h incubation, AR42-mediated increase of CD22 was highest at 0.5 μM; * p < 0.05. (C) Treatment with 0.5 μM AR42 for 24 h significantly inhibited CD22 internalization induced by up to 4.8 μg/ml HB22.7. Internalization of CD22 was assessed by measuring residual surface-bound HB22.7 following incubation at either 37 °C or 4 °C. * p < 0.01.
This increase was achieved with a dose of HB22.7 (1.2 μg/ml) that is well below its IC₅₀ for any of the cell lines in this study. The combined effect of AR42 and HB22.7 was synergistic, as determined by isobologram analysis and combination index (CI). CI values <1, indicative of synergism [30], were obtained in all 6 cell lines (data not shown).

3.2. Effect of HB22.7 and AR42 on CD22 expression

Previous work showed that HB22.7 initially increased CD22 expression, followed by the internalization of CD22 [31,32]. Since HDAC is capable of regulating expression of various cell surface receptors [27,33], we tested the effect of AR42, alone or in combination with HB22.7, on CD22 expression. Surface CD22 was measured in Raji cells by flow cytometry. As reported before, treatment with HB22.7 resulted in a transient increase of CD22 (12 h), followed by a decrease in surface staining (due to internalization); surface CD22 levels returned to baseline levels at 24 h (Fig. 2A). As expected surface CD22 continued to decrease after 48 and 72 h of treatment with HB22.7 due to mAb-mediated internalization [31]. Interestingly, AR42 also initially increased CD22 cell surface expression; this effect persisted for up to 24 h of treatment. CD22 surface levels then decreased to baseline at 48 h, and a slight further decrease was observed after 72 h when compared to HB22.7 alone. Treatment of Raji cells with the combination of both drugs resulted in an intermediate effect: surface levels decreased more slowly and reached a higher plateau after 72 h. These results suggest that AR42 antagonizes HB22.7-mediated internalization of CD22. To confirm this observation, we evaluated the effect of different concentrations of AR42 and HB22.7 on CD22 surface expression. After a 24-h incubation, AR42 (0.5 μM) significantly increased CD22 levels (Fig. 2B); however, increasing the concentration abrogated this effect: at 3 μM AR42, surface CD22 levels were not significantly different from those of untreated controls. Treatment with HB22.7 resulted in twice as much internalization when compared to AR42 (Fig. 2C). The presence of AR42 inhibited CD22 internalization induced by HB22.7 (Fig. 2C), and this effect was HB22.7 concentration-dependent and completely reversed at the highest dose (19.2 μg/ml).

3.3. AR42 and HB22.7 inhibit colony formation in soft agar

To further validate the anti-cancer activity of AR42 and HB22.7, we performed in vitro soft agar anchorage-independent growth assays [34]. Treatment with AR42 effectively inhibited anchorage-independent growth as assessed by the reduced number of colonies when compared to untreated controls, Fig. 3. Treatment with HB22.7 resulted in a slightly reduced number of colonies, which was not significantly different than controls. However, the combination treatment of HB22.7 plus AR42 resulted in more inhibition when compared to AR42 alone (3.3 ± 0.3 versus 7.7 ± 0.3, respectively, p = 0.0008), indicating that HB22.7 potentiated the activity of AR42.

3.4. In vivo lymphomacidal activity of AR42 and HB22.7

In order to validate the translational potential of the previous in vitro experiments, nude mice bearing Raji xenografts were used to assess the pre-clinical efficacy the combination of HB22.7 and AR42 (Fig. 4). Two weeks after cell implantation, when tumors reached 340–380 mm³, mice were treated weekly using intraperitoneal injections of HB22.7 (1.4 mg/mouse), AR42 (10 mg/kg), or both, for 6 weeks. When compared to control mice (PBS), HB22.7 dramatically blocked tumor progression when used alone, showing independent lymphomacidal activity as described previously [13] (Fig. 4A). In agreement with its reported anti-tumor activity in other cancer models, AR42 also significantly inhibited progression of Raji lymphoma cell xenografts. The combination of HB22.7 and AR42 was even more effective than either drug alone. Mice treated with HB22.7 and AR42 showed tumor regression after two weeks (Fig. 4A) and the response persisted for the duration of the study. After 45 days of treatment, mice were euthanized and tumors dissected and weighed (Fig. 4B). The average weight of tumors from untreated control mice were 1.55 ± 1.48 g, while tumors for mice treated with HB22.7 or AR42 were 0.59 ± 0.47 g and 0.47 ± 0.31 g, respectively, which is a 60% reduction in volume when compared to
controls. The average weight of the tumors from mice treated with the combination of AR42 and HB22.7 was 0.08 ± 0.13 g, a reduction of 95%, on average, over control animals and 87% or 83% when compared to mice treated with HB22.7- or AR42 alone, respectively. To determine if this drug combination was also safe, body weight was monitored throughout treatment and blood was collected for cell counts and chemistries. There was some weight loss in the AR42 group during the second week of treatment, but this was not observed in the HB22.7 alone or the combination treatment group. No treatment group showed a significant difference in weight when compared to controls (Fig. 5A). Blood cell counts were normal for all groups (Fig. 5B–E). AST and ALT levels were normal without evidence of hepatotoxicity (Fig. 5F and G), and normal BUN and creatinine indicated preserved renal function (Fig. 5H and I).

4. Discussion

This is, to our knowledge, the first study to look at a combination treatment for NHL using an HDACi (AR42) and an anti-CD22 mAb (HB22.7). Our results demonstrate that the in vitro cytotoxicity and in vivo efficacy of the combination of AR42 and HB22.7 is greater than either one alone.

Combination treatments are a common approach in oncology in order to improve efficacy by combining agents that use different mechanisms of action but have non-overlapping toxicity. New combinations are sought to further increase the efficacy of therapy for NHL without increasing toxicity. HB22.7 has demonstrated tumoricidal activity in pre-clinical models of human NHL [13,35–40], and is an ideal for targeted therapy, as CD22 is expressed on the surface of most B-cell NHL and is rapidly internalized upon binding. On the other hand, AR42 has been shown to be effective against B-cell malignancies through induction of caspase-dependent apoptosis [22,41]. Moreover the efficacy of HB22.7 may be dampened due to its internalizing effects. HDACi have also been shown to up regulate receptor surface expression. Based on this we hypothesized the AR42 not only uses a distinct and different mechanism than HB22.7 but also has the potential to decrease CD22 internalization and maintain target density. Thus, the combination of HB22.7 and AR42 warranted further investigation as it may represent a valid therapeutic approach to increase efficacy and reduce toxicity in NHL.

The results presented herein indicate that the combination of AR42 and HB22.7 has higher cytotoxic activity in Raji cells than either agent alone. This is further supported by our results showing similar effects in other B cell-NHL cell lines that represent several different NHL subtypes. All six NHL cell lines assessed had similar sensitivity to AR42 and HB22.7, and the drug combination had the most potent effect in all cases. The IC50s presented in Table 1 closely match IC50s of earlier studies examining AR42 and HB22.7 alone [22]. Combining both reduced IC50 5–10 fold, strongly supporting our hypothesis that when these agents are more effective when combined.

Several epigenetic studies have shown that HDACi effectively modulate the expression of surface molecules such as gp100 [42] and NKG2D [43,44]. Of particular interest to this study, Shimizu et al. [27] demonstrated that valproic acid enhanced CD22 surface expression on Burkitt’s lymphoma cell lines and enhanced rituximab’s cytotoxicity. This effect becomes more relevant when considering internalizing receptors such as CD22 due to the potential for loss of target. Our data demonstrates that AR42 also increases surface expression of CD22 as early as 12 h after treatment. However, both prolonged exposure (72 h) and higher
doses of AR42 (1–3 μM) did not result in additional increases in CD22 surface levels. Notably, treatment with AR42 inhibited HB22.7-mediated CD22 internalization (Fig. 3C). This encumbering effect was overcome by increased concentrations of HB22.7. On the one hand, reducing CD22 internalization may mean reduced efficacy if the antibody is to be used as a therapeutic drug carrier, and hence inhibition of CD22 internalization by AR42 may be detrimental. On the other hand, maintaining CD22 on the surface may mean increased availability for HB22.7 binding, hence enhancing its inherent cytotoxic properties based on CD22 engagement/crosslinking, onset of pro-apoptotic signals, and recruitment of host immune effector mechanisms [10,12,13,31,32]. Nonetheless, the results presented herein support our hypothesis that the combination of HB22.7 and AR42 improved activity via a number of different mechanisms.

Neoplastic cells can proliferate independently of external signals. Transformed cells acquire anchorage-independent growth capabilities, which can be evaluated in vitro by the ability to form colonies in soft agar, a well-established and stringent assay for oncogenic potential [34]. In this study, we examined AR42 and HB22.7’s efficacy in inhibiting Raji cells anchorage-independent proliferation. Consistent with its anti-tumor activity, AR42 effectively inhibited colony formation when used alone. HB22.7 did not show statistically significant inhibitory activity in this assay when used alone. However, when used in combination with AR42, HB22.7 doubled AR42’s inhibitory effect, suggesting a synergistic combination.

There is a direct correlation between in vitro clonogenic assays and assessing efficacy in pre-clinical models [45] and clinical responsiveness [46], however the current results show that the AR42 and HB22.7 combination could be an effective NHL therapy and justified further evaluation of the combination in a in vivo human NHL xenograft model. Using a Raji xenograft model we demonstrated that the combination of AR42 and HB22.7 had improved pre-clinical efficacy when compared to either agent alone. This combination demonstrated no significant toxicity assessed by mouse body weights, hematologic parameters, kidney or liver function. Previous studies with AR42 showed a marked increase in survival in murine models of several human cancers [21,22,47,48]. Pertinent to this study, AR42 increased median survival with negligible toxicity in mice bearing Raji NHL xenografts, and was more potent than the maximum tolerated dose of another HDACi, suberoylanilide hydroxamic acid (SAHA or vorinostat) [22]. It has also been hypothesized that HB22.7 has independent therapeutic potential for NHL due to its specific binding of distinct epitopes on CD22 [13,31,32,35,38,49,50].

Based on our findings, we hypothesize that AR42 enhances HB22.7’s efficacy by increasing surface levels of CD22 and utilizing a distinct cytotoxic mechanism.

Conflict of interest statement

The authors report no conflict of interest.

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References


