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Anti-CD19 and Anti-CD22 Monoclonal Antibodies Increase the Effectiveness of Chemotherapy in Pre-B Acute Lymphoblastic Leukemia Cell Lines

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Abstract

The monoclonal antibodies (MAbs) HD37 and RFB4 bind to receptors on precursor B acute lymphoblastic leukemia (ALL) cells. These MAbs were tested alone and in combination with chemotherapy for their anti-leukemic activity. HD37 and not RFB4 increased the *in vitro* cytotoxicity of daunorubicin (DNR) and vincristine (VCR) in three Pre-B ALL cell lines. HD37 alone induced apoptosis in 30% of the cells vs. 2% for RFB4. The treatment of SCID/ALL mice with either chemotherapy agent minimally prolonged their mean survival time (MST) vs. controls but HD37 or RFB4 plus VCR significantly extended the MST. 40% of the mice treated with HD37 plus VCR survived. In conclusion, chemotherapy was made more effective when combined with HD37, and less so with RFB4.

Keywords

Monoclonal antibodies; HD37; RFB4; anti-CD19; anti-CD22; Pre-B ALL; Immunotherapy; Chemotherapy; Childhood Leukemia; Vincristine; Daunorubicin

Introduction

The CD19 cell surface antigen is a B-lineage specific receptor that is expressed on the surface of leukemia cells in >90% of children (1) and adults (2) with acute lymphoblastic leukemia (ALL). It is also expressed on tumor cells of patients with B-cell Non-Hodgkin's lymphoma (NHL) (3), and chronic lymphocytic leukemia (CLL) (4). Flow cytometric analysis of marrow specimens obtained from patients with B-lineage leukemia has demonstrated that there are > 50,000 molecules per cell (5). CD19 is a 95 kDA transmembrane protein consisting of two

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extracellular immunoglobulin (Ig)-like domains and an extensive cytoplasmic tail containing numerous tyrosine residues (6,7). The cytoplasmic tail is physically associated with a family of protein tyrosine kinases, namely Lyn, Lck, Fyn, and Blk that couple CD19 to downstream signaling pathways (8,9). This receptor is not shed from the cell surface and undergoes antibody-induced internalization (10). Certain chemotherapy agents have been shown to affect the modulation of the CD19 antigen induced by anti-CD19 Mabs in lymphoma (11). The anti-CD19 MAb, HD37 can directly inhibit the growth of lymphoma cells by inducing cell cycle arrest (12) and by synergising with an immunotoxin (IT) (13).

The CD22 cell surface antigen is also restricted to B-lineage cells (14). It is prevalent on B-lineage lymphoma cells, and is present on >90% of B-precursor ALL blasts (15). The RFB4 anti-CD22 MAb has been coupled to deglycosylated ricin-A chain (dgRTA) and the resulting ITs have been used to treat adult patients with CD22⁺ lymphomas (16,17,18). More recently, chimeric anti-CD22 Mabs using RFB4 have been developed by the same research team (19).

HD37 and RFB4 have little or no inherent cytotoxic activity on lymphoma cell lines unless they are crosslinked (20) or used as homodimers (21). They have not been tested for their anti-tumor activity as individual agents or in combination with chemotherapy in the setting of leukemia. The purpose of this study was to determine the *in vitro* and *in vivo* anti-tumor effects of the HD37 and RFB4 alone and in combination with chemotherapeutic agents used in the treatment of childhood ALL.

Materials and Methods

MAbs

HD37 is a murine IgG₁ MAb directed against the CD19 molecule and was obtained from hybridoma cells originating from Dr. B. Dorken's laboratory (Heidelberg, Germany), and purified in the Cancer Immunobiology Center, Dallas, TX. RFB4 is a murine IgG₁ MAb that was developed, prepared, and purified in the Cancer Immunobiology Center, Dallas, TX. The anti-CD25 MAb RFT5 is an IgG₁ isotype-matched control MAb obtained from Dr. Peter Amlot in the Department of Immunology, Royal Free and University College Medical School, London, UK.

Chemotherapy

The vinca alkaloid vincristine, (VCR) was purchased from the Faulding Pharmaceutical Co. (Paramus, NJ). The anthracycline daunorubicin, (DNR) was purchased from BenVenue Labs (Bedford, OH). These agents were used for the *in vitro* and *in vivo* experiments.

Leukemia cell lines

The human Pre-B ALL cell lines Nalm-6-UM1, REH, and JM-1 were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and maintained by serial passages in RPMI-1640 medium (GIBCO Laboratories, Grand Island, NY, USA) containing 10% heat-inactivated fetal bovine serum (FBS), 25mM Hepes buffer, 100 µg/ml streptomycin, 100 units/ml penicillin, and 100 µM glutamine. The cells were grown in a humidified atmosphere of 5% CO₂ and air and expanded every other day. The cell viability was determined by trypan blue exclusion to ensure greater than 90% viability prior to use. These three cell lines were used for the *in vitro* combination Mab plus chemotherapy assays and the Nalm-6-UM1 cell line was used for the *in vivo* experiments.

Cytotoxicity assays

Assays were performed in 96 well microtiter plates. Cells were incubated at 10⁵ cells/well with varying concentrations of the HD37 MAb, RFB4 MAb, DNR, VCR, or a combination of the

two MAbs and the two chemotherapy agents for 48 h at 37°C in complete medium. During the final 4 h of incubation, the cells were pulsed with 1.0 µCi of [³H]-thymidine (MP Biomedicals, Inc., Costa Mesa, CA). The radiolabeled cells were harvested using a PHD Cell Harvester (Cambridge Technology, Inc., Cambridge, MA) and the radioactivity on the filters was determined using the Packard Tri-Carb 1600TR Scintillation Analyzer (Packard Instrument Co., Meriden, CT). The % reduction in [³H]-thymidine incorporation, as compared to untreated controls, was used to assess killing. For the combination experiments, a fixed concentration of either the MAb (IC₅₀ or the concentration determined to inhibit [³H]-thymidine incorporation in 50% of the cells for HD37 or an equivalent concentration for RFB4) was combined with three different concentrations of either DNR or VCR. For these experiments 50 µl of each of the agents were added to 100 µl of the cells. Cells were incubated, pulsed, harvested, and counted in the same manner as for the individual agents.

Apoptosis Assays

Cells were cultured at 10⁵ cells/well with varying concentrations of either HD37, RFB4, DNR, or VCR at 37° C for 48 h in a humidified atmosphere containing 5% CO₂. At 24 h intervals, treated and untreated cells were harvested, washed, labeled with fluorescein isothiocyanate (FITC)-Annexin-V and counter-stained with propidium iodide (PI), according to the manufacturer's instructions (Biosource International, Inc., Camarillo, CA). 10⁴ cells were analyzed using a FACSCalibur flow cytometer (BD Immunocytometry Systems, San Jose, CA). The results were represented as dot plots of FITC-Annexin V⁺ cells (FL-1) versus PI⁺ cells (FL-2). Annexin V⁺, PI⁻ cells were considered to be in early apoptosis. The final results were expressed as the percentages of early apoptotic cells in each of the cell suspensions. For the combination experiments, a fixed concentration of HD37 (IC₅₀) or RFB4 (equivalent IC₅₀) was combined with three different concentrations of either DNR or VCR. For these experiments 50 µl of each of the agents were combined with 100 µl of the cells. Cells were incubated, harvested, washed, labeled, and analyzed by flow cytometry.

SCID/NALM-6-UM1 mice

Eight to ten week old male SCID mice (CB-17 SCID/SCID) weighing 25–30 g., and originating from Dr. Mel Bosma's colony at Fox Chase Cancer Center (Philadelphia, PA) were obtained from Taconic Laboratories (Germantown, NY). They were housed and maintained in a specific pathogen-free (SPF) facility. They were provided with autoclaved food and sterile water. All manipulations were performed in a laminar flow hood. To ensure consistent engraftment, the mice received 150 cGy of total body irradiation from a cesium source (JL Shepard Model 143 Irradiator) 24 h prior to receiving the leukemia cells. The mice were inoculated in the tail vein with 5 × 10⁶ Nalm-6-UM1 cells, and monitored daily for weakness or paralysis of their front or back legs, symptoms that preceded death. The mice were then euthanized using an intraperitoneal (i.p.) injection of pentobarbital.

Therapy of mice

The dose of HD37 administered was modeled after the 375 mg/m² dose of Rituxan, (an anti-CD20 MAb) currently approved for the treatment of various B-lineage malignancies. The dose for a 20 g mouse with a body surface area of 0.007 m² would be 2.6 mg. We chose to give 50% of this dose of either HD37 or RFB4. The maximum tolerated dose (MTD) of VCR and DNR was determined by administering each agent at increasing dosages to pairs of normal SCID mice (data not shown). The MTDs (as defined by 25% weight loss or death) for DNR and VCR were 1 µg/g and 3 µg/g, respectively. The mice were divided into the following treatment groups: 1) saline, 2) DNR, 3) VCR, 4) HD37 or RFB4, 5) HD37 or RFB4 plus DNR, 6) HD37 or RFB4 plus VCR, and 7) RFT5. The groups receiving a single agent were injected i.p. with either MAb or 50% of the MTD of the chemotherapeutic drug divided into four equal doses on d 1, 2, 3

and 4 after tumor cell inoculation. Those groups receiving two agents received both agents simultaneously at the same dosages and time intervals. The control groups received either RFT5 or saline. The mice were observed daily for the onset of paresis or paralysis.

Results

Effect of combination therapy on DNA synthesis

The cytotoxicities of HD37, RFB4, VCR and DNR on the leukemia cell lines were determined by incubating the cells with increasing concentrations of the agents separately and in combination. The percentage of [³H]-thymidine incorporation relative to control was determined for each of the agents. All of the agents, with the exception of RFB4 were effective in killing the cells. The most effective agent was VCR with a mean IC₅₀ value of 1.0–2.0 × 10^{−9} mol/L, followed by DNR, and then HD37. This was true for all three cell lines tested (data not shown). RFB4 did not have any inhibitory activity and was similar to RFT5 and saline. The IC₁₀, IC₅₀ and IC₉₀ values for each of the agents on the Nalm-6-UM1 cell line are shown in Table 1. In the combination therapy experiments, the IC₁₀, IC₅₀ and IC₉₀ dose of each of the chemotherapy agents was combined with the IC₅₀ dose of HD37 or an equivalent dose of RFB4. The addition of HD37 to either chemotherapeutic agent resulted in a profound increase in cytotoxic activity at the lower dose levels for all three cell lines (Figs 1A, B, C, D, E, and F). The combination of RFB4 and chemotherapy was no more effective than chemotherapy alone (data not shown).

Effect of combination therapy on apoptosis

HD37 can induce apoptosis in leukemia cells. The extent of apoptosis is dose and time dependent. At 24 h there was no significant difference in the percentage of apoptotic cells following culture with medium vs. HD37. At 48 h the lowest and highest concentration of HD37 induced apoptosis in 2.7% and almost 30% of the Nalm-6-UM1 cells, respectively. The percentage of apoptosis in the REH and JM-1 cells at the same time interval were very similar and ranged from 2.7–32.2 (data not shown). In contrast, RFB4 induced apoptosis in fewer than 4% of the cells even at the highest concentration. HD37 also increased the percentage of necrotic cells. The percentage of necrotic cells ranged from 4–29 % at 48 h. DNR induced apoptosis in up to 24% of the cells and reached maximal levels at 48 h. VCR induced apoptosis in up to 18% of cells at 24 h. The results for the Nalm-6-UM1 cell line are depicted in Table 2. When HD37 (IC₅₀) was combined with varying concentrations of VCR, (IC₁₀, IC₅₀, and IC₉₀) there was a significant increase in apoptotic cells compared to the individual agents. The greatest effect was observed at the lowest concentration of VCR at the 48 h interval (p=.00005) (Fig 2A). Similarly, the combination of HD37 and DNR resulted in a significant increase in % apoptotic cells compared to the individual agents (p=.00032) (Fig 2B). Similar results were seen in the REH and JM-1 cell line (Fig 2C, D, E, and F). In contrast, the combination of RFB4 with either DNR or VCR did not result in an increase in % apoptotic cells in any of the cell lines tested (data not shown).

Effect of combination therapy on survival of mice

The *in vivo* efficacy of HD37 and RFB4 in combination with VCR and DNR was evaluated in our previously established model of early disseminated disease (23). The mice were sacrificed when they developed either paresis or paralysis. Kaplan-Meier plots were constructed from the data. Mice receiving either saline or the irrelevant MAAb RFT5 had no statistical difference in MSTs of 36.6 and 35.7 d, respectively (p=.41). Treatment with HD37 alone resulted in a significant (p=.00002) increase in the MST to 48 d. Mice treated with RFB4 had a MST of 39.2 d (p=.26). Treatment with DNR resulted in a comparable MST of 39.6 d (p=.17). In contrast, VCR prolonged survival to 51.8 d (p<.00001). Despite the improvement, there were no long term survivors in any of the single agent treatment groups. Mice receiving HD37 plus

DNR had a significant survival advantage over DNR alone at 54.5 d ($p=.00016$); but was not significantly different from HD37 alone ($p=.15$). More importantly, the addition of HD37 to VCR resulted in a profound increase in MST (100.3 d) as compared to VCR alone (51.8 d) ($p<.00001$) or HD37 (48 d) ($p<.0001$). Forty percent of the mice receiving this combination were long-term survivors (survival up to 120 d at which point the experiments were terminated). The addition of RFB4 to VCR resulted in a minor, but statistically significant ($p=.00001$) improvement in MST (57.2d) as compared to mice that received VCR as a single agent. Mice that received a combination of RFB4 and DNR had a MST of 45.4 d. This was not statistically different from the mice that received DNR alone ($p=.54$), and there were no long term survivors (Figs 3A, 3B).

Discussion

CD19 and CD 22 are lineage specific receptors highly expressed in B cell leukemia and lymphoma (23). They have been useful markers for identifying pre-B ALL, B-ALL, hairy cell leukemia, and B-cell chronic lymphocytic leukemia, and have been studied as immunoconjugates in limited Phase I and II clinical trials for leukemia (24). A number of chimaeric or humanized “naked” MAbs including RituximabTM (26,27), ErbituxTM (28), HerceptinTM (29), anti-insulin-like growth factor-1 receptor (30), anti-FAS (31), and anti-CD33 (32) have been shown to enhance or sensitize malignant cells to chemotherapy. The predominant theory is that the combination of agents that inhibit different cellular pathways results in more effective destruction of tumor cells. The intracellular signaling pathways used by MAbs to enhance the cytotoxic effect of chemotherapy are now being explored. Potential mechanisms on how a MAb may enhance the effect of chemotherapy include, but are not limited to shared intracellular pathways leading to apoptosis, facilitation of drug uptake, or cell cycle arrest.

The major role of CD19 in normal B cells is to promote their survival and proliferation. The signaling cascade created by the ligation of the CD19 cell surface receptor, and its interaction with the (B cell antigen receptor) BCR complex in normal B cells is well documented (33), but there is very little information available on the cellular effects or the signaling cascade that result from ligation of CD19 in malignant cells (35,36,37), and no information on the intracellular signaling events that take place upon binding of the CD19 antigen on leukemia cells. The binding of HD37 to the CD19 cell surface receptor can inhibit the function of the P-glycoprotein pump in a multi-drug resistant B-lymphoma cell line (38), and can inhibit the growth of various lymphoma cell lines by inducing cell cycle arrest (39). Crosslinking of HD37 has been shown to increase the level of tyrosine phosphorylation (40) and inhibit anti-Ig-induced B cell activation (41). Our *in vitro* data demonstrate that HD37 can inhibit the growth of Pre-B ALL cell lines in the absence of effector cells or complement. We show that by targeting CD19, that these cells can be killed as demonstrated by inhibition of [³H]-thymidine incorporation and that death is partially due to the induction of apoptosis as measured by the Annexin V assays. The increase in apoptosis noted at the higher concentrations may be due to enhanced negative intracellular signaling through dimerization of the CD19 antigen.

CD22, like CD19, is a coreceptor to the BCR complex and is thought to regulate BCR-mediated signal transduction pathways (42). Studies performed using CD22 (−/−) mice have demonstrated that CD22 is a negative regulator of BCR signaling (43). The effects of binding CD22 in malignant cells that lack the BCR complex is unknown. RFB4 has been shown to have no effect on the Daudi lymphoma cell line (44) and our results also demonstrate that RFB4 had no activity *in vitro* in the leukemia cells and therefore appears to lack the negative intracellular signaling potential of HD37. The lack of activity of RFB4 on the NALM-6-UM1 cell line may be explained in part on the fewer number of CD22 molecules/cell present (approximately 40,000 vs 10,000), but we have determined that the number of molecules of

CD22 on the REH and JM-1 cells are similar to the number of CD19 molecules (data not shown).

Our *in vivo* data demonstrate that although HD37 as a single agent provides only a minimal survival advantage for mice with leukemia, its addition to VCR, and not DNR results in a marked survival advantage. RFB4 as a single agent provided no survival advantage over mice receiving no treatment, but when combined with VCR, there was an improvement in survival suggesting that it could chemosensitize cells via an unknown pathway. The *in vivo* finding that the addition of HD37 to VCR was the most effective in prolonging and curing mice may be due to shared negative signaling pathways leading to cell death. The finding that both HD37 and RFB4 when combined with VCR improved survival may be due to the stabilization of both antigens on the cell surface by VCR resulting in more effective recruitment of effector cells or complement to the cell surface. Our results show that the ability of MAbs to enhance chemotherapy is specific for both the Mab and the chemotherapy agent. In conclusion, our findings demonstrate that HD37 markedly enhances the effect of VCR. This would suggest that the dose of VCR needed to effect tumor cell kill could be reduced thereby decreasing the side effects that are all too common for this chemotherapeutic agent. The potential clinical utility of HD37 and RFB4 for the treatment of leukemia in combination with chemotherapy will be further explored.

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Figure 1. Decrease in [³H]-thymidine incorporation using a combination of HD37 or RFB4 with VCR or DNR in three Pre-B ALL cell lines
Cells (10⁵) were incubated for 48 hr at 37°C with three different concentrations (IC₁₀, IC₅₀, and IC₉₀) of either VCR (left column of figures) or DNR (right column of figures) alone (open fig.) or combined with a fixed concentration (IC₅₀) of HD37 (closed fig.). The cells were washed, and then pulsed for 4 hr at 37°C in 5% CO₂ in 5% CO₂ with 1 μCi [³H]-thymidine. The graphs represent the Nalm-6-UM1 (A and B); REH (C and D); and JM-1 (E and F) cell lines. The values are of three separate experiments represented as percentage of control ± standard error of the mean. Significant differences between mean results with and without HD37 are indicated: *, P<.1; **, P<.05; ***, P<.01.

Figure 2. Apoptosis due to Combination of HD37 with VCR or DNR in three Pre-B ALL cell lines Cells (10^5) were incubated for 24 and 48 hr with three different concentrations (IC_{10} , -black bar; IC_{50} , -gray bar; and IC_{90} -white bar) of either VCR (Fig A, C, and E) or DNR (Fig B, D, and F) alone or in combination with a fixed concentration of HD37 (IC_{50}). The percentage of cells that were Annexin V⁺, PI⁻ (apoptotic) were determined for each treatment using flow cytometry. The results are cumulative data of three separate experiments with results represented as % apoptosis over time $\pm$ SEM.

Figure 3. Survival of SCID/human ALL mice treated with HD37 or RFB4 in combination with DNR or VCR

Pre-irradiated SCID mice were injected in the tail vein with 5×10^6 Nalm-6-UM1 cells 24 hr prior to treatment. Mice received 4 daily i.p. injections of HD37 (3A) or RFB4 (3B) alone or in combination with VCR or DNR. In four separate experiments, mice received either saline (●) (n=15), RFT5 (○) (n=20), HD37 (■) (n=10), HD37 + DNR (□) (n=10), HD37 + VCR (◆) (n=10), VCR (△) (n=20), DNR (▼) (n=20), RFB4 (■) (n=10), RFB4 + DNR (□) (n=10), and RFB4 + VCR (◆) (n=10).

Table 1**Decrease in [³H]-thymidine incorporation by HD37 (Anti-CD19 Mab), RFB4 (Anti-CD22 Mab), VCR, and DNR in the Nalm-6-UM1 cell line**

Cells (10^5) were incubated for 48 hr at 37°C with six different concentrations of each agent, washed, and then pulsed for 4 hr at 37°C in 5% CO₂ with 1 μ Ci [³H]-thymidine. The IC₁₀, IC₅₀, and IC₉₀ values are extrapolated from inhibition curves of three separate experiments of each treatment.

TREATMENT (Mab or Drug)	INHIBITORY CONCENTRATION (IC)		
	[IC ₁₀] (M)	[IC ₅₀] (M)	[IC ₉₀] (M)
HD37	2.9×10^{-7}	5.2×10^{-7}	1.0×10^{-6}
RFB4	ND	ND	ND
DNR	9.0×10^{-9}	1.7×10^{-8}	2.4×10^{-8}
VCR	5.8×10^{-10}	1.0×10^{-9}	3.0×10^{-9}

Table 2**Apoptosis due to HD37, RFB4, DNR, and VCR in the Nalm-6-UM1 cell line**

Cells (10^5) were incubated for 24 and 48 hr with three different concentrations (IC_{10} , IC_{50} , and IC_{90}) of each of the four agents. The percentage of cells that were Annexin V⁺, PI⁻ (apoptotic) were determined for each treatment using flow cytometry. The results are cumulative data of three experiments represented in tabular form as the percentage of apoptotic cells $\pm$ standard deviation.

<i>Treatment</i>	<i>Concentration</i>	<i>Time Interval</i>	
<i>MAb or Drug</i>		<i>(24*)</i>	<i>(48*)</i>
HD37	(IC 10)	3.7 $\pm$.7	2.7 $\pm$.1
HD37	(IC 50)	3.4 $\pm$ 1.0	17.3 $\pm$ 2.4
HD37	(IC 90)	4.6 $\pm$.2	29.4 $\pm$ 4.7
RFB4	(IC 10)	3.5 $\pm$.1	2.4 $\pm$.3
RFB4	(IC 50)	3.43 $\pm$.5	1.93 $\pm$.2
RFB4	(IC90)	3.9 $\pm$.7	1.91 $\pm$.1
DNR	(IC10)	2.8 $\pm$.3	4.74 $\pm$ 1.0
DNR	(IC50)	6.57 $\pm$.4	17.2 $\pm$ 2.3
DNR	(IC90)	9.39 $\pm$ 1.0	23.3 $\pm$.2
VCR	(IC10)	2.66 $\pm$.1	3.07 $\pm$.4
VCR	(IC50)	5.21 $\pm$.8	7.66 $\pm$ 1.5
VCR	(IC90)	17.9 $\pm$.6	9.19 $\pm$.5