CYTOKINE-MEDIATED SUPRESSION OF CYP ENZYMES BY THE TOLL-LIKE RECEPTOR 9 AGONIST, TILSOTOLIMOD, IN CULTURED HUMAN HEPATOCYTES

Paul Tarantino[1], Tim Sullivan[1], Brian W. Ogilvie[2] , Maciej Czerwinski[2]

[1]Idera Pharmaceuticals, Inc., 167 Sidney Street, Cambridge, MA 02139; [2]XenoTech LLC, 1101 W. Cambridge Cir. Drive, Kansas City, KS 66103

INTRODUCTION

The innate immune response to some drugs may involve the release of proinflammatory cytokines, such as IL-6, which have the ability to suppress xenobiotic-metabolizing CYP enzymes and affect the pharmacokinetics of co-administered small molecules. The 2012 FDA Drug-Drug Interaction (DDI) guidance recommended the evaluation of therapeutic proteins such as cytokines or cytokine modulators for their ability to modulate the expression of CYP enzymes or transporters (Food and Drug Administration (US), 2012). Although not a peptide drug, it is possible that an immuno-modulator, such as the oligonucleotide, Tilsotolimod (IMO-2125), an investigational agonist of toll-like receptor 9 designed to enhance T-cell responses to tumor antigens, could precipitate DDIs. Tilsotolimod alters the tumor microenvironment by improving antigen presentation by dendritic cells and macrophages with subsequent proliferation of antigen-specific cytotoxic T lymphocytes (CD8+ T cells) in both injected and distant tumors resulting in tumor cell death (Figure 1). In this study, we investigated the potential of Tilsotolimod to cause direct and cytokine-mediated effects on CYP mRNA expression and enzyme activity in primary human hepatocytes according to a published in vitro method (Czerwinski et al., 2014). The method comprised stimulation of human blood with a drug or a therapeutic protein followed by separation and subsequent incubation of human hepatocytes co-cultured with Kupffer cells with the drug-treated plasma to assess the potential of the drug to alter the expression of drug metabolizing enzymes and transporters through its effect on cytokines. All cultures of hepatocytes used in this study were co-cultures of hepatocytes and Kupffer cells isolated from the same donors in routine perfusion and plating procedures. The test system accounts for the combined effects of all cytokines stimulated by the drug in blood on CYP expression in primary cultures of human hepatocytes (Figure 2).

MATERIALS & METHODS

Idera Pharmaceuticals provided Tilsotolimod. Other reagents used in this study were obtained from suppliers described in other literature (Czerwinski et al., 2014). Blood was donated by 10 healthy volunteers who gave informed consent to participate in the study. Blood was treated with Tilsotolimod (10 or 100 µg/mL) or LPS (10 ng/mL) in the room air at 37°C. The blood and drug were mixed gently and incubated for 24 h. For the quantification of cytokines, samples were diluted by a factor of four and analyzed in duplicate by sandwich immunoassay with electrochemiluminescence detection according to the manufacturer's instructions (Meso Scale Discovery, Gaithersburg, MD, USA). Interferon gamma-induced protein 10 (IP-10), monocyte chemoattractant protein 1 (MCP-1) and macrophage inflammatory protein 1-alpha (MIP-1α) were measured with the Human Chemokine V-PLEX assay interleukin-2 (IL-2), interleukin-6 (IL-6), interleukin-12 (IL-12p70) and tumor necrosis factor alpha (TNF-α) with the Human Proinflammatory V-PLEX assay, and interferon alpha (IFN-α2a) with the U-PLEX assay.

Hepatocytes and Kupffer cells were isolated from three non-transplantable livers by a two-step collagenase perfusion method and plated on collagen-coated 24-well plates as previously described (Madan et al., 2003). These isolation and plating procedures routinely result in a co-culture of hepatocytes and liver macrophages (Lambert-Li et al., 2014). For treatment of cell cultures, equal volumes of drug-treated plasma from 10 donors were pooled.

Treatments of hepatocyte cultures and evaluation of CYP mRNA levels and enzymatic activity were conducted as previously described (Czerwinski et al., 2014).

RESULTS

The effects of incubation of blood with or without saline, LPS or Tilsotolimod on IP 10, MCP-1, MIP-1α, IL-2, IL-6, IL-12p70, TNF-α and IFN-α2a are compared in Table 1. After 24 h incubation, the levels of MCP-1, MIP-1α and TNF-α in the untreated plasma were increased by 30, 57 and 9.3 fold, respectively. Incubation of blood with saline (1% v/v) had no effect on the plasma level of any of the cytokines as compared to the untreated control. The plasma level of IFN-α2a in saline treated blood was below the limit of quantification (BLQ). Treatment of blood with 10 ng/mL LPS increased the plasma level of IP-10, IL-2, IL-6, IL-12 and TNF-α by 72, 3.5, 62, 28 and 230 fold relative to the saline control, respectively. Treatment of blood with 10 ng/mL LPS decreased the plasma level of MCP-1 down to 23% of the saline control. LPS also increased the plasma levels of MIP-1α and INF-α2a, but statistical significance of these increases was not determined as several data points were above or below the limit of quantification (ALQ or BLQ). Treatment of blood with up to 100 µg/mL Tilsotolimod elevated plasma levels of IP-10, MCP-1, MIP-1α and TNF-α by 61, 1.7, 3.4 and 2.8 fold of the saline control, respectively. Tilsotolimod also increased the plasma level of IFN-α2a, approximately as high as 1500 fold relative to the saline control.

Direct and cytokine-mediated effects of Tilsotolimod, LPS or the controls on CYP1A2, CYP2B6 and CYP3A4 in primary cultures of human hepatocytes are presented in Table 2 and Figure 3. Treatment of three cultures of hepatocytes with enzyme inducers omeprazole, phenobarbital or rifampin caused expected increases in CYP1A2, CYP2B6 and CYP3A4 mRNA expression, on average, of 37, 11 and 10 fold over the vehicle control, respectively. Corresponding increases in the enzyme activities were observed. Flumazenil, a negative control for CYP induction, had no effect on the CYP mRNA or enzyme activity levels. Direct treatment of hepatocytes with up to 100 µg/mL Tilsotolimod had little or no effect on the CYP mRNA or enzyme activity levels with changes ranging from 0.813 to 1.52 fold over the vehicle control. Treatment of hepatocytes with 40% v/v of plasma from blood incubated with up to 100 µg/mL Tilsotolimod changed CYP1A2, CYP2B6 and CYP3A4 mRNA, on average, 1.50, 0.633 and 0.520-fold over the saline plasma control, respectively. These changes were accompanied by reduction of CYP1A2, CYP2B6 and CYP3A4 enzyme activity, on average, 0.474, 0.132 and 0.862-fold over the saline plasma control, respectively.

Table 1. Effects of incubation, saline, LPS and Tilsotolimod on the release of cytokines in human blood

Cytokine	Treatment					
	Incubation 0 h, untreated	Incubation 24 h, untreated	Saline[a] 1 %, v/v	LPS 10 ng/mL	Tilsotolimod 10 µg/mL	Tilsotolimod 100 µg/mL
	[Cytokine] (pg/mL)					
IP-10	323 ± 211	367 ± 268	353 ± 269	25400 ± 38500 (9[b])**	21600 ± 34900 (7 [b])**	5650 ± 3850 (9 [b])**
MCP-1	191 ± 30.6	5550 ± 1770*	6840 ± 2250	1590 ± 803**	11200 ± 3220**	11500 ± 3110**
MIP-1 α	17.5 ± 9.92 (7[c])	998 ± 1170*	732 ± 855	ALQ	991 ± 1013	2510 ± 1210**
IL-2	0.335 ± 0.167 (8[c])	0.386 ± 0.198 (9[c])	0.325 ± 0.205	1.15 ± 0.285**	0.370 ± 0.206 (9[c])	0.417 ± 0.197
IL-6	2.75 ± 3.33 (5[c])	147 ± 243	135 ± 229	8400 ± 2760**	163 ± 237	64.4 ± 33.2
IL-12p70	1.31 ± 0.704 (7[c])	3.15 ± 3.04 (8[c])	2.67 ± 2.66 (9[c])	74.0 ± 27.8**	2.72 ± 2.63	1.98 ± 0.921
TNF-α	1.98 ± 1.02	18.5 ± 17.0*	15.6 ± 17.4	3590 ± 1120**	19.7 ± 19.0	43.4 ± 25.9**
IFN-α2a	BLQ	0.325 (1[c])	BLQ	0.325 (1[c])	166 ± 145	487 ± 319

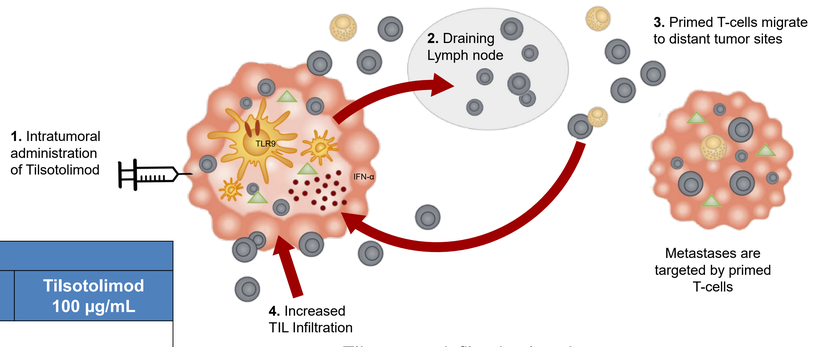
ALQ Above the limit of quantification
BLQ Below the limit of quantification
[a] Values are the mean ± standard deviation of duplicate determinations in blood from 10 donors
[b] (n) ALQ results not reported
[c] (n) BLQ results not reported
*Statistically different from 0 h incubation control (t test: two samples, equal variance, one-tail, $P < 0.05$);
**Statistically different from saline control (t test: two samples, equal variance, one-tail, $P < 0.05$)

Table 2. Direct and cytokine-mediated effects of Tilsotolimod on CYP enzyme mRNA and activity in primary cultures of human hepatocytes (n = 3)

Treatment	mRNA	Enzyme activity
Direct effects	**CYP1A2**	
Vehicle control	1.00 ± 0.00[a]	1.00 ± 0.00[b]
10 µg/mL Tilsotolimod	1.09 ± 0.34	1.01 ± 0.11
30 µg/mL Tilsotolimod	1.01 ± 0.11	0.960 ± 0.039
100 µg/mL Tilsotolimod	0.813 ± 0.256	0.884 ± 0.094
25 µM Flumazenil	1.14 ± 0.30	1.08 ± 0.09
50 µM Omeprazole	36.7 ± 2.7	59.2 ± 22.6
Cytokine-mediated effects		
10 µg/mL Tilsotolimod plasma	1.50 ± 0.58	0.581 ± 0.278
100 µg/mL Tilsotolimod plasma	1.16 ± 0.51	0.474 ± 0.281
10 ng/mL LPS plasma	0.188 ± 0.194	0.213 (n = 2)
Direct effects	**CYP2B6**	
10 µg/mL Tilsotolimod	1.11 ± 0.24	0.868 ± 0.073
30 µg/mL Tilsotolimod	1.52 ± 0.39	0.888 ± 0.071
100 µg/mL Tilsotolimod	1.34 ± 0.41	0.803 ± 0.077
25 µM Flumazenil	1.02 ± 0.13	1.16 ± 0.09
750 µM Phenobarbital	11.1 ± 4.0	20.3 ± 12.2
Cytokine-mediated effects		
10 µg/mL Tilsotolimod plasma	0.804 ± 0.276	0.263 ± 0.012
100 µg/mL Tilsotolimod plasma	0.633 ± 0.059	0.132 ± 0.051
10 ng/mL LPS plasma	0.253 ± 0.138	0.161 ± 0.095
Direct effects	**CYP3A4**	
10 µg/mL Tilsotolimod	1.28 ± 0.13	1.18 ± 0.12
30 µg/mL Tilsotolimod	1.52 ± 0.17	1.35 ± 0.14
100 µg/mL Tilsotolimod	1.45 ± 0.21	1.49 ± 0.24
25 µM Flumazenil	0.967 ± 0.033	1.03 ± 0.07
20 µM Rifampin	9.51 ± 3.60	8.38 ± 3.62
Cytokine-mediated effects		
10 µg/mL Tilsotolimod plasma	0.520 ± 0.049	0.875 ± 0.073
100 µg/mL Tilsotolimod plasma	0.562 ± 0.106	0.862 ± 0.054
10 ng/mL LPS plasma	0.0903 ± 0.0672	0.706 ± 0.044

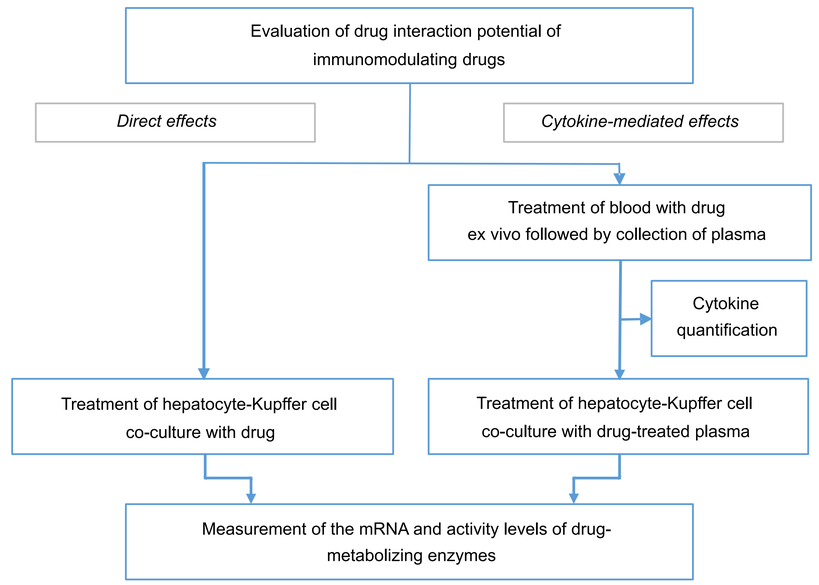
[a] Values, the ratio of treatment vs. control groups, are the mean ± standard deviation of Relative Quantification Factor;
[b] Values, the ratio of treatment vs. control groups, are the mean ± standard deviation of triplicate determinations of phenacetin O-dealkylation (CYP1A2), bupropion hydroxylation (CYP2B6) or midazolam 1´-hydroxylation (CYP3A) expressed as percent of control. Control values in vehicle control and saline plasma were 1.77 ± 0.91 and 0.842 ± 0.214 pmol/incubation/min for CYP1A2, 1.04 ± 0.50 and 1.11 ± 0.10 pmol/incubation/min for CYP2B6 and 5.06 ± 2.49 and 1.58 ± 1.58 pmol/incubation/min for CYP3A, respectively;
* Plasma was applied to hepatocytes at 40% v/v of cell culture medium

Figure 1. Mode of action of Tilsotolimod



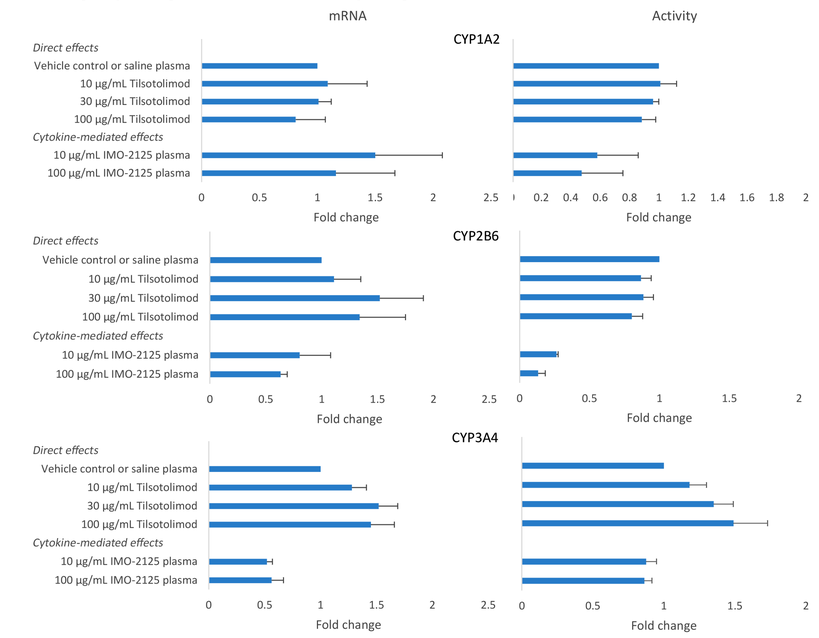
TIL – tumor-infiltrating lymphocytes

Figure 2. Experimental approach to an in vitro evaluation of direct and cytokine-mediated drug interaction potential of immuno-modulating drugs



To examine the direct effects of Tilsotolimod on CYP enzyme levels in primary co-cultures of hepatocytes and Kupffer cells, the drug was added to cells in the absence of peripheral blood mononuclear cells (PBMC). To examine the cytokine-mediated effects, IMO-2125 was incubated ex vivo with whole blood containing PBMCs after which plasma was prepared and added to the co-culture of hepatocytes and Kupffer cells (final concentration equal to 40% v/v of cell culture medium). Plasma levels of selected cytokines were evaluated by sandwich immunoassay with electrochemiluminescence detection (Meso Scale Discovery). After 72-h incubation, changes in CYP expression were determined by RT-PCR or enzyme activities toward CYP-selective probes in situ.

Figure 3. Direct and cytokine-mediated effects of Tilsotolimod on CYP enzyme mRNA and activity in primary cultures of human hepatocytes



CONCLUSIONS

Tilsotolimod, a toll-like 9-receptor agonist, stimulated release of IP-10, MCP-1, MIP-1α, TNF-α and IFN-α2a in blood of healthy donors. Strong increases of MCP-1 and IFN-α2a, as well as a lack of effect on IL-2, IL-6 and IL-12p70, differentiated the Tilsotolimod response from that of the prototypical toll-like 4-receptor agonist, LPS.

Tilsotolimod was free of direct inductive or suppressive effects (i.e., the changes were between 0.5 and 2-fold) on the CYP1A2, 2B6 or 3A4 mRNA or enzyme activity levels in primary cultures of human hepatocytes, but the plurality of cytokines released into the plasma in response to the drug suppressed CYP1A2 and 2B6 activities. The most pronounced response to the Tilsotolimod plasma was a reduction of CYP2B6 enzyme activity down to 13% of the saline control. This response of hepatocytes to the Tilsotolimod plasma was more pronounced than the effect of the LPS plasma.

REFERENCES

Czerwinski M, Kazmi F, Parkinson A, and Buckley DB (2014) Anti-CD28 Monoclonal Antibody-stimulated Cytokines Released from Blood Suppress CYP1A2, CYP2B6 and CYP3A4 in Human Hepatocytes In Vitro. *Drug Metab Dispos* **43**:42–52.

Food and Drug Administration (US), Center for Drug Evaluation and Research (2012) Guidance for Industry: Drug Interaction Studies--Study Design, Data Analysis, Implications for Dosing, and Labeling Recommendations, Draft Guidance, pp 79, Food and Drug Administration (US), Center for Drug Evaluation and Research, Silver Spring, Maryland.

Lambert-Li D, Lyon KC, Czewinski M, and Buckley DB (2014) Endotoxin up-regulates the proinflammatory cytokines TNF-a and IL-6 in freshly-isolated human Kupffer cells. *Drug Metab Rev* **45**(S1): 145–146.

Madan A, Graham RA, Carroll KM, Mudra DR, Burton LA, Krueger LA, Downey AD, Czerwinski M, Forster J, Ribadeneira MD, Gan LS, LeCluyse EL, Zech K, Robertson P, Koch P, Antonian L, Wagner G, Yu L, and Parkinson A (2003) Effects of prototypical microsomal enzyme inducers on cytochrome P450 expression in cultured human hepatocytes. *Drug Metab Dispos* **31**:421-431.

XENOTECH
A BioIVT Company