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Recombinant immunotoxins targeting B-cell maturation antigen are cytotoxic to myeloma cell lines and myeloma cells from patients

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Novel antibody-based therapies for cancer are predictably effective if they can target cancer cells without damaging normal organs.[1] We have developed various recombinant immunotoxins (RITs) against different targets on cancer cell surfaces.[2] RITs are hybrid proteins that consist of antibody variable fragments attached to a truncated portion of *Pseudomonas* Exotoxin A (PE). Several immunotoxins are currently in clinical trials or preclinical development.[2–4] We have reported previously that immunotoxin moxetumomab paseudotox targeting CD22 produces complete remissions in many patients with refractory hairy cell leukemia.[5] This agent has recently completed a phase 3 trial. In addition, a RIT that targets mesothelin showed promising clinical responses in patients with chemotherapy-resistant malignant mesothelioma.[4]

Multiple myeloma (MM) is a B-cell malignancy that originates in the bone marrow (BM). Although there are FDA-approved antibody-based therapies available for the treatment of some B-cell malignancies, no very effective antibody-based therapy is yet available for MM.[6] The B-cell maturation antigen (BCMA) belongs to the tumor necrosis factor receptor (TNFR) superfamily and is highly expressed in all MM cells from patients.[7] Because of the restricted expression of BCMA to plasma cells and its role in growth as well as cell survival of MM, the BCMA antigen has been investigated as the targets in various immunotherapeutic strategies. These include antibody-based therapy,[8] chimeric antigen receptor therapy[9] and therapy with BiTEs.[10]

To develop immunotoxins that target BCMA we have generated a panel of monoclonal antibodies (mAbs) by immunizing mice with recombinant BCMA protein using hybridoma technology. We produced hybridomas producing anti-BCMA mAbs as described (Supplementary Materials and Methods). Because BCMA, TACI and BAFFR and BCMA share the same natural ligands, we tested the reactivity of each anti-BCMA mAb with two structurally closely-related TNFRs (TACI or BAFFR) expressed on transfected 293 T cells by flow cytometry (Supplementary Figure S1A) and TNFR-rFc fusion proteins by enzyme-linked immunosorbent assay (Supplementary Figure S1B). Based on this analysis, we selected BM24 and BM306 because they bind to BCMA antigen on the cell surface with high affinity and specificity. The binding affinity (KD) of both mAbs are $< 1 \times 10^{-10}$ M. We cloned the VH and the VL from BM24- and BM306-expressing hybridomas using IgG1 isotype-specific oligo primers[11] and used the LR version of the PE toxin.[12] A schematic of the Fab-immunotoxin protein and genes

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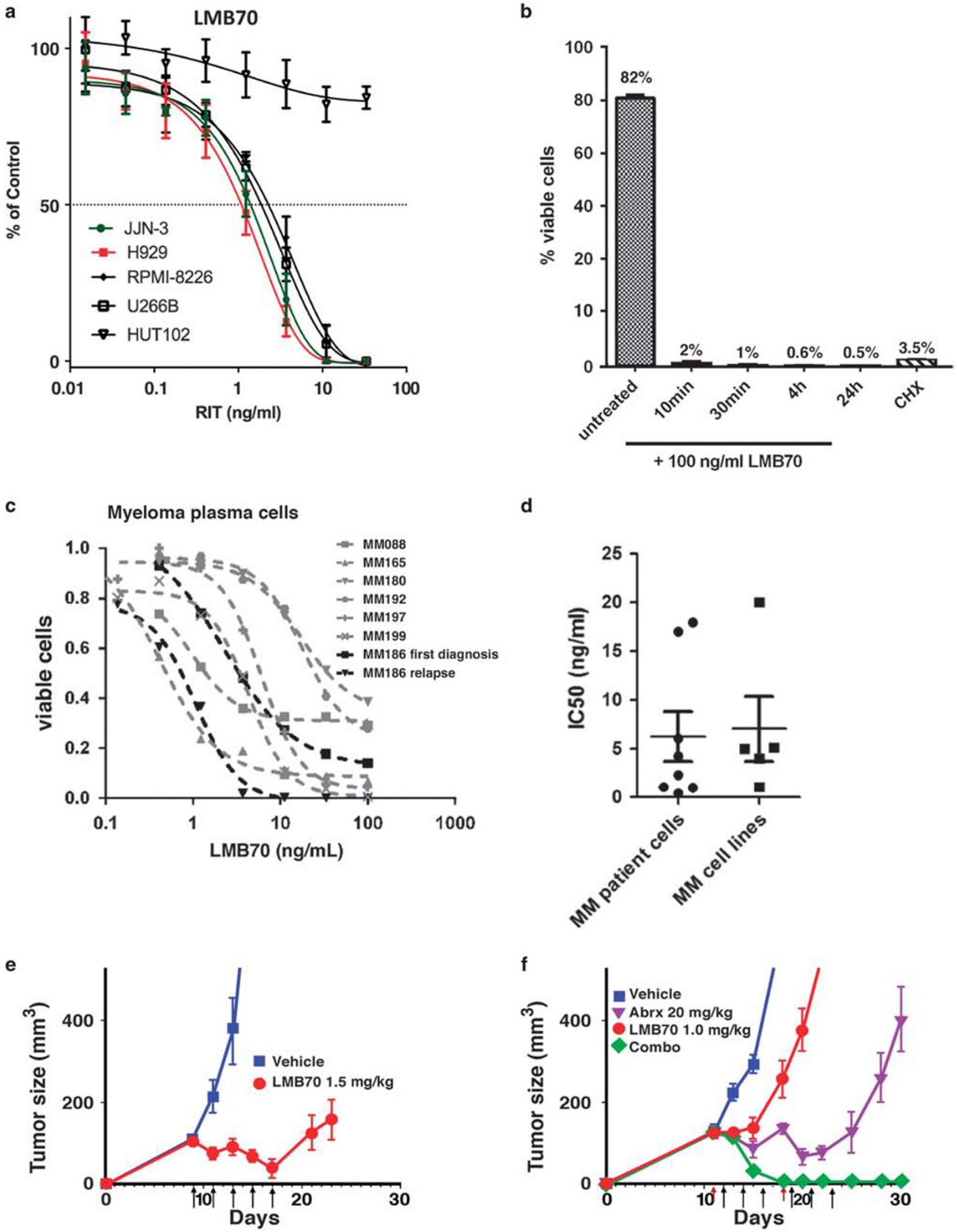

encoding the immunotoxins is shown (Supplementary Figure S1C and D). After expression and purification, we obtained highly purified RITs for both BM24 and BM306. The corresponding immunotoxins are named LMB38 and LMB70, respectively. A sodium dodecyl sulfate gel showing that the immunotoxins are highly purified is shown (Supplementary Figure S1E).

We tested the cytotoxic activity of LMB38 and LMB70 on BCMA expressing cell lines using a cell proliferation assay (WST-1).

Figure 1. Activity of anti-BCMA immunotoxin LMB70. (**a**) Representative cytotoxicity assay on BCMA-positive cells using WST8 reagent after three days incubation with RIT. (**b**) Cell viability assay using Flow cytometry analysis. BCMA expressing H929 cells are incubated with 100 ng/ml of LMB70 for indicated time, washed three times and incubated further with complete media. Three days after seeding, cells were stained with Annexin V PE and 7-ADD and cell viability was analyzed using flow cytometry. Annexin V- and 7AAD-negative cells were considered viable. (**c**) Normalized viability of myeloma plasma cells, identified as illustrated in Supplementary Figures S2A and B. Cells were incubated with different concentrations of LMB70 for 3 days and viability was measured using 7AAD. (**d**) IC_{50} value of LMB70 on myeloma plasma cells from different myeloma patients and from myeloma cell lines. (**e**) Antitumor activity of LMB70 on BCMA expressing H929 xenograft ($P < 0.001$ for LMB70 vs PBS on day 17). (**f**) Antitumor activity of LMB70 and Abraxane combination on BCMA expressing H929 xenograft ($P < 0.001$ for Combo vs LMB70 and $P < 0.002$ for Abrx on day 19).

Table 1. Activity of LMB38 and LMB70 on BCMA-positive cell determined by 3 days WST assay

		IC_{50} (ng/ml)			
Cell line	*Cell type*	*LMB38*	*LMB70*	*RG7787*	*BM306*
U266B	Myeloma	1.9 ± 0.2	5.0 ± 0.4	>1000	>1000
H929	Myeloma	1.2 ± 0.3	1.1 ± 0.2	>1000	>1000
RPMI-8226	Myeloma	6.9 ± 0.8	5.1 ± 0.4		>1000
LP-1	Myeloma	25.0 ± 2	20.0 ± 2		
JJN3	Plasma cell leukemia	2.5 ± 0.8	4.0 ± 0.6		>1000
Jeko-1	Mantle cell Lymphoma	>100	>100		
HUT-102	T-cell lymphoma	>100	>100		
Cells from patient					
MM-165	Myeloma		0.4		
MM-180	Myeloma		17.0		
MM-186	Myeloma		2.3		
MM-186 Relapse	Myeloma		1.0		
MM-192	Myeloma		17.9		
MM-088	Myeloma		1.0		
MM-197	Myeloma		6.0		
MM-199	Myeloma		4.3		

Representative cell-killing curves are shown for LMB70 (Figure 1a) and LMB38 (Supplementary Figure S1F). The IC_{50} values are summarized in Table 1. The IC_{50} of LMB38 on H929, U266B, JJN3, RPMI-8226, LP-1 and KMS-18 are 1.2, 1.9, 2.5, 6.9, 25 and 55 ng/ml, respectively. Similarly, the IC_{50} values for LMB70 on those cell lines are 1.1, 5.0, 4.0, 5.1, 20 and 65 ng/ml, respectively. LMB38 and LMB70 have no activity on Jeko-1 and the HUT-102 cell line that is BCMA negative.

Because WST-1 assays measure both cell growth inhibition and cell death, we measured the cell killing by flow cytometry in which Annexin V and 7AAD staining was simultaneously used to analyze apoptosis and cell death at the same time. Over 95% of cells were Annexin V/7AAD positive when exposed to LMB70 for only 10 min, indicating a very short exposure to LMB70 is sufficient to kill almost all of the antigen-positive target cells (Figure 1b).

To determine whether LMB70 would kill cells from patients with MM, we analyzed BMMNCs from seven patients with active disease who had considerable numbers of myeloma plasma cells in the BM. A summary of the activity results with all patients analyzed are shown (Figure 1c, 1d and Table 1). Typical cell analysis results for three patients are shown in Supplementary Figure S2A and B. The myeloma plasma cells of all patient samples were killed by LMB70 in a dose-dependent manner as shown (Figure 1c) but not the non-myeloma by standard cells (Supplementary Figure S2C). Figure 1d shows that the IC_{50} values for the MM cell lines varied between 0.4 and 18 ng/ml and the IC_{50} values of myeloma plasma cells from patients were in the same range as from myeloma cell lines.

To determine whether the apoptosis pathway is induced after exposure of H929 cells to LMB70, we performed western analysis of proteins involved in apoptosis. As shown in Supplementary Figure S2D, the level of Mcl-1 and Bcl-XL was markedly diminished after 6-h exposer of immunotoxin. Also, Caspase 3, 8 and 9 underwent cleavage during the 6-h period. These changes are consistent with rapid induction of apoptosis.

Next, we grew tumor in severe combined immunodeficient (SCID) mice using the most sensitive cell line H929 to test *in vivo* efficacy of LMB38 and LMB70 in mice. Based on mouse safety data of other previous immunotoxins, we treated mice with 1.5 mg/kg every other day × 5 doses. As shown for LMB70 (Figure 1e) and LMB38 (Supplementary Figure S2E), the growth of tumors in immunotoxin treated groups are delayed or the tumors decrease in size up to 50%, but they grew back once the treatment was stopped. We used the H929 cell line for animal studies because it grows in SCID mice and develops uniform tumors when injected subcutaneously with matrigel. We found that both LMB38 and LMB70 caused tumors to shrink and slowed tumor progression but alone did not produce a complete response, although both are very active on H929 cells *in vitro*. The basis of the incomplete responses is being studied but is not owing to antigen loss, and cells isolated from treated tumors are fully responsive when returned to cell culture.

Paclitaxel, an anti-microtubule agent that promotes microtubule assembly has been studied in MM as a chemotherapeutic agent. Nab-paclitaxel (Abraxane) is a modified version of paclitaxel that has distinct pharmacologic properties with greater uptake by and retention within tumors which made it efficacious against some solid tumors. Several clinical studies have reported modest response by paclitaxel as a single agent in patients with newly diagnosed MM.[13] A recent phase II trial of nab-paclitaxel in patients with relapse of refractory MM showed 15% (2/13) overall response rate.[14] We also previously found that the combination of immunotoxin and a taxane acted synergistically to cause complete regressions of mesothelin expressing malignancies (cancers of cervix, pancreas and stomach).[15] Therefore, we treated H929 xenograft bearing mice with LMB70 and Abraxane. The combination of Abraxane (20 mg/kg) and LMB70 (1 mg/kg) gave complete remissions that lasted over 30 days (Figure 1f), whereas each agent alone did not. These doses of drug were well tolerated with no loss of weight in the treated mice (Supplementary Figures 3A-D).

In summary, we have produced two RITs that kill myeloma cell lines and produce complete remissions in mice with myeloma tumors. Our data suggest that further preclinical development of the agents for myeloma therapy is warranted.

CONFLICT OF INTEREST

IP is an inventor on several patents on immunotoxins that have all been assigned to the NIH. The remaining authors declare no conflict of interest.

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