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, James Jeffry Howbert2, Mary L. Disis1, and Robert M. Hershberg2

Abstract

Purpose: We aim to characterize VTX-2337, a novel Toll-like receptor (TLR) 8 agonist in clinical development, and investigate its potential to improve monoclonal antibody–based immunotherapy that includes the activation of natural killer (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 peripheral blood mononuclear cells (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. Finally, we tested the potential for VTX-2337 to augment antibody-dependent cell-mediated cytotoxicity (ADCC), especially in individuals with low-affinity FcγR3A single-nucleotide polymorphism (SNP).

Results: VTX-2337 selectively activates TLR8 with an EC50 of about 100 nmol/L and stimulates production of TNFα and interleukin (IL)-12 from monocytes and myeloid dendritic cells (mDC). VTX-2337 stimulates IFNγ production from NK cells and increases the cytotoxicity of NK cells against K562 and 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 PBMCs with different FcγR3A genotypes (V/V, V/F, and F/F at position 158).

Conclusions: VTX-2337 is a novel small-molecule TLR8 agonist that activates monocytes, DCs, and NK cells. Through the activation of NK cells, it has the potential to augment the effectiveness of monoclonal antibody treatments where a polymorphism in FcγR3A limits clinical efficacy. Clin Cancer Res; 18(2); 1–11. ©2011 AACR.
A number of Toll-like receptor (TLR) agonists, especially agonists of TLR7 and TLR9, are being developed for their potential to enhance antitumor immunity. The therapeutic potential of TLR8 in cancer has not been fully assessed because of the lack of highly selective TLR8 agonists. Here, we report the characterization of a novel TLR8 agonist, VTX-2337. Our studies show that this agonist effectively activates mDCs and is more potent than imiquimod (TLR7) and CpG (TLR9) in inducing IL-12 and TNFα production by mDCs. 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 monoclonal antibodies. Our results highlight the potential of using this novel TLR8 agonist to induce an immune response to tumors and improve clinical responses to clinically approved monoclonal antibody therapies, especially in individuals who show reduced ADCC activity.

**Materials and Methods**

**Reagents**

VTX-2337 is a synthetic small-molecule agonist of TLR8 [molecular weight (MW), 458.6; Supplementary Fig. S1] that is based on a 2-aminobenzazepine core structure and has been evaluated in a phase I oncology trial (NCT00688415) sponsored by VentiRx Pharmaceuticals. Imiquimod, CpG ODN2006 (5′-TCG TCG TTT CGT TTT TTC GTT-3′), and CPG79 were purchased from InvivoGen. RPMI culture media for culturing human peripheral blood mononuclear cells (PBMC) were purchased from Invitrogen. Cell surface–specific, phospho-protein–specific, and cytokine-specific fluorochrome-labeled Abs for flow cytometry were obtained from BD Biosciences.

**NF-κB activation in HEK cells transfected with TLRs**

Human embryonic kidney cells (HEK293) expressing TLR2, 3, 4, 5, 7, 8, or 9 were purchased from InvivoGen. The cells were cultured in Dulbecco’s Modified Eagle’s Media (Cambrex) containing 4.5 g/L d-glucose (Sigma-Aldrich) 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) after TLR agonist treatment was carried out similarly as described previously (24).

**Measurement of cytokine and chemokine secretion from human PBMCs following stimulation with TLR agonists**

Blood was collected from 10 healthy human donors after obtaining appropriate informed consent. The PBMCs 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 PBMCs (200,000 per well) were plated in 96-well round-bottom culture plates and treated with serial dilutions of the TLR agonists: imiquimod (39–50,000 nmol/L), VTX-2337 (6–6,400 nmol/L), or CpG ODN2006 (23–3,000 nmol/L) 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; IFNγ kit from PBL InterferonSource) 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. The procedures for determining cytokine/chemokine levels by ELISA and Luminex methods were carried out following the
manufacturer’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 PBMCs was assessed by intracellular staining, using methods similar to those previously described (25). PBMCs were cultured in vitro in polypropylene tubes in the absence (unstimulated control) or presence of CpG ODN2006 (5,000 nmol/L), VTX-2337 (50–800 nmol/L), or imiquimod (1,000–50,000 nmol/L). 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- to 18-hour activation period, the cells were initially stained with fluorochrome-conjugated antibodies to surface markers CD3 + CD19 AmCyan, HLA-DR APC-H7, CD11c V450, CD123 PerCP-Cy5.5, CD56 PE-Cy7, CD16 PE-Cy7, and CD14 Alexa 700 (BD Biosciences). After subsequent fixation and permeabilization (BD Cytofix/CytoPerm Fixation/Permeabilization Solution Kit), the cells were stained for intracellular markers using TNFα fluorescein isothiocyanate (FITC), IFNα Alexa 647, and IL-12 phycocerythrin (PE; BD Biosciences). Samples were analyzed on a BD LSRII flow cytometer using FACSDiva software. Monocytes were defined as CD14+ cells, pDCs were defined as CD3− CD14− CD19− CD16− CD35− (lineage negative), HLA-DR+, CD11c−, CD123−, and mDC were defined as lineage negative, HLA-DR+, CD11c+ cells. To measure IFNγ production in NK cells, both overnight and short-term (6 hours) incubation periods were used. 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**

PBMCs 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 × 10⁶ to 4 × 10⁶/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 (50–800 nmol/L), imiquimod (1,000–50,000 nmol/L), or CpG ODN2006 (10–6,000 nmol/L). 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% bovine serum albumin, 0.5% NaN₃) and stained with HLA-DR PerCP-Cy5.5 and p-NF-κB PE (BD Biosciences) for 1 hour at room temperature in the dark, then washed again twice in wash buffer. Flow cytometric analysis was conducted on a BD FACSCantoII flow cytometer using BD FACSDiva software (BD Biosciences). The definition of NK cells, 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**

PBMCs 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, PBMCs or purified NK cells were cultured for 48 hours in RPMI medium in the presence of VTX-2337 (167 or 500 nmol/L) 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: [(experimental release — spontaneous release)/(maximal release — spontaneous release)] × 100%.

**Measurement of FcyR3A single-nucleotide polymorphism**

The FcyR3A-158 genotype was determined using a method similar to what has been previously published (20). DNA was extracted from human PBMCs with a QIA DNA mini kit (Qiagen) as per the manufacturer’s instructions. TaqMan genotyping assay with pre-made primer and probes from Applied Biosystems was used to determine the FcyR3A-158 single-nucleotide polymorphism (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 conducted 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 EC₅₀ 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) = E₀ + (Eₘ₋ₐₓ₋₀) × (C/C + EC₅₀). The percentages of IFNγ-positive NK cells in VTX-2337–treated and unstimulated PBMCs were compared using 2-tailed Mann–Whitney test. A P value of ≤0.05 was considered significant.
Results

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

The selectivity and potency of VTX-2337 was initially evaluated and compared with the TLR7 agonist imiquimod and TLR9 agonist CpG ODN2006 using HEK293 cells transfected with various human TLRs. Among the TLRs tested (TLR2, 3, 4, 5, 7, 8, and 9), VTX-2337 selectively activated TLR8. As shown in Fig. 1A, imiquimod and CpG ODN2006 selectively activated TLR7 and TLR9 respectively, as expected. Only VTX-2337 activated TLR8. Although 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 (~5,000 nmol/L).

To further characterize the immunostimulatory activity of this novel TLR8 agonist, human PBMCs 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 Fig. 1B, VTX-2337 stimulated the production of both TNFα (EC50 = 140 ± 30 nmol/L based on 10 donors) and IL-12 (EC50 = 120 ± 30 nmol/L based on 10 donors) in PBMCs. Imiquimod stimulated low levels of TNFα and IL-12 but only at concentrations exceeding 3,000 nmol/L. However, imiquimod also stimulated secretion of IFNα, as previously reported, whereas VTX-2337 did not (data not shown; ref. 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 with 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 (Supplementary Fig. S1B). Consistent with the HEK data, the 2 compounds show similar profiles for TNFα and IFNα induction in PBMCs (Supplementary Fig. S1C), although VTX-2337 is more potent than CL075 in inducing TNFα (Supplementary Fig. S1C).

VTX-2337 drives mDCs and monocytes to produce IL-12 and TNFα via NF-κB activation

To elucidate the cell subsets in PBMCs that were the source of the TNFα, IL-12, and IFNα, intracellular staining by flow cytometry was carried out on PBMC following TLR7, 8, and 9 stimulation. Agonists were evaluated at concentrations corresponding to approximately their EC50 in the HEK transfectant assay. As shown in Fig. 2A, VTX-2337 (800 nmol/L) stimulated the production of both TNFα and IL-12 in a high percentage of monocytes (59% ± 10% positive for TNFα and 14% ± 4% positive for IL-12, n = 10) and mDCs (57% ± 8% positive for
TNFα and 15% ± 3% positive for IL-12, n = 10), but not in pDCs. In contrast, imiquimod (25,000 nmol/L) and CpG ODN2006 (5,000 nmol/L) stimulated TNFα production in a high percentage of pDC (77% ± 4% for imiquimod and 55% ± 7% for CpG ODN2006, n = 10) but not in mDCs or monocytes. Both imiquimod and CpG ODN2006 stimulated IFNα production in a low percentage of pDCs (7% ± 2% of imiquimod and 4% ± 1% for CpG ODN2006, n = 10), whereas VTX-2337 did not stimulate IFNα in pDCs. Representative histograms showing intracellular staining levels of IL-12, TNFα, and IFNα in monocytes, pDC, and mDC subsets in control and VTX-2337-stimulated PBMCs are shown in Fig. 2B. The selective activation of mDCs and monocytes, but not of pDCs, by VTX-2337 was further shown by intracellular detection of phosphorylated signaling proteins by PhosFlow analysis. VTX-2337 stimulated NF-κB phosphorylation in a high percentage of monocytes and mDCs (44% ± 7% and 81% ± 4%, respectively), whereas imiquimod and CpG ODN2006 stimulated NF-κB phosphorylation mainly in pDCs (18% ± 2% and 32% ± 3%, respectively; Fig. 2C). Representative histograms showing the change in levels of phosphorylated NF-κB in the different cell populations following VTX-2337 treatment are shown in Fig. 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 whether other important differences exist, the repertoire of other cytokines and chemokines induced in PBMCs following VTX-2337 activation was compared with 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 (Fig. 1). VTX-2337 at a concentration of 1,600 nmol/L 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 nmol/L or CpG ODN2006 at 1,500 nmol/L, as shown in Table 1. In contrast, VTX-2337 and imiquimod induced comparable levels of IL-10 and all 3 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 with VTX-2337, the magnitude of induction by VTX-2337 was generally much greater. For example, the EC50 values for MIP-1β induction were 60 nmol/L for VTX-2337 and 30 nmol/L for CpG ODN2006, yet VTX-2337 induced a maximum MIP-1β response that was about 10-fold higher (4,262 ± 1,011 ng/mL for VTX-2337 vs. 472 ± 169 ng/mL for CpG ODN2006), as shown in Table 1. Another mediator of interest was IFNγ, where VTX-2337 resulted in a 1,000-fold induction over the unstimulated control, whereas imiquimod induced only a 15-fold increase and CpG ODN2006 failed to stimulate this cytokine (Table 1).

VTX-2337 activates NK cells to produce IFNγ

To determine the cellular source of the IFNγ seen in activated PBMCs, intracellular staining was carried out on different cell populations, including CD4 and CD8 T cells, γδ T cells, and NK cells, after incubating PBMCs with VTX-2337 800 nmol/L for 24 hours. As shown in Fig. 3A, the major source of IFNγ in PBMCs 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 (6 hours) in the presence of brefeldin A, which blocks cytokine release from activated cells. Under these conditions, a robust induction of IFNγ in NK cells (10.6% ± 4.5% CD69+IFNγ+ NK cells in VTX-2337–treated PBMCs vs. 1.1% ± 0.4% in the unstimulated controls, \( P = 0.004; \) Fig. 3B and C) was observed, showing a direct effect of VTX-2337 on NK cells. To determine whether 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 PBMCs with VTX-2337 (0.8 μmol/L). The response to IL-12 blockade was variable between donors, and IL-12 blockade did not significantly decrease VTX-2337–induced IFNγ production (data not shown). IL-18 blockade significantly decreased IFNγ production (Fig. 3D). However, there was significant induction of IFNγ by VTX-2337 even in the presence of anti-IL-18 mAb, suggesting the existence of a direct effect of VTX-2337 on NK cells (Fig. 3D). To confirm the direct effect of VTX-2337 on NK cells, purified NK cells were treated with VTX-2337. In 3 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 PBMCs (Fig. 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 show 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 reverse transcriptase (RT)-PCR using RNA from fluorescence-activated cell-sorted (FACS) cells. As shown in...
Supplementary Fig. S2, TLR8 was absent on pDCs and was expressed at increasing levels on NK cells, monocytes, and mDCs. In contrast, TLR7 and TLR9 were highly expressed on pDCs, but not on mDCs.

**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 Fig. 4A, PBMCs pretreated with VTX-2337 (167 or 500 nmol/L, 24 hours) showed enhanced lysis of the K562 target cells. To determine whether TLR8 activation can also augment NK cell–mediated ADCC, VTX-2337–stimulated, or unstimulated PBMCs 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 Fig. 4B and C, VTX-2337–stimulated PBMCs had enhanced ADCC 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 (Supplementary Fig. S3). 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 (Fig. 4D). Purified NK cells activated with VTX-2337 (500 nmol/L, 24 hours) showed enhanced ADCC as compared with unstimulated NK cells, indicating that VTX-2337 acts directly on this cell population (Fig. 4E).
Enhancement of ADCC in FcγR3A variants

Previous studies have found that a polymorphism in the FcγR3A 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 whether this common polymorphism affects the baseline ADCC response and/or response following VTX-2337 activation, donors were genotyped for the 2 alleles encoding the F and V isoforms, respectively. Rituximab-mediated ADCC using unstimulated and VTX-2337–stimulated PBMCs 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 Fig. 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 PBMCs were stimulated with VTX-2337, ADCC was significantly enhanced in both F/F and F/V and V/V genotypes (Fig. 5). The level of ADCC was enhanced from 20.5% to 40.0% in the higher affinity FcγR3A phenotype (P = 0.0007) and from 31.7% ± 2.9% to 55.5% ± 2.6 in the higher affinity FcγR3A corresponding with the V/V genotype (P = 0.0003). The level of ADCC seen with VTX-2337–stimulated PBMCs from F/F and F/V donors was also found to be comparable with the level of ADCC seen in unstimulated PBMCs 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 show 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. In addition, 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 mDCs, monocytes, and NK cells, whereas TLR7 and TLR9 are expressed in pDCs. 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 that induced by 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 antitumor 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 anticancer effect of standard chemotherapy. It has been previously reported that TLR7/8 agonist resiquimod synergizes with TLR4 agonist in activating DCs 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 DCs 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 mDCs and monocytes, VTX-2337 appears to have a direct effect on NK cells, as shown by increased IFNγ production, enhanced cytotoxicity toward 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 has been documented in previous publications (21, 38–40). 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 antitumor 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 (Supplementary Fig. S3) 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 and colleagues 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 and colleagues 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 showed that 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 shown 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 (Supplementary Fig. S4A–S4C). 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 (Supplementary Fig. S4D and S4E), 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). Because 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 PBMCs 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 and 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 3 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 show 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, 2 agents that have been extensively evaluated in multiple cancer types. VTX-2337 directly activates mDCs, monocytes, and NK cells, resulting in the production of high levels of mediators including TNFα, IL-12, and IFNγ, known to orchestrate adaptive antitumor 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.

Disclosure of Potential Conflicts of Interest

H. Lu has received commercial research grant from VentiRx Pharmaceuticals. M.J. Disis is a consultant/advisory board member for VentiRx Pharmaceuticals. The other authors disclosed no potential conflicts of interest.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received June 23, 2011; revised November 4, 2011; accepted November 7, 2011; published OnlineFirst November 29, 2011.

References


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


Clin Cancer Res  Published OnlineFirst November 29, 2011.

Updated version
Access the most recent version of this article at:
doi:10.1158/1078-0432.CCR-11-1625

Supplementary Material
Access the most recent supplemental material at:
http://clincancerres.aacrjournals.org/content/suppl/2011/11/29/1078-0432.CCR-11-1625.DC1

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.