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IMMU-140, a Novel SN-38 Antibody–Drug Conjugate Targeting HLA-DR, Mediates Dual Cytotoxic Effects in Hematologic Cancers and Malignant Melanoma

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Abstract

HLA-DR is a member of the MHC class II antigen family expressed on hematologic and solid tumors. Antibodies directed against HLA-DR have demonstrated some clinical success, but toxicities limited development. IMMU-140 is an anti–HLA-DR antibody–drug conjugate composed of the active metabolite of irinotecan, SN-38, conjugated to a humanized anti–HLA-DR IgG_4 antibody (IMMU-114); the IgG_4 naked antibody is devoid of immune functions. Our aim was to determine if SN-38, the metabolite of a drug not commonly used in hematopoietic cancers, would be effective and safe when targeted to HLA-DR–expressing tumors. IMMU-140 had dual-therapeutic mechanisms, as evidenced by its retention of nonoverlapping anti–HLA-DR nonclassical apoptotic signaling and classical apoptosis mediated by its SN-38 payload. In seven human disease models [acute lymphocytic leukemia (ALL), chronic lymphocytic leukemia (CLL), multiple myeloma (MM), acute myeloid leukemia (AML), diffuse large B-cell lymphoma (DLBCL), Hodgkin lymphoma (HL), and melanoma], IMMU-140 provided significant therapeutic efficacy compared with controls, *in vitro*, in 3D spheroid models, and *in vivo*. Except for MM and HL, IMMU-140 imparted significantly improved antitumor effects compared with parental IMMU-114. Even in intractable AML and ALL, where IMMU-114 only had modest antitumor effects, IMMU-140 therapy mediated >80% improvement in survival. Therapy was well tolerated, as demonstrated by no marked loss in body weight. Combined with doxorubicin, IMMU-140 produced significantly greater antitumor effects in HL than with monotherapy and without any added toxicity. The dual-therapeutic action of IMMU-140 resulted in promising therapeutic activity in a range of hematopoietic tumors and melanoma, and therefore warrants clinical development. *Mol Cancer Ther; 17(1); 150–60. ©2017 AACR.*

Introduction

Human leukocyte antigen-DR (HLA-DR) is a member of the major histocompatibility complex (MHC) class II antigen family that is a heterodimer comprised of an α- and β-chain expressed on both normal and malignant hematopoietic cells (1). In addition to hematopoietic-lineage neoplasia, HLA-DR is likewise expressed by certain solid tumors, including malignant melanoma (2). Thirty-years ago, efforts to exploit this target with antibody-based therapeutics demonstrated that an anti–MHC class II antibody in a syngeneic mouse lymphoma model could cure animals without permanent damage to the immune system (3). However, a humanized anti–HLA-DR β-chain–specific monoclonal antibody (hu1D10) produced severe side effects upon infusion into Hodgkin lymphoma patients, due in part to IgG_1 Fc-dependent mechanisms, including complement activation (4). Studies in cynomolgus monkeys infused with anti–HLA-DR antibodies, either an IgG_1 or IgG_2, demonstrated severe infusion reactions resulting in death at a dose of 1.5 mg/kg [human equivalent dose (HED) of ∼0.5 mg/kg]. Complement activation was the main cause of this toxicity (5). Nagy and colleagues (6) found that by utilizing the human IgG_4 backbone for an anti–HLA-DR antibody, it was still effectively cytotoxic to human lymphoma and leukemia cells both *in vitro* and *in vivo*. Furthermore, when injected into primates with relevant HLA-DR normal tissue distribution and cross-reactivity, there were neither infusion-related toxicities nor long-lasting adverse effects to the immune system.

IMMU-114 is a humanized anti–HLA-DR IgG_4 monoclonal antibody specific for the α-chain that was engineered to lack effector-cell functions, but retains binding and a broad range of antitumor effects in diverse hematologic neoplasms (7, 8). When administered subcutaneously, it has encouraging efficacy in an initial phase I clinical trial in relapsed or refractory non-Hodgkin lymphoma (NHL) and chronic lymphocytic leukemia (CLL), with a good safety profile (ref. 9; ClinicalTrials.gov, NCT01728207). Preclinically, IMMU-114 demonstrated a range of antitumor effects *in vitro* in several different human acute lymphocytic leukemia (ALL), multiple myeloma (MM), Hodgkin lymphoma

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(HL), diffuse large B-cell lymphoma (DLBCL), CLL, and NHL cell lines; it was also efficacious in xenograft disease models of NHL (7, 8). Despite such a wide range of hematopoietic malignancies responsive to IMMU-114, acute myeloid leukemia (AML) remained resistant. Results suggest that cell killing mediated by IMMU-114 and other anti–HLA-DR antibodies may be linked to a nonclassical apoptotic pathway through direct signaling that bypasses the caspase cascade and poly(ADP-ribose) polymerase (PARP) cleavage (6, 8, 10, 11). This signaling was lacking in AML cell lines, regardless of HLA-DR expression levels, suggesting that this direct pathway may be defective in these AML cell lines, thus posing a further therapy challenge (8).

Antibody–drug conjugates (ADC) have gained much interest as a means of targeting specific cytotoxic drugs to both liquid and solid tumors (12). In an effort to improve the antitumor activity of IMMU-114, an ADC (IMMU-140) was developed by conjugating IMMU-114 with the active metabolite of irinotecan, SN-38. Other ADCs utilizing SN-38 (sacituzumab govitecan and labetuzumab govitecan) being studied in solid tumors have been well tolerated, having clinically significant objective responses in patients given multiple cycles over >6 months, and with manageable neutropenia being the major toxicity (13–17). Cytotoxicity mediated by SN-38 is through DNA breakage triggering the intrinsic apoptotic pathway, resulting in activation of the caspase cascade, PARP cleavage, and further DNA degradation (18–20). Thus, our goal was to determine if SN-38, the active metabolite of a drug (irinotecan) not commonly used in hematopoietic cancers, would prove to be an effective and safe therapeutic when targeted with the anti–HLA-DR antibody, IMMU-114. Given the potential of delivering nonoverlapping, dual-apoptotic signaling through nonclassical and intrinsic apoptotic pathways, we hypothesized that IMMU-140 could provide a superior antitumor effect in HLA-DR–expressing tumors.

Materials and Methods

Cell lines and chemotherapeutics

Human cell lines U266B1, GDM-1, SU-DHL-6, A-375, and SK-MEL-28 were purchased from the American Type Culture Collection. MN-60, MOLM-14, JVM-3, REH, L-540, and MEC-1 were purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany). A CAG human multiple myeloma cell line was developed at the Arkansas Cancer Research Center (21). ATCC authenticates all their cell lines by short tandem repeat (STR) assay prior to sale. DSMZ likewise authenticates all their cell lines via cytogenetics, immunophenotyping, and cancer-type specific mutations. All cell lines were passaged in culture less than 6 months. Any cell line with an unknown passage number was authenticated by the STR assay by the ATCC. CAG cells were authenticated as human by STR and positive FACS staining for CD138 and CD38 and negative for CD45 and CD19 (22). Each cell line was maintained according to the recommendations of ATCC or DSMZ, and routinely tested prior to experimentation for *mycoplasma* using MycoAlert Mycoplasma Detection Kit (Lonza). Chemotherapeutics were purchased for use in studies described herein with source and methods of dilution provided in the Supplementary Data.

Antibodies and ADCs

Development of humanized anti–HLA-DR IgG_4 monoclonal antibody (IMMU-114) has been characterized and described previously (8). Other humanized antibodies developed by Immunomedics, Inc., for control ADCs, consisted of anti-CD20 (veltuzumab), anti-CEACAM5 (hMN-14, labetuzumab), or an anti-histamine–succinyl–glycine (HSG) monoclonal antibody (h679). Preparations of CL2A-SN-38 drug linker-drug molecule and its IMMU-114 conjugate, IMMU-140, serum stability, and binding studies, were by procedures described previously (23, 24, 31) and are presented in Supplementary Fig. S1.

HLA-DR expression on cell lines via FACS or IHC

Detailed staining procedures used for FACS analysis for HLA-DR α-chain expression and IHC of formalin-fixed, paraffin-embedded tissues are described in Supplementary Data.

In vitro cytotoxicity of tumor cell monolayers and 3D spheroids

Cytotoxicity *in vitro* was determined using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium dye-reduction assay (MTS dye reduction assay; Promega), as described in Supplementary Data. Dose–response curves were generated from the mean of triplicate determinations using best-fit curves for the data, and IC_{50} values were calculated using PrismPad software. Three independent assays were run and calculated IC_{50} values compared using a two-tailed *t* test. Significance was set at $P < 0.05$. Development of spheroids from A-375 cells for the evaluation of cytotoxicity was produced as previously described (25) and is presented in Supplementary Data.

Western blot assessment of ERK1/2 phosphorylation, PARP cleavage, and double-stranded DNA breaks *in vitro*

Evaluation of ERK1/2 phosphorylation, PARP cleavage, and dsDNA breaks has previously been described (8, 18–20) and presented in Supplementary Data. IMMU-140 and IMMU-114 concentrations and incubation times are indicated in the figures or figure legends.

In vivo therapeutic studies

All animal studies were approved by Rutgers School of Biomedical and Health Sciences, Rutgers IACUC protocol number (14037E0717) and by Montclair (2016-032) State University Institutional Animal Care and Use Committees, respectively. Details for each disease model are presented in Supplementary Data. Mice used to establish experimental AML and MM received 2 Gy irradiation 24 and 48 hours prior to inoculation of cells, respectively. Therapy began 5 days after tumor cell inoculation for disseminated disease models. Mice were deemed to have succumbed to disease progression and euthanized once hind-limb paralysis developed or if they otherwise became moribund. Additionally, if mice lost more than 15% of initial body weight, they were sacrificed. For solid tumors, mice were randomized into treatment groups and therapy begun when tumor volumes (TV) were approximately 0.3 cm^3. Mice were euthanized for disease progression once tumors grew to greater than 1.0 cm^3 in size. For melanoma, mice were euthanized once tumors exceeded 2.0 cm^3.

RECIST criteria for solid tumor measurements were used to assess tumor response to therapy (26). A partial response in this type model was defined as shrinking the tumor >30% from initial size. Stable disease was when the TV remained between 70% and 120% of initial size. Time-to-tumor progression (TTP) was determined as time when tumor grew more than 20% from its nadir.

All treatment regimens, dosages, and number of animals in each experiment are described in the Results, tables, and figure legends. Lyophilized IMMU-140 and control ADCs were reconstituted and diluted as required in sterile saline. IMMU-114 was administered as s.c. injections, while IMMU-140 was administered i.p.

Statistical analysis of *in vivo* data

A Grubbs test was performed on data of treatment and control groups with $P \leq 0.05$ for any mouse deemed an outlier. Such mice were removed from further statistical analysis and are noted in the Results. Survival studies were analyzed using Kaplan–Meier plots, using Prism GraphPad Software (v7.02; Advanced Graphics Software, Inc.). Statistical analysis of solid tumor growth was based on area under the curve (AUC). Profiles of individual tumor growth were obtained through linear-curve modeling. An *f* test was used to determine equality of variance between groups prior to statistical analysis. A two-tailed *t* test was used to assess statistical significance between various treatment groups and controls, except for saline control, where a one-tailed *t* test was used in the comparison. Significance was set at $P \leq 0.05$.

Results

In vitro characterization of IMMU-140

Conjugation of SN-38 to IMMU-114 through TCEP-reduction allowed for site-specific conjugation of 6 to 8 CL2A-SN-38 molecules per molecule of IMMU-114 (Fig. 1A). Size-exclusion HPLC analysis of both the unmodified IMMU-114 and final IMMU-140 product demonstrated similar elution times when detected at either A_{280nm} for IMMU-114 or A_{360nm} for IMMU-140, with a resulting conjugate that was >98% monomeric and a drug to antibody substitution ratio (DAR) of 6.1 (Fig. 1B). Stability of the conjugated SN-38 on IMMU-114 demonstrated a half-life of 21 and 23 hours when incubated in normal mouse or normal human serum, respectively (Fig. 1C). This was doubled to 42 hours when IMMU-140 was incubated in pH 7.4 PBS. Furthermore, there was no evidence in loss of targeting of the ADC, as shown by comparable binding of IMMU-140 and IMMU-114 on an HLA-DR^{pos} human melanoma cell line, A-375 (Fig. 1D). Both have calculated K_D values in the subnanomolar range and were not significantly different.

In vitro cytotoxicity of IMMU-140 versus IMMU-114 on various hematopoietic and melanoma cell lines

Human hematopoietic tumor and melanoma cell lines exposed to either IMMU-140 or free SN-38 demonstrated IC_{50} values in the nanomolar range (Table 1). Among the hematopoietic cell lines, only in MOLM-14 (AML) and U266B1 (MM) did free SN-38 produce a significantly lower IC_{50} than IMMU-140 ($P < 0.0266$). This was also true in the two melanoma cell lines ($P < 0.0201$). These data confirm that the SN-38 carried by IMMU-140 is active in all these various hematopoietic neoplastic and melanoma cell lines.

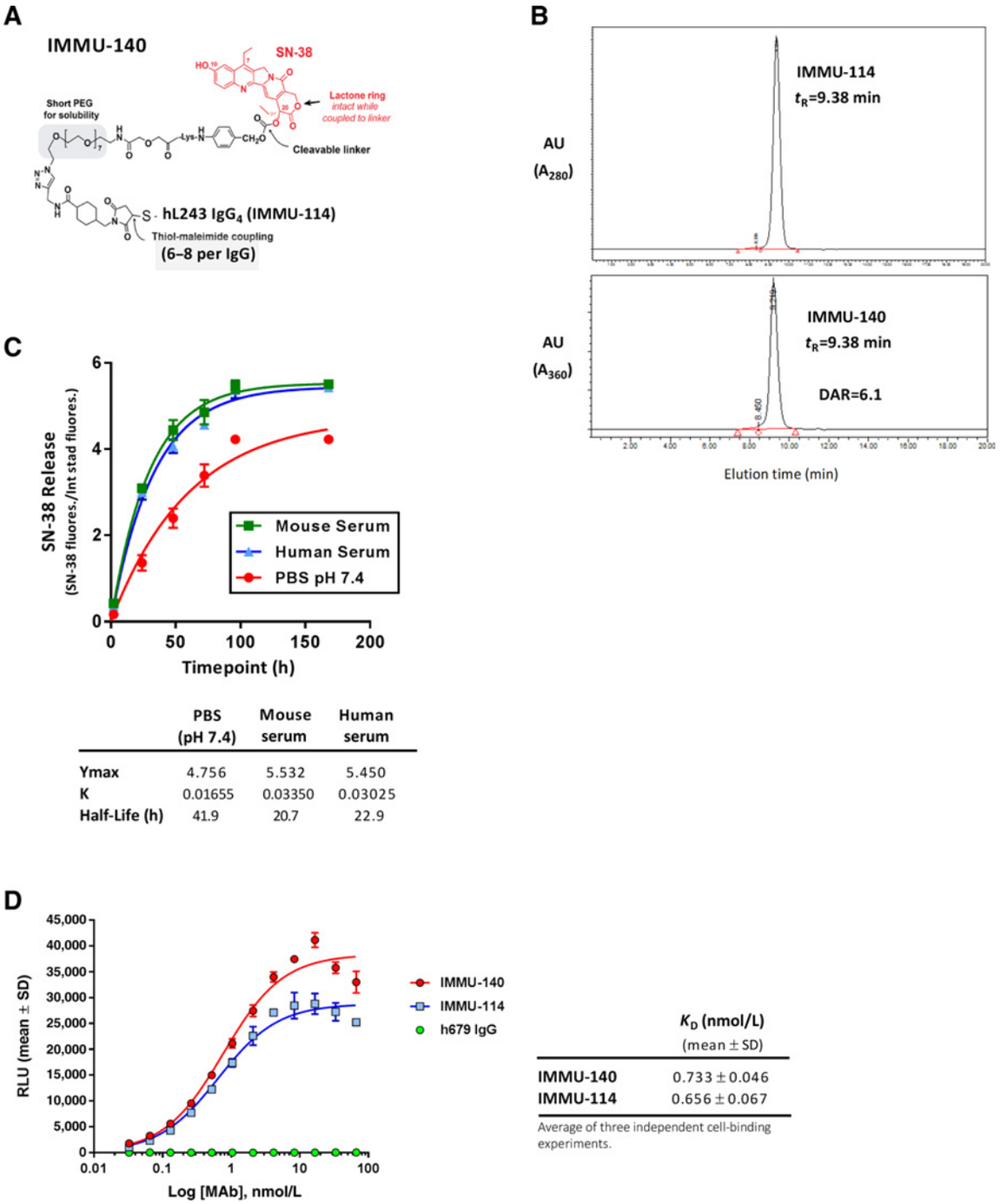
Similar to what was reported previously (8), IMMU-114 produced significant growth inhibition compared with a nonspecific control antibody in the various CLL, ALL, MM, and melanoma cell lines (Table 1; $P < 0.0061$). However, unlike IMMU-140, incubation with IMMU-114 resulted in IC_{50} values >40 nmol/L. In terms of protein concentrations, this represents a >316-fold higher concentration for IMMU-114 than that achieved with IMMU-140. Only in JVM-3 did IMMU-114 mediate growth inhibition with an IC_{50} in the low nanomolar range and a maximum 65% inhibition at the highest concentration. However, even this IC_{50} achieved for IMMU-114 represents a >12-fold higher protein concentration than that obtained with IMMU-140 in this same cell line. In addition, IMMU-114 demonstrated a small (15%), but significant, growth-inhibitory effect in one of two melanomas tested, A-375 ($P = 0.009$). As has been noted previously, IMMU-114 demonstrated no cytotoxic effects in either of the AML cell lines tested in this assay (8). These data indicate that while IMMU-140 demonstrated >50% growth inhibition in all 10 cell lines tested, IMMU-114 was limited to only 6 of 10 showing any significant growth inhibition, and of these only 1 of 10 was greater than 50%.

Enhanced killing of A-375 spheroids by IMMU-140

Single spheroids of A-375 were evaluated for cytolysis by IMMU-140 (DAR = 6.1) and nonspecific control h679-SN-38 (DAR = 6.8) at two concentrations of conjugated SN-38 (200 and 100 nmol/L). As shown by the fluorescent images taken at 48 hours after treatment, the intensity of PI staining of dead cells (Supplementary Fig. S2A) was much higher for the spheroids treated with 33 nmol/L of IMMU-140 (200 nmol/L SN-38 equivalents) than the spheroids treated with 30 nmol/L of h679-SN-38 (200 nmol/L SN-38 equivalents), indicating IMMU-140 was more effective than the nonspecific h679-SN-38 for killing HLA-DR–expressing A-375 cells. The increase in cell death was also discernible in Supplementary Fig. S2B, which shows the superimposed images of the same spheroids stained with both PI (red) and calcein AM (green). Similar images of PI-stained A-375 spheroids demonstrated enhanced cytolysis by IMMU-140 in comparison with h679-SN-38 at even lower concentrations of 100 nmol/L SN-38 equivalents (Supplementary Fig. S2C). Because the IMMU-114 targeting moiety of IMMU-140 also has cytotoxic activity, spheroids treated with 33 nmol/L IMMU-114 likewise demonstrated a greater degree of dead cells than those treated with nontargeting h679 IgG (Supplementary Fig. S2D). Further, a comparison between IMMU-140-treated spheroids (Sp3 and Sp4) to those treated with the equivalent protein dose of IMMU-114 (Sp11 and Sp12) likewise demonstrates a higher amount of dead cells in those treated with IMMU-140. These data are indicative of the higher degree of specific cell killing mediated by IMMU-140 compared with a nonspecific ADC and to IMMU-114.

Dual apoptotic signaling pathways triggered by IMMU-140: anti–HLA-DR- and SN-38–mediated signals

While the cytotoxic effects of IMMU-114 are believed to be dependent on phosphorylation of ERK1/2 to trigger apoptosis (8), IMMU-140 has the added benefit of carrying the topoisomerase I inhibitor <, SN-38. Cytotoxicity imparted by SN-38 is through impaired DNA replication resulting in activation of the caspase cascade, ultimately leading to the cleavage of PARP and double-stranded DNA (dsDNA) breaks (18–20). Accordingly, two different mechanisms of action leading to cell death can be triggered by HLA-DR–targeting of IMMU-140. Conjugation of SN-38 to IMMU-114 did not alter IMMU-114–mediated signaling, as evidenced by similarly increased phospho-ERK1/2 (p-ERK1/2) levels in both JVM-3 and MN-60 upon exposure to IMMU-114 and IMMU-140 (Fig. 2A). Interestingly, while it was reported that treatment with IMMU-114 did not result in activation of this signaling pathway in AML (8), reconfirmed in GDM-1,



	PBS (pH 7.4)	Mouse serum	Human serum
Ymax	4.756	5.532	5.450
K	0.01655	0.03350	0.03025
Half-Life (h)	41.9	20.7	22.9

	K_D (nmol/L) (mean ± SD)
IMMU-140	0.733 ± 0.046
IMMU-114	0.656 ± 0.067

Average of three independent cell-binding experiments.

Figure 1.

In vitro characterization of IMMU-140 ADC. **A,** CL2A-SN-38 linker contains a short polyethylene glycol (PEG) moiety to confer aqueous solubility; a maleimide group was incorporated for fast thiol–maleimide conjugation to mildly reduced antibody; a benzylcarbonate site provided a pH-mediated cleavage site to release the drug from the linker; and the cross-linker was attached to SN-38's 20-hydroxy position, to keep the lactone ring of the drug from opening to the less active carboxylic acid form under physiological conditions. **B,** Size-exclusion HPLC of unmodified IMMU-114 (top) and IMMU-140 conjugate with a drug/antibody molar substitution of 6.1 (bottom). Unmodified IMMU-114 was detected at A_{280nm}, while IMMU-140 was detected at the absorbance wavelength of SN-38 (A_{360nm}). Peak elution near the antibody position corresponds to antibody substituted with SN-38. The conjugate was >98% monomeric. In the figure, t_R represents retention time. **C,** Stability of IMMU-140 in PBS, normal mouse serum, and normal human serum was determined as described in Materials and Methods and Supplementary Data. Triplicate samples were analyzed for SN-38 content at 2, 24, 48, 72, and 96 hours. **D,** Binding of IMMU-140 and IMMU-114 to an HLA-DR–positive human melanoma cell line (A-375) via a cell-based ELISA, as described in Materials and Methods and Supplementary Data. Mean K_D values are shown in the table to the right. Negative control (h679) is a humanized anti-HSG IgG.

Table 1. *In vitro* cytotoxicity of IMMU-140 and IMMU-114 in various human hematopoietic tumor and malignant melanoma cell lines

			Cytotoxicity of IMMU-140 vs. free SN-38 IC_{50} (mean ± SD)		IMMU-114–mediated growth inhibition	
Disease	Cell line	HLA-DR expression (MFI)	IMMU-140[a] (nmol/L)	Free SN-38 (nmol/L)	IC_{50} (nmol/L; mean ± SD)	Maximum percent inhibition at 40 nmol/L
CLL	JVM-3	78,700 ± 3,676	0.77 ± 0.15	0.51 ± 0.18	1.52 ± 0.84	[b]65 ± 11
	MEC-1	112,777 ± 4,509	1.56 ± 0.17	1.41 ± 0.26	>40	[b]39 ± 2
MM	CAG	80,733 ± 2,715	7.05 ± 2.73	7.02 ± 1.77	>40	[b]28 ± 6
	U266B1	[c]2,059 ± 92	11.22 ± 1.02	[d]4.14 ± 0.66	>40	0 ± 3
ALL	MN-60	65,800 ± 1,664	1.29 ± 0.27	0.86 ± 0.09	>40	[b]37 ± 10
	REH	74,610 ± 1,769	0.67 ± 0.10	0.66 ± 0.07	>40	[b]41 ± 2
AML	MOLM-14	5,753 ± 254	1.21 ± 0.08	[d]0.90 ± 0.13	>40	11 ± 5
	GDM-1	31,377 ± 5,129	0.89 ± 0.11	0.82 ± 0.12	>40	2 ± 3
Melanoma	A-375	64,310 ± 400	2.99 ± 0.06	[d]2.17 ± 0.37	>40	[b]15 ± 4
	SK-MEL-28	3,419 ± 150	43.16 ± 5.86	[d]26.06 ± 1.68	>40	3 ± 6

Abbreviation: MFI, mean fluorescent intensity of IMMU-114-Alexa-647–stained cells as described in Materials and Methods.
[a]Concentration of IMMU-140 shown as SN-38 drug-equivalents. Protein concentrations of IMMU-140 would be 6.1-fold lower based on a DAR of 6.1.
[b]Significant inhibition compared with control antibody ($P < 0.009$).
[c]Represents only HLA-DR–positive cell population (36%).
[d]IC_{50} of free SN-38 is significantly different compared with IMMU-140 in MOLM-14 ($P = 0.0266$).

a different AML cell line, MOLM-14, did show enhanced levels of p-ERK1/2 when exposed to both IMMU-114 and IMMU-140. However, as already shown above, both GDM-1 and MOLM-14 were resistant to the *in vitro* growth-inhibitory effects of IMMU-114, suggesting that the signal induced in MOLM-14 is insufficient to cause cell death, whereas IMMU-140 was very potent in both these cell lines (Table 1). In the A-375 human melanoma line, there was constitutive phosphorylation of ERK1/2 that was not altered by IMMU-114 or IMMU-140 exposure.

In addition to the phosphorylation of ERK1/2 mediated by the IMMU-114 moiety of IMMU-140, the SN-38 of this ADC was shown to be fully functional. In all four cell lines, including both AML lines, IMMU-140 exposure induced PARP cleavage within 24 hours (Fig. 2A). JVM-3 appeared to be particularly sensitive to IMMU-140, with PARP cleavage evident within 4 hours. Consistent with the hematopoietic cell lines, IMMU-140 also mediated PARP cleavage in the A-375 melanoma, suggesting that in both hematopoietic and solid cancer lineage cell lines, the SN-38 resulted in a similar mechanism of action.

Both IMMU-114 and IMMU-140 ultimately kill the targeted cell through DNA degradation. As seen in both melanoma and hematopoietic lineage neoplastic cells, IMMU-114 incubation resulted in dsDNA breaks, as evidenced by increased levels of phosphorylated histone H2A.X (p-H2A.X, Fig. 2B). Comparing the sensitive CLL cell line, JVM-3, to-insensitive AML cells, GDM-1, as early as 4 hours after IMMU-114 incubation, dsDNA breaks increase >20-fold in JVM-3 versus almost no change in GDM-1. Even after 24 hours, GDM-1 only demonstrates ∼3-fold increase in dsDNA breaks, which was similar to the A-375 melanoma (2.5-fold). Conversely, IMMU-140 mediated an even greater degree of dsDNA breaks when compared with IMMU-114, at >9-fold in A-375 to a high of >450-fold in JVM-3 at 24 hours. Even in GDM-1, IMMU-140 increased dsDNA breaks by >20-fold after a 24-hour incubation. Altogether, these data clearly indicate that both the anti–HLA-DR signaling activity of IMMU-140 as well as the SN-38-payload were functional and provide a dual cytotoxic potential for this particular ADC.

Therapeutic efficacy of IMMU-140 vs. IMMU-114 in disseminated disease models of ALL, AML, MM, and CLL

Individual survival curves for each treatment in the various disease models are shown (Supplementary Fig. S3). In all four models, including AML, IMMU-140 provided a significantly superior survival benefit compared with control ADC and saline control animals ($P < 0.0031$, Table 2). Further, in all but MM, IMMU-140 was superior to unconjugated parental IMMU-114 in improving survival ($P < 0.0053$). While not significantly better than IMMU-114 in MM, IMMU-140 was approaching significance at the time the experiment was ended on day 151 ($P = 0.0612$). Combining bortezomib treatment did not provide any significant improvement in survival in the MM disease model (Supplementary Fig. S3E). Given that IMMU-140 still provided a greater than 60% increase in median survival, it is not likely that CAG is resistant to SN-38, but rather less sensitive relative to the antitumor activity provided by the HLA-DR–targeting function of IMMU-140.

Importantly, mice bearing disseminated AML (MOLM-14) succumbed to disease progression quickly, with median survival times (MST) of only 14 and 15 days for saline control and IMMU-114–treated mice, respectively (Supplementary Fig. S3C). While this one day advantage for IMMU-114 was significant ($P = 0.0031$), animals treated with IMMU-140 had a >1.5-fold increase in survival (MST = 37 days, $P = 0.0031$). A dose reduction to 12.5 mg/kg IMMU-140 (HED = 1 mg/kg) still provided a >80% improvement in survival compared with saline and control ADC at the same dose ($P = 0.0031$).

Although JVM-3 CLL cells were injected i.v., mice that succumbed to disease progression were found to have large tumor masses within their abdominal and thoracic cavities upon necropsy. In two mice treated with IMMU-140 (12.5 mg/kg), although they had prolonged survival (euthanized on days 112 and 117, respectively), tumor masses were found in the thoracic cavity attached to the lungs. Because these mice succumbed to disease >81 days after their last IMMU-140 injection, it may be possible that further cycles of therapy would have resulted in prolonged survival or possibly cures. As to whether these tumors would have been susceptible to such further treatment, IHC analysis was performed to determine expression of HLA-DR (Supplementary Fig. S3G). In both, expression of the IMMU-140 antigen (i.e., HLA-DR α-chain) was clearly present and no different than that observed from JVM-3 tumor taken from an ADC control mouse that succumbed to disease progression on day 61. Likewise, a tumor removed from a mouse on day 112 that was treated with 5 mg/kg of IMMU-114 still expressed HLA-DR

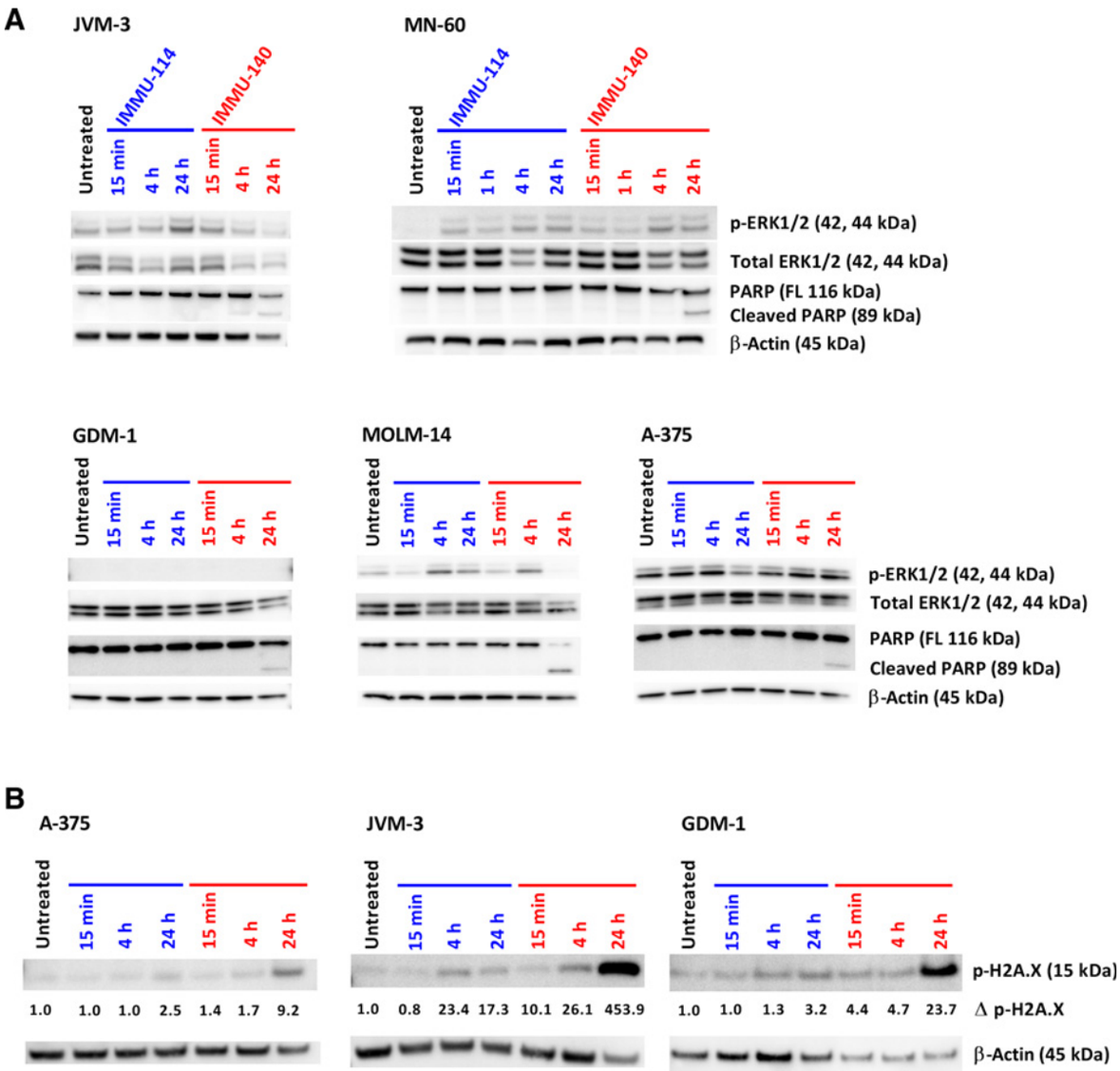


Figure 2.

Dual apoptotic signaling pathways triggered by IMMU-140: Anti–HLA-DR- and SN-38–mediated signals. Cells were plated and IMMU-114 and IMMU-140 were added as described in Materials and Methods and Supplementary Data. Both IMMU-114 and IMMU-140 were added at equal 10 nmol/L protein doses for the indicated times before cells were harvested for Western blot analysis. All incubation times shown in blue indicate IMMU-114 and those in red indicate IMMU-140. **A,** JVM-3 (CLL), MN-60 (ALL), GDM-1 (AML), MOLM-14 (AML), and A-375 (melanoma) cell lines analyzed for full-length PARP (PARP FL), cleaved PARP fragment (Cleaved), and phosphorylated ERK1/2 (p-ERK1/2) levels. Total ERK1/2 and β-actin served as protein loading controls. **B,** Levels of dsDNA breaks were determined by release of phosphorylated histone H2A.X (p-H2A.X). Assessment of changes in dsDNA breaks was calculated as ratios relative to untreated control, normalized to β-actin protein loading control (Δp-H2A.X).

α-chain and may have benefited from further treatment cycles. While it cannot be ruled out that resistance played a role in tumor progression, it is unlikely that resistance to SN-38 occurred in such a short period of time, because previous efforts to make SN-38–resistant tumor lines has taken >9 months *in vitro* under optimal conditions (27). These data suggest that disease progression in these mice was not due to loss of antigen or acquired resistance, but rather likely due to residual disease that remained at the time therapy was terminated.

IMMU-140 therapy was well tolerated by the animals, with no significant loss in body weight (Supplementary Fig. S3B). Even in mice that received 2 Gy irradiation prior to cell inoculation (MOLM-14), therapy with IMMU-140 was well tolerated, with no significant loss in body weight in any of the treated animals (Supplementary Fig. S3D). Additionally, MM tumor–bearing mice administered the combination of IMMU-140 plus bortezomib had no greater weight loss than those mice that received just bortezomib (Supplementary Fig. S4A).

Table 2. Efficacy of IMMU-140 therapy in four different models of disseminated hematologic cancers (ALL, AML, MM, and CLL)

Disease (cell line)	ADC treatment (25 mg/kg; twice weekly x 4 weeks)	*N*	Median survival (days)	IMMU-140 vs. controls (*P*)	IMMU-114 vs. controls (*P*)	Superior therapeutic
ALL (MN-60)	IMMU-140	10	66.5	n.a.	n.a.	IMMU-140
	IMMU-114	10	37	<0.0001	n.a.	
	Control ADC	10	26	<0.0001	<0.0001	
	Saline	10	22.5	<0.0001	<0.0001	
AML (MOLM-14)	IMMU-140	5	37	n.a.	n.a.	IMMU-140
	IMMU-114	5	15	0.0015	n.a.	
	Control ADC	5	21	0.0031	0.0077[a]	
	Saline	5	14	0.0031	0.0031	
MM (CAG)	IMMU-140	9[b]	>151	n.a.	n.a.	IMMU-140 and IMMU-114 equivalent
	IMMU-114	10	94.5	0.0612	n.a.	
	Control ADC *plus* Bortezomib[c]	10	32.5	<0.0001	<0.0001	
	Bortezomib[c]	10	32.5	<0.0001	<0.0001	
	Saline	10	32	<0.0001	<0.0001	
CLL (JVM-3)	IMMU-140	10	>168	n.a.	n.a.	IMMU-140
	IMMU-114	10	108	0.0053	n.a.	
	Control ADC	10	44.5	0.0002	<0.0001	
	Saline	10	41	<0.0001	<0.0001	

Abbreviation: n.a., not applicable.
[a]Control ADC provided superior survival benefit when compared with IMMU-114 in the AML model.
[b]One mouse censored as an outlier via the Grubbs test.
[c]Bortezomib was administered at 0.89 mg/kg weekly for 4 weeks.

IMMU-140 efficacy in xenograft models of DLBCL, Hodgkin lymphoma, and malignant melanoma

IHC of SU-DHL-6 (DLBCL) and L-540 (HL) tumor xenografts demonstrated strong staining for HLD-DR α-chain, consistent with FACS analysis of these two cell lines (Supplementary Fig. S5). All treatments administered to mice bearing SU-DHL-6 tumors (Fig. 3A), including the anti-HSG ADC control (h679-SN-38), provided significant antitumor effects when compared with saline control animals ($P < 0.0051$). However, the highest dose of IMMU-140 (25 mg/kg) resulted in significant tumor regressions compared with mice treated with comparable doses of either IMMU-114 or the ADC control, with all 10 mice tumor-free within 18 days of therapy initiation ($P < 0.0202$).

Mice bearing L-540 (HL) tumors (Fig. 3B) and administered doxorubicin chemotherapy demonstrated significant antitumor effects compared with saline and control ADC treatment ($P < 0.0251$). Likewise, all three doses of IMMU-140 and IMMU-114 resulted in significant tumor growth inhibition relative to saline and control ADC groups ($P < 0.0388$). There were no significant differences between mice administered IMMU-140 versus IMMU-114 at comparable doses in this disease model, although it did approach significance at the highest doses ($P = 0.0662$). However, only mice administered IMMU-140 at 25 mg/kg resulted in improved antitumor effects, compared with animals treated with doxorubicin chemotherapy ($P = 0.0118$). When doxorubicin was combined with the lowest dose of IMMU-140 (5 mg/kg), there were significantly greater antitumor effects than in mice given monotherapy with either agent ($P < 0.0094$; Fig. 3C). It should be noted that at the time the combination group began their treatment, tumors were significantly larger than in mice that received only doxorubicin or IMMU-140 (0.287 ± 0.044 cm^3 and 0.287 ± 0.038 cm^3 vs. 0.345 ± 0.02 cm^3, respectively; $P = 0.0058$), further demonstrating the superior effect of the combination. There was no added toxicity observed in the mice, as evidenced by no significant differences in body weight between the combination group and those treated only with doxorubicin (Supplementary Fig. S4B).

Because HLA-DR has been shown to be expressed in many different solid tumors, including malignant melanoma (2), mice bearing HLA-DR^{pos} melanomas (A-375) were treated with IMMU-140 (Fig. 3D). Only mice treated with IMMU-140 demonstrated a significant antitumor effect when compared with all other groups ($P < 0.0244$; Supplementary Table S1). Even therapy with irinotecan (3.75 mg/kg), at a dose calculated to deliver 10-fold more SN-38 than with IMMU-140, or combining a dose of irinotecan equal to the amount of SN-38 on IMMU-140 with IMMU-114 (0.375 mg/kg irinotecan), did not equal the activity of IMMU-140 ($P < 0.0001$). All mice treated with IMMU-140 were positive responders, with two mice tumor-free when the experiment ended on therapy day 70, resulting in a >3-fold delay in TTP when compared with all the non-ADC control groups ($P < 0.0005$; Table 3). Even though this tumor was sensitive to the nonspecific anti-CD20 ADC, treatment with IMMU-140 imparted a >80% delay in TTP (15.6 ± 7.7 days vs. 28 ± 9.9 days, respectively; $P = 0.012$). These results demonstrate that even in a murine disease model of an aggressive human melanoma, therapy with IMMU-140 resulted in significant tumor regression and delay in disease progression.

Discussion

While many ADCs are currently in clinical trials, only four have been approved by the FDA, and of these, three were developed against liquid tumors (gemtuzumab ozogamicin, inotuzumab ozogamicin, and brentuximab vedotin) and one against solid tumor (ado-trastuzumab emtansine; refs. 12, 28). A common hallmark for these early ADCs is the use of highly potent drugs (e.g., auristatin derivatives with picomolar IC_{50}) and stable linkers (29). A different approach is to utilize a moderately potent drug (e.g., SN-38 with nanomolar IC_{50}) and a linker designed with a limited stability of ~24 hours. This approach uses SN-38 site specifically conjugated to 8 possible interchain thiols of the antibody, yielding a substitution of 6 to 8 drugs per antibody, with a carbonate linker that is cleavable at low pH, resulting in

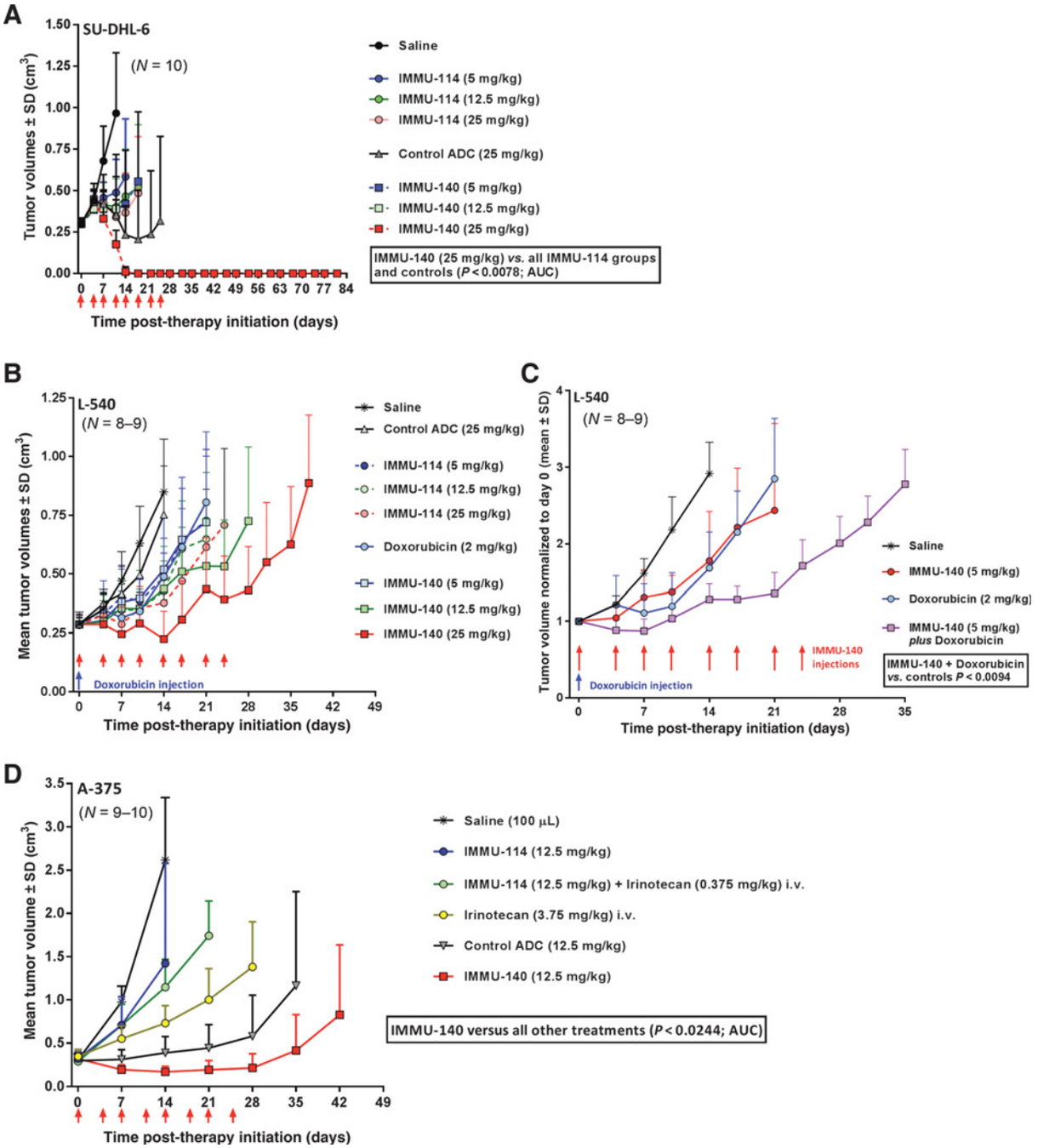


Figure 3.

Therapeutic efficacy of IMMU-140 versus IMMU-114 in solid xenograft disease models of human DLBCL, Hodgkin lymphoma, and malignant melanoma. Changes in mean tumor volumes of mice bearing s.c. tumors. Time = 0 on the graphs indicates time when therapy began in mice with established s.c. tumors. Red arrows indicate time when animals were administered an ADC, antibody, or irinotecan. Statistical analysis performed on area under-the-curve (AUC) data as described in Materials and Methods. Significance set at $P < 0.05$. **A,** SU-DHL-6 DLBCL tumor–bearing mice received IMMU-140 (i.p.) and IMMU-114 (s.c.) at the indicated concentrations twice weekly for 4 weeks. **B,** Likewise, mice bearing L-540 Hodgkin lymphoma received IMMU-140 and IMMU-114 as indicated, twice weekly for 4 weeks. Doxorubicin (blue arrow) was administered as a single i.v. injection (2 mg/kg) at time = 0. **C,** Combination of doxorubicin and IMMU-140 in the L-540 tumor–bearing mice. Tumor volume normalized to day 0 to compensate for larger tumor volumes in the mice that received the combination compared with doxorubicin and IMMU-140 monotherapy groups. **D,** A-375 melanoma–bearing mice also received IMMU140 and IMMU-114 twice weekly for 4 weeks. One control group also received irinotecan i.v. twice weekly alone or with IMMU-114.

Table 3. TTP for A-375 melanoma–bearing mice treated with IMMU-140

Treatment	*N*	% PR (TF)	TTP (days)	IMMU-140 vs. controls (*P*)
IMMU-140	10	100 (2)	28.0 ± 9.9	N.A.
Control ADC	10	30 (1)	15.6 ± 7.0	0.0120
Irinotecan	10	0 (0)	8.4 ± 4.4	0.0005
IMMU-114 + irinotecan	10	0 (0)	7.0 ± 0.0	0.0005
IMMU-114	10	0 (0)	7.0 ± 0.0	0.0005
Saline	9[a]	0 (0)	7.0 ± 0.0	0.0003

Abbreviations: *N*, number of mice per group; % PR, percent of mice that exhibited a positive response to treatment; TF, number of mice tumor-free when the experiment ended; TTP for mice not tumor-free as defined in Materials and Methods; N.A., not applicable.
[a]One mouse censored as an outlier via the Grubbs test.

drug release with a half-life in serum of ∼24 hours (23, 24). The moderately stable linker in these unconventional ADCs enables the drug to be released in the acidic pH of tumor microenvironment, while internalization further increases the drug's bioavailability. These design features, while not amenable to showing specificity *in vitro*, have resulted in encouraging clinical activity of other ADCs (anti–Trop-2 ADC, anti-CEACAM5 ADC) in solid cancer therapies (14–17, 30). In particular, the same conjugation method has been used to create an anti–Trop-2 SN-38-ADC (sacituzumab govitecan, IMMU-132) that targets Trop-2 on solid tumors, and likewise has been shown to be superior to parental antibody both *in vitro* and *in vivo* (18, 19, 31). Further, it has demonstrated efficacy in heavily pretreated metastatic triple-negative breast cancer, as well as small-cell and non–small-cell lung cancer patients (14–16), suggesting that IMMU-140 may prove to be superior to its parental antibody clinically.

Results from this SN-38-conjugation process to produce IMMU-140 achieved a DAR of 6.1 with no loss of binding activity and a serum stability of ∼22 hours, all of which are consistent with previous ADCs made by the same methodology (18, 23, 24). In terms of pharmacokinetics (PK), we have previously demonstrated that the DAR number does not affect PK of the antibody in mice (19). Clearance and half-lives were similar for the ADC and parental antibody. Because IMMU-140 does not cross-react with murine tissues, there is no reason to expect any difference from what we have already demonstrated in mice with other ADCs. However, this will be a consideration clinically, and as such, before any clinical trials are performed with IMMU-140, such toxicokinetic studies would need to be performed in monkeys.

Due to the optimal linker-drug design in this unconventional ADC, it is difficult to show specificity *in vitro*, particularly in 96-hour incubations. Nevertheless, *in vitro* growth inhibition data provide proof that the drug is not modified by conjugation to some inactive form, because the released drug maintains the SN-38's cytotoxicity. Growth inhibition mediated by IMMU-140 was in the nanomolar range against an array of different hematologic tumors, including AML, as well as in melanoma, and was superior to parental IMMU-114.

Given the challenges of demonstrating specificity of IMMU-140 *in vitro* using cell monolayers, 3D cell culture techniques were utilized to confirm the specific nature of cell killing mediated by IMMU-140. Use of 3D cell cultures has recently gained favor as a bridge between *in vitro* monolayer cytotoxicity assays and *in vivo* efficacy studies in tumor-bearing animals (32, 33). Many aspects of tumor physiology are thought to be provided through these 3D models, such as changes in cellular morphology effecting tumor microenvironment and changes in gene expression resulting in metabolic and adaptive responses that, taken together, better mimic the *in vivo* system (33). In this 3D tumor–cell assay, A-375 melanoma spheroids treated with IMMU-140 clearly demonstrated a higher degree of cell death within the spheroid compared with nonspecific ADC. Likewise, IMMU-114 demonstrated more dead cells within the spheroid than control antibody, although to a much lesser extent than IMMU-140. Both these observations were borne out *in vivo* in which IMMU-140 provided significantly greater antitumor effects than control ADC or IMMU-114 in mice bearing A-375 tumors, and where IMMU-114 treatment proved to be superior to control IgG.

The decision to use IMMU-114 as the HLA-DR–targeting antibody to construct IMMU-140 was based in part on its ability to directly mediate cell death signals in many HLA-DR–expressing hematologic and solid tumors (2, 6, 8, 10, 11, 34). In hematopoietic cells, this anti–HLA-DR-mediated signaling is thought to be through changes in mitochondrial membrane potential and generation of reactive oxygen species (ROS) that is devoid of the caspase cleavage cascade, which is normally associated with the intrinsic apoptosis pathway (6, 8, 10, 11). In particular, IMMU-114 was shown to mediate an increase in phosphorylation of ERK1/2, JNK, and p38 in response to the increase in ROS (8). Conversely, SN-38, as a topoisomerase I (topo-I) inhibitor, impedes DNA replication, resulting in triggering of the intrinsic apoptosis pathway that leads to activation of the caspase–cleavage cascade, PARP cleavage, and ultimately to dsDNA breaks and cell death (18–20). Given these two different effects mediated by binding to HLA-DR and SN-38 delivery, IMMU-140 was predicted to have nonoverlapping, dual-signaling effects on targeted neoplastic cells. In fact, we demonstrated that IMMU-140 mediated both anti–HLA-DR signaling, as evidenced through p-ERK1/2, and topo-I inhibition (as observed by PARP cleavage and dsDNA breaks). Increased p-ERK1/2 mediated by both IMMU-114 and IMMU-140 in ALL and CLL was expected, because ligation of HLA-DR by IMMU-114 was reported previously, as was the lack of p-ERK1/2 in the GDM-1 AML cell line (8). Unexpectedly, in a second AML cell line, MOLM-14, both IMMU-140 and IMMU-114 were capable of inducing p-ERK1/2, although both GDM-1 and MOLM-14 were resistant to the growth-inhibitory effects of IMMU-114 *in vitro*. Likewise, in A-375 melanoma, there were no changes in p-ERK levels mediated by either IMMU-114 or IMMU-140. This is consistent with reports that in solid tumors, including melanoma, HLA-DR signaling is through phosphorylation of p125 focal adhesion kinase and not ERK (2, 30). In AML, it has been suggested that increased expression of Bcl-2 proteins, in particular Mcl-1, act to attenuate apoptosis (35, 36). Using drugs that induce DNA damage decreased levels of Mcl-1 and increased apoptosis in AML cells (36). Because IMMU-140 is capable of inducing significant damage to DNA in targeted cells through its SN-38 payload, including in AML, it is possible that an added benefit of IMMU-140 is to make targeted AML cells more sensitive

to the direct signaling activity mediated by HLA-DR. These possible signaling effects mediated by IMMU-140 in both solid tumors and AML are being further evaluated to better understand this dual-signaling effect in targeted cells.

At the highest dose of 25 mg/kg (HED = 2 mg/kg), IMMU-140 treatment was well tolerated, with no evidence of off-target toxicity in the form of weight loss. Clinically, other SN-38 ADCs made with this linker technology are administered at up to 10 mg/kg with neutropenia being the major adverse event, though this is manageable (14–17). Even in the AML disease model, IMMU-140 did not produce any obvious signs of toxicity, despite the fact that the mice were irradiated prior to AML engraftment. This is an important observation, given that relapsed/refractory AML patients undergo stem cell transplantation and are likewise severely immunocompromised to allow successful engraftment (37, 38). Finally, not only was IMMU-140 effective as a monotherapeutic, but combining it with doxorubicin in HL provided a significantly improved antitumor effect than monotherapy with either agent. This combination was also well tolerated, with no evidence of added toxicity, suggesting the prospect of such combinations clinically.

In summary, conjugating 6 to 8 SN-38 molecules via a cleavable linker to IMMU-114 to produce IMMU-140 does not alter its binding to HLA-DR–positive cells, while providing an added benefit of a dual therapeutic. This dual effect is through the direct antitumor activity mediated by the IMMU-114-HLA-DR–binding moiety (nonclassical apoptotic pathway) and the added cytotoxic effect of SN-38 delivery to the cells (intrinsic apoptotic pathway). Additionally, by utilizing an IgG_4 backbone to alleviate the infusion challenges previously observed with IgG_1 anti–HLA-DR antibodies (4), IMMU-140 should be well tolerated in patients, as is the IMMU-114 parental antibody (6, 9). IMMU-140 demonstrated higher potency than naked IMMU-114 preclinically in ALL, AML, CLL, and DLBCL, and an added, if not significant, survival benefit in experimental HL and MM. Likewise, in malignant melanoma, IMMU-140 provided a significant antitumor effect compared with IMMU-114. It was well tolerated, even in irradiated SCID mice, and did not add to the toxicity of doxorubicin or bortezomib therapy when combined. This dual therapeutic potential of IMMU-140 allows for the ability to treat a range of HLA-DR–positive hematopoietic and solid cancers, and therefore warrants clinical development.

Disclosure of Potential Conflicts of Interest

D.M. Goldenberg has ownership interest (including patents) in Immunomedics, Inc. No potential conflicts of interest were disclosed by the other authors.

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Writing, review, and/or revision of the manuscript: T.M. Cardillo, S.V. Govindan, C.-H. Chang, D.M. Goldenberg
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): T.M. Cardillo, S.V. Govindan, M.B. Zalath, D.M. Goldenberg
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