Milatuzumab-SN-38 conjugates for the treatment of CD74⁺ cancers

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Abstract

CD74 is an attractive target for antibody-drug conjugates (ADC), because it internalizes and recycles after antibody binding. CD74 mostly is associated with hematological tumors, but is expressed also in solid cancers. Therefore, ADCs of the humanized anti-CD74 antibody, milatuzumab, were examined for the therapy CD74-expressing solid tumors. Milatuzumab-doxorubicin, and two milatuzumab-SN-38 conjugates with cleavable linkers, differing in their stability in serum and how they release SN-38 in the lysosome, were prepared. CD74 expression was determined by flow cytometry and immunohistology. In vitro cytotoxicity and in vivo therapeutic studies were performed in the human cancer cell lines A-375 (melanoma), HuH-7 and Hep-G2 (hepatoma), Capan-1 (pancreatic), NCI-N87 (gastric), and Raji Burkitt lymphoma. The milatuzumab-SN-38 ADC was compared to SN-38 ADCs prepared with anti-Trop-2 and anti-CEACAM6 antibodies in xenografts expressing their target antigens. Milatuzumab-doxorubicin was most effective in the lymphoma model, while in A-375 and Capan-1 solid tumors, only milatuzumab-SN-38 showed a therapeutic benefit. Despite much lower surface expression of CD74 than Trop-2 or CEACAM6, milatuzumab-SN-38 had similar efficacy in Capan-1 as anti-Trop-2-SN-38, but in NCI-N87, anti-CEACAM6 and anti-Trop-2 conjugates were superior. Studies in 2 hepatoma lines at a single dose level showed significant benefit over saline controls, but not against an irrelevant IgG conjugate. CD74 is a suitable target for ADCs in some solid tumor xenografts, with efficacy largely influenced by uniformity of CD74 expression, and with SN-38 conjugates providing the best therapeutic responses; SN-38 conjugates were preferable in solid cancers, while doxorubicin ADC was better in lymphoma tested.
Introduction

CD74, referred to as invariant chain or Ii, is a type II transmembrane glycoprotein that associates with HLA-DR and inhibits the binding of antigenic peptides to the class II antigen presentation structure (1-3). It serves as a chaperone molecule, directing the invariant chain complexes to endosomes and lysosomes, an accessory molecule in the maturation of B cells, using a pathway mediated by NF-κB (4), and in T-cell responses via interactions with CD44 (5). It is also a receptor for the pro-inflammatory cytokine, macrophage migration inhibitory factor (6), which is involved in activating cell proliferation and survival pathways.

In normal human tissues, CD74 is primarily expressed in B cells, monocytes, macrophages, dendritic cells, Langerhans cells, subsets of activated T cells, and thymic epithelium (data on file, Immunomedics, Inc.), and it is expressed in over 90% of B-cell tumors (7, 8). Early studies had conflicting data on whether CD74 is present on the membrane, in part because the antibodies to the invariant chain were specific for the cytoplasmic portion of the molecule (9), but also because there are relatively few copies on the surface, and its half-life on the cell surface is very short. Approximately 80% of the CD74 on the cell surface is associated with the MHC II antigen, HLA-DR (3). Using the murine anti-CD74 antibody, LL1, the Raji Burkitt lymphoma cell line was estimated to have 4.8 x 10⁴ copies/cell, but because of rapid intracellular transit, ~8 x10⁶ antibody molecules were internalized and catabolized per cell per day (10). Thus, CD74 internalization is highly dynamic, with the antibody being moved quickly from the surface and unloaded inside the cell, followed by CD74 re-expression on the surface. Fab’ internalization occurs just as rapidly as IgG binding, indicating that bivalent binding is not required (3, 10). Later studies with a CDR-grafted version of murine LL1, milatuzumab (hLL1), found that the antibody could alter B-cell proliferation, migration, and adhesion molecule
expression (8, 11, 12), but the exceptional internalization properties of the anti-CD74 antibody made it an efficient carrier for the intracellular delivery of cancer therapeutics (13-16). Based on preclinical efficacy and toxicology results, Phase I clinical trials with milatuzumab in multiple myeloma (17), as well as milatuzumab-doxorubicin in multiple myeloma, non-Hodgkin lymphoma, and chronic lymphocytic leukemia, have been initiated.

Interestingly, CD74 also is expressed in non-hematopoietic cancers, such as gastric, renal, urinary bladder, non-small cell lung cancers, certain sarcomas, and glioblastoma (18-25), and therefore it may be a therapeutic target for solid tumors expressing this antigen. Since a milatuzumab-doxorubicin conjugate was highly active in models of hematological tumors (13, 14), it was a logical choice for this assessment. However, we developed procedures for coupling the highly potent topoisomerase I inhibitor, SN-38, to antibodies (26, 27). SN-38 is the active form of irinotecan, whose pharmacology and metabolism are well known (28, 29). These conjugates have nanomolar potency in solid tumor cell lines, and were found to be active with antibodies that were not actively internalized (26, 30). Prior studies indicated a preference for a linker (CL2A) that allowed SN-38 to dissociate from the conjugate in serum with a half-life of ~1 day, rather than other linkers that were either more or less stable in serum. However, given milatuzumab’s exceptional internalization capability, a new linker that is highly stable in serum, but can release SN-38 when taken into the lysosome, was developed (31).

The current investigation examines the prospects for using these three milatuzumab anti-CD74 conjugates, one with doxorubicin, and two as SN-38 conjugates, for effective therapy primarily against solid tumors.
Materials and Methods

Human tumor cell lines

Raji Burkitt lymphoma, A-375 (melanoma), Capan-1 (pancreatic adenocarcinoma), NCI-N87 (gastric carcinoma), Hep-G2 hepatoma and MC/CAR myeloma cell lines were purchased from American Tissue Culture Collection (Manassas, VA). HuH-7 hepatoma cell line was purchased from Japan Health Science Research Resources Bank (Osaka, Japan). All cell lines were cultured in a humidified CO₂ incubator (5%) at 37 °C in recommended media containing 10% to 20% fetal-calf serum and supplements. Cells were passaged <50 times and checked regularly for mycoplasma.

Antibodies and conjugation methods

Milatuzumab (anti-CD74 MAb) (8, 10, 11, 32), epratuzumab (anti-CD22) (33), veltuzumab (anti-CD20) (34, 35), labetuzumab (anti-CEACAM5) (36), hMN15 anti-CEACAM6 (37), and hRS7 anti-Trop-2 (30) are humanized IgG₁ monoclonal antibodies. CL2A and CL2E linkers and their SN-38 derivatives were prepared and conjugated to antibodies as reported earlier (30, 31), as were the milatuzumab-doxorubicin conjugates (13, 14). All conjugates were prepared by disulfide reduction of the IgG, followed by reaction with the corresponding maleimide derivatives of these linkers (Fig. 1). Spectrophotometric analyses estimated the drug:IgG molar substitution ratio was 5-7 (1.0 mg of the protein contains ~16 μg of SN-38 or 25 μg of doxorubicin equivalent).
In vitro cell binding and cytotoxicity

Assays used to compare cell binding of the unconjugated and conjugated milatuzumab to antigen-positive cells, and cytotoxicity testing using the MTS dye reduction method (Promega, Madison, WI), have been reported previously (27).

Flow cytometry and immunohistology

Flow cytometry was performed using the primary humanized monoclonal antibodies listed earlier, which were then revealed with a goat anti-human IgG-FITC conjugate (see Supplementary Materials). Cells from culture were processed in a manner that provided an assessment of only membrane-bound or membrane and cytoplasmic antigen (Supplementary Materials). Immunohistology was performed on formalin-fixed, paraffin-embedded sections of subcutaneous tumor xenografts, staining without antigen retrieval methods, using the primary humanized monoclonal antibodies listed earlier (except hRS7 anti-Trop-2), at 10 μg/mL, that were revealed with an anti-human IgG conjugate (19). Trop-2 expression was assessed using a goat polyclonal antibody, as indicated in Supplementary Fig. S1.

SN-38 release from linker and in vitro serum stability of SN-38 conjugates

The stability of SN-38 conjugates under acidic conditions (pH 5.0) with or without cathepsin B, and in serum, are provided in the Supplementary Materials.

In vivo studies

All studies were performed in accordance with institutional-approved animal welfare protocols. Female nude mice (4-8 weeks old) or female SCID mice (7 weeks old) were purchased from Taconic (Germantown, NY) and used after a 1-week quarantine. Animals
bearing solid tumor xenografts were treated intraperitoneally with test and control articles twice-weekly for 4 weeks. Specific doses are given in Results. Toxicity was assessed by weekly weight measurements.

For the Raji Burkitt lymphoma model, SCID mice were injected intravenously with 2.5×10^6 Raji cells in 0.1 mL media. Five days later, animals received a single intravenous injection (0.1 mL) of the conjugate or saline (N = 10/group). Mice were observed daily for signs of distress and paralysis, and were euthanized when either hind-limb paralysis developed, >15% loss of initial weight, or if otherwise moribund (surrogate survival endpoints).

Subcutaneous tumors were measured by caliper in two dimensions, and the tumor volume (TV) calculated as \( L \times w^2/2 \), where \( L \) is the longest diameter and \( w \) is the shortest. Measurements were made at least once weekly, with animals terminated when tumors grew to 1.0 cm^3 (i.e., surrogate survival end-point). The A-375 melanoma cell line (6 x 10^6 cells in 0.2 mL) was implanted in nude mice and therapy was initiated when tumors averaged 0.23 ± 0.06 cm^3 (N = 8/group). Capan-1 was implanted subcutaneously in nude mice using a combination of tumor suspension from serially-passaged tumors (0.3 mL of a 15% w/v tumor suspension) combined with 8×10^6 cells from tissue culture. Treatments were initiated when TV averaged 0.27 ± 0.05 cm^3 (N = 10/group). NCI-N87 gastric tumor xenografts were initiated by injecting 0.2 mL of a 1:1 (v/v) mixture of matrigel and 1×10^7 cells from terminal culture subcutaneously. Therapy was started when the TV averaged 0.249 ± 0.045 cm^3 (N = 7/group). The same procedure was followed for developing the Hep-G2 and HuH-7 hepatoma xenografts in nude mice. Therapy was started when Hep-G2 averaged 0.364 ± 0.062 cm^3 (N = 5/group) and HuH-7 averaged 0.298 ± 0.055 cm^3 (N = 5/group).
Efficacy is expressed in Kaplan-Meier survival curves, using the surrogate end-points mentioned above for determining the median survival times. Analysis was performed by a log-rank (Mantel-Cox) test using Prism GraphPad software (LaJolla, CA), with significance at $P < 0.05$.

**Results**

**CD74 expression in human tumor cell lines and xenografts**

Surface CD74 expression in Raji was 19.6-fold higher than background staining, whereas in most solid tumor the MFI of membrane-only CD74 was often $\leq 2$-fold higher than the background MFI (**Table 1**). Therefore, identification of CD74-positivity for solid tumors was made primarily in permeabilized cells. In permeabilized cells, the MFI for 6 solid tumor cell lines was similar to Raji.

Immunohistology showed Raji subcutaneous xenografts had a largely uniform and intense staining, with prominent cell surface labeling (**Fig. 2A**). The Hep-G2 hepatoma cell line had the most uniform uptake of the solid tumors, with moderately strong, but predominantly cytoplasmic, staining (**Fig. 2D**), followed by the A-375 melanoma cell line that had somewhat less uniform staining with more intense, yet mostly cytoplasmic, expression (**Fig. 2B**). The Capan-1 pancreatic (**Fig. 2C**) and NCI-N87 (**Fig. 2F**) gastric carcinoma cell lines had moderate (Capan-1) to intense (NCI-N87) CD74 staining, but it was not uniformly distributed. The HuH-7 hepatoma cell line (**Fig. 2E**) had the least uniform and the weakest staining.
Immunoreactivity of the conjugates

$K_d$ values for unconjugated milatuzumab, milatuzumab-CL2A- and CL2E-SN-38 conjugates were not significantly different, averaging 0.77 nM, 0.59 nM, and 0.80 nM, respectively. $K_d$ values for the unconjugated and doxorubicin-conjugated milatuzumab measured in the MC/CAR multiple myeloma cell line were 0.5 ± 0.02 nM and 0.8 ± 0.2 nM, respectively (14).

In vitro drug release and serum stability of conjugates

Fig. 3A illustrates the release mechanisms of SN-38 from the mercaptoethanol-capped CL2A and CL2E linkers in an environment, partially simulating lysosomal conditions; namely, low pH (pH 5.0), and in the presence or absence of cathepsin B. The CL2E-SN-38 substrate was inert at pH 5 in the absence of the enzyme (Supplementary Table S1), but in the presence of cathepsin B, cleavage at the Phe-Lys site proceeded quickly, with a half-life of 34 min (Fig. 3A, left panel). The formation of active SN-38 requires intra-molecular cyclization at the carbamate bond at the 10th position of SN-38, which occurred more slowly, with a half-life of 10.7 h (Fig. 3A, left panel; additional data in Supplementary Table S1 and Supplementary Fig. S2).

As expected, cathepsin B had no effect on the release of active SN-38 in the CL2A linker. However, CL2A has a cleavable benzyl carbonate bond, releasing active SN-38 at a rate similar to the CL2E linker at pH 5.0, with a half-life of ~ 10.2 h (Fig. 3B, right panel; additional data Supplementary Table S2). The milatuzumab-doxorubicin conjugate, which has a pH-sensitive acylhydrazone bond, had a half-life of 7 to 8 h at pH 5.0 (Supplementary Fig. S3A and Supplementary Table S3).

While all of these linkers release the drug at relatively similar rates under lysosomally-relevant conditions, they have very different stability in serum. Milatuzumab-CL2A-SN-38
released 50% of free SN-38 in 21.55 ± 0.17 h (Fig. 3C), consistent with other CL2A-SN-38 conjugates (30, 31). The CL2E-SN-38 conjugate, however, was highly inert, with a half-life extrapolated to ~2100 h. The milatuzumab-doxorubicin conjugate released 50% of the doxorubicin in 98 h, which was similar to 2 other antibody-doxorubicin conjugates (Supplementary Fig. S3B and Supplementary Table S3).

Cytotoxicity

A significant issue related to the evaluation of these conjugates was the relative potency of free doxorubicin and SN-38 in hematopoietic and solid tumor cell lines. Our group reported previously that SN-38 was active in several B-cell lymphoma and acute leukemia cell lines, with potencies ranging from 0.13 to 2.28 nM (31). SN-38 potency in 4 of the solid tumor cell lines that were later used for in vivo therapy studies ranged from 2.0 to 6 nM (Table 2). Doxorubicin had a mixed response, with 3-4 nM potency in the Raji lymphoma and the A-375 melanoma cell lines, but it was nearly 10 times less potent than SN-38 against Capan-1, NCI-N87, and Hep G2 cell lines. Other studies comparing the potency of SN-38 to doxorubicin found 2 additional cell lines with similar potency for both drugs: LS174T colon cancer, 18 vs. 18 (nM potency of SN-38 vs. doxorubicin, respectively) and MDA-MB-231 breast cancer, 2 vs. 2 nM. In 4 other cell lines, SN-38 was 5-20-fold more potent than doxorubicin: SK-OV-4 ovarian cancer: 18 vs. 90 nM; Calu-3 lung adenocarcinoma, 32 vs. 582 nM; Capan-2 pancreatic cancer, 37 vs. 221 nM; and NCI-H466 small cell lung cancer, 0.1 vs. 2 nM. Collectively, these data suggest that doxorubicin may be less effective against solid tumors than SN-38, while SN-38 appears to be equally effective in solid and hematopoietic tumors.

As expected, the 3 conjugate forms were often some order of magnitude less potent than the free drug in vitro, since both drugs are expected to be transported readily into the cells, while
drug conjugates require antibody binding to transport drug inside the cell, and with the solid tumor cell lines having such low surface expression, this was expected (Table 2). The CL2A-linked SN-38 conjugate is an exception, since more than 90% of the SN-38 is released from the conjugate into the media over the 4-day assay period (30, 31). Thus, even if this conjugate was internalized rapidly, it would be difficult to discern differences between the free drug and the CL2A-linked drug.

The stable CL2E-linked SN-38 performed well in the Raji cell line, compared to free SN-38, but it had substantially (7- to 16-fold) lower potency in the 4 solid tumor cell lines, suggesting the relatively low surface expression of CD74 may be playing a role in minimizing drug transport in these solid tumors. The milatuzumab-doxorubicin conjugate had substantial differences in its potency when compared to the free doxorubicin in all cell lines, which was of similar magnitude as the CL2E-SN-38 conjugates to free SN-38 in the solid tumor cell lines.

In the 6 additional cell lines mentioned above, the milatuzumab-CL2A-SN-38 conjugate was 9- to 60-times more potent than the milatuzumab-doxorubicin conjugate (not shown), but again, this result was influenced largely by the fact that the CL2A-linked conjugate releases most of its SN-38 into the media over the 4-day incubation period, whereas the doxorubicin conjugate would at most release 50% of its drug over this same time. The CL2E-linked milatuzumab was not examined in these other cell lines.

In vivo therapy of human tumor xenografts

Previous in vivo studies with the milatuzumab-doxorubicin or -SN-38 conjugates prepared with various antibodies had indicated they were efficacious at doses far lower than their maximum tolerated dose (13, 14, 26, 30, 31), and thus in vivo testing focused on comparing similar, but fixed, amounts of each conjugate at levels that were tolerated. For example, weight
loss never exceeded 10% of the starting weight, except in the IV Raji model, where weight loss was often indicative of progressive disease, not toxicity. It also is important to note that milatuzumab does not bind to murine blood cells (i.e., murine CD74).

Initial studies first examined the doxorubicin and SN-38 conjugates in a disseminated Raji model of lymphoma in order to gauge how the milatuzumab-doxorubicin conjugate compared to the 2 SN-38 conjugates (Fig. 4A). All specific conjugates were significantly better than non-targeting labetuzumab-SN-38 conjugate or saline-treated animals, which had a median survival of only 20 days ($P<0.0001$). Despite in vitro studies indicating as much as an 8-fold advantage for the SN-38 conjugates in Raji, the best survival was seen with the milatuzumab-doxorubicin conjugate, where all animals given a single 17.5 mg/kg (350 μg) dose and 7/10 animals given 2.0 mg/kg (40 μg) were alive at the conclusion of the study (i.e., 17.5 mg/kg dose milatuzumab-doxorubicin vs. milatuzumab-CL2A-SN-38 with median survival >112 days vs. 78.5 days, respectively; $P = 0.0012$). Survival was significantly lower for the more stable CL2E-SN-38 conjugate than with the CL2A-SN-38 conjugate ($P<0.0001$ and $P = 0.0197$, 17.5 and 2.0 mg/kg doses, respectively, for the CL2A vs. CL2E), even though in vitro studies suggested that both conjugates would release active SN-38 at similar rates when internalized.

Five solid tumor cell lines were examined, starting with the A-375 melanoma cell line, since it had the best in vitro response to both doxorubicin and SN-38. A-375 xenografts grew rapidly (tumor sizes in individual animals shown in Fig. 4D), with saline-treated control animals having a median survival of only 10.5 days (Fig. 4B). A 12.5 mg/kg (0.25 mg per animal) twice-weekly dose of the milatuzumab-CL2A-SN-38 conjugate extended survival to 28 days ($P = 0.0006$), which was significantly better than the control epratuzumab-CL2A-SN-38 conjugate having a median survival of 17.5 days ($P = 0.0089$), with the latter not being significantly
different from the saline-treated animals ($P = 0.1967$). The milatuzumab-CL2A conjugate provided significantly longer survival than the milatuzumab-CL2E-SN-38 conjugate ($P = 0.0014$), which had the same median survival of 14 days as its control epratuzumab-CL2E-SN-38 conjugate. Despite giving a 2-fold higher dose of the milatuzumab-doxorubicin than the SN-38 conjugates, the median survival was no better than the saline-treated animals (10.5 days).

As with the A-375 melanoma model, only the CL2A-linked SN-38 conjugate was effective in Capan-1, with a median survival of 35 days, significantly different from untreated animals ($P < 0.036$) (Fig. 4C), even at a lower dose (5 mg/kg;100 μg per animal) ($P<0.02$). Neither the milatuzumab-CL2E nor the non-targeting epratuzumab-CL2A-SN-38 conjugates, or a 2-fold higher dose of the milatuzumab-doxorubicin conjugate, provided any survival advantage ($P = 0.44$ vs. saline). It is noteworthy that in the same study with animals given the same dose of the internalizing anti-Trop-2 CL2A-SN-38 conjugate (hRS7-SN-38; IMMU-132), the median survival was equal to milatuzumab-CL2A-SN-38 (Fig. 5A). The hRS7-CL2A-SN-38 conjugate had been identified previously as an ADC of interest for treating a variety of solid tumors (30). The MFI for surface-binding hRS7 on Capan-1 was 237, compared to 22 for milatuzumab (see Table 1). Thus, despite having a substantially lower surface antigen expression, the milatuzumab-CL2A-SN-38 conjugate performed as well as the hRS7-CL2A-SN-38 conjugate in this model.

With the milatuzumab-doxorubicin conjugate having inferior therapeutic results in 2 of the solid tumor xenografts, the focus shifted to comparing the milatuzumab-SN-38 conjugates to SN-38 conjugates prepared with other humanized antibodies against Trop-2 (hRS7) or CEACAM6 (hMN-15), which are more highly expressed on the surface of many solid tumors (38, 39). Three additional xenograft models were examined.
In the gastric tumor model, NCI-N87, animals given 17.5 mg/kg/dose (350 μg) of milatuzumab-CL2A-SN-38 provided some improvement in survival, but it failed to meet statistical significance compared to the saline-treated animals (31 vs. 14 days; \( P = 0.0760 \)) or to the non-binding veltuzumab anti-CD20-CL2A-SN39 conjugate (21 days; \( P = 0.3128 \)) (Fig. 5B). However, the hRS7- and hMN-15-CL2A conjugates significantly improved the median survival to 66 and 63 days, respectively (\( P = 0.0001 \)). The MFI for surface-expressed Trop-2 and CEACAM6 were 795 and 1123, respectively, much higher than CD74 that was just 5 (see Table 1). Immunohistology showed a relatively intense cytoplasmic expression of CD74 in the xenograft of this cell line, but importantly it was scattered, appearing only in defined pockets within the tumor (Fig. 2F). CEACAM6 and Trop-2 were more uniformly expressed than CD74 (Supplementary Fig. S1 D and E, respectively), with CEACAM6 being more intensely present both cytoplasmically and on the membrane, and Trop-2 primarily found on the membrane. Thus, the improved survival with the anti-CEACAM6 and anti-Trop-2 conjugates most likely reflects higher antigen density and more uniform expression in NCI-N87.

In the Hep-G2 hepatoma cell line, immunohistology showed a very uniform expression with moderate cytoplasmic staining of CD74 (Fig. 2D), and flow cytometry indicated a relatively low surface expression (MFI = 9). The MFI with hMN-15 was 175 and immunohistology showed a fairly uniform membrane and cytoplasmic expression of CEACAM6, with isolated pockets of very intense membrane staining (Supplementary Fig. S1B). A study in animals bearing Hep-G2 xenografts found the milatuzumab-CL2A-SN-38 extended survival to 45 days compared to 21 days in the saline-treated group (\( P = 0.0048 \)), while the hMN-15-CL2A-SN-38 conjugate improved survival to 35 days (Fig. 5C). There was a trend favoring the milatuzumab conjugate over hMN-15-CL2A-SN-38, but it did not achieve statistical significance (46 vs. 35
days; \( P = 0.0802 \)). However, the non-binding veltuzumab-CL2A-SN-38 conjugate provided a similar survival advantage as the milatuzumab conjugate. We previously observed that therapeutic results with non-binding conjugates could be similar to the specific CL2A-linked conjugate, particularly at higher protein doses (30), but titration of the specific and control conjugates usually revealed selectively. Thus, neither of the specific conjugates provided a selective therapeutic advantage at these doses in this cell line.

Another study using the HuH-7 hepatoma cell line, which had similar surface expression, but slightly lower cytoplasmic levels as Hep-G2 (see Table 1), found the hMN-15-SN-38 conjugate providing a longer (35 vs. 18 days), albeit not significantly different, survival advantage than the milatuzumab-CL2A conjugate \( (P = 0.2944) \) (Fig. 5D). While both the hMN-15 and milatuzumab conjugates were significantly better than the saline-treated animals \( (P = 0.008 \) and 0.009, respectively), again neither conjugate was significantly different from the non-targeted veltuzumab-SN-38 conjugate at this dose level \( (P = 0.4602 \) and 0.9033, respectively). CEACAM6 surface expression was relatively low in this cell line (MFI = 81), and immunohistology showed that both CD74 (Fig. 2E) and CEACAM6 (Supplementary Fig. S1C) were very faint and highly scattered.

Discussion
ADCs have been of considerable research interest for many years, but only recently peaked, primarily due to the clinical success of 2 conjugates prepared with so-called “supertoxic” agents that have subnanomolar potency, which replaced many of the earlier ADCs prepared using chemotherapeutic agents that had potencies in the nanomolar levels (40-46). However,
drug potency or even its specific mechanism of action is not the only defining property that affords optimal performance of an ADC.

CD74 is expressed at relatively low levels on the cell surface (2, 3, 10), but its unique internalization and surface re-expression allows milatuzumab anti-CD74 ADCs to be effective in hematopoietic cancer xenograft models, even with a moderately toxic drug, such as doxorubicin (13, 14). This conjugate is currently being studied clinically in patients with hematopoietic cancers (NCT01101594 and NCT01585688), but with evidence that CD74 is expressed on several types of solid tumors, additional preclinical studies were initiated to assess its potential utility in these cancers. Additionally, since SN-38 and other camptothecins are used to treat solid tumors, the utility of milatuzumab-SN-38 conjugates was assessed as well. Promising efficacy has been seen with SN-38 conjugates prepared with several antibodies against other antigens expressed in solid and hematological tumor models (13, 14, 30, 31, 47), and this has led to the development of 2 new SN-38 conjugates being pursued in Phase I clinical trials of colorectal and diverse epithelial cancers (NCT01270698 and NCT01631552).

*In vitro* studies revealed unconjugated doxorubicin and SN-38 had similar potency in the Raji lymphoma cell line, but SN-38 was more potent in several of the solid tumor cell lines, suggesting SN-38 was potentially preferred for solid tumors. Despite the similarities in potency of the free drugs against Raji *in vitro*, the milatuzumab-doxorubicin conjugate provided a significantly better response in mice bearing Raji xenografts than the milatuzumab-SN-38 conjugates. In contrast, even though *in vitro* testing had indicated that A-375 melanoma was equally sensitive to free doxorubicin as to free SN-38, when tested *in vivo*, milatuzumab-doxorubicin was less effective than the CL2A-linked SN-38 milatuzumab conjugate in A-375 as well as in xenografts of Capan-1 human pancreatic cancer. These results and the *in vitro* studies
showing unconjugated SN-38 had a 5- to 20-fold higher potency than doxorubicin in more solid tumor cell lines led to our decision to abandon further evaluation of the doxorubicin conjugate for solid tumor therapy. However, in order to gauge the utility of the milatuzumab-SN-38 conjugates, we performed additional comparative assessments to antibody-SN-38 conjugates against other antigens present in a variety of solid tumors.

The internalizing hRS7 anti-Trop-2 CL2A-linked SN-38 conjugate was evaluated previously in the Capan-1 cell line (30), and therefore the efficacy of milatuzumab and hRS7 SN-38 conjugates was examined. Milatuzumab and hRS7 CL2A-linked SN-38 conjugates had similar median survivals that were significantly higher than with control conjugates, and better than their respective CL2E-linked conjugates. Flow cytometry had indicated Trop-2 expression was ~10-fold higher than CD74 in Capan-1, which suggested that the transport capabilities of CD74, which were known to be exceptional (10), were more efficient than Trop-2. However, it is well known that other factors, such as antigen accessibility (i.e., membrane vs. cytoplasm, physiological, and “binding-site” barriers) and distribution among cells within a tumor are critical factors influencing every form of targeted therapy, particularly those that depend on adequate intracellular delivery of a product to individual cells (48). For example, the binding-site barrier could potentially impede tumor penetration when antigen expression is high. However, if the payload could be released from the conjugate after localizing in the tumor, such as with the CL2A-linked conjugates, the drug could diffuse to non-targeted bystander cells, thereby enhancing its efficacy range. This mechanism also is thought to aid the efficacy of other CL2A-SN-38 conjugates that we examined using poorly internalizing antibodies, such as anti-CEACAM5 (26) and the anti-CEACAM6 used herein. Conjugates based on milatuzumab rely more on the antibody’s direct interaction with the tumor cell, taking advantage of CD74’s rapid
internalization and re-expression that can compensate for its lower abundance on the surface of cells. Naturally, this advantage would be reduced when CD74 is highly scattered within the tumor, and without direct binding to the tumor antigen to encourage retention, the benefit of the drug’s slow release from the conjugate would be lost. These observations suggest a pre-assessment of the distribution of CD74 within solid tumors may be required before selecting a CD74-targeted agent. A previous review of human gastrointestinal tumors by our group suggests that they often have a high level of expression with good uniformity (19).

We previously evaluated a ‘CL2E-like’ linker that was coupled at the 20-hydroxyl position of SN-38, similar to the CL2A linker, but that antibody conjugate lacked sufficient antitumor activity and was not pursued (unpublished data). Given the exceptional internalization properties of milatuzumab, we revisited the SN-38-linker chemistry, hypothesizing that a more serum-stable linker might be preferred with such a rapid internalizing antibody. In order to release SN-38 in an active form, we surmised that if the leaving group was phenolic, this could promote cyclization, and therefore the CL2E-linker was designed to join at the phenolic 10-position of SN-38. We included a cathepsin B cleavage site in the CL2E linker to cleave the monocarbamate derivative of SN-38 and N,N’-dimethylethylenediamine from the antibody-linker, but for the SN-38 to be active, cyclization was still required. In vitro studies proved the CL2E-linked SN-38 was highly stable in serum, but under lysosomal conditions (pH 5.0 and in the presence of cathepsin B), active SN-38 was released with a half-life of ~ 11 h, similar to the release rate measured for CL2A-linked SN-38 at lysosomal pH (i.e., pH 5.0).

The CL2E-linked SN-38 conjugate had a similar IC$_{50}$ as the CL2A conjugate in the Raji cell line, which was consistent with the view that if rapidly internalized, both conjugates would release the active form of SN-38 at approximately the same rate. However, as already
mentioned, the *in vitro* activity of the CL2A conjugate is influenced largely by the release of SN-38 into the media, and does not necessarily reflect uptake by the intact conjugate, whereas cytotoxicity of the CL2E-linked conjugate reflected internalization of the intact conjugate (presumably by selective binding to CD74). When the CL2E-linked conjugate was found to be much less potent in the solid tumor cell lines than the CL2A conjugate, this suggested that the lower surface expression of CD74 on the solid tumor cell lines reduced the internalization of SN-38 via milatuzumab binding. However, when *in vivo* studies in Raji also showed the milatuzumab-CL2A-SN-38 was superior to the CL2E conjugate, other factors had to be affecting CL2E-based conjugate’s efficacy.

One possibility was that the linker design in CL2E-SN-38 left the 20-position of the drug underivatized, rendering the lactone group susceptible to ring-opening. Studies with irinotecan have shown the carboxylate form of SN-38 is only 10% as potent as the lactone form (49). The CL2A-linked SN-38 is derivatized at the 20-hydroxyl position, a process that stabilizes the lactone group in camptothecins under physiological conditions (50). Since the *in vitro* stability studies and the analysis of serum stability were performed under acidic conditions, we do not have a direct measure of the carboxylate form of SN-38 in either of these conjugates, but it is reasonable to suspect that destabilization of the lactone ring could have contributed to CL2E’s diminished efficacy *in vivo*. Another explanation for the different activity of the CL2A- and CL2E-linked SN-38 conjugates may be related to the multiple roles that CD74 plays in cell biology. For example, in antigen-presenting cells, it may have a more dominant role in processing antigenic peptides, where is solid tumors, its role might be related more to survival. This could affect intracellular trafficking and processing, thereby affecting the conjugate’s potency.
In conclusion, \textit{in vitro} and \textit{in vivo} results indicate that the milatuzumab-doxorubicin conjugate is superior to the CL2A-SN-38 conjugate in the Raji lymphoma cell line, which may reflect the improved serum stability of the doxorubicin conjugate compared to the CL2A-linked SN-38. The serum-stable CL2E-linked SN-38 conjugate was again found to be inferior to the less stable CL2A-linked SN-38 (31), and therefore it appears that at least with SN-38, a linker that allows the drug to be released in serum (half-life ~1 day) is preferred. Finally, antigen accessibility appears to have a dominant role in defining milatuzumab-CL2A-SN-38’s potency when measured against conjugates prepared with other internalizing (hRS7) or poorly internalizing antibodies (hMN15) that were more accessible (surface expressed) and abundant. We suspect this finding is universal for targeted therapies, but these studies have at least shown that the unique internalization properties of a CD74-targeted agent can provide significant efficacy even when surface expression of the target antigen is minimal.

\textbf{Acknowledgement}

We thank Dr. Jennifer Pickett for performing \textit{in vitro} stability studies on the milatuzumab-doxorubicin conjugate, and Mr. R. Arrojo, Ms. A. Nair, and Ms. N. Sathyanarayan for expert technical assistance. This work was presented in part at the 2012 ASCO Annual Meeting [J Clin Oncol 30, 2012 (suppl; abstr 3091)].
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47. Sharkey RM, Karacay H, Govindan SV, Goldenberg DM. Combination radioimmunotherapy and chemoimmunotherapy involving different or the same targets improves therapy of human pancreatic carcinoma xenograft models. Mol Cancer Ther 2011;10:1072-81.


Table 1. CD74 expression by flow cytometry expressed as mean fluorescent intensity (MFI) of milatuzumab-positive gated cells.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Surface</th>
<th>Surface and cytoplasmic</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>hLL1 (bkgd)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>MFI Ratio hLL1:bkgd</td>
</tr>
<tr>
<td>Panc CA&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Capan-1</td>
<td>22 (12)</td>
</tr>
<tr>
<td>Gastric</td>
<td>Hs746T</td>
<td>17 (8)</td>
</tr>
<tr>
<td></td>
<td>NCI-N87</td>
<td>5 (4)</td>
</tr>
<tr>
<td>Melanoma</td>
<td>A-375</td>
<td>16 (3)</td>
</tr>
<tr>
<td>Hepatoma</td>
<td>Hep-G2</td>
<td>9 (6)</td>
</tr>
<tr>
<td></td>
<td>HuH-7</td>
<td>8 (5)</td>
</tr>
<tr>
<td>Lymphoma</td>
<td>Raji</td>
<td>59 (3)</td>
</tr>
</tbody>
</table>

<sup>a</sup>Background MFI of cells incubated with GAH-FITC only.
Table 2. *In vitro* cytotoxicity in human cancer cell lines.

<table>
<thead>
<tr>
<th>Drug or conjugate</th>
<th>Melanoma (A-375)</th>
<th>Pancreatic (Capan-1)</th>
<th>Gastric (NCI-N87)</th>
<th>Hepatic (Hep G2)</th>
<th>NHL (Raji)</th>
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</thead>
<tbody>
<tr>
<td>SN-38</td>
<td>2</td>
<td>6</td>
<td>6</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>Milatuzumab-SN-38</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CL2A linker(^a)</td>
<td>5</td>
<td>13</td>
<td>15</td>
<td>8</td>
<td>2</td>
</tr>
<tr>
<td>CL2E linker(^a)</td>
<td>34</td>
<td>210</td>
<td>130</td>
<td>78</td>
<td>4</td>
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<tr>
<td>Doxorubicin</td>
<td>3</td>
<td>43</td>
<td>29</td>
<td>46</td>
<td>4</td>
</tr>
<tr>
<td>Milatuzumab-doxorubicin(^a)</td>
<td>29</td>
<td>540</td>
<td>280</td>
<td>628</td>
<td>32</td>
</tr>
</tbody>
</table>

\(^a\) Drug/IgG mole ratio: CL2A, 6.5; CL2E, 6.6; doxorubicin, 7.3
FIGURE LEGENDS

Fig 1. Schematic representation of the CL2A- and CL2E-SN-38 linkers and the doxorubicin linker used to prepare the antibody conjugates.

Fig 2. CD74 expression in specimens of several human cancers grown as subcutaneous xenografts in athymic SCID nude mice: (A) Raji Burkitt lymphoma, (B) A-375 melanoma, (C) Capan1 pancreatic carcinoma, (D) Hep-G2 hepatoma, (E) HuH-7 hepatoma, and (F) NCI-N87 gastric carcinoma. Scale bar in (A) corresponds to 200 μm (50 μm the higher magnification insets). A non-binding, isotype matched antibody showed no evidence of staining (shown only for the Raji xenograft, upper right insert in “A”; scale 200 μm).

Fig 3. Cleavage of ME-capped CL2A-SN-38 and CL2E-SN-38 derivatives with or without cathepsin-B at pH 5, and conjugates in human serum in vitro. (A) The liberation of SN-38 from CL2E linker is a 2-step process, while being a 1-step process for CL2A linker; (B) drug release kinetics for ME-capped CL2E-SN-38 (left) and CL2A-SN-38 (right); (C) in vitro stability of antibody conjugates in human serum at 37 °C.

Fig 4. Comparing milatuzumab-SN-38 and doxorubicin ADCs in 3 human tumor xenograft models. (A) Disseminated model of lymphoma (Raji) (10/group) was given a single IV dose of the agents listed 5 days after intravenous injection of tumor cells. (B) subcutaneous A-375 melanoma xenografts (8/group) and (C) Capan-1 pancreatic adenocarcinoma xenografts (10/group) using Mmab (milatuzumab) conjugates given intraperitoneally on the days indicated. (D) Individual animal data from the A375 study in panel B, showing tumor size progression.
(dashed lines), plotted with the average ± SEM (solid line). Dotted horizontal line at 1.0 cm³ marks the time when animals were removed from study due to tumor progression (i.e., survival time). Other abbreviations include: Lmab, labetuzumab humanized anti-CEACAM5 IgG and Emab, epratuzumab, humanized anti-CD22 IgG.

**Fig. 5. Therapeutic efficacy of antibody-SN-38 conjugates in various tumor models.** (A) hRS7 anti-Trop-2 conjugates given intraperitoneally twice-weekly for 4 weeks in nude mice bearing subcutaneous Capan-1 human pancreatic cancer xenografts (N = 10/group); (B) animal bearing subcutaneous NCI-N87 gastric carcinoma xenografts (7/group) treated with CL2A-SN-38 conjugates prepared with milatuzumab, hRS7 or hMN15 anti-CEACAM6 IgG; (F) Hep-G2 and (G) HuH-7 human hepatoma xenografts (5/group) treated with milatuzumab or hMN15-CL2A-SN-38 conjugates (Vmab, veltuzumab humanized anti-CD20 IgG).
**Figure 1**

**A** CL2A-SN-38:

O-{2-[4-{4-(N-Maleimidomethyl)cyclohexane-1-carboxamidomethyl]-1,2,3-triazolyl}ethyl}-O'-{[(N-diglycolyl-2-aminoethyl)-lysyl-\(\rho\) aminobenzoxycarbonyl-20-O-SN-38]heptaethyleneglycol

**B** CL2E-SN-38:

O-{2-[4-{4-(N-Maleimidomethyl)cyclohexane-1-carboxamidomethyl]-1,2,3-triazolyl}ethyl}-O'-{[(N-diglycolyl-2-aminoethyl)-phenylalanyl-lysyl-\(\rho\) aminobenzoxycarbonyl-[N-methyl-N-[2-(N-methyl-N-(carbonyl-10-O-SN-38))amino]ethylamino}]heptaethyleneglycol

**C** Doxorubicin-MCC hydrazone
Figure 3

A

Cathepsin-B
(t1/2 = 34 min)

ME-CL2E-SN-38

buffer control
(does not release SN-38)

ME-CL2A-SN-38

Cathepsin-B
(t1/2 = 9.3 h);
buffer control
(t1/2 = 10.2 h)

SN-38

B

ME-capped CL2E-SN-38

\[\text{% SN-38 vs. Hours}\]

- Cathepsin B cleavage (t1/2 = 34 min)
- SN-38 release (t1/2 = 10.7 h)

ME-capped CL2A-SN-38

\[\text{% SN-38 vs. Hours}\]

- Cathepsin B (t1/2 = 9.3 h)
- Buffer pH 5.0 (t1/2 = 10.2 h)

C

\[\text{SN-38/Int Std vs. Hours}\]

- hLL1-CL2A-SN-38
- hLL1-CL2E-SN-38

<table>
<thead>
<tr>
<th></th>
<th>hLL1-CL2A-SN-38</th>
<th>hLL1-CL2E-SN-38</th>
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</thead>
<tbody>
<tr>
<td>Ymax</td>
<td>4.965</td>
<td>4.769</td>
</tr>
<tr>
<td>K</td>
<td>0.03218</td>
<td>0.0003337</td>
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<tr>
<td>HalfLife</td>
<td>21.54</td>
<td>2077</td>
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Figure 4

Cell line: Raji

<table>
<thead>
<tr>
<th>Inject Cells</th>
<th>Therapy</th>
<th>Median Survival (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D0; D5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mmab-Dox (17.5 mg/kg)</td>
<td>&gt; 112</td>
<td></td>
</tr>
<tr>
<td>Mmab-Dox (2 mg/kg)</td>
<td>&gt; 112</td>
<td></td>
</tr>
<tr>
<td>Mmab-CL2A-SN38 (17.5 mg/kg)</td>
<td>78.5</td>
<td></td>
</tr>
<tr>
<td>Mmab-CL2A-SN38 (2 mg/kg)</td>
<td>37.5</td>
<td></td>
</tr>
<tr>
<td>Mmab-CL2E-SN38 (17.5 mg/kg)</td>
<td>34.5</td>
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</tr>
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<td>Mmab-CL2E-SN38 (2 mg/kg)</td>
<td>27.5</td>
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</tr>
<tr>
<td>Lmab-CL2A-SN38 (17.5 mg/kg)</td>
<td>20</td>
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<tr>
<td>Saline</td>
<td></td>
<td>20</td>
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Cell line: A-375

<table>
<thead>
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<th>Median survival (days)</th>
</tr>
</thead>
<tbody>
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<td>0, 3, 7, 10, 14, 17, 21, 24</td>
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</tr>
<tr>
<td>Mmab-CL2A-SN38 (12.5 mg/kg)</td>
<td>28</td>
</tr>
<tr>
<td>Mmab-CL2E-SN38 (12.5 mg/kg)</td>
<td>17</td>
</tr>
<tr>
<td>Mmab-DOX (25 mg/kg)</td>
<td>17</td>
</tr>
<tr>
<td>Saline</td>
<td>17</td>
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</table>

Cell line: Capan-1

<table>
<thead>
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<th>Treatment days</th>
<th>Median survival (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0, 3, 7, 10, 14, 17, 21, 24</td>
<td></td>
</tr>
<tr>
<td>Mmab-CL2A-SN38 (12.5 mg/kg)</td>
<td>35</td>
</tr>
<tr>
<td>Mmab-CL2A-SN38 (5 mg/kg)</td>
<td>28</td>
</tr>
<tr>
<td>Mmab-CL2E-SN38 (12.5 mg/kg)</td>
<td>21</td>
</tr>
<tr>
<td>Mmab-CL2E-SN38 (5 mg/kg)</td>
<td>21</td>
</tr>
<tr>
<td>Mmab-Dox (25 mg/kg)</td>
<td>21</td>
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<tr>
<td>Emab-CL2A-SN38 (12.5 mg/kg)</td>
<td>21</td>
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<tr>
<td>Saline</td>
<td>21</td>
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Cell line: Cell line: A-375

<table>
<thead>
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<th>Treatment days</th>
<th>Median survival (days)</th>
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<tbody>
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<td></td>
</tr>
<tr>
<td>Mmab-CL2A-SN38 (12.5 mg/kg)</td>
<td>28</td>
</tr>
<tr>
<td>Mmab-CL2E-SN38 (12.5 mg/kg)</td>
<td>17</td>
</tr>
<tr>
<td>Mmab-DOX (25 mg/kg)</td>
<td>17</td>
</tr>
<tr>
<td>Saline</td>
<td>17</td>
</tr>
</tbody>
</table>

Tumor volume (cm³)

Days from start of treatment
Molecular Cancer Therapeutics

Milatuzumab-SN-38 conjugates for the treatment of CD74+ cancers

Serengulam V. Govindan, Thomas M. Cardillo, Robert M Sharkey, et al.

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