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Pharmacokinetic and Pharmacodynamic Properties of GS-9620, a Novel TLR7 Agonist, Demonstrates ISG Induction without Detectable Serum Interferon at Low Oral Doses

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Running Title: PK and PD of the TLR7 Agonist GS-9620

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No. of text pages: 23

No. of tables: 4

No. of figures: 6

No. of references: 31

No. of words in Abstract: 250

No. of words in Introduction: 679

No. of words in Discussion: 1329

Nonstandard Abbreviations: AUC, area under the concentration-time curve; cDCs, conventional dendritic cells ; CL, clearance; C_{\max} , maximum plasma/serum concentration; EC_{\max} , maximum effective concentration; E_{\max} , maximum amount of cytokine produced; F, bioavailability; GALT, gut-associated lymphoid tissue; HBV, hepatitis B virus; HCV, hepatitis C virus; IFN, interferon; ISGs, interferon-stimulated genes; LLOQ, lower limit of detection; MEC,

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minimum effective concentration; MX1, Myxovirus resistance 1; OAS1, 2'5'-oligoadenylate synthetase 1; OD, optical density; PBMC, peripheral blood mononuclear cell; pDCs, plasmacytoid dendritic cells; SEAP, secreted alkaline phosphatase; SFBR, Southwest Foundation for Biomedical Research; $t_{1/2}$, half-life; TLR7, Toll-like receptor 7; T_{max} , time to reach C_{max} ; TNF, tumor necrosis factor; V_{ss} , volume of distribution at steady state.

Recommended Section Assignment: Drug Discovery and Translational Medicine

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ABSTRACT

GS-9620 is a potent, orally bioavailable small molecule agonist of Toll-like receptor 7 (TLR7) being developed for finite treatment of chronic hepatitis B viral (HBV) infection with the goal of inducing a liver-targeted antiviral effect without inducing the adverse effects associated with current systemic IFN- α therapies. We characterized the pharmacodynamic response of GS-9620 in CD-1 mice and cynomolgus monkeys following intravenous or oral administration and showed that GS-9620 induces the production of select chemokines and cytokines, including interferon- α (IFN- α) and interferon-stimulated genes (ISGs). Importantly, we also demonstrate in animals and healthy human volunteers that oral administration GS-9620 can induce a type I interferon-dependent antiviral innate immune response, as measured by whole blood mRNA of the ISGs 2'5'-oligoadenylate synthetase 1 (OAS1) and Myxovirus resistance 1 (MX1), without the induction of detectable systemic IFN- α i.e. a presystemic response. Additionally, presystemic induction of hepatic OAS1 and MX1 mRNA was observed in CD-1 mice in the absence of detectable systemic IFN- α . We propose that the mechanism of this presystemic response is likely its high intestinal absorption, which facilitates localized activation of TLR7, likely in plasmacytoid dendritic cells at the level of gut-associated lymphoid tissue and/or the liver. This localized response is further supported by data that indicate only minimal contributions of systemic immune stimulation to the overall pharmacodynamic response to orally administered GS-9620. These data demonstrate that GS-9620 can induce an antiviral innate immune response without inducing a systemic IFN- α response and thus suggest the therapeutic potential of this approach in the treatment of chronic HBV infection.

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INTRODUCTION

Toll-like receptor 7 (TLR7) is a pathogen recognition receptor that plays an important role in the detection of, and the innate immune response to, pathogens. TLR7 is predominantly activated by viral single-stranded RNA and is localized within the endolysosomal compartments of plasmacytoid dendritic cells (pDCs) and B lymphocytes in humans and non-human primates (Ketloy et al 2008, Hornung et al 2002, Jarrossay et al 2001, Kadowaki et al 2001). Activation of pDCs plays an important role in the innate response to viral pathogens and are responsible for the majority of type I interferon (IFN) production during the acute phase of a viral infection. The induction and secretion of endogenous IFNs (e.g., IFN- α , IFN- β) also facilitate the development of an efficient adaptive immune response (Colonna et al 2004, Feld and Hoofnagle 2005, Kanzler et al 2007, Barbalat et al 2011). Interferons induce the transcription of interferon-stimulated genes (ISGs) which generate an antiviral state within cells (de Veer et al 2001, Schoggins et al 2011), as well as induce the production of other cytokines and chemokines which facilitate intercellular communication and cellular trafficking.

Administration of exogenous IFN- α (e.g., recombinant IFN- α or pegylated IFN- α [PEG]), provides therapeutic benefit in patients with chronic hepatitis B (HBV) and C (HCV) infections. In HBV, durable responses, normalization of alanine aminotransferase (ALT), and sustained reduction in viremia have been attained in a small percentage (less than 20%) of patients treated for one year with exogenous IFN- α (Moucari et al 2009, Marcellin et al 2009, Reijnders et al 2011). A key observation is loss of HBV surface antigen (HBsAg) and seroconversion for anti-HBsAg antibody, following IFN- α treatment increases for several years after discontinuation.

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This supports the hypothesis that viral control is due to immune modulation and slow induction of a protective antiviral immune response.

However, exogenous IFN- α treatment has several notable barriers to patient acceptance. These include the necessity of subcutaneous administration, and treatment-limiting adverse effects (Mourcari et al 2009, Marcellin et al 2009, Reijnders et al 2011). The low rate of HBsAg loss and seroconversion and high rate of adverse events associated with current therapies illustrate the need for new approaches to induce a protective antiviral immune response and durable cure in patients with chronic HBV. Consequently, orally administered immunomodulatory therapies are an attractive approach to develop tolerable, safer therapies that are more broadly applicable and potentially associated with higher long-term viral eradication rates in a larger percentage of treated patients.

GS-9620, a dihydropteridinone derivative, was designed to induce a presystemic response, defined as local stimulation of innate immune cells, likely in plasmacytoid dendritic cells (pDCs) in gut-associated lymphoid tissue (GALT) and/or the liver without the induction of a systemic IFN- α response. GS-9620 possesses high absorption properties from the gastrointestinal tract but has moderate clearance during first-pass hepatic metabolism. These properties minimize systemic exposure of GS-9620 following oral administration while targeting exposure to, and stimulation of, innate immune cells within the gastrointestinal tract and/or liver. This presystemic response can be detected in whole blood by the presence of 2'5'-oligoadenylate synthetase 1 (OAS1) and Myxovirus resistance 1 (MX1) mRNA. Both OAS1 and MX1 are specific and sensitive biomarkers of IFN- α induction (Roers et al 1994, von Wussow et al 1990, Schattner et al 1981). The therapeutic benefit of TLR7 stimulation by oral GS-9620 in animal models of chronic HBV infection has been shown in both chimpanzees infected with HBV and

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the woodchuck hepatitis virus (WHV) model. In both models, oral administration of GS-9620 reduced viremia and induced an antiviral immune response, and in woodchucks, GS-9620 induced an antibody response to viral surface antigen with subsequent clearance of viral S-antigen (Lanford et al 2013, Menne et al 2011).

We conducted a series of studies to explore the pharmacodynamic and pharmacokinetic properties of GS-9620, a selective TLR7 agonist, and to determine whether oral administration of low doses of GS-9620 could induce OAS1 and MX1 mRNA in peripheral blood in the absence of detectable systemic IFN- α in mice and cynomolgus monkeys. Additionally, we also assessed the relative contribution of systemic immune stimulation to the pharmacodynamic response to GS-9620.

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MATERIALS and METHODS

In addition to the information below, details on experimental approaches and reagents are available in the supplemental materials.

TLR7 Agonist

GS-9620 is a dihydropteridinone derivative and was synthesized by the department of medicinal chemistry Gilead Sciences, Inc. (Supplemental Figure 1; Roethle et al 2013).

NF- κ B Reporter Assay

Murine TLR activity assays were performed at InvivoGen (San Diego CA <http://www.invivogen.com/hek-blue-tlr-cells>).

Splenocyte and PBMC In Vitro Cytokine Release Assays

Cytokine release in mouse (splenocytes) and cynomolgus monkey (peripheral blood mononuclear cells; PBMC) was performed by Eurofins (Bothell, WA) using standard methods. Secreted proteins in cell culture supernatants were assessed by multiplex immunoassay using species-specific, standard Luminex and/or ELISA technologies.

In Vivo Studies

Animals

In vivo studies were conducted at Covance Laboratories, Inc. All protocols were approved by the respective Institutional Animal Care and Use Committees.

Blood collection

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Blood was collected at various timepoints and processed for serum and/or RNA. In the mouse studies, 3-5 mice were euthanized at each time point via cardiac puncture under CO₂ anesthesia. For cynomolgus monkeys, serial blood samples were collected from conscious animals via the femoral vein or other suitable veins.

Cytokine analysis

Cytokine evaluation was performed by Eurofins (Bothell, WA). Cytokine detection in serum was accomplished by a multiplex immunoassay using species-specific, standard Luminex and/or ELISA technologies.

ISG Analysis

Whole blood was collected as described above into tubes containing K2EDTA. Isolated whole blood total RNA was quantified by absorbance spectroscopy, and equal quantities were used as template for 1-step RT-PCR TaqMan[®] reactions to measure the level of RNA transcripts of the interferon-stimulated genes OAS1 and MX1 and the control genes GAPDH or 18S. Individual primer/probe sets were purchased from Life Technologies (Carlsbad, CA). Each mRNA signal was normalized to the mRNA signal of the endogenous control (GAPDH or 18S ribosomal RNA) in the same sample using a duo-plex approach.

Data Analysis

Unless otherwise stated, male and female animals administered the same dose were combined, and values below LLOQ were excluded from analysis. Fold increases in ISG values were calculated for each individual animal relative to the predose value if available, or were calculated relative to the ISG mean from animals treated concurrently with vehicle. Mean \pm SD fold

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increases in ISG for each dose level was calculated using individual fold change values. Where data are presented in the absence of systemic IFN- α , the absence of systemic IFN- α is defined as IFN- α levels below the LLOQ at all timepoints where IFN- α was evaluated for the individual animal.

Human Sample Analysis. Human blood samples were collected from healthy volunteers and analyzed for IFN- α , MX1, and OAS1 as previously described (Lopatin et al, 2013).

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RESULTS

GS-9620 is Selective for TLR7 over other TLRs in a NF- κ B dependent cell based reporter assay

To assess the selectivity of GS-9620 for TLR7 versus other TLRs, GS-9620 was evaluated in a NF- κ B-dependent reporter cell based assays with murine TLRs. In this assay, GS-9620 activated mouse TLR7 at concentrations ≥ 100 nM (Fig. 1A) with no detectable activation of the other TLRs, including TLR8, up to the maximum concentration tested of 100 μ M (Fig. 1B), whereas all TLRs were activated by the respective positive control agents. Prior studies evaluated the selectivity of GS-9620 for human TLR7 and demonstrated an approximately 30-fold selectivity of GS-9620 for activation of human TLR7 over TLR8 with no detectable activity on other human TLRs in similar reporter cell based assays (Tumas et al 2011). However, it should be noted that NF- κ B is only one of several transcription factors activated by TLR7 signaling (He et al 2013), and therefore the reporter assay employed in this study does not necessarily permit extrapolation about GS-9620 function in different cells or species but merely served as a tool to investigate receptor selectivity.

GS-9620-Dependent In Vitro Cytokine Secretion

The pharmacodynamic response of GS-9620 was initially assessed in splenocytes isolated from CD-1 mice and in PBMCs isolated from healthy cynomolgus monkeys. By comparing the pattern of cytokines and chemokines induced in these primary cellular assays, we assessed the selectivity of GS-9620 for TLR7, as measured by induction of IFN- α , versus TLR8, as measured by the induction of TNF- α , in a PBMC assay with a complete complement of immune competent cells. Additionally, we assessed how species specific differences in the expression of TLR7 and

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TLR8 can affect the observed GS-9620 selectivity in primary cells. It has been reported that murine TLR7 expression is notable on both cDCs and pDCs, which is in contrast to cynomolgus monkeys and humans, where TLR7 expression is largely restricted to pDCs and TLR8 expression is predominantly expressed in cDCs (Edwards et al 2003, Ketloy et al 2008).

Not unexpectedly and consistent with the reported differences in TLR7/8 expression in the mouse, the pattern of cytokines and chemokines induced by GS-9620 was notably different in murine splenocytes when compared to the pharmacodynamic response in cynomolgus monkey PBMCs. In murine splenocytes, the minimum effective concentration (MEC) of GS-9620 to induce IFN- α was 30 μ M whereas the MEC for the induction of TNF- α was 30 nM (Table 1). The relative amount of TNF- α induced was also higher than the induced levels of IFN- α in these cultures and dose-response relationships appeared roughly linear (Fig. 2A).

In contrast to the mouse, GS-9620 preferentially induced IFN- α over TNF- α at low concentrations in cynomolgus monkey PBMCs. The amount of TNF- α induced was markedly lower than IFN- α at GS-9620 concentrations less than 10 μ M and dose-dependent induction of TNF- α was only observed at concentrations greater than 1 μ M (Fig. 2B). This is consistent with GS-9620 selectivity for TLR7 versus TLR8 at lower concentrations and combined TLR7 and 8 stimulation at higher concentrations of GS-9620. TLR7 agonism preferentially mediates secretion of IFN- α in PBMC cultures whereas TLR8 stimulation in cDC and monocyte PBMC subsets is viewed as the dominant trigger for secretion of inflammatory cytokines, e.g., TNF- α , (Gorden et al 2005, Thomas et al 2007). Although GS-9620 induced IFN- α at a mean (\pm SD) MEC of 308 \pm 463 nM and induced TNF- α at a mean (\pm SD) MEC of 285 \pm 478 nM (Table 1),

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the similar MEC for IFN- α and TNF- α is not unexpected, as TLR7-dependent activation of pDCs can result in proinflammatory cytokine secretion on a small scale, as was observed here. This observed pattern and profile of induced cytokines in the cynomolgus monkey was generally comparable to that we have reported in human PBMCs where we have shown that the mean MEC for induction IFN- α and TNF- α were 66 nM and 3650 nM, respectively, in the same set of donors (Tumas et al 2011).

GS-9620 has high intestinal absorption, but low to intermediate metabolic stability and bioavailability

GS-9620 was designed to have high intestinal absorption and moderate clearance, primarily via hepatic metabolism by cytochrome P450 3A4 (CYP3A4), during hepatic first-pass extraction to maximize exposure to interferon-producing cells in the GALT and/or liver while minimizing systemic exposure. In vitro experiments with hepatic microsomal fractions from CD-1 mouse, cynomolgus monkey, and human revealed a GS-9620 half life ($T_{1/2}$) of 4.9 min, 5.9 min, and 30.5 minutes, respectively, indicating that GS-9620 will have moderate to high hepatic metabolic clearance in all species. Consistent with the in vitro hepatic microsomal prediction, GS-9620 has low oral bioavailability in mouse (0.2%), monkey (1.1%) and moderate bioavailability in dog (20%) (Table 2). However, data from a study in portal vein-cannulated male beagle dogs demonstrated that the amount of GS-9620 entering the portal vein (F_{ABS}) was high (82%; Supplement Table 1), indicating that GS-9620 is readily absorbed from the gastrointestinal tract following oral administration.

IFN- α Response is higher following oral versus i.v. administration despite similar systemic exposure

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To assess the relative contribution of systemic GS-9620 exposure to the peripheral induction of IFN- α , the IFN- α response after a single i.v. versus oral administration of GS-9620 was directly compared in CD-1 mouse and cynomolgus monkey. Comparable systemic exposure (assessed by both C_{\max} and AUC) following i.v. and oral administration resulted in notably higher peripheral IFN- α concentration after oral administration in both mouse and cynomolgus monkey (Table 3). In mouse, the mean GS-9620 C_{\max} following an oral dose at 100 mg/kg and i.v. infusion at 1 mg/kg was similar: 277 versus 305 ng/ml, respectively. However, despite this similar systemic exposure, the mean peak serum IFN- α levels following oral administration was more than 100-fold higher than that from i.v. infusion: 6572 versus 60 pg/ml, respectively (Table 3).

A similar pattern of pharmacodynamic response was observed in cynomolgus monkeys. An oral dose at 2 mg/kg resulted in a C_{\max} that was approximately 2-fold lower than achieved after i.v. infusion of 0.1 mg/kg GS-9620 to male cynomolgus monkeys: 66 vs. 128 ng/ml, respectively (Table 3). However, the mean peak IFN- α serum level induced after oral dosing was more than 60-fold higher than that produced after i.v. infusion: 119,000 versus 1840 pg/ml, respectively (Table 3). These data suggest that systemic exposure to GS-9620 via the intravenous route generates a minimal pharmacodynamic response when compared to exposure to GS-9620 via the oral route.

Profiling of Pharmacodynamic Response to GS-9620 Following Oral Administration

Consistent with observations from in vitro experiments described above, oral administration of single ascending oral doses of GS-9620 to CD-1 mice and cynomolgus monkeys resulted in dose-dependent induction of serum cytokines and chemokines. In CD-1 mouse, single ascending

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oral doses of GS-9620 ranging from 0.1 mg/kg to 50 mg/kg resulted in dose-dependent induction of IFN- α and cytokines and chemokines, generally at doses ≥ 1 mg/kg (Fig. 3A). Similar to the findings attained from in vitro mouse splenocyte cultures, the proinflammatory cytokines IL-1 β and TNF- α were induced in mouse concurrently with the induction of IFN- α which is consistent with the different expression of TLR7 in the mouse.

In contrast to the in vivo mouse pharmacodynamic profile, but consistent with the results from cynomolgus monkey in vitro PBMC studies, in vivo administration of single oral doses of GS-9620 (0.05 to 10 mg/kg) to cynomolgus monkeys preferentially induced dose-dependent amounts of IFN- α at low oral doses, whereas induction of TNF- α was only observed at doses ≥ 1.5 mg/kg (Fig. 3B). These data indicate that GS-9620 can induce a selective, TLR7-dependent pharmacodynamic response in vivo with TLR8-dependent cytokines and chemokines only observed after administration of high oral doses.

Relationship of GS-9620 Exposure, Serum IFN- α and Induction of ISGs in Whole Blood

As a biomarker for an antiviral immune response, peripheral whole blood mRNA expression of the ISGs OAS1 and MX1 were evaluated and we characterized the kinetic relationship of ISG induction to GS-9620 serum exposure and peripheral blood IFN- α concentration in cynomolgus monkey after a single oral dose of 0.15 mg/kg (Fig. 4). The induction of peripheral IFN- α and ISG was transient and appeared sequential following the rapid absorption and clearance of GS-9620 ($C_{max} = 1.6 \pm 1.1$ ng/ml; $T_{max} = 0.8 \pm 0.3$ hours). Peak induction of peripheral IFN- α was observed at approximately 4 hours postdose and was transient; levels rapidly decreased following 4 hours post dose and returned to base line levels by 12 hours post dose. This peak peripheral IFN- α concentration was followed induction of peripheral ISGs which were maximal

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at approximately 8 hours post dose. ISG induction was also transient and returned to baseline by 24 hours post dose. This data is consistent with a single, transient activation of TLR7, followed by the sequential induction of IFN- α which in turn induces the ISGs OAS1 and MX1.

GS-9620–Dependent ISG Induction is Independent of Detectable Peripheral Interferon- α

Importantly, we also observed dose-dependent induction of ISG expression in peripheral blood without concurrent induction of systemic IFN- α in individual cynomolgus monkeys and mice after a single oral dose of GS-9620 (Table 4, Fig. 5). This data, described below, suggests local induction of IFN- α induces ISGs in blood cells trafficking out of tissues, which then can be detected as peripheral ISG induction in whole blood.

In mice orally dosed with 0.3 mg/kg GS-9620, elevated peripheral blood and hepatic gene expression of OAS1 and MX1 was observed in a time dependent manner in the absence of concurrently detectable systemic IFN- α (Table 4, Fig. 5). Hepatic gene expression of MX1 and OAS1 was induced in the absence of detectable systemic IFN- α (i.e., >3-fold increase from vehicle control) in individual animals at 4 (MX1 only), 8 and 12 hours post dose (Fig. 5).

Importantly, no ISG induction was observed in any animal at 24 hours post dose. This data is consistent with the sequential induction of ISGs following TLR7 activation described above.

However, the possibility that systemic IFN- α was induced at an earlier timepoint in these animals cannot be excluded as serial sampling was not feasible in this experiment.

In cynomolgus monkey, MX1 and OAS1 were induced (i.e., >3 fold increase from vehicle-treated monkeys) in the absence of systemic IFN- α following administration of a single dose of GS-9620 as low as 0.05 mg/kg, the lowest dose evaluated (Table 4). Despite the lack of detectable systemic IFN- α in these cynomolgus monkeys, induction of MX1 and OAS1 mRNA

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was dose dependent. Interestingly, when the ISG response was compared between animals with and without detectable systemic IFN- α , mean MX1 and OAS1 fold increase in animals with systemic IFN- α was within 3-fold of mean ISG induction observed animals without detectable serum IFN- α (Table 4). This is despite mean systemic IFN- α as high as 28,186 pg/mL in animals administered 1.5 mg/kg. These data suggest that systemic IFN- α is not a dominant driver of GS-9620-dependent induction of ISGs in peripheral blood. This, along with the observations following oral versus i.v. administration, further support the hypothesis the pharmacodynamic response is driven by presystemic activation of TLR7, either in the gut-associated lymphoid tissue and/or liver.

The induction of an ISG response in whole blood, without induction of detectable levels of serum IFN- α , was also observed in a single dose study in healthy human volunteers (Lopatin et al 2013). After single ascending oral doses of GS-9620 ranging from 0.3 mg to 12 mg, dose-dependent induction of MX1 and OAS1 mRNA relative to predose was observed following GS-9620 oral doses 2 mg and above (Lopatin et al 2013). Of note, no subject had detectable systemic IFN- α at doses of \leq 6 mg (LLOQ = 15.6 pg/ml). One subject at 8 mg and 4 subjects at 12 mg had detectable IFN- α . Similar to the cynomolgus monkeys, induction of detectable IFN- α did not appear to have a notable effect on ISG induction. In the 4 subjects with detectable IFN- α at 12 mg, ISG induction was not notably different than in subjects without detectable systemic IFN- α (Table 4). This data suggests that, at the level of peripheral blood gene expression, a type I IFN-independent anti-viral response can be invoked after oral administration of GS-9620 in humans without concurrent induction of detectable systemic IFN- α .

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DISCUSSION

Profiling of GS-9620-Induced Cytokine and Chemokine Secretion In Vitro and In Vivo

In vitro stimulation of cynomolgus monkey PBMCs with GS-9620 at concentrations less than 10 μ M results in the induction of a pattern of cytokines and chemokines that are consistent with the biology of TLR7. At concentrations of GS-9620 higher than 10 μ M, additional cytokines such as TNF- α were induced in PBMCs, which is consistent with stimulation of TLR8 (Gorden et al 2005, Thomas et al 2007). Similarly, oral administration of GS-9620 to cynomolgus monkeys in vivo preferentially induced IFN- α and a pattern of antiviral serum cytokines and chemokines at low oral doses whereas TNF- α was only observed following relatively high oral doses. In general, the cytokine and chemokine patterns observed across ascending doses in vivo was similar to or overlapped with to the cytokine/chemokine profile induced in in vitro PBMC studies. The differences could be a result of differentials in the half life or distribution of cytokines in vivo versus in vitro or may reflect the response to tissues other than PBMCs which are exposed to GS-9620 in vivo.

The in vitro and in vivo pharmacodynamic profile induced by GS-9620 in the mouse was notably different than that seen in cynomolgus monkeys and to that which we have previously reported in humans (Tumas et al 2011, Lopatin et al 2013). Oral administration of GS-9620 to CD-1 mice resulted in dose-dependent induction of IFN- α and other cytokines and chemokines; however, in contrast to the cynomolgus monkey, induction of the proinflammatory cytokines IL-1 β and TNF- α occurred at relatively low doses and were observed concurrent with the induction of IFN- α in mouse (Fig. 2, Fig. 3A). Unlike human and cynomolgus monkey dendritic cell subsets where TLR7 is exclusively expressed by pDCs and TLR8 expression is predominantly found in cDC,

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the pattern of murine TLR7 expression is notably different as expression of murine TLR7 is found in both pDCs and cDCs (Edwards et al 2003, Ketloy et al 2008). As a consequence, the response profiles observed with a TLR7 agonist in mice or mouse splenocytes are more reminiscent of profiles observed with dual TLR7/TLR8 agonists in monkeys and humans (Gorden et al 2005, Ito et al 2002, Thomas et al 2007).

The pattern of cytokine and chemokine induced in human PBMCs (Tumas et al 2011) by GS-9620 in vitro was similar to the pattern we described for cynomolgus monkey PBMCs. Likewise, the in vivo responses to GS-9620 after oral dosing to human volunteers (Lopatin et al 2013) was similar to the in vivo responses in cynomolgus monkey described herein. In contrast, induction of cytokines and chemokines in vitro and in vivo in mice was generally consistent with the notion of different TLR7 expression profile in murine DC subsets. Protein sequence comparison of mouse (mo), human (hu) and cynomolgus (cy) TLR7 and TLR8 showed that sequence identity between huTLR7 and moTLR7 or cyTLR7 is approximately 81% or 98%, respectively; sequence identity between huTLR8 and moTLR8 or cyTLR8 is approximately 70% or 97%, respectively (Supplemental Fig. 2). This analysis suggests that selectivity for TLR7 versus TLR8, as observed with the human TLRs, is likely maintained in the cynomolgus monkey.

GS-9620-Dependent Induction of Antiviral Gene Expression In Vivo

Oral administration of GS-9620 induces pre-systemic IFN- α and induction of MX1 and OAS1 mRNA in peripheral blood. The sequential time course for induction of IFN- α and these ISGs is consistent with activation of TLR7 leading to the release of IFN- α in the tissues containing pDCs which in turn triggers the induction of IFN- α response elements in adjacent cells in paracrine fashion. However, we also observed that single, low oral doses of GS-9620 caused dose-

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dependent induction of OAS1 and MX1 without concurrent detectable serum IFN- α in individual cynomolgus monkeys and mice (Table 4, Fig. 5). In addition, elevated intra-hepatic expression of OAS1 and MX1 mRNA was observed in the mouse after a single oral dose of GS-9620 despite the absence of detectable peripheral IFN- α concurrently (Fig. 5). These data suggest that TLR7 agonism can occur in tissues during oral absorption of GS-9620 resulting in local induction of a response although IFN- α is not detectable in serum but is clearly active in these local tissues as evidenced by ISG induction. In addition, despite notable induction of systemic IFN- α induction following higher doses of GS-9620 to cynomolgus monkeys, OAS1 and MX1 mRNA induction were only less than 3 fold higher when compared to induction in individuals with undetectable systemic IFN- α (Table 4). Of note, these observations in animals are further corroborated by findings in healthy human subjects, where >3-fold ISG mRNA induction over pre-dose occurred after administration of single doses of ≥ 2 mg, despite undetectable peripheral IFN- α (Lopatin et al 2013). In addition, in human volunteers with systemic IFN- α , ISG induction was not notably different than for those subjects without detectable systemic IFN- α , which again is similar to our results in cynomolgus monkey (Table 4).

These data suggests that GS-9620-dependent ISG induction may mainly be due to a localized, presystemic response rather than due to systemic immune stimulation and further suggest that the contribution of systemic immune stimulation to the overall response is low. This local innate immune response without a systemic IFN- α response might indicate a potential for harnessing the benefit of an innate immune response in the liver without systemic adverse side effects. Although the possibility that systemic IFN- α is produced at levels below the limit of detection for the assays used in these studies cannot be excluded, this data suggests that GS-9620-dependent ISG induction can be mounted independent of appreciable concentrations of systemic

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IFN- α . In contrast, induction of ISGs with therapeutic doses of recombinant IFN- α is dependent on systemic IFN- α , which is obviously associated with flu-like symptoms and other adverse events. In the studies described here with GS-9620, IFN- α levels at or below the LLOQ are considerably lower than what is currently administered for approved exogenous IFN- α therapies. Therefore, it can be extrapolated that even if low (undetectable) systemic IFN- α levels are present, systemic levels are unlikely to approach concentrations where adverse events associated with existing interferon therapies were observed. Indeed, healthy human volunteers who were orally administered a single dose of GS-9620 had minimal adverse events that were consistent with systemic IFN- α exposure and these were only observed at dose levels ≥ 8 mg (Lopatin et al., 2013). We hypothesize that the induction of an antiviral immune signature devoid of detectable systemic IFN- α is caused by presystemic stimulation of TLR7, likely in pDCs at the level of the GALT and/or in the liver, e.g., presystemic response. Fig. 6 presents the postulated underlying mechanism for the presystemic response to oral GS-9620.

The high absorption of GS-9620 allows for the targeted exposure to interferon producing cells in the GALT and/or liver following oral administration. Interferon-producing cells present in the GALT and/or liver, likely pDCs, are activated by local exposure to GS-9620 and produce IFN- α . This localized production of IFN- α stimulates ISG induction in lymphocytes and other cells as they circulate through the GALT and/or liver, which can then be detected in the peripheral blood (Fig. 6). ISG induction may occur in the liver by a similar effect – through either local IFN- α produced from stimulated pDCs residing in the liver or from a first-pass effect on the liver from portal blood containing IFN- α produced from GALT-resident cells.

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The therapeutic benefit of TLR7 stimulation by oral GS-9620 in animal models of chronic HBV infection has been shown in both chimpanzees infected with HBV and the woodchuck hepatitis virus model (Lanford et al 2013, Menne et al 2011). Importantly, in uninfected and chronic HBV infected chimpanzees, GS-9620 induced ISGs in the liver and/or in PBMC with no detectable increase in serum levels of IFN- α at low doses. Consequently, GS-9620 may be an effective therapy for viral hepatitis at doses that produce no detectable serum IFN- α levels and, therefore, minimize tolerability limitations associated with systemic IFN- α therapy. Currently, weekly administration of GS-9620 is being evaluated in patients infected with chronic HBV.

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ACKNOWLEDGEMENTS

The authors acknowledge Paul Duatschek for in vitro pharmacodynamic analysis, Dr. Robert Lanford for ISG sample analysis, Bing Lu for PK sample analysis, Covance Laboratories for study conduct, Eurofins Laboratories for cytokine/chemokine sample analysis.

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AUTHORSHIP CONTRIBUTIONS

Participated in research design: AF, JZ, SP, CF, JH, RH, DT, GW

Conducted experiments: CF, JH, SP

Performed data analysis: AF, JZ, SP, CF, JH, DT, GW

Wrote or contributed to the writing of the manuscript: AF, JZ, SP

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FOOTNOTES

This work was supported by Gilead Sciences, Inc., Foster City, CA.

Presented previously, in part, at two meetings:

Tumas D, Zheng X, Lu B, Rhodes G, Duatschek P, Hesselgesser J, Frey C, Henne I, Fosdick A, Halcomb R, Wolfgang G. Preclinical characterization of GS-9620, a potent and selective oral TLR7 agonist. 46th Annual Meeting of the European Association for the Study of the Liver. 30 March – 03 April 2011. Berlin, Germany. Abstract 1776.

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FIGURE LEGENDS

Fig. 1. GS-9620 selectively targets TLR7 over other TLRs.

(A) Dose-response relationship of GS-9620 activity on murine TLR7-expressing NF- κ B-dependent reporter cell line; data points represent mean \pm SD of the OD values from three separate experiments. (B) Activity of 100 μ M GS-9620 was assessed in an NF- κ B dependent reporter assays with several murine TLRs, including TLR7. Vertical bars represent mean OD values from two separate experiments. Controls were used for TLR2 (heat-killed *Listeria monocytogenes*), TLR3 (poly I:C), TLR4 (*Escherichia coli* K12 LPS), TLR5 (*Salmonella typhimurium* flagellin), TLR7 (CL097), TLR8 (CL075), TLR9 (CpG ODN 1826) and NF- κ B reporter (human TNF- α).

Fig. 2. In vitro induction of IFN- α and TNF- α in murine cultured splenocytes (A) and primary PBMC cultures from cynomolgus monkeys (B). IFN- α concentrations in the control incubations without GS-9620 were 58 pg/ml, and 500 pg/ml for mouse and cynomolgus monkey, respectively, and TNF- α concentrations in the control incubations without GS-9620 were 117 pg/ml, and 4.9 pg/ml for mouse, and cynomolgus monkey, respectively. Mean \pm error of the mean is presented.

Fig. 3. GS-9620-dependent induction of peripheral cytokines.

(A) In vivo cytokine induction in male and female mice following a single oral administration of GS-9620. IFN- α and cytokine induction was determined at various timepoints up to 24 hours postdose. Due to blood collection limits in mice, each sample was a terminal sample. Therefore,

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the mean maximal induction for each cytokine was determined for each dose level. Fold change from vehicle control mean is presented below. Values below the LLOQ were excluded from analysis with the exception of IFN- α vehicle control. As all vehicle control animals were below the limit of detection, the LLOQ of 39 pg/mL was used to calculate fold change.

(B) In vivo mean cytokine induction in male and female cynomolgus monkeys following a single oral dose of GS-9620. IFN- α and cytokine induction was determined at various timepoints through 24 - 72 hours postdose. Individual maximal induction for each cytokine for each individual animal and the results averaged for each dose group. Values below the LLOQ were excluded from analysis with the exception of IFN- α vehicle control. As all vehicle control animals were below the limit of detection, the mean LLOQ (64 pg/mL) was used to calculate fold change.

Fig. 4. Peripheral blood PK of GS-9620 and kinetics of pharmacodynamic markers IFN- α , MX1 and OAS1 following oral administration of a single dose of 0.15 mg/kg GS-9620 to male cynomolgus monkeys (n=5). Mean fold change relative to pre-dose are shown on the left y-axis, serum concentration of GS-9620 in ng/mL is shown on the right y-axis. As all pre-dose IFN- α values were below the limit of detection, the LLOQ for this study, 25 pg/mL, was used to calculate fold change.

Fig. 5. Murine MX1 and OAS1 expression in liver and peripheral blood without concurrently detectable systemic IFN- α after a single oral dose of 0.3 mg/kg GS-9620. Gene expression of MX1 (left panel) or OAS1 (right panel) relative to the control gene 18S was assessed from terminal samples at indicated time points through 24 hours postdose in liver (top panel) or

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peripheral blood (bottom panel). All changes in gene expression are plotted relative to mean vehicle; dotted horizontal line indicates 3-fold induction relative to mean vehicle. N=5 animals per group were euthanized at each indicated time point; and mice with detectable serum IFN- α were excluded from analysis. Note that the number of animals per time point shown in liver maybe smaller than in peripheral blood: poor RNA quality in a subset of liver samples precluded analysis by qRT-PCR.

* denotes statistical significance in differential gene expression compared to vehicle ($p < 0.05$, Tukey test).

Fig. 6. Intravenous administration of GS-9620 to cynomolgus monkeys (n=3 males/dose group) results in lower IFN- α induction than oral administration despite comparable GS-9620 exposure. GS-9620 was administered as a single oral or i.v. infusion at 2 mg/kg or 0.1 mg/kg, respectively. IFN- α was evaluated at time points through 24 hours postdose.

After i.v. dosing, systemic exposure to GS-9620 was 2-fold higher, but serum levels of IFN- α induced after i.v. infusion were <2% of that produced after oral dosing. After oral dosing, the high bioavailability of GS-9620 results in targeted exposure to interferon producing cells in the GALT and/or liver, and the relative hepatic instability and high first-pass clearance of GS-9620 minimizes systemic exposure.

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TABLES

Table 1. Summary of in vitro induction of selected cytokines induced in cultured normal mouse splenocytes or PBMC from cynomolgus monkeys

Cytokine	Mouse Splenocytes (nM)	Cynomolgus Monkey PBMC (nM)
CCL3	Not evaluated	35 ± 45
CCL5	30	Not evaluated
CCL4	3	773 ± 1485
CXCL10	30	Not evaluated
G-CSF	1000	Not evaluated
IFN- α	30000	308 ± 463
IFN- γ	10	3334 ± 4933
IL-1 α	> 30,000 ^a	Not evaluated
IL-1 β	> 30,000 ^a	1025 ± 1391
IL-1Ra	Not evaluated	18 ± 14
IL-2	Not evaluated	11000 ± 16522
IL-4	Not evaluated	> 30,000 ^a
IL-5	Not evaluated	> 30,000 ^a
IL-6	30	15 ± 17
IL-10	10	> 30,000 ^a
IL-12p40	> 30,000 ^a	91 ± 140
IL-12p70	100	Not evaluated
IL-15	Not evaluated	> 30,000 ^a
IL-17	Not evaluated	> 30,000 ^a
IL-18	Not evaluated	1001 ± 1732
sCD40L	Not evaluated	> 30,000 ^a

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TNF- α	30	285 \pm 478
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G-CSF, granulocyte colony-stimulating factor; IFN, interferon; IL, interleukin; PBMC, peripheral blood mononuclear cell; TNF, tumor necrosis factor.

^a The MEC was not calculable as no levels of these cytokines were induced that exceeded the vehicle control by at least three fold at the highest concentration tested, 30 μ M

Mean minimum effective concentration (MEC) \pm SD is presented. Values below the limit of detection of the assay were excluded from calculations.

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Table 2. Key pharmacokinetic parameters following a 30-minute intravenous infusion of GS-9620 to male CD-1 mice and male cynomolgus monkeys

Parameter	CD-1 Mouse		Cynomolgus Monkey	
	IV Infusion	PO	IV Infusion	PO
Dose (mg/kg)	1	10	0.1	0.3
N	3/timepoint	3/timepoint	3	3
CL (L/h/kg)	5.8	—	0.89 ± 0.29	—
V _{ss} (L/kg)	2.8	—	4.1 ± 2.2	—
t _{1/2} (h)	1.9	2.4	7.3 ± 3.8	2.4 ± 0.7
F	—	0.2%	—	1.2%

CL, clearance; F, bioavailability; IV, intravenous; PO, oral; t_{1/2}, half-life; V_{ss}, volume of distribution at steady state.

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Table 3. Mean serum pharmacokinetic/pharmacodynamics parameters for GS-9620 following 30-minute intravenous infusion and single ascending oral administration.

Dose Route	Dose (mg/kg)	Pharmacokinetics		Pharmacodynamics
		GS-9620 C _{max} ^a (ng/ml)	GS-9620 AUC ^a (ng•h/ml)	Mean Peak Serum IFN-α ^b (pg/ml)
CD-1 mouse				
Intravenous infusion	1	305 ± 130	178	60 ± 28.9
Oral	0.3	BLQ	NC	BQL
	1.0	0.25 ± 0.20	0.25	174 ± 192
	3.0	0.53 ± 0.25	1.1	106 ± 48.3
	10.0 (A)	2.95 ± 4.43	3.5	567 ± 290
	10.0 (B)	12.5 ± 12.4	28.6	1728 ± 2219
	30.0	65.6	163	989 ± 820
	100.0	277 ± 123	641	6572 ± 2187
Cynomolgus monkey				
Intravenous infusion	0.1	128 ± 43	117 ± 43	1840 ± 1495
Oral	0.1	1.6 ± 0.7	1.9 ± 0.8	140 ± 17
	0.3	4.6 ± 3.0	3.9 ± 1.1	763 ± 72
	1.0	20.7 ± 9.4	25.0 ± 1.5	22,367 ± 16,685
	2.0	66.4 ± 57.8	80.3 ± 40.6	119,000 ± 131,275

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AUC, area under the concentration-time curve; BLQ, below the LLOQ; C_{\max} , maximum plasma/serum concentration; IFN, interferon; LLOQ, lower limit of quantitation; NC, not calculated; all values below the LLOQ.

Mean \pm SD, n = 3/timepoint for mouse and n=3/dose for cynomolgus monkey.

^a The LLOQ for GS-9620 was 0.04 ng/ml.

^b The LLOQ for IFN- α was 25 pg/ml for mouse and was 130 pg/mL for monkey.

A and B are two independent experiments at 10 mg/kg oral dosing.

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Table 4. Mean Peak Induction of MX1 and OAS1 gene expression in healthy human volunteers, cynomolgus monkeys, and mice after a single dose of GS-9620

Species	Dose	HED (mg) ^a	ISG Induction without Detectable Serum			ISG Induction with Detectable Serum IFN- α			
			Total	IFN- α		Total	Mean \pm SD ^b		
				MX1 Fold Increase (n)	OAS1 Fold Increase (n)		IFN- α pg/mL (n)	MX1 Fold Increase (n)	OAS1 Fold Increase (n)
Human									
	8 mg	N/A	5	21.7 \pm 13.2 (5)	10.9 \pm 5.0 (5)	1	19	27.8	12.4
	12 mg	N/A	2	28.2 (2)	11.8 (2)	4	132 \pm 201 (4)	31.6 \pm 17.0 (4)	13.7 \pm 5.2 (4)
Cynomolgus monkey									
	0.05 mg/kg	1	13	5.6 \pm 3.3 (5)	12.2 (2)	1	1792 (1)	25.6 (1)	70.4 (1)
	0.15 mg/kg	2.9	13	16.1 \pm 7.1 (11)	30.4 \pm 25.8 (10)	6	457 \pm 259 (6)	19.4 \pm 23.2 (6)	19.6 \pm 30.9 (6)
	0.5 mg/kg	9.6	5	23.8 \pm 17.2 (5)	41.3 \pm 32.7 (3)	23	6052 \pm 7551 (23)	38.5 \pm 15.9 (23)	87.5 \pm 62.1 (23)
	1.5 mg/kg	29	0	N/A	N/A	13	28186 \pm 23788 (13)	33.2 \pm 15.0 (13)	70.2 \pm 50.6 (13)
Mouse									

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Species	Dose	HED (mg) ^a	ISG Induction without Detectable Serum		ISG Induction with Detectable Serum IFN- α			
			IFN- α		Total	Mean \pm SD ^b		
			Total (n)	MX1 Fold Increase (n)		OAS1 Fold Increase (n)	IFN- α pg/mL (n)	MX1 Fold Increase (n)
0.3 mg/kg	1.4	N/A	17.6 \pm 7.8 (4)	3.4 \pm 0.5 (4)	N/A	N/A	N/A	N/A

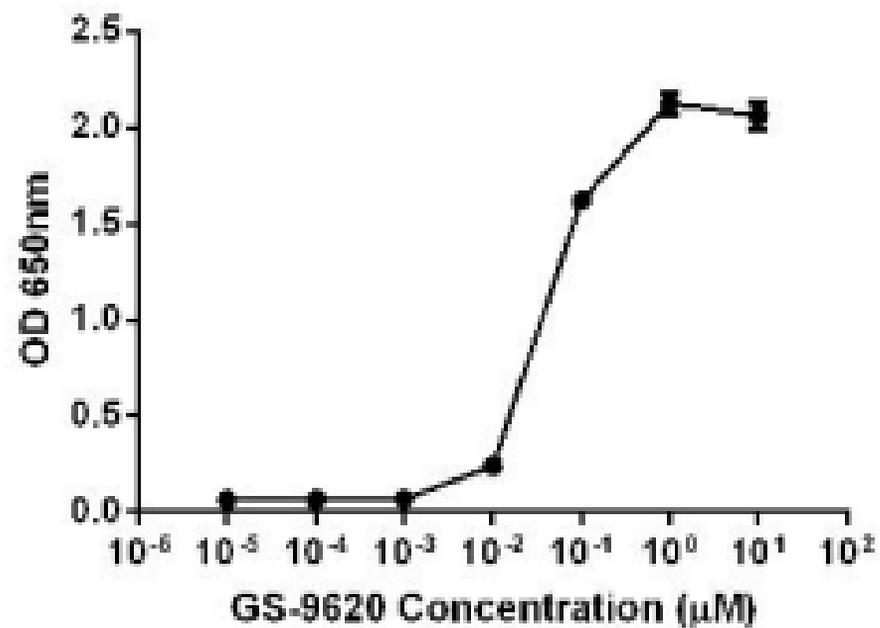
HED, human equivalent dose; IFN, interferon; ISG, interferon-stimulated genes.

^a HED was calculated by multiplying the animal dose in mg/kg by 0.32 and 0.08 conversion factors for cynomolgus monkey and mouse, respectively, and then converted to a total human dose (mg) by multiplying by a standard human body weight of 60 kg (FDA Guidance for Industry).

^b Animals were included in calculation if ISG induction was ≥ 3 fold. Fold increases in MX1 and OAS1 were determined by comparison of pre- and post-treatment levels or comparison to control groups.

Figure 1

A



B

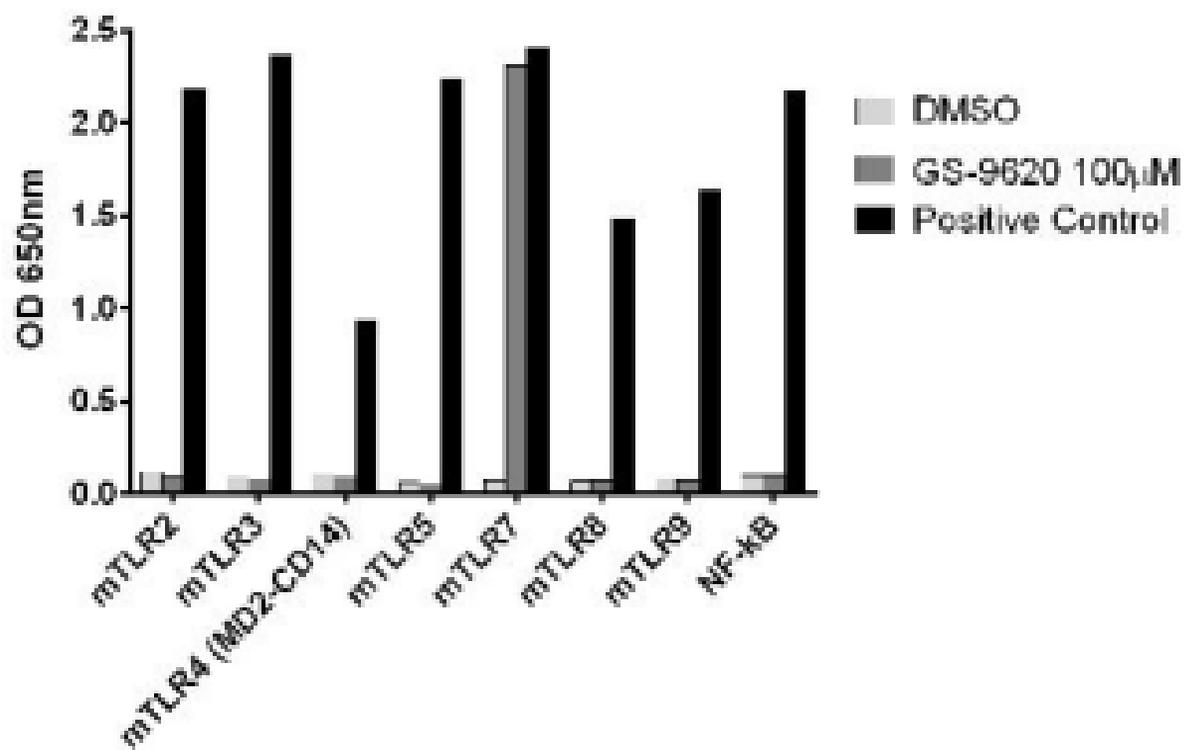
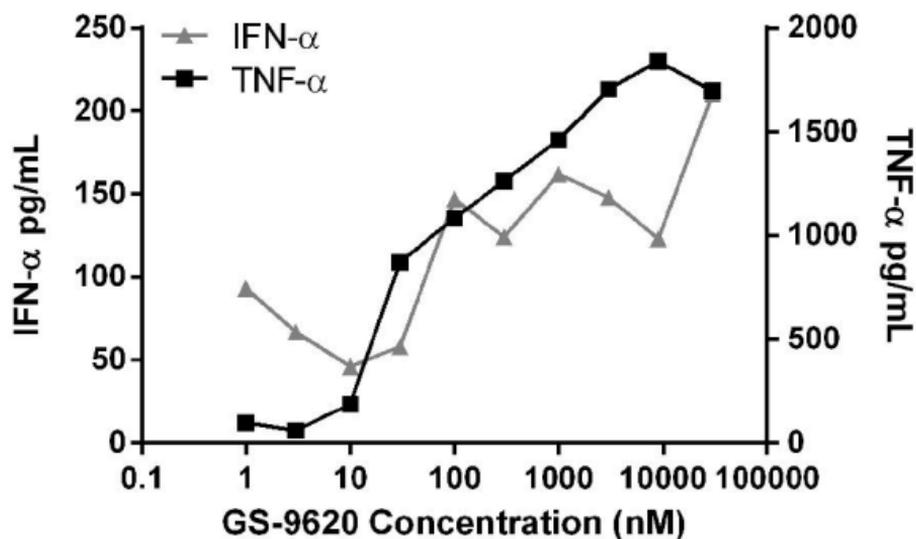


Figure 2

A



B

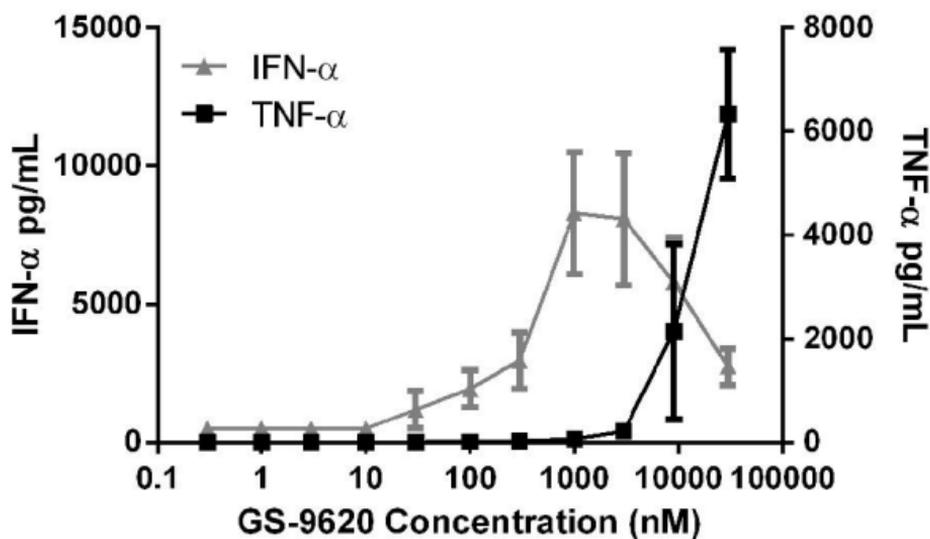
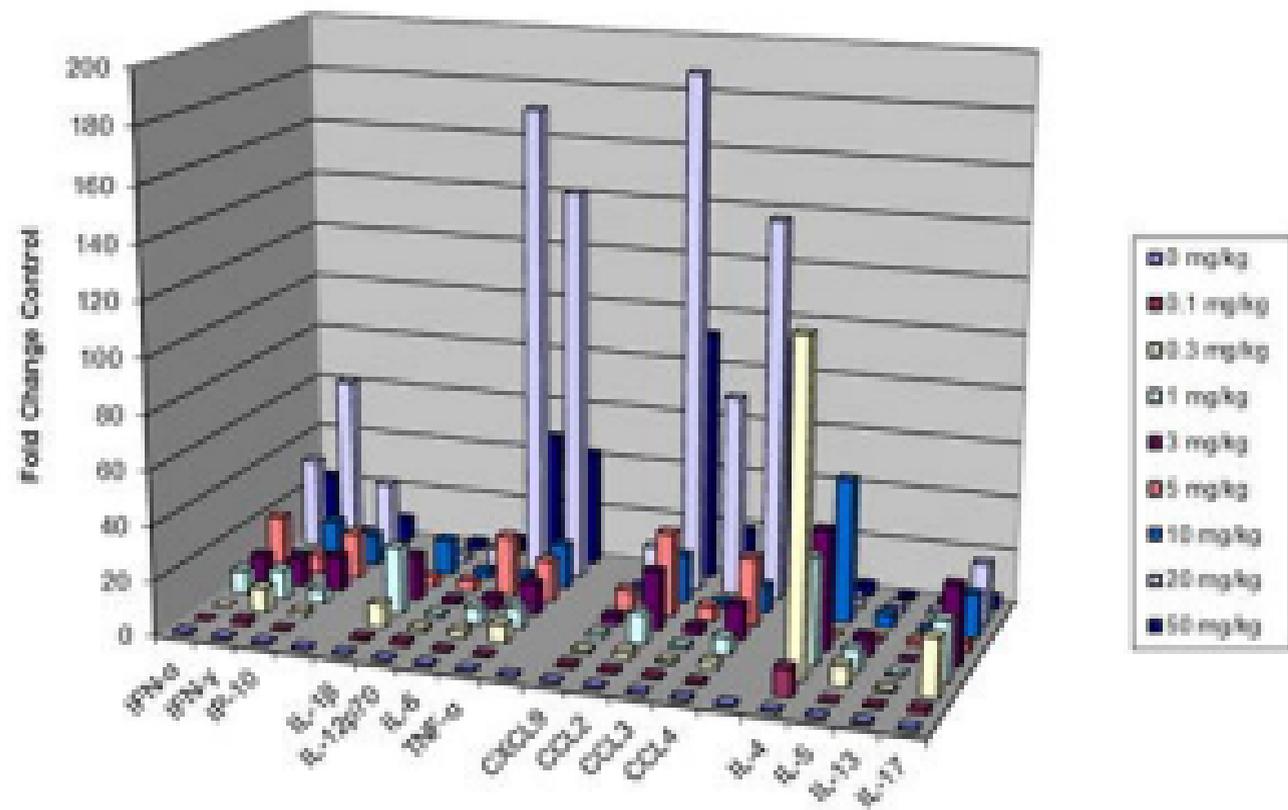


Figure 3

A



B

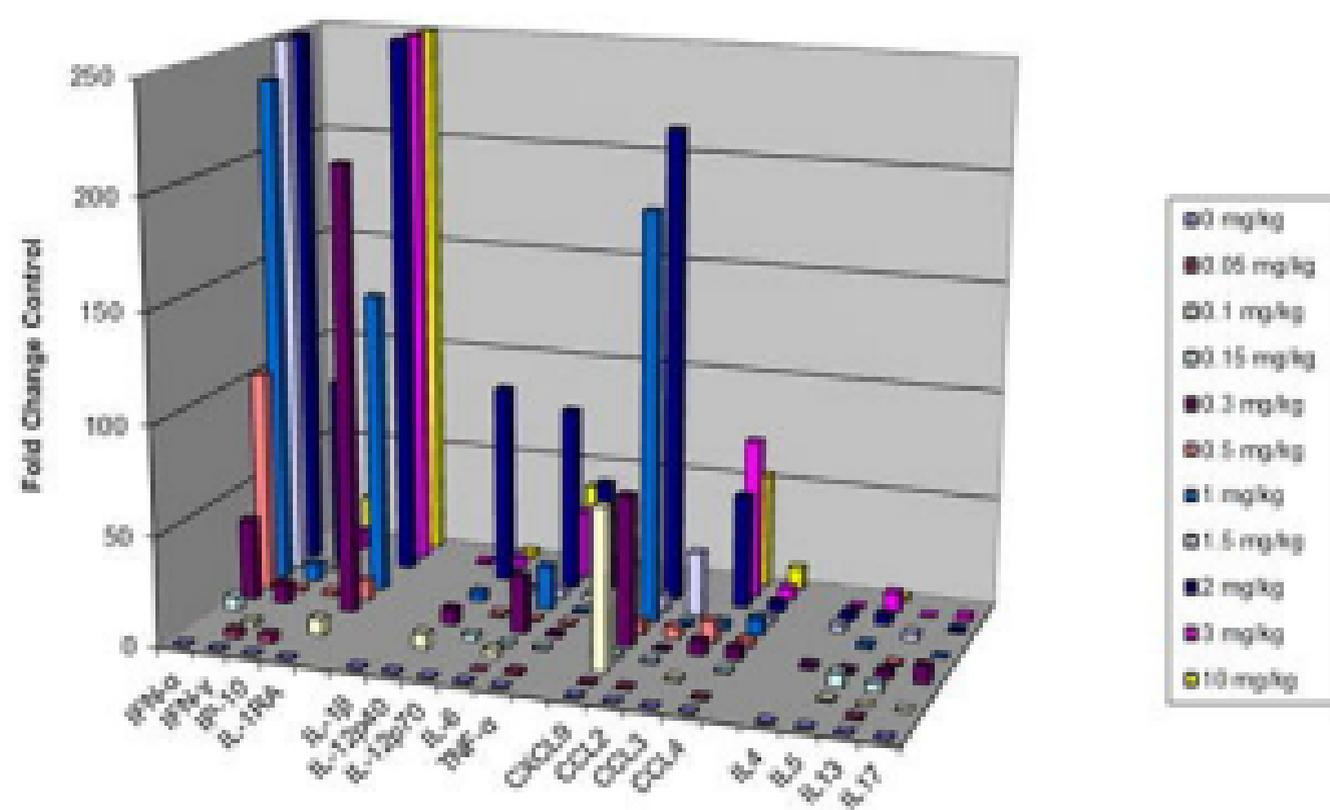


Figure 4

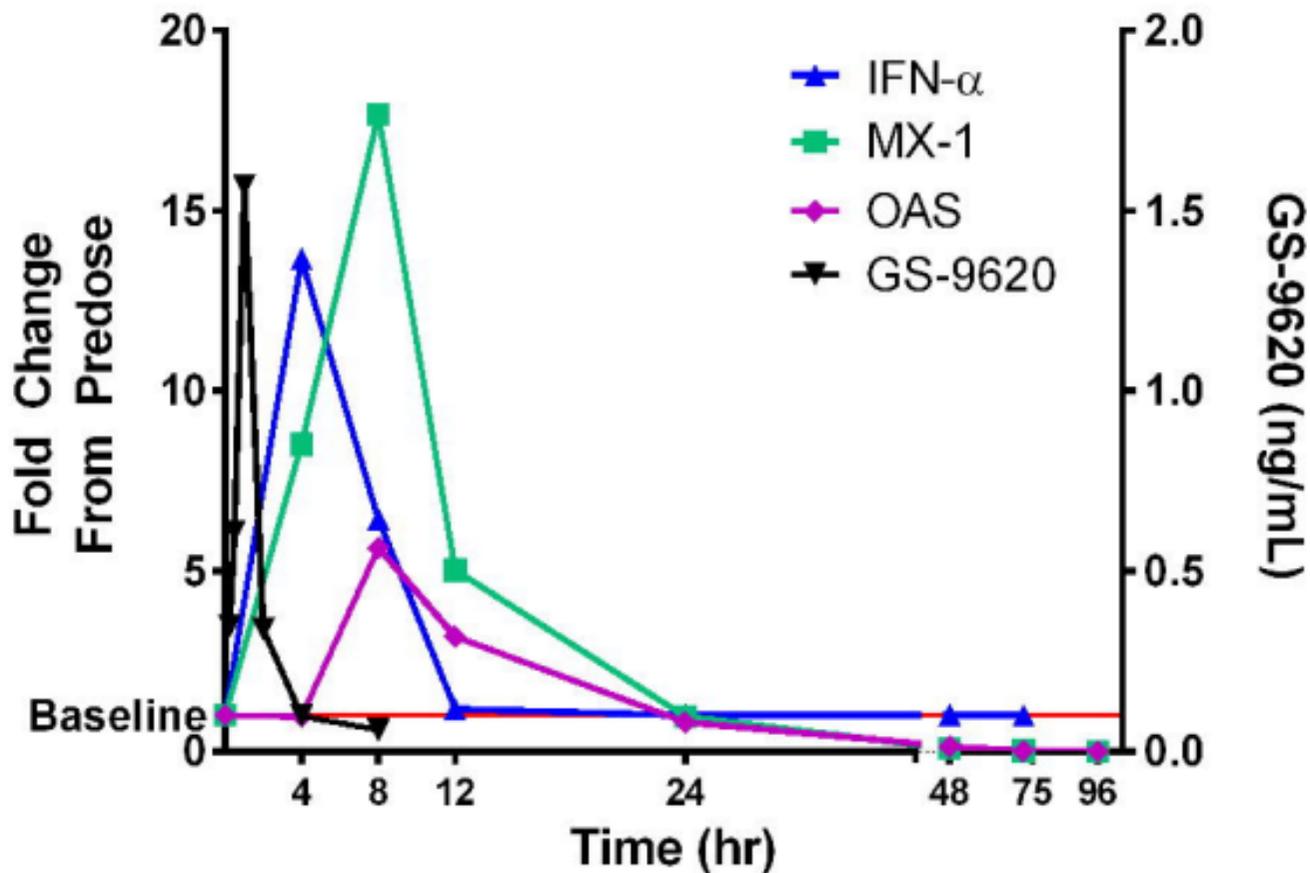
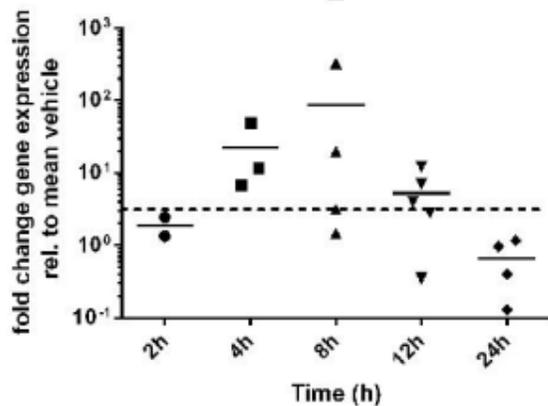
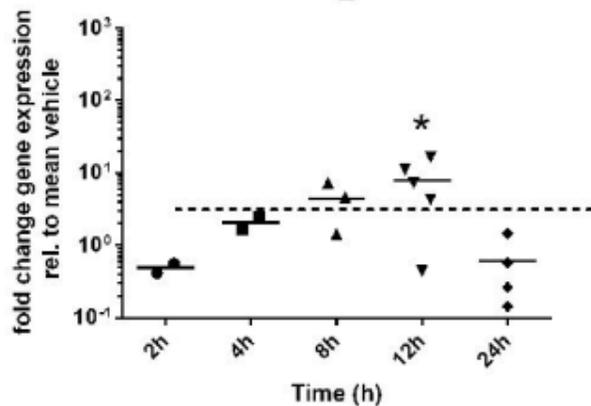


Figure 5

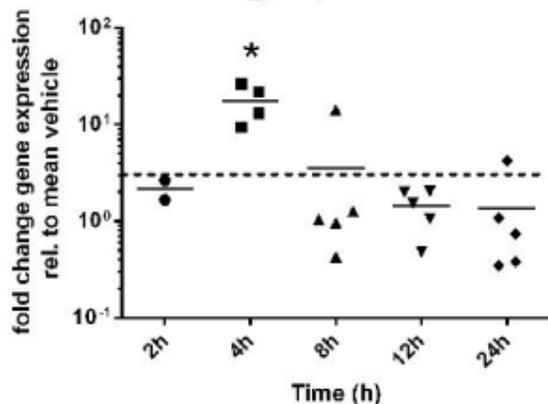
MX1_liver



OAS1_liver



MX1_peripheral blood



OAS1_peripheral blood

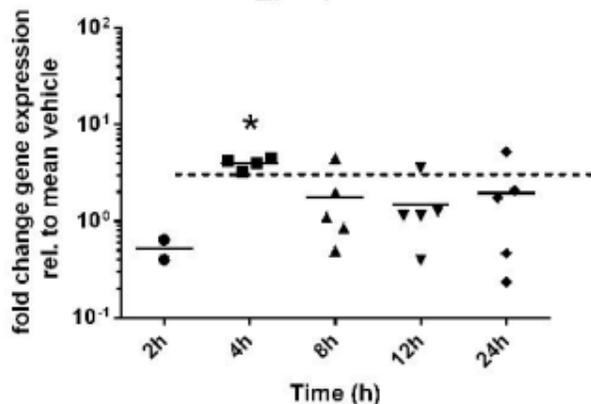
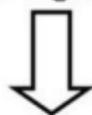


Figure 6

Oral Dose

2 mg/kg



Gut

GALT

High Oral Absorption

High liver extraction

Liver

Low Systemic Exposure = 66 ng/mL
(bioavailability < 2%)

High IFN- α = 119,000 pg/ml

IV Infusion

0.1 mg/kg



High Systemic Exposure = 128 ng/mL

Low IFN- α = 1,800 pg/ml

( = Plasmacytoid Dendritic Cells)

