VTX-2337 Is a Novel TLR8 Agonist that Activates NK Cells and Augments ADCC

Hailing Lu¹, Gregory N. Dietsch², Maura-Ann H. Matthews², Yi Yang¹, Smita Ghanekar³, Margaret Inokuma³, Maria Suni³, Vernon C. Maino³, Katherine E. Henderson¹, J. Jeffry Howbert², Mary L. Disis¹, and Robert M. Hershberg², 4

1: Tumor Vaccine Group, Center for Translational Medicine in Women’s Health, University of Washington, 815 Mercer St., Seattle, WA 98195

2: VentiRx Pharmaceuticals, 1700 7th Avenue, Suite 1900, Seattle, WA 98101

3: BD Biosciences, 2350 Qume Drive, San Jose, CA 94536

4: To whom correspondence should be addressed: Robert Hershberg, VentiRx Pharmaceuticals, 1700 7th Avenue, Suite 1900, Seattle, WA 98101, Phone (206) 689-2269, Email: rhershberg@ventirx.com

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Abbreviations:

ADCC: antibody-dependent cell-mediated cytotoxicity
CpG: cytosine phosphate guanine
CTL: cytotoxic T lymphocyte
DC: dendritic cell
EC$_{50}$: effective concentration 50 (the dose to induce half of the maximal response)
EC$_{90}$: effective concentration 90 (the dose to induce 90% of the maximal response)
E:T: effector:target ratio
HEK: human embryonic kidney
IFN$\alpha$: interferon alpha
IFN$\gamma$: interferon gamma
IL: interleukin
mAb: monoclonal antibody
mDC: myeloid DC
MHC: major histocompatibility complex
MIP: macrophage inflammatory protein
NK: natural killer cells
OD: optical density
ODN: oligodeoxynucleotide
PAMP: pathogen-associated molecular pattern
PBMC: peripheral blood mononuclear cells
pDC: plasmacytoid DC
SEAP: secretory embryonic alkaline phosphatase

SNP: single nucleotide polymorphism

Th: T helper cells

TLR: toll-like receptor

TNFα: tumor necrosis factor alpha
Translational Relevance:

A number of TLR agonists, especially agonists of TLR7 and TLR9, are being developed for their potential to enhance anti-tumor immunity. The therapeutic potential of TLR8 in cancer has not been fully assessed due to the lack of highly selective TLR8 agonists. Here we report the characterization of a novel TLR8 agonist, VTX-2337. Our studies demonstrate that this agonist effectively activates mDCs and is more potent than imiquimod (TLR7) and CpG (TLR9) in inducing IL-12 and TNFα production by mDC. VTX-2337 activates NK cells, leading to increased IFNγ production and increased cytolytic activity. Furthermore, VTX-2337 enhances rituximab-mediated ADCC, including individuals with FcγR3A genotypes associated with a reduced affinity for therapeutic mAbs. Our results highlight the potential of using this novel TLR8 agonist to induce the immune response to tumors and improve clinical responses to clinically approved mAb therapies, especially in individuals that demonstrate reduced ADCC activity.
Abstract

Purpose: We aim to characterize VTX-2337, a novel TLR8 agonist in clinical development, and investigate its potential to improve monoclonal antibody based immunotherapy that includes the activation of NK cells.

Experimental Design: HEK-TLR transfectants were used to compare the selectivity and potency of VTX-2337, imiquimod, CpG ODN2006, and CL075. The ability of VTX-2337 to induce cytokine and chemokine production from human PBMC and activation of specific immune cell subsets was examined. The potential for VTX-2337 to activate NK cell activity through direct and indirect mechanisms was also investigated. Lastly, we tested the potential for VTX-2337 to augment ADCC, especially in individuals with low affinity FcγR3A SNP.

Results: VTX-2337 selectively activates TLR8 with an EC50 of ~100 nM and stimulates production of TNFα and IL-12 from monocytes and mDCs. VTX-2337 stimulates IFNγ production from NK cells and increases the cytotoxicity of NK cells against K562 and antibody-dependent cell-mediated cytotoxicity (ADCC) by rituximab and trastuzumab. Effects of VTX-2337 on NK cells were in part from direct activation as increased IFNγ production and cytotoxic activity were seen with purified NK cells. Finally, VTX-2337 augments ADCC by rituximab in PBMC with different FcRγ3A genotypes (V/V, V/F and F/F at position 158).

Conclusion: VTX-2337 is a novel small molecule TLR8 agonist that activates monocytes and mDC, and NK cells. Through the activation of NK cells it has the potential to augment the effectiveness of mAb treatments where a polymorphism in FcγR3A limits clinical efficacy.
Introduction

The TLRs are a family of pathogen recognition receptors expressed broadly on hematopoietic cells (e.g. mDCs, pDCs, monocytes, and B cells) that recognize pathogen associated molecular patterns (PAMPs), activate innate immune responses and facilitate the development of adaptive responses (1). There are 10 unique TLRs expressed in humans (1), with TLR1, 2, 4, 5, and 6 being expressed on the cell surface where they primarily serve to recognize extracellular macromolecular ligands from bacteria and fungi. In contrast, TLR3, 7, 8, and 9 are expressed within the endo-lysosomal compartmental pathway of various cells where they function in the recognition of foreign nucleic acids from intracellular pathogens. It is the endosome localized TLRs, particularly TLR7, 8, and 9, which have recently emerged as important targets for anticancer immunotherapies (2-5).

The engagement of specific TLRs leads to the activation of different cell populations (6) and the production of distinct patterns of cytokines and other inflammatory mediators (7), resulting in alternative immune response profiles. For example, TLR7 activation of pDC in response to viral infections induces high levels of IFNα and enables these cells to prime adaptive T cell responses to endogenous viral antigens (8). TLR8 is more widely distributed on subsets of immune cells than TLR7 and TLR9, and selective agonists effectively activate both mDC and monocytes (9). mDC activated by TLR8 are well-suited for the generation of adaptive immune responses directed at tumor cells (10, 11). Activated mDC phagocytose both apoptotic and necrotic tumor cells, then cross-present tumor-associated antigens to CD8+ CTLs more effectively than pDC (12, 13). Additionally, TNFα and IL-12 released upon activation of mDCs can stimulate T cell
We and others have hypothesized that the addition of TLR8 agonists to some standard-of-care anti-cancer agents (e.g. anthracycline chemotherapy, monoclonal antibody therapy, or radiation therapy) may dramatically augment the anti-tumor response (2, 3, 5). In particular, enhancement of tumor cell killing through ADCC may represent an important therapeutic opportunity for TLR8 specific agonists.

Monoclonal therapies are widely used in the treatment of some cancer types, and ADCC is considered a component of the clinical efficacy for some mAbs, including rituximab and trastuzumab (17). NK cells are the major mediators of ADCC, and NK cell function has been shown to impact the clinical response to monoclonal antibody based therapy (18-20). The potential of using TLR agonists to activate NK cells and enhance their function through mechanism including ADCC has been shown in some previous publications (21-23). We hypothesize that the addition of a TLR8 agonist to a mAb treatment should enhance ADCC and increase efficacy of mAb treatments.

Herein, we describe a novel, small molecule agonist of TLR8, referred to as VTX-2337, which has recently completed a Phase I study in oncology patients. We compare the selectivity, potency, cellular specificity, and in vitro pharmacodynamic activity of VTX-2337 to imiquimod, a TLR7 agonist, and CpG ODN2006 (PF-3512676), a TLR9 agonist, two agents that have been extensively tested as anti-cancer agents in human clinical trials. In addition, we focus on the ability of VTX-2337 to activate NK cells and enhance ADCC in the context of a common genetic variant in the FcγR3A gene that has been associated with affecting the clinical response.
to several mAb therapeutics (18, 19). Collectively, these data highlight the potential value of TLR8 as a target for immunotherapy in cancer patients.

Materials and Methods:

Reagents

VTX-2337 is a synthetic small molecule agonist of TLR8 (MW 458.6, Figure S1), that is based on a 2-aminobenzazepine core structure, and has been evaluated in a Phase I oncology trial (NTC00688415) sponsored by VentiRx Pharmaceuticals (Seattle, WA). Imiquimod, CpG ODN2006 (5'-TCG TCG TTT TGT CGT TTT GTC GTT-3'), and CL075 were purchased from InvivoGen (San Diego, CA). RPMI culture media for culturing human peripheral blood mononuclear cells (PBMC) was purchased from Invitrogen (Carlsbad, CA). Cell-surface-specific, phospho-protein-specific and cytokine-specific fluorochrome-labeled Abs for flow cytometry were obtained from BD Biosciences (San Jose, CA).

NF-κB activation in HEK cells transfected with TLRs

Human embryonic kidney cells (HEK293) expressing TLR2, TLR3, TLR4, TLR5, TLR7, TLR8, or TLR9 were purchased from InvivoGen. The cells were cultured in DMEM (Cambrex, Walkersville, MD) containing 4.5 g/l L-glucose (Sigma–Aldrich, St. Louis, MO) and 10% FBS. The activity of specific TLR agonists was assessed using the secretory embryonic alkaline phosphatase (SEAP) reporter gene that is linked to NF-κB activation in response to TLR stimulation. Measurement of SEAP activity using the Quanti-blue substrate (InvivoGen, San Diego, CA) after TLR agonist treatment was carried out similarly as described previously (24).
Measurement of cytokine and chemokine secretion from human PBMC following stimulation with TLR agonists

Blood was collected from ten healthy human donors after obtaining appropriate informed consent. The PBMC were isolated within 4 hours of the blood collection using a Ficoll gradient separation and resuspended in RPMI at 1 million cells per ml in RPMI + 2% heat-inactivated FBS. Isolated PBMC (200,000 per well) were plated in 96-well round bottom culture plates and treated with serial dilutions of the TLR agonists: imiquimod (39 to 50,000 nM), VTX-2337 (6 to 6,400 nM), or CPG ODN2006 (23 to 3,000 nM), for 24 hours. The cell culture supernatants were harvested and levels of various cytokines and chemokines were measured using either ELISA kits (TNFα kit from eBiosciences (San Diego, CA); IFNα kit from PBL InterferonSource (Piscataway, NJ)) or Luminex (for IL-1α, IL-1β, IL-6, IL-8, IL-10, IL-12p40, IL-12p70, MIP-1α, MIP-1β, G-CSF, and IFNγ); plate purchased from Millipore (Pittsburgh, PA). The procedures for determining cytokine/chemokine levels by ELISA and Luminex methods were carried out following the manufacture’s protocols. Of the various mediators (cytokines and chemokines) evaluated in the supernatants, those that were significantly increased following the activation with one or more TLR agonist (p<0.05) or increased by more than 1 log over unstimulated controls are described.

Intracellular cytokine staining to measure cytokine production in PBMC subsets following stimulation with TLR agonists

The production of TNFα, IL-12, and IFNα by specific cell subsets present in PBMC was assessed by intracellular staining, using methods similar to those previously described (25). PBMC were cultured in vitro in polypropylene tubes in the absence (unstimulated control) or
presence of CpG ODN2006 (5,000 nM), VTX-2337 (50 to 800 nM), or imiquimod (1,000 to 50,000 nM). Brefeldin-A (5 μg/mL, Sigma-Aldrich), a protein secretion inhibitor, was added 2 hours after the addition of TLR agonists to allow for intracellular staining of cytokines. Following a 16-18 hour activation period, the cells were initially stained with fluorophore-conjugated antibodies to surface markers CD3+CD19 AmCyan, HLA-DR APC-H7, CD11c V450, CD123 PerCP-Cy5.5, CD56 PE-Cy7, CD16 PE-Cy7, CD14 Alexa 700 (BD Biosciences, CA). After subsequent fixation and permeabilization (BD Cytofix/Cytoperm™ Fixation/Permeabilization Solution Kit), the cells were stained for intracellular markers using TNFα FITC, IFNα Alexa 647 and IL-12 PE (BD Biosciences). Samples were analyzed on a BD LSR II flow cytometer using FACSDiva software. Monocytes were defined as CD14+ cells, pDC were defined as CD3-CD14-CD19-CD16-CD56- (lineage negative), HLA-DR+, CD11c+, CD123+ cells, and mDC were defined as lineage negative, HLA-DR+, CD11c+ cells. To measure IFNγ production in NK cells, we have used both overnight incubation and short-term (6 hours) incubation. For overnight incubation, brefeldin A was included in the last 6 hours. For short-term incubation, brefeldin A was added at the beginning of the 6 hour incubation time. NK cells were defined as CD3-CD56+ cells. For any given marker, background staining obtained in the unstimulated sample was subtracted from that in the stimulated sample.

**PhosFlow assays to detect phosphorylation of signaling molecules.**

PBMC were resuspended in PBS and then stained for 15 minutes at room temperature with CD3 FITC, CD14 FITC, CD19 FITC, CD11c V450, CD56 PE-Cy7 and CD16 PE-Cy7. After staining, cells were diluted to 2-4x10^6/ml in warm PBS and dispensed at 1 ml/well into polypropylene 24-well blocks (Qiagen). Cells were either left unstimulated or stimulated for 20
minutes at 37°C with VTX-2337 (50 to 800 nM), imiquimod (1,000 to 50,000 nM), or CpG ODN2006 (10 to 6,000 nM). Following activation, cells were fixed in paraformaldehyde at a final concentration of 5% for 10 minutes at 37°C. Cells were then washed in PBS and permeabilized for 30 minutes at room temperature in 3 ml of Custom Perm Buffer (BD Biosciences). Permeabilized cells were washed twice in wash buffer (PBS, 1% BSA, 0.5% NaN₃) and stained with HLA-DR PerCP-Cy5.5, and pNF-κB PE (BD Biosciences) for 1 hour at room temperature in the dark, then washed again twice in wash buffer. Flow cytometric analysis was performed on a BD FACSCantoII flow cytometer using BD FACSDiva software (BD Biosciences). The definition of NK, monocytes, pDC, and mDC subsets by surface CD markers was the same as described above for intracellular cytokine staining.

Measurement of NK cell activity and ADCC of tumor cells

PBMC or purified NK cells were prepared as previously described, and the purity of NK cells was approximately 99% (26). NK cell mediated cytotoxicity was assessed by Calcein AM release from labeled target cells (26). In brief, PBMC or purified NK cells were cultured for 48 hours in RPMI medium in the presence of VTX-2337 (167 or 500 nM) before incubation with target cells.

To assess tumor cell lysis by ADCC, target cell lines were coated with mAbs (5 μg/ml) that recognize specific cell surface antigens expressed by the target cells (rituximab, an anti-CD20 IgG1 mAb for HS-Sultan lymphoma cells; trastuzumab, an anti-HER2 IgG1 mAb for MDA-MB-231 breast cancer cells) or control IgG1 for 30 minutes at 4°C. Triplicate wells were set up for each effector: target (E:T) ratio. The percentage of specific lysis was calculated according to the
formula: \[ \frac{(\text{experimental release} - \text{spontaneous release})}{(\text{maximal release} - \text{spontaneous release})} \times 100\% \].

**Measurement of FcγR3A single nucleotide polymorphism (SNP)**

The FcγR3A-158 genotype was determined using a method similar to what has been previously published (20). DNA was extracted from human PBMC with a QIA DNA mini kit (Qiagen, Valencia, CA) as per the manufacturer’s instructions. Taqman genotyping assay with pre-made primer and probes from Applied Biosystems (Foster City, CA) was used to determine the FcγR3A-158 SNP for the various donors. The allelic discrimination reactions were carried out in standard 384-well reaction plate in 5 μL volume on the Prism 7900 HT (Applied Biosystems).

**Statistical Analysis**

Statistical analyses were performed using Graphpad Prism software or SPSS v16.0. Cytokine and chemokine induction by VTX-2337, imiquimod, or CpG ODN2006 was analyzed using one-way ANOVA with post hoc analysis. An estimate of the EC\(_{50}\) for each cytokine and chemokine induced by the different agonists was calculated with WinNonlin Professional version 5.2.1, using a pharmacodynamic response model (Model 102) where: Effect (E) = E\(_0\) + (E\(_{\text{max}}\) – E\(_0\)) \times \frac{(C/C + EC_{50})}{\text{(C/C +EC}_{50})}. The percentages of IFNγ positive NK cells in VTX-2337 treated and unstimulated PBMC was compared using 2 tailed Mann-Whitney test. A p value of \( \leq 0.05 \) was considered significant.
Results:

VTX-2337 is a selective and potent TLR8 agonist that stimulates PBMC to produce TNFα and IL-12.

The selectivity and potency of VTX-2337 was initially evaluated and compared to the TLR7 agonist imiquimod and TLR9 agonist CpG ODN2006 using HEK293 cells transfected with various human TLRs. Among the TLRs tested (2, 3, 4, 5, 7, 8, and 9), VTX-2337 selectively activated TLR8. As shown in Figure 1A, imiquimod and CpG ODN2006 selectively activated TLR7 and TLR9 respectively, as expected. Only VTX-2337 activated TLR8. While VTX-2337 has TLR7 agonist activity in the HEK system, it is only at concentrations more than 30-fold above levels that activate TLR8. For subsequent experiments characterizing VTX-2337 activity on TLR8, the compound was evaluated at concentrations well below levels needed to activate TLR7 (~5000 nM).

To further characterize the immunostimulatory activity of this novel TLR8 agonist, human PBMC were treated with VTX-2337, imiquimod, or CpG ODN2006 and levels of TNFα, IL-12, and IFNα in the media were measured. As shown in Figure 1B, VTX-2337 stimulated the production of both TNFα (EC50=140±30 nM based on 10 donors) and IL-12 (EC50=120±30 nM based on 10 donors) in PBMC. Imiquimod stimulated low levels of TNFα and IL-12, but only at concentrations exceeding 3,000 nM. However, imiquimod also stimulated secretion of IFNα, as previously reported, while VTX-2337 did not (data not shown) (27). CpG ODN2006 did not stimulate the secretion of TNFα, IL-12, or IFNα over the concentration range in which it effectively activated the HEK TLR9 transfectants.
We also compared VTX-2337 to CL075, a well characterized TLR8 agonist that has previously been described (15, 28). In HEK transfectant cells, VTX-2337 was approximately 10-fold more potent than CL075 in activating TLR8 and both compounds had weak TLR7 agonist activity (Supplemental Figure 1B). Consistent with the HEK data, the two compounds show similar profiles for TNF\(\alpha\) and IFN\(\alpha\) induction in PBMC (Supplemental Figure 1C), although VTX-2337 is more potent than CL075 in inducing TNF\(\alpha\) (Supplemental Figure 1C).

**VTX-2337 drives mDCs and monocytes to produce IL-12 and TNF\(\alpha\) via NF-\(\kappa\)B activation.**

To elucidate the cell subsets in PBMC that were the source of the TNF\(\alpha\), IL-12, and IFN\(\alpha\), intracellular staining by flow cytometry was performed on PBMC following TLR7, 8, and 9 stimulation. Agonists were evaluated at concentrations corresponding to approximately their EC\(_{90}\) in the HEK transfectant assay. As shown in Figure 2A, VTX-2337 (800 nM) stimulated the production of both TNF\(\alpha\) and IL-12 in a high percentage of monocytes (59±10% positive for TNF\(\alpha\) and 14±4% positive for IL-12, n=10) and mDC (57±8% positive for TNF\(\alpha\) and 15±3% positive for IL-12, n=10), but not in pDC. In contrast, imiquimod (25,000 nM) and CpG ODN2006 (5,000 nM) stimulated TNF\(\alpha\) production in a high percentage of pDC (77±4% for imiquimod and 55±7% for CpG ODN2006, n=10), but not in mDC or monocytes. Both imiquimod and CpG ODN2006 stimulated IFN\(\alpha\) production in a low percentage of pDC (7±2% of imiquimod and 4±1% for CpG ODN2006, n=10), while VTX-2337 did not stimulate IFN\(\alpha\) in pDC. Representative histograms showing intracellular staining levels of IL-12, TNF\(\alpha\), and IFN\(\alpha\) in monocytes, pDC, and mDC subsets in control and VTX-2337 stimulated PBMC are shown in Figure 2B. The selective activation of mDC and monocytes, but not of pDC by VTX-2337 was further demonstrated by intracellular detection of phosphorylated signaling proteins.
proteins by PhosFlow™ analysis. VTX-2337 stimulated NF-κB phosphorylation in a high percentage of monocytes and mDC (44±7% and 81±4%, respectively), while imiquimod and CPG ODN2006 stimulated NF-κB phosphorylation mainly in pDC (18±2% and 32±3%, respectively) (Figure 2C). Representative histograms showing the change in levels of phosphorylated NF-κB in the three cell populations following VTX-2337 treatment are shown in Figure 2D.

**VTX-2337 stimulates the production of other immune mediators including IFNγ.**

The robust induction of both IL-12 and TNFα distinguishes TLR8 activation by VTX-2337 from TLR7 and TLR9 activation by imiquimod and CpG ODN2006, respectively. To determine if other important differences exist, the repertoire of other cytokines and chemokines induced in PBMC following VTX-2337 activation was compared to the responses seen with imiquimod and CpG ODN2006 over a range of relevant concentrations based on each compounds potency and selectivity as seen in the HEK TLR assay (Figure 1). VTX-2337 at a concentration of 1600 nM induced considerably higher levels of G-CSF, IL-1α, IL-1β, IFNγ, IL-6, IL-12p40, IL-12p70, MIP-1α, and MIP-1β, than either imiquimod at 25,000 nM or CpG ODN2006 at 1,500 nM, as shown in Table 1. In contrast, VTX-2337 and imiquimod induced comparable levels of IL-10, and all three agonists induced IL-8. As expected, concentrations of VTX-2337 that stimulated a half maximal response (EC50) for most mediators were considerably lower than that for imiquimod, due to the compound’s higher potency. Although CpG ODN2006 induced some mediators at EC50 concentrations comparable to VTX-2337, the magnitude of induction by VTX-2337 was generally much greater. For example, the EC50 values for MIP-1β induction were 60 nM for VTX-2337 and 30 nM for CpG ODN2006, yet VTX-2337 induced a maximum MIP-1β
response that was ~10-fold higher (4,262±1,011 ng/mL for VTX-2337 versus 472±169 ng/mL for CpG ODN2006), as shown in Table 1. Another mediator of interest was IFN\(\gamma\), where VTX-2337 resulted in a 1,000 fold induction over the unstimulated control, while imiquimod induced only a 15 fold increase and CpG ODN2006 failed to stimulate this cytokine (Table 1).

**VTX-2337 activates NK cells to produce IFN\(\gamma\).**

To determine the cellular source of the IFN\(\gamma\) seen in activated PBMC, intracellular staining was performed on different cell populations, including CD4 and CD8 T cells, gamma delta (\(\gamma\delta\)) T cells, and NK cells, after incubating PBMC with VTX-2337 800 nM for 24 hr. As shown in Figure 3A, the major source of IFN\(\gamma\) in PBMC stimulated with VTX-2337 was the NK cell population. To prevent secondary activation of NK cells through cytokines released by accessory cells, intracellular staining was done following a short-term activation period (6hr) in the presence of brefeldin A, which blocks cytokine release from activated cells. Under these conditions, we continued to observe robust induction of IFN\(\gamma\) in NK cells (10.6±4.5% CD69\(^+\)IFN\(\gamma\)^+ NK cells in VTX-2337-treated PBMC versus 1.1±0.4% in the unstimulated controls, \(p=0.004\), Figure 3B and C), demonstrating a direct effect of VTX-2337 on NK cells.

To determine if IL-12 and/or IL-18 play a role in the activation of NK cells by VTX-2337, these cytokines were blocked with neutralizing antibodies during the 24 hour treatment of PBMC with VTX-2337 (0.8 \(\mu\)M). The response to IL-12 blockade was variable between donors and IL-12 blockade did not significantly decrease VTX-2337-induced IFN\(\gamma\) production (data not shown). IL-18 blockade significantly decreased IFN\(\gamma\) production (Figure 3D). However, there was significant induction of IFN\(\gamma\) by VTX-2337 even in the presence of anti-IL18 mAb, suggesting the existence of a direct effect of VTX-2337 on NK cells (Figure 3D). To confirm the direct
effect of VTX-2337 on NK cells, purified NK cells were treated with VTX-2337. In 3 out of the 4 donors tested, VTX-2337 stimulated IFNγ production in purified NK cells to a level that is similar to what was observed in PBMC (Figure 3E). Using the same purification protocol, we have recently shown that purified NK cells do not respond to a TLR2 agonist unless exogenous IL-12 was included (26), thus it is very unlikely that contaminating DC/monocytes contributed to VTX-2337 induced IFNγ production in the purified NK cells. Altogether, these results demonstrate that VTX-2337 has a direct effect on NK cells, although the IFNγ response can be enhanced by mediators such as IL-18, which may be produced by other cell populations in response to TLR8 activation by VTX-2337 (data not shown). Expression of TLR8 on NK cells was evaluated by RT-PCR using RNA from FACS-sorted cells. As shown in Supplemental Figure 2, TLR8 was absent on pDC and was expressed at increasing levels on NK cells, monocytes, and mDC. In contrast, TLR7 and TLR9 were highly expressed on pDC, but not on mDC.

**VTX-2337 augments the lytic function of NK cells and enhances ADCC.**

The cytolytic activity of NK cell was initially assessed on K562 cells, a NK cell sensitive leukemia cell line. As shown in Figure 4A, PBMC pretreated with VTX-2337 (167 or 500 nM, 48 hours) demonstrated enhanced lysis of the K562 target cells. To determine whether TLR8 activation can also augment NK cell mediated ADCC, VTX-2337-stimulated or unstimulated PBMC were incubated with HS-Sultan lymphoma cells coated with anti-CD20 mAb rituximab or MDA-MB-231 breast cancer cells coated with anti-HER2 mAb trastuzumab. As shown in Figures 4B-C, VTX-2337 stimulated PBMC had enhanced ADCC activity against both HS-Sultan and MDA-MB-231 tumor cells. Imiquimod and CpG2006 did not enhance ADCC in our
system, although the dual TLR7/8 agonist resiquimod, did enhance ADCC at concentrations where TLR8 is activated (Supplemental Figure 3). The depletion of NK cells from the activated PBMC population resulted in a loss of target cell lysis, confirming that the enhancement of ADCC by VTX-2337 is mediated by NK cells (Figure 4D). Purified NK cells activated with VTX-2337 (500 nM, 48 hours) showed enhanced ADCC as compared to unstimulated NK cells, indicating that VTX-2337 acts directly on this cell population (Figure 4E).

**Enhancement of ADCC in FcyR3A variants.**

Previous studies have found that a polymorphism in the FcyR3A molecule (158F/V), which affects the receptor’s affinity for IgG1, is an important factor determining the level of clinical efficacy for some mAbs used in the treatment of cancer (18-20). To determine if this common polymorphism affects the baseline ADCC response and/or response following VTX-2337 activation, donors were genotyped for the two alleles encoding the F and V isoforms, respectively. Rituximab-mediated ADCC using unstimulated and VTX-2337-stimulated PBMC from 15 donors, including 10 donors with the F/F or F/V genotypes and 5 donors with V/V genotype was assessed. As shown in Figure 5, the F/F and F/V donors have significantly reduced rituximab-mediated ADCC activity relative to individuals with the V/V genotype (20.5±2.5% specific lysis for F/F and F/V vs. 31.7±2.9% specific lysis for V/V, p=0.017). When PBMC were stimulated with VTX-2337, ADCC was significantly enhanced in both F/F and F/V and V/V genotypes (Figure 5). The level of ADCC was enhanced from 20.5±2.5% to 40.0±4.1% in the F/F and F/V genotypes with the lower affinity FcyR3A phenotype (p=0.0007) and from 31.7±2.9% to 55.5±2.6 in the higher affinity FcyR3A corresponding with the V/V genotype (p=0.0003). The level of ADCC seen with VTX-2337 stimulated PBMC from F/F and F/V
donors was also found to be comparable to the level of ADCC seen in unstimulated PBMC from V/V donors. This indicates that for individuals with the lower affinity genotypes, TLR8 activation by VTX-2337 increases the level of NK mediated ADCC to the level typically seen in donors with the high affinity V/V genotypes.
Discussion:

In this study we demonstrate that the novel TLR8 agonist VTX-2337 stimulates human mDCs and monocytes to produce high levels of IL-12 and TNFα, as well as other inflammatory cytokines and chemokines. VTX-2337 also stimulates NK cells to produce IFNγ, increases their lytic activity against K562, and enhances their ability to lyse tumor cells through ADCC. Additionally, TLR8 stimulation by VTX-2337 enhances ADCC in individuals with F/F and F/V FcγR3A genotypes, who do not respond as robustly to some mAb therapeutics as individuals with the higher affinity V/V FcγR3A genotype. Collectively, these activities highlight the potential of VTX-2337 as an immunotherapeutic approach in various oncology indications.

A direct comparison of VTX-2337 as a prototypic TLR8 agonist to the clinically characterized TLR7 and TLR9 agonists, imiquimod and CpG ODN2006, respectively, underscores important differences between TLRs in human immune responses. One observation confirmed in these studies was the reciprocal pattern of TLR expression on mDC and pDC populations. Specifically, TLR8 is expressed in mDC, monocytes, and NK cells whereas TLR7 and TLR9 are expressed in pDC. This differential pattern of TLR expression and selective activation by the different agonists was documented by RT-PCR, intracellular cytokine staining, and phosphorylated NF-κB in PBMC subpopulations. The repertoire and magnitude of the cytokine/chemokine response induced by TLR8 also differs considerably from TLR7 and TLR9, providing additional evidence for the unique immune stimulating activities of VTX-2337 relative to imiquimod and CpG ODN2006.
TLR8 activation of mDC and monocyte cell populations leading to the robust production of TNFα and IL-12 is consistent with previous reports (9, 15), and distinguishes VTX-2337 activity from both the TLR7 agonist imiquimod and the TLR9 agonist CpG ODN2006, which have been used as immunotherapies in some types of cancer (29-31). The induction of IL-12 by VTX-2337 is a desirable feature for a cancer immunotherapy. This cytokine enhances the development of both Th and CTL responses (32), and has anti-tumor activity (33) that is enhanced by TNFα (34). The production of high levels of IL-12 and TNFα are distinguishing features of TLR8 activation and indicate that VTX-2337 may be an effective agent for cancer immunotherapy. TLR8 activation by VTX-2337 may be particularly suited to enhance the anti-cancer effect of standard chemotherapy. It has been previously reported that TLR7/8 agonist resiquimod synergize with TLR4 agonist in activating DC and priming T cell response (35, 36). Dying cancer cells from chemotherapy or radiotherapy can release high-mobility-group box 1 (HMGB1) which acts on TLR4 on DC and stimulates cross-priming of tumor antigens (37). Therefore, we hypothesize that TLR8 agonists may work synergistically with standard chemotherapy or radiotherapy by enhancing the immunogenic effect.

In addition to the activation of mDC and monocytes, VTX-2337 appears to have a direct effect on NK cells, as shown by increased IFNγ production, enhanced cytotoxicity towards NK sensitive target cells and increased ADCC. This suggests the opportunity of using VTX-2337 in combination with approved mAbs, where ADCC contributes to the clinical efficacy (17). The potential for TLR agonists to enhance NK cell function and increase ADCC have been documented in previous publications (21, 38, 39, 40). For example, CpG ODN has been
reported to increase IFNγ production of NK cells and enhance trastuzumab-mediated lysis of breast cancer cells and rituximab-mediated lysis of lymphoma cells (21, 39). The TLR7/8 agonist resiquimod has also been shown to enhance FcγR function and ADCC, and enhance the anti-tumor effect of HER2-targeted mAb therapy in a mouse model (40). Interestingly we did not observe IFNγ induction (Table 1) or the enhancement of ADCC by CpG ODN2006 (Supplemental Figure 3) in our experimental system. This might be due to the different ODNs that were tested in previous publications from ours.

Previous studies have not identified a consistent pathway of NK activation by TLR8 agonists. Gorski et al showed that NK cells did not express TLR8, and that IL-18 and IL-12p70 were required for TLR8 agonist induced IFNγ production by NK cells (15). Yet, Hart et al showed that human NK cells expressed functional TLR8, but the cytokine production and cytotoxicity in response to resiquimod were mediated primarily through accessory cells (14). In our study, we demonstrated there is a direct effect of VTX-2337 on NK cells, as shown by VTX-2337-induced IFNγ production and increased ADCC in purified NK cells. However, our studies also show that released mediators can modify the NK response as demonstrated by IL-18 neutralization decreasing VTX-2337-induced IFNγ production in PBMC cultures. The expression of TLR8 mRNA on NK cells observed in our studies is consistent with previous report (14).

We also investigated whether VTX-2337 has activity on murine TLR8. Using HEK cells transfected with murine TLR7 and TLR8, we found that VTX-2337 has some weak activity on TLR7, but does not activate murine TLR8, unless poly(dT) is included (Supplemental Figure 4A-C). Similar results were observed with CL075. These observations are consistent with
previous report that murine TLR8 can be activated by a combination of imidazoquinoline and poly(T) oligodeoxynucleotides (41). VTX-2337 was also shown to stimulate IFNγ and TNFα production from mouse splenocytes (Supplemental Figure 4D-E), presumably through the activation of murine TLR7. Whether VTX-2337 can enhance the mAb therapy in mouse models remain to be investigated in future studies.

The binding of IgG to receptors for the Fc region of IgG (FcγR) on effector cells is a critical step in the lysis of tumor cells by ADCC. It is known that a polymorphism at amino acid position 158 of FcγR3A influences the affinity of the receptor for IgG1. The V residue at position 158 confers a higher affinity for IgG, relative to the F residue, and individuals with the V/V genotype are reported to have better clinical responses in cancers where rituximab, trastuzumab, and cetuximab are used as part of the treatment regimen (18-20). Since the low affinity F/F genotype exists in approximately 50% of the population, augmentation of the ADCC response mediated through therapeutic mAbs in these individuals could have a large impact on clinical response rates. Consistent with published reports, we found that ADCC activity mediated through rituximab was lower in individuals with the F/F or F/V genotypes, relative to individuals with the V/V genotype. Activation of PBMC with VTX-2337 resulted in statistically significant, 2-fold increase in mean tumor cell lysis for both individuals with the F/F and F/V genotypes as well as those with the V/V genotype. This enhancement of ADCC through TLR8 activation suggests that VTX-2337 could improve the clinical response in individuals with all three FcγR3A genotypes, although the greatest clinical benefit may be in the F/F and F/V genotypes due to the lower baseline response currently achieved with mAb therapies.
In summary, results presented in these studies demonstrate that VTX-2337 is a novel, highly potent, and selective TLR8 agonist. Activation of the innate immune system using VTX-2337 differs from what was seen with the TLR7 agonist imiquimod and the TLR9 agonist CpG ODN2006, two agents that have been extensively evaluated in multiple cancer types. VTX-2337 directly activates mDC, monocytes, and NK cells, resulting in the production of high levels of mediators including: TNFα, IL-12, and IFNγ, known to orchestrate adaptive anti-tumor responses. VTX-2337 activation of NK cells also augments ADCC of tumor cells by mAbs used in the treatment of some cancers. Importantly, VTX-2337 augmented ADCC activity in individuals with F/F and F/V FcγR3A genotypes, who have a less robust clinical response than individuals with the V/V genotype. Enhancement of the ADCC response has the potential to increase the effectiveness of clinically approved mAbs currently used in the treatment of some cancers. VTX-2337 has been tested in a first-in-man clinical trial evaluating the pharmacokinetics, pharmacodynamic responses as well as safety and tolerability in late stage oncology patients, and subsequent clinical oncology studies assessing VTX-2337 in combination with mAb therapies or anthracycline chemotherapy have been initiated.
Figure Legends:

Figure 1. VTX-2337 is a selective and potent TLR8 agonist that induces TNFα and IL-12 production. (A) Shown is the activation of TLR7, 8, and 9 HEK transfectants by VTX-2337 (●), imiquimod (□), and CpG ODN2006 (△). The y-axis shows the level of NF-κB-driven SEAP activity in the Quanti-blue assay. The x-axis shows the concentration of each compound. Each data point represents the mean±sem of optical density (OD) at 650nm of triplicate culture wells. Results shown are representative of three independent experiments. (B) Shown are TNFα and IL-12 levels in culture supernatants of human PBMC stimulated with VTX-2337 (●), imiquimod (□), and CpG ODN2006 (△). Each data point represents mean±sem of cytokine levels in duplicate wells as measured by ELISA. Results shown are representative of 10 independent experiments using PBMC from different donors.

Figure 2. VTX-2337 selectively induces the production of TNFα and IL-12 and activates NF-κB phosphorylation in monocytes and mDC, but not pDC. (A) Summary graphs showing the percentage of monocytes, pDC, and mDC that are positive for intracellular IL-12 (●), TNFα (■), and IFNα (▲) in PBMC after stimulation with VTX-2337 (800 nM), imiquimod (25,000 nM), or CpG ODN2006 (5,000 nM) for 18 hours. Each data point represents the response from an individual donor (n=10), while the horizontal bar represents the group mean. (B) Representative overlay histograms from one donor showing the change in intracellular cytokine levels in monocytes, pDC, and mDC populations from PBMC following VTX-2337-stimulation. The shaded histograms show intracellular cytokine staining in unstimulated cell populations, while the unshaded histograms with the solid line show staining in the same cell
populations following stimulation with VTX-2337 (800 nM, 18 hours). (C) Summary graph showing the percentages of monocyte, pDC, and mDC cell populations positive for phosphorylated NF-κB (∗) after stimulation with VTX-2337, imiquimod, or CpG ODN2006. Each data point represents the response from an individual donor (n=10), and the horizontal bar represents the group mean. (D) Representative overlay histograms from one donor showing the phosphorylation of NF-κB in unstimulated monocyte, pDC, and mDC populations in PBMC (shaded histograms) and following stimulated with VTX-2337 (unshaded histograms with solid line).

Figure 3. VTX-2337 stimulates IFNγ production from NK cells. (A) Representative flow cytometry graphs showing intracellular staining of IFNγ in CD4, CD8 T cells, γδ T cells, or NK cells in VTX-2337-treated PBMC (800 nM, 24 hour). The numbers in each graph represents the percentage of IFNg+ cells in each cell population. (B) Representative flow cytometry graphs showing intracellular staining of IFNγ in NK cells among control and VTX-2337 stimulated PBMC under conditions that allow for only the activation of cell populations that respond directly to TLR8 agonists. PBMC were treated with VTX-2337 (800 nM) for 6 hour and brefeldin A was included throughout the incubation. The numbers in each dot plot indicate the percentage of the gated NK cells that are positive for IFNγ and CD69. (C) Summary graph showing the percentages of IFNγ positive NK cells in control and VTX-2337-stimulated PBMC, where each dot represents the data from an individual normal donor and the horizontal bar represents the group mean (n=7 in each group). (D) Summary graph showing IFNγ levels in culture supernatant from PBMC as measured by ELISA. PBMC were treated with control medium alone, VTX-2337 (800 nM) or VTX-2337 and anti-IL18 (10 μg/ml) for 24 hr. Each dot
represents the data from an individual normal donor and the horizontal bar represents group mean (n=7 in each group). (E) IFNγ levels in the culture supernatant from purified NK cells isolated from 4 different donors, as measured by ELISA. NK cells (60,000 cells/well) were treated with VTX-2337 (800 nM, 24 hour) or control medium. Each column represents the mean±SD of triplicate culture wells. *, p<0.05; **, p<0.01; ***, p<0.001 by student t test or Mann-Whitney test.

**Figure 4. VTX-2337 enhances the NK cell lytic activity and augments rituximab and trastuzumab-mediated ADCC.** (A) Lysis of NK sensitive K562 cells by unstimulated or VTX-2337 treated (167 nM or 500 nM, 48 hours) PBMC. (B) Rituximab-mediated ADCC of HS-Sultan cells by unstimulated or VTX-2337 treated PBMC. (C) Trastuzumab-mediated ADCC of MDA-MB-231 breast cancer cells by unstimulated or VTX-2337 treated PBMC. (D) Rituximab-mediated ADCC using PBMC depleted of NK cells. (E) Rituximab-mediated ADCC using purified NK cells subsequently activated with VTX-2337. X axis shows effector:target (E:T) ratio; Y axis shows the percentage of specific lysis. ●, unstimulated PBMC or purified NK cells; ▲, PBMC treated with low dose VTX-2337 (167 nM, 48 hours); ■, PBMC or purified NK cells treated with high dose VTX-2337 (500 nM, 48 hours). Each data point represents mean±sem of triplicate treatment wells. **, p<0.01 from control; ***, p<0.001 from control by ANOVA analysis. Similar results have been obtained in 3 or more independent experiments with PBMC from different donors.

**Figure 5. VTX-2337 enhances ADCC in donors with different SNP on FcγR3A.** Shown is rituximab-mediated ADCC of HS-Sultan cells using PBMC from donors with low affinity for FcγR3A.
genotypes (F/F or F/V) and high affinity genotypes (V/V) with or without stimulation with VTX-2337 prior to ADCC analysis. PBMC were treated with VTX-2337 (500 nM) or control PBS for 48 hours before mixing with target cells. The box and whisker plot shows the minimum and maximum observations, the lower and upper quartiles, and the mean in each group. The graph summarizes results obtained from 5 independent ADCC analyses using PBMC from a total of 15 donors. *, p<0.05; ***, p<0.001
References:


34. Sabel MS, Arora A, Su G, Mathiowitz E, Reineke JJ, Chang AE. Synergistic effect of intratumoral IL-12 and TNF-alpha microspheres: systemic anti-tumor immunity is mediated by both CD8+ CTL and NK cells. Surgery. 2007;142:749-60.


Figure 1
Figure 2

A

VTX-2337  Imiquimod  CpG ODN2006

Monocytes

pDC

mDC

Percent cytokine positive

IL-12  TNFα  IFNα

B

VTX-2337-induced cytokines

Monocytes

pDC

mDC

IL-12  TNFα  IFNα

C

D

VTX-2337  Imiquimod  CpG ODN2006

Monocytes

pDC

mDC

Percent positive

pNFκB

pNFκB

pNFκB

pNFκB

pDC

mDC
Figure 3

A) Flow cytometry analysis of CD4 T cells, CD8 T cells, γδ T cells, and NK cells.

B) CD69 and IFN-γ expression in NK cells from PBS and VTX-2337 treated groups.

C) Percent cytokine positive (of total NK cells) comparison between Control and VTX-2337.

D) IFN-γ levels in NK cells from different donors treated with Control, VTX-2337, VTX-2337 + anti-IL-18.

E) IFN-γ levels in NK cells from different donors treated with PBS and VTX-2337.
Figure 4
Figure 5
Table 1. Production of proinflammatory mediators from human PBMCs activated with VTX-2337, Imiquimod and CpG ODN2006.

<table>
<thead>
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<th>Analyte</th>
<th>Mediator Level (mean±SEM, pg/mL)</th>
<th>EC_{50} (nM)</th>
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<tbody>
<tr>
<td></td>
<td>Control (1600 nM)</td>
<td>VTX-2337 (25,000 nM)</td>
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<tr>
<td>G-CSF</td>
<td>1.4±0.9</td>
<td>97±23.7*</td>
</tr>
<tr>
<td>IL-1α</td>
<td>1.3±0.7</td>
<td>321±127</td>
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<td>IL-1β</td>
<td>9.1±5.7</td>
<td>4,989±1,560</td>
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<tr>
<td>IFNγ</td>
<td>0.5±0.3</td>
<td>785±227*</td>
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<tr>
<td>IL-6</td>
<td>62±26</td>
<td>9,583±3,461</td>
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<tr>
<td>IL-8</td>
<td>211±119</td>
<td>9,850±4,720</td>
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<tr>
<td>IL-10</td>
<td>0.9±0.6</td>
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<td>IL-12p40</td>
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<td>IL-12p70</td>
<td>&lt;0.5</td>
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<tr>
<td>MIP-1α</td>
<td>188±91</td>
<td>15,258±3,547*</td>
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<tr>
<td>MIP-1β</td>
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* p<0.05 relative to Control
N/C = not calculated
# Clinical Cancer Research

## VTX-2337 Is a Novel TLR8 Agonist that Activates NK Cells and Augments ADCC


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