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RXDX-107, A Dodecanol Alkyl Ester of Bendamustine, Demonstrates Greater Stability and Broad Antitumor Activity in Multiple Pre-Clinical Models of Solid Tumor

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Abstract

Purpose: RXDX-107 is a dodecanol alkyl ester of bendamustine encapsulated in human serum albumin (HSA) to form nanoparticles. The anti-tumor activity of bendamustine in solid tumor malignancies has been less impressive, partially due to short half-life. RXDX-107 was designed to extend the half-life and improve tissue biodistribution over bendamustine, which may result in superior efficacy and tolerability in patients with solid tumors.

Experimental Design: The anti-tumor activity of RXDX-107 was measured in cellular anti-proliferation assay, cell-line derived xenograft (CDX) models and patient-derived xenograft (PDX) models. The mechanism of action and pharmacodynamics properties were measured by comet assay. The tumor accumulation was measured by a novel LC-MS/MS method.

Results: In *in vitro* anti-proliferative studies, RXDX-107 displayed dose-dependent cytotoxicity against multiple solid tumor cell lines. While the IC50 of RXDX-107 were comparable to those of bendamustine, RXDX-107 displayed more complete cell killing. RXDX-107 exhibited enhanced pharmacodynamics properties, including stronger induction of pH2AX (a biomarker for DNA damage) and higher interstrand crosslinks (ICLs) formation. RXDX-107 significantly reduces tumor growth in human NSCLC xenograft models. RXDX-107 also showed single agent anti-tumor activities, including tumor regression in multiple PDX models of solid tumors including breast, lung, and ovarian cancer. Furthermore, the mode of action data exhibit slow and sustained release of bendamustine from RXDX-107, and high intratumoral accumulation of RXDX-107.

Conclusions: Our preclinical data demonstrate potent and broad anti-tumor activity of RXDX-107 across a variety of solid tumor types, and support further clinical development of this novel drug candidate for the treatment of solid tumors.

Keywords: Solid tumor; Breast; Hematological; Preclinical; Antimetabolic

Statement of Translational Relevance

The bendamustine is an effective therapy in various hematological malignancies, but it has been less impressive in solid tumor setting, due to short half-life. The manuscript provides a novel scientific rationale and approach to improve half-life and bio-distribution properties of bendamustine, which may results in significant benefits for solid tumor patients.

RXDX-107 showed enhanced pharmacokinetics and pharmacodynamics properties of RXDX-107 *vs.* bendamustine, including stronger induction of pH2AX, high interstrand crosslinks formation, extended half-life of bendamustine and high intratumoral enrichment of RXDX-107. The manuscript demonstrates the superior anti-tumor property of RXDX-107 in preclinical models, including high tumor growth inhibition in cell line-derived xenograft (CDX) models of human NSCLC and multiple patient-derived xenograft (PDX) models of advanced solid tumors, such as breast, lung, and ovarian cancer. RXDX-107 is currently in Phase 1 clinical trial and

capable of effectively targeting several solid tumor patients. Thus, the manuscript supports further clinical development of this novel compound in solid tumors.

Introduction

Bendamustine HCL is an alkylating agent that induces interstrand DNA crosslinks (ICLs) and causes cell death via several pathways, including intrinsic apoptosis. Bendamustine was originally developed in 1963 in the former East German Democratic Republic with the intent to produce an antitumor and antimetabolic agent with superior pharmacological properties compared to other nitrogen mustards. Unlike other alkylating agents, Bendamustine exhibits a unique structure and pattern of activity. Bendamustine contains three structural elements: 1) a chloroethylamine alkylating group, which is shared with other member of nitrogen mustard family, including cyclophosphamide, chlorambucil and melphalan; 2) a butyric acid side chain, which is shared with chlorambucil and melphalan; 3) a benzimidazole central ring system is unique to bendamustine [1-3]. The complete mechanism of action of bendamustine in humans has not been fully characterized, but unique mechanisms of action include DNA damage stress response, inhibition of mitotic checkpoint,

activation of mitotic catastrophe, and induction of extensive and durable DNA damage [2-5].

Bendamustine has been extensively tested in hematological malignancies in both Europe and the United States and has proven to be a highly effective treatment for patients with chronic lymphocytic leukemia (CLL) and rituximab-refractory indolent non-Hodgkin's lymphoma (NHL), with clinical activity also seen in other B-cell malignancies and in multiple myeloma (MM) [2,6,7]. Emerging preclinical and clinical research indicate bendamustine may also serve as a backbone for the development of novel combination regimens including targeted therapies such as second generation anti-CD20 monoclonal antibodies, BTK inhibitors, and PI3K-delta inhibitors [8-10]. Bendamustine has also shown activity against chemoresistant cancer cells [5]. However, the activity of bendamustine in the treatment of solid tumor malignancies has been less impressive, possibly due to the pharmacokinetic and biodistribution properties of bendamustine. Bendamustine displays very short half-life, approximately 40 mins in human [1,11-13].

RXDX-107 is a dodecanal (C12) alkyl ester of bendamustine encapsulated in human serum albumin (HSA) to form nanoparticles. The alkyl ester was incorporated to reduce the rate of hydrolysis of the alkylating moiety and is designed to increase the plasma circulation time of bendamustine to enable a longer half-life and greater distribution to tissues, resulting in enhanced efficacy and tolerability, thus potentially provide meaningful benefit to patients with solid tumors. The aim of the current study was to demonstrate anti-tumor activity of RXDX-107 in preclinical models of solid tumors. This report demonstrate enhanced pharmacokinetic properties and single agent antitumor activity of RXDX-107 across a multiple solid tumor preclinical models.

Materials and Methods

Cell lines and reagents

Non-small cell lung cancer (NSCLC; NCI-H460 and NCI-H1944), ovarian (A2780) and breast (MD-MB-231) cell lines were obtained from ATCC and cultured in RPMI (Gibco) supplemented with 10% fetal bovine serum (FBS) and 100 U/ml penicillin-streptomycin (Gibco). Squamous cancer cell line (TE10) was obtained from Riken BRC and cultured in RPMI supplemented with 10% FBS. All cell lines were obtained directly, within 6 months of the studies, from the respective cell banks with certificate of Short Tandem Repeat (STR) authentication.

Western blotting

Cells were seeded in a density of 1×10^6 cells/10 cm dish and incubated at 37°C overnight. After incubation, cells were treated with bendamustine and RXDX-107 for 6 and 9 hrs. Cells were lysed in RIPA buffer, 30 µg/lane were separated by 4-20% SDS-PAGE (Biorad, 4561096) and transferred to PVDF membrane (Biorad, 1704157). Blots were probed with antibodies against phosphorylation of H2AX (Cat.No: 9718, Cell Signaling Technology), Beta-actin (Cat.No: 4967, Cell Signaling Technology) and cleaved PARP (Cat.No: ab136812, Abcam).

Cell viability and apoptosis assay

Cells were seeded in 96 well plates at a density of 5×10^3 cells/well in medium and incubated for 24 hrs and then, treated with different concentrations of RXDX-107 and bendamustine. After 72 hours, cell viability was determined by standard CellTiter-GLO according to manufacturer's protocol (CellTiter-Glo® Luminescent Cell Viability Assay, Promega).

An apoptotic marker, caspase-3/7 was evaluated using IncuCyte Caspase-3/7 reagent. Cells were seeded in 96 well plates at a density of 5×10^3 cells/well in medium containing IncuCyte Caspase-3/7 reagent at a final concentration of 5 µM and incubated overnight. Then compounds were added at different concentrations to the plates. Plates were incubated in the IncuCyte, and images were captured using IncuCyte ZOOM live cell imaging system.

Comet assay

Exponentially growing cells were seeded at a density of 1×10^6 cells/10 cm dish. The cells were incubated overnight, and then treated with different concentrations of RXDX-107 and bendamustine for 7 hrs. Cells were then trypsinized and resuspended in culture medium, and cell numbers were adjusted to 2×10^4 cells per mL of culture medium. Cells were irradiated (2.5 Gy/min), mixed with low melting agarose at 37°C and immobilized on CometSlide (Trevigen). The comet assay was carried out according to manufacturer's protocol (Trevigen). The cells were stained with SYBR Green I (ThermoFisher) and imaged.

Colony formation assay

Sensitivity of NSCLC cell line (H460) to RXDX-107 was measured using a colony formation assay. The 5000 cells per well in a 6-well plate was split, cells were allowed to adhere overnight at 37°C, and the cells were treated with RXDX-107 (25 µM). After treatment, cells were collected every day for 7 days. Cells were washed twice with PBS and fixed with 4% paraformaldehyde for 30 mins. After fixation, cells were stained with 0.1% crystal violet for 30 mins and allowed to dry in room air. Images were captured using Biorad hood imaging system, and the crystal violet was de-stained using 10% acetic acid, and OD was measured.

In vitro drug stability measurement

Stability in medium: RXDX-107 and/or bendamustine were added to medium containing 10% fetal bovine serum at final concentration of 200 µM. The mixture was incubated at 37°C for 6 hrs. After incubation, the medium was injected into an LC-MS/MS system, and stability was evaluated.

Stability in cells: The cells were seeded at a density of 1×10^6 cells per 10 cm dish and incubated at 37°C overnight. Cells were treated with RXDX-107 and/or bendamustine for 3.5 hrs. and 7 hrs. The medium was collected, and RXDX-107 and bendamustine were assessed by LC-MS/MS. Similarly, after treatment, the cells were collected and lysed in RIPA buffer. The cell lysate was injected into an LC-MS/MS system, and intracellular RXDX-107 and bendamustine were determined.

Animal tumor model, tumor efficacy determination, and analysis of intratumoral accumulation of RXDX-107

The human cancer cell derived xenografts were established using methods reported previously. The exponentially growing cells in monolayer were trypsinized, harvested, washed in serum-free medium and resuspended in the same medium with matrigel basement membrane matrix at a 3:1 ratio. The cells (2×10^6 cells in 0.2 ml) were injected subcutaneously into the left inguinal area of the nude mice. Tumor growth was monitored and measured by caliper measurement. For the efficacy study, tumor bearing animals were randomized and divided into various treatment and control groups. The animals were dosed intravenously on a Day 1 and Day 2 schedule. The animals were dosed with three different concentrations of bendamustine (37.5 mg/kg, 54.8 mg/kg and 68.5 mg/kg) and corresponding equimolar doses of RXDX-107 (55 mg/kg, 80 mg/kg and 100 mg/kg). For the intratumoral drug accumulation study, tumor bearing animals were dosed only on Day 1 with bendamustine (68.5 mg/kg) and equimolar dose of RXDX-107 (100 mg/kg). Plasma and tumor were collected 2 and 6 hrs following dosing. RXDX-107 and bendamustine in plasma and tumor tissue were determined by LC-MS/MS.

Activity of RXDX-107 in patient derived tumor xenograft model (PDX)

Animal information: Immunocompromised female mice between 5-8 weeks of age were implanted bilaterally in the flank region with tumor fragments harvested from the appropriate number of host animals, each implanted from a P3 passage. Pre-study tumor volumes were recorded for each experiment, beginning about one week prior to its estimated start date. When tumors reached approximately 250-300 mm^3, animals were matched by tumor volume into treatment and control groups. Dosing occurred on a Days 0 and 1 schedule.

Tumor growth inhibition: Starting Day 0, tumor dimensions were measured twice weekly by digital caliper, and data including individual and mean estimated tumor volumes (Mean TV ± SEM) were recorded for each group. Tumor volume was calculated using the formula (1): TV=width^2 × length × 0.5. At study completion, percent of tumor growth inhibition (%TGI) values were calculated and reported for each treatment group (T) versus control (C), using initial (i) and final (f) tumor measurements by the formula (2): %TGI=[1-(Tt-Ti)/(Ct-Ci)] × 100%.

Results

RXDX-107 shows more complete anti-proliferative activity *vs.* bendamustine

RXDX-107 is a dodecanal (C12) alkyl ester of bendamustine encapsulated in human serum albumin (HSA) to form nanoparticles (Figure 1A). The C12 alkyl ester was incorporated to reduce the rate of hydrolysis of the alkylating moiety and stabilize the molecule when combined with albumin. A C12 alkyl ester was selected as it optimized the encapsulation efficiencies with HSA to form nanoparticles and the ester pro-drug hydrolysis time.

The effect of RXDX-107 on cellular proliferation was initially assessed across multiple cell lines, including A2780 (ovarian), H460 (NSCLC), H1944 (NSCLC) and TE10 (Squamous). Cells were treated with RXDX-107 and/or bendamustine for 72 hours, and cell viability was measured using a Cell Titer-Glo luminescent assay. Figure 1B shows that RXDX-107 inhibited cell viability in a dose dependent manner, with complete inhibition of cell viability at the highest dose, whereas bendamustine only partially inhibited cell proliferation across multiple solid tumor cell lines.

Figure 1A and 1B : RXDX-107 shows more complete anti-proliferative activity; A) Structure of RXDX-107 showing the alkyl ester component and the active bendamustine component; B) Cell viability/dose response curve to bendamustine and RXDX-107 in A2780, MCF7, H1944 and TE10 cell lines.

RXDX-107 displays enhanced pharmacodynamics properties and extensive apoptosis *vs.* bendamustine

To determine whether RXDX-107 induces enhanced pharmacodynamics properties compared to bendamustine, we performed the comet assay to measure drug-induced interstrand crosslinks (ICLs). We treated H460 cells for 7 hrs with RXDX-107 and/or equimolar concentration of bendamustine. The data shows that the control irradiated cells showed comet tail formation, indicating damaged DNA. The length of the tail was significantly decreased with bendamustine treatment, indicating interstrand crosslinks (ICLs)

formation; interestingly, further tail decrease was also observed with equimolar concentration of RXDX-107 treatment (Figure 2A).

Figure 2A: RXDX-107 displays enhanced pharmacodynamics properties and extensive apoptosis *vs.* bendamustine; A: Comet assay showing the decrease in tail length in H460 with bendamustine and RXDX-107.

Decrease in DNA tail length occurred in a dose-dependent manner, of note, a greater decrease in tail length was observed in RXDX-107 treated *vs.* bendamustine treated cells, suggesting greater formation of interstrand crosslinks (ICLs) formation with RXDX-107 than with bendamustine. Furthermore, we also analyzed phosphorylation of H2AX to confirm the enhanced pharmacodynamics properties of RXDX-107 compared to bendamustine. H460 (NSCLC) and MDA-MB-231 (breast cancer) cells were treated with RXDX-107 and/or

equimolar concentration of bendamustine for 6 and 9 hrs. Figure 2B displays induced phosphorylation of H2AX after drug treatment, a biomarker reflecting DNA damage. Notably, significantly higher phosphorylation of H2AX was observed in RXDX-107 treated H460 and MDA-MB-231 cells *vs.* bendamustine treated cells at both 6 and 9 hrs. The increased phosphorylation of H2AX indicates extensive DNA damage caused by RXDX-107 treatment.

Figure 2B: Representative immunoblots of phosphorylation of H2AX in H460 and MDA-MB-231 with bendamustine and RXDX-107.

Next, we investigated whether extensive DNA damage leads to drug-induced apoptosis. We treated MDA-MB-231 cells for 6 and 9 hrs with RXDX-107 and/or bendamustine, and levels of known apoptotic factor, cleaved PARP, was evaluated to measure apoptosis. Figure 2C displays high levels of cleaved PARP at 9 hrs post-treatment with

RXDX-107. We also assessed caspase-3 activation using IncuCyte caspase-3/7 reagent. A non-fluorescent (DEVD) was subjected to cleaving by activated caspase-3/7, to release a DNA-binding green fluorescent label. Apoptotic cells were identified by the appearance of green labeled-nuclei. The MDA-MB-231 cells were treated with

RXDX-107 and/or equimolar doses of bendamustine. After 48 hrs of drug treatment, greater numbers of green cells was detected in RXDX-107 treated cells *vs.* bendamustine treatment (Figure 2C). Taken together, these results demonstrate that relative to bendamustine, RXDX-107 exhibits higher anti-proliferation activity, enhanced pharmacodynamics properties and extensive induction of apoptosis. Furthermore, we evaluated whether the enhanced ICLs, extensive DNA damage, and thereby apoptosis leads to reduced colony formation after RXDX-107 treatment. We treated a non-small cell lung cancer cell line (H460) with RXDX-107 and monitored colony formation every day using crystal violet staining. Figure 2D shows high colony formation in the control untreated cells after 4 days, but a fewer colonies formed under RXDX-107 treatment condition after 4 days (left panel). We also monitored colony formation for 7 days using OD based quantitation. The colony formation curves (lower panel) display high colony formation in the control group. In contrast, RXDX-107 significantly reduced colony formation in H460 cells.

Figure 2C and 2D: Representative immunoblot of cleaved PARP after RXDX-107 treatment in MDA-MB-231 cells (top) and IncuCyte caspase-3/7 assay showing the induced caspase-3/7 after bendamustine and RXDX-107 treatment in MDA-MB-231 cells; 2D) the colony growing assay showing reduced colony growth after RXDX-107 treatment in H460 cells.

RXDX-107 demonstrates potent anti-tumor activity in cell line-derived solid tumor xenograft models (CDX) and patient derived solid tumor xenograft models (PDX)

The *in vivo* anti-tumor efficacy of RXDX-107 was evaluated in nude mice bearing H460 subcutaneous xenografts. Doses of 55 mg/kg, 80 mg/kg and 100 mg/kg of RXDX-107 and/or equimolar doses of bendamustine were injected on a Day 1 and Day 2 dose schedule. Tumor growth and body weights were monitored. Figure 3A illustrates that treatment with 55 mg/kg and 80 mg/kg RXDX107 induced regression of tumor growth. Treatment with 100 mg/kg RXDX-107 induced higher regression of tumor growth. Bendamustine at 37.5 mg/kg demonstrated comparable *in vivo* activity. However, RXDX-107 displayed better tolerability over bendamustine. Specifically, at the two higher bendamustine doses (54.8 and 68.5 mg/kg), irreversible weight loss and significant mortality were observed (Figure 3B). On the other hand, RXDX-107 did not show notable weight loss at the lower doses and at higher corresponding molar equivalent doses to bendamustine, showed more significant, but still reversible weight loss. In summary, RXDX-107 shows comparable anti-tumor activity, and of greater significance, better tolerability than bendamustine.

RXDX-107 possesses robust single agent anti-tumor activity as illustrated by potent tumor regression in a range of PDX models established from patients with advanced solid tumors, including breast, lung, ovarian and pancreatic cancer (Figure 3C and Table 1). For example, in a small cell lung cancer (SCLC) model CTG-0198, 55 mg/kg RXDX-107 produced a tumor growth inhibition (TGI) value of 121% (regression). Similarly, RXDX-107 produced a TGI value of 109% (regression) in PDX model CTG-0502 of human NSCLC (Figure 3C).

Model	Tumour Type	TGI (%)
CTG-0670	Breast	129
CTG-0012	Breast	127
CTG-0257	Ovarian	126
CTG-0258	Ovarian	122
CTG-0198	SCLC	121
CTG-0958	Ovarian	120
CTG-0888	Breast	114

CTG-0869	Breast	113
CTG-0502	NSCLC	109
CTG-0828	NSCLC	109
CTG-0158	NSCLC	106
CTG-0486	Ovarian	106
CTG-1017	Breast	101

Table 1: Tumor growth inhibition by RXDX-107 in PDX models of various tumor types that showed more than 100% TGI; Only models

with >100% TGI (regression) are shown Tumor volume was calculated using the formula (1): $TV = width^2 \times length \times 0.52$. at study completion, percent tumor growth inhibition (%TGI) values were calculated and reported for each treatment group (T) versus control (c) using initial (i) and final (t) tumor measurement by the formula (2): $\%TGI = [1 - (T_t - T_i)/(C_t - C_i)] \times 100\%$.

Figure 3: RXDX-107 demonstrates potent anti-tumor activity in cell line derived solid tumor xenograft models and patient derived solid tumor xenograft models; A: H460 tumors treated with vehicle control, bendamustine and RXDX-107 on Day 1 and Day 2 dosing schedule; B: H460 tumor bearing animals were treated with vehicle control, bendamustine and RXDX-107 on Day 1 and Day 2 dosing schedule, and body weight loss was determined. Data are expressed as mean ± SEM; C: Animals bearing small cell lung cancer and non-small cell lung cancer patient derived xenografts were treated with RXDX-107 on Day 1 and Day 2 dosing schedule. Tumor dimensions were measured twice weekly.

RXDX-107 displays greater stability *vs.* bendamustine, due to integration of alkyl ester, and exhibits multiple mechanisms of cell entry, resulting in high intracellular accumulation

A C12 alkyl ester was incorporated into RXDX-107 to reduce the rate of hydrolysis of the alkylating moiety thereby increasing plasma half-life. To test this hypothesis, the stability of RXDX-107 and

bendamustine was studied *in vitro*. In particular, we incubated RXDX-107 and/or bendamustine in tissue culture medium at 37°C for 6 hrs. The bendamustine was determined using LC-MS/MS. Figure 4A displays the rapid degradation of bendamustine in bendamustine treated medium. In contrast, a significant quantity of bendamustine was observed in RXDX-107 treated medium consistent with the notion that RXDX-107 provides slow and sustained release of bendamustine.

Figure 4A: RXDX-107 displays greater stability *vs.* bendamustine due to integration of alkyl ester exhibits multiple mechanisms of cell entry and results in high intracellular accumulation; A: Representative graph shows slow and sustained release of bendamustine.

Fetal bovine serum in culture medium is known to contain significant esterase activity, thus potentially shortening half-life of RXDX-107. Hence, we compared the release of bendamustine in the presence and absence of serum in culture medium. A2780 (ovarian) cells were treated with RXDX-107 in presence or absence of medium, followed by measurement of bendamustine and RXDX-107 levels and corresponding phosphorylation levels of H2AX and 2, 4, 6 hrs post treatment. Figure 4B shows release of bendamustine in presence of

serum, resulting in DNA damage or high phosphorylation of H2AX. In the absence of serum, no bendamustine was detected, yet notably, high phosphorylation of H2AX or DNA damage was still observed. The data suggest that, in the presence of serum, RXDX-107 releases bendamustine and the resulting released bendamustine and remaining RXDX-107 causes DNA damage. Interestingly, in the absence of serum, RXDX-107 still penetrates into cells and causes DNA damage.

Figure 4B: Top graph represents release of bendamustine in culture medium containing serum, and immunoblot shows the phosphorylation of H2AX with RXDX-107 treatment. The bottom graphs show no released bendamustine in serum-free culture medium, and the immunoblot represents high phosphorylation of H2AX.

Next we sought to assess intracellular accumulation of RXDX-107 and bendamustine *in vitro*. We treated A2780 (ovarian) cells with different doses of RXDX-107 and/or bendamustine for 3.5 and 7 hrs. After treatment, cells were lysed, and levels of RXDX-107 and bendamustine were measured in cell lysate and medium by LC-MS/MS (Figure 4C).

Figure 4C: Schematic diagram represents experimental design.

The bendamustine was rapidly degraded as expected after 3.5 hrs of bendamustine treatment. In contrast, a significant amount of bendamustine was detected in a dose dependent manner in RXDX-107 treated medium, representing released bendamustine from RXDX-107 hydrolysis. This released bendamustine was still detectable after 7 hrs treatment, consistent with notion that RXDX-107 hydrolysis provides sustained release of bendamustine in medium (Figure 4D). Moreover, Figure 4E demonstrates that intracellular bendamustine was also detected following RXDX-107 treatment. Interestingly, a significantly high amount of RXDX-107 was detectable in both medium and cells. Specifically, intracellular levels of RXDX-107 exceeded that of bendamustine by 92 fold suggesting penetration of RXDX-107 into cells *in vitro* and suggesting that RXDX-107 is potentially the dominant molecular species causing intracellular DNA damage.

Figure 4D and 4E: Representative graph of RXDX-107 and released bendamustine in culture medium; Representative graph of intracellular RXDX-107 and released bendamustine.

RXDX-107 exhibits high intratumoral drug distribution

Next, we extended our study to tumor bearing nude mice after single administration of each compound (details in "Materials and Method" section). Plasma and tumor tissue were collected at 2 hrs and 6 hrs after administration, and the concentration of RXDX-107 and bendamustine levels were measured by LC-MS/MS. A high amount of bendamustine was present in plasma after 2 hrs of RXDX-107 treatment compared to bendamustine-treatment. After 6 hrs of treatment, a comparable amount of bendamustine was detected in plasma of both bendamustine and RXDX-107 treatment. In addition, there was a minimal level of the parent compound, RXDX-107 was also detectable in plasma after 6 hrs of RXDX-107 treatment (Figure 5A). In tumor tissue, a comparable amount of bendamustine was present following 2 hrs treatment of bendamustine or RXDX-107 (Figure 5B). In contrast, Figure 5B displays a significantly high concentration of RXDX-107 in tumor tissue even after 6 hrs of RXDX-107 treatment. The data suggest that RXDX-107 penetrates into tumor, and high intratumoral enrichment may explain the superior efficacy *in vivo*.

Figure 5: A high enrichment of RXDX-107 in tumor; A: animals were dosed with a single dose of bendamustine (68.5 mg/kg) and RXDX-107 (100 mg/kg); The graphs indicates RXDX-107 releases comparable amounts of bendamustine in plasma and tumor tissue; B: Sustained accumulation of RXDX-107 in tumor tissues.

Discussion

Despite recent progress made in molecularly targeted and immune-oncology therapies, emergence of drug resistance remains an unsolved challenge and hence, there is still a high unmet clinical need for developing broadly acting novel chemotherapies that extend survival for cancer patients. Bendamustine is an alkylating agent in used to treat various hematological malignancies, but it has never demonstrated objective in patients with solid tumors likely due to very short half-life [1,12,13]. This report demonstrates single agent antitumor activity of RXDX-107 in solid tumor preclinical models with enhanced pharmacodynamics and pharmacokinetic properties. As previously described, bendamustine failed to show complete anti-proliferation activity in multiple solid tumor cell lines. Interestingly, RXDX-107 exhibits more complete and superior anti-proliferative activity *vs.* bendamustine across multiple solid tumor cell lines. Bendamustine is believed to act primarily as an alkylating agent that induces interstrand crosslinks [14,15]. In our study, increased phosphorylation of H2AX and higher interstrand crosslinks suggest superior alkylating properties of RXDX-107. The interstrand crosslinks and phosphorylation of H2AX are known DNA damage markers, induced by multiple chemo agents, including bendamustine [4,16-18]. However, the degree of DNA damage is rate limiting, the moderate DNA damage can be repaired in many cancer cell lines, [19-21]. Extensive DNA damage can induce apoptosis in cancer cells and lead to efficient therapy [4,5]. Thus we sought to evaluate induction of apoptosis in RXDX-107 treated cells compare to bendamustine treated cells. The higher and early induction of cleaved PARP and caspase-3/4 in RXDX-107 treated cells compared to bendamustine treated cells suggest RXDX-107 induces extensive apoptosis due to higher level of intrinsic DNA damage.

The potent anti-tumor activity of RXDX-107 was also demonstrated *in vivo* using H460 human non-small cell lung cancer xenograft model. A marked antitumor activity without toxicity was observed over a range of doses from 55 to 100 mg mg/kg injection. However, the moderate and high doses of bendamustine (equimolar doses to RXDX-107) exhibited significant weight loss (>30%), whereas the molar equivalent dosses of RXDX-107 exhibited around 10% weight loss, which recovered quickly. The data suggests that RXDX-107 demonstrates similar anti-tumor activity with higher tolerability profile than bendamustine. Furthermore, RXDX-107 demonstrates single agent anti-tumor activity in multiple patient derived xenograft solid tumor models. A complete tumor regression or more than 100% TGI was achieved when animals with patient derived xenografts from breast, ovarian, SCLC and NSCLC were dosed with 55 mg/kg of RXDX-107 on a Day 1 and Day 2 schedule. The data suggest that RXDX-107 has broad anti-tumor activity in multiple solid tumor preclinical models. Notably, the dodecanal ester of bendamustine is subject to targeted esterase activity, and higher esterase activity in rodents compared to human have been reported [22,23]. Hence, the lower esterase activity in human would be expected to provide greater therapeutic benefit as manifested in potentially enhanced stability, higher efficacy and better tolerability.

The short half-life of bendamustine may represent a key factor in limiting its clinical activity in patient with solid tumors. A recent report also indicates that short esters of bendamustine showed high enrichment in tumor cells *in vitro* and were more effective than bendamustine [24]. Esters of bendamustine exhibited improved stability in *ex vivo* experiments [22]. However, the studies did not explore *in vivo* pharmacokinetic properties and therapeutic index in *in vivo* preclinical solid tumor models. RXDX-107 is designed to improve half-life of bendamustine by covalently linking dodecanal ester (C12 ester). In this report we demonstrated that RXDX-107 provides prolonged exposure of bendamustine to cells. A significant amount of bendamustine was detected in RXDX-107 treated medium, whereas no bendamustine was detected in bendamustine treated medium, suggesting that RXDX-107 hydrolysis enables the sustained release and consequent prolonged *in vivo* exposure of bendamustine. Furthermore, intracellular accumulation of RXDX-107 and bendamustine are consistent with the notion that RXDX-107 readily penetrates tumor cells. In contrast, likely due to its short half-life, no bendamustine was detected in bendamustine treated cells. These data is consistent with previous findings that show penetration of bendamustine esters into cells [24]. Our observation in tumor bearing nude mice suggests that RXDX-107 provides comparable amount of bendamustine in plasma and tumor tissue. More interestingly, RXDX-107 is highly enriched in tumor tissue in RXDX-107 administered animals. A high enrichment of RXDX-107 and together with released bendamustine in tumor tissue represents a distinct properties of RXDX-107 and correlates with enhanced interstrand crosslinks and extensive DNA damage. Taken together, the data suggest that the superior anti-tumor activity of RXDX-107 in solid tumors is due to the distinct prolonged exposure of bendamustine and high tumor enrichment properties of RXDX-107. Given that both

compounds share the same chloroethylamine alkylating group, the alkylating properties of RXDX-107 should be similar to that of bendamustine [24]. Nevertheless, a further confirmation study would be beneficial.

Conclusion

In conclusion, RXDX-107 shows anti-tumor activity in cell line-based and across multiple patient-derived xenograft models of advanced solid tumors, such as lung, breast and ovarian cancer. RXDX-107 penetrates readily into cells, and results in high intratumoral accumulation and extensive DNA damage over bendamustine. Our preclinical data support further clinical development of this novel drug candidate for the treatment of solid tumors.

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