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PHARMACOKINETICS, SAFETY AND INDUCIBLE CYTOKINE RESPONSES DURING A PHASE 1 TRIAL OF THE HISTONE DEACETYLASE INHIBITOR ITF2357 (GIVINOSTAT)

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ABSTRACT

Inhibitors of histone deacetylases (HDAC) are used widely in medicine. ITF2357 is a hydroxamic acid-containing, orally active HDAC inhibitor with anti-inflammatory properties. In endotoxin-stimulated human PBMC, ITF2357 at 50 to 125 nM inhibited the release of TNFα and IL-1β by more than 70%, respectively. The induction of IFNγ by the combination of IL-18 plus IL-12 was also reduced by ITF2357. We report here a Phase I safety and pharmacokinetic trial. After an oral dose of 50 mg, the maximal plasma concentration was 104 nM at 2 hours post dosing with a half-life of 6.9 hours. For the 100 mg dose, the Cmax was 199 nM at 2.1 hours and a half life of 6.0 hours. For a dose of 200 mg, the Cmax was 470 nM after 2.1 hours and a half-life of 6.0 hours. Repeat daily doses of ITF2357 for 7 consecutive days of 50, 100 or 200 mg resulted in nearly the same Cmax and half-life each day as was observed following a single dose. Side effects were limited to reductions in platelets (10-25% of baseline) within the first week after 7 days of treatment, reached a nadir point after 2 to 3 weeks and resolved fully within 2 to 3 weeks. Whole blood was cultured with endotoxin (10 ng/mL). Production of TNFα progressively decreased reaching a nadir 4 hours after oral dosing (45 and 52% of the pre-drug level for 50 and 100 mg, respectively). Similar decreases were observed after 4 hours for IL-1 beta, IL-6 and interferon gamma but not for IL-8. The IL-1 receptor antagonist did not decrease in the same samples. After 24 hours, the levels had returned to base line measurements. In a one week-long multiple dosing schedule, the fall in cytokine production in blood cultures observed on day 7 was nearly the same as that of the first day. We conclude that ITF2357 is a safe and effective therapy for reducing cytokine production following oral dosing.

RIASSUNTO

Gli inibitori delle istone deacetilasi (HDAC) sono stati recentemente introdotti nella terapia medica oncologica. ITF2357 (Givinostat) è un inibitore delle HDAC, attivo per via orale e con proprietà anti-infiammatorie. In colture di PBMC umani stimolati con LPS, ITF2357 alle concentrazioni da 50 a 125 nM ha dimostrato efficacia nell'inibire il rilascio di TNFα ed IL-1β. Anche l'induzione di IFNγ mediante la combinazione di IL-18 ed IL-12 è ridotta in vitro. Il seguente rappresenta uno studio di Fase I relativo alla sicurezza e alla farmacocinetica di ITF2357 in volontari sani. Dopo una dose orale di 50 mg, la concentrazione plasmatica massima è stata di 104 nM a 2 ore dalla somministrazione, con una emivita di 6,9 ore. Per la dose di 100 mg, la Cmax è stata di 199 nM a 2,1 ore e con un'emivita di 6,0 ore. Per la dose di 200 mg, la Cmax è stata di 470 nM dopo 2,1 ore e una $t_{1/2}$ di 6,0 ore. Ripetute dosi giornaliere di ITF2357 per 7 giorni consecutivi (50, 100 o 200 mg) hanno generato la stessa Cmax ed emivita osservate dopo una singola dose. Gli effetti collaterali si sono limitati ad una riduzione delle piastrine (10-25% del basale) entro la prima settimana di assunzione con un nadir al settimo giorno ed un ripristino completo dopo 3 settimane. Il sangue intero dei soggetti in studio è stato incubato con lipolisaccaride (10 ng/mL). La produzione di TNFα è risultata ridotta raggiungendo un nadir a 4 ore dall'assunzione orale (-45 e -52% comparati con i livelli pre-assunzione per i dosaggi di 50 e 100 mg, rispettivamente). diminuzioni simili sono state osservate dopo 4 ore per IL-1β, IL-6 ed IFNγ ma non per IL-8 ed IL-1Ra. Nei campioni di sangue prelevati a 24 ore dall'assunzione, i livelli citochinici sono tornati comparabili al basale. Nello studio con dosi multiple, l'effetto anti-citochinico osservato al settimo giorno è stato sovrapponibile a quello del primo. In conclusione ITF2357 è una terapia sicura ed efficace nel ridurre la produzione di citochine in un modello ex-vivo.

ABBREVIATIONS

AEs – Adverse Events

AUC – Area under curve

BMT – bone marrow transplantation

CaMK – calmodulin-dependent protein kinase

HDAC – Histone deacetylases

HDACi – Histone deacetylases inhibitors

FDA – Food and drug administration

FOXP3 - forkhead box P3

IL-1β – Interleukin 1 beta

IL-6 – Interleukin 6

TNFα – Tumor necrosis factor alpha

IFNγ – Interferon gamma

PBMCs – Peripheral blood mononuclear cells

HAT - Histone acetyl transferases

RPD3 - Yeast transcriptional regulator

NLS - Nuclear localization signal

PCNA - Proliferating cell nuclear antigen

SAHA - Suberoylanilide hydroxamic acid

SMRT - Retinoid and thyroid receptors

GM-CSF - Granulocyte/macrophage colony-stimulating factor

HSMCs - Human vascular smooth muscle cells

TSA - Trichostatin A

TPX - Trapoxin

HDLP - Histone deacetylase-like protein

MEL - Murine erythroleukemia

CHAP - Cyclic hydroxamic acid containing peptide

SCOP - Sulfur containing cyclic peptide

HMR - Homeodomain regulator

GVHD - Graft-versus-host disease

NF-kB – Nuclear Factor k B

STAT3 - Signal transducer and activator of transcription 3

IDO - Indoleamine 2,3-dioxygenase

TGFβ – Transforming grow factor beta

SMAD3 - Mothers against decapentaplegic homolog 3

ET - Essential Thrombocythaemia

PV – Polycythemia Vera

MF – Myelofibrosis

RPMI – Roswell Park Memorial Institute

IL-1Ra – Interleukin 1 receptor antagonist

IL-10 – Interleukin 10

SEM – Standard error of mean

ECG – Electrocardiogram

LPS – Lipopolysaccharide

INTRODUCTION

Eukaryotic DNA is packed in a high level structure called chromatin, resulting from the assembly of an elementary unit, the nucleosome, an octameric structure obtained from eight proteins called histones. About 150 base pairs of DNA are sequentially folded around one nucleosome unit. The interactions between the DNA and the histones terminal tails control the activation or repression of gene transcription and several chemical modifications can change the status of histones with impact on gene transcription.

In particular the N-ε-acetylation of lysine residues found in histones is equilibrated by two enzymes: the histone acetyl transferases (HAT) and the histone deacetylases (HDAC).

While the base sequence of DNA provides the fundamental code for proteins, post-translational modification of proteins plays a major role in the control of gene transcription. The amino acid tails of the core nucleosomal histones are subject to post-translational modifications by acetylation of lysines, methylation of lysines and arginines, phosphorylation of serines, and ubiquitination of lysines. HDAC inhibitors (HDACi) modulate the expression of multiple genes [1].

HISTONE DEACETYLASES (HDAC)

Excessive deacetylated level of these histones has been linked to cancer pathologies by promoting the repression of tumor regulatory genes. HDACi activities resulted in an increase of the acetylated level of histones, promoting in turn the re-expression of silenced

regulatory genes. These compounds are a promising family of small molecule-based anti-cancer therapies.

Table 1 - Properties of zinc dependant HDAC. Size is expressed in amino acid number, N: nuclear, C: cytoplasm, TC.: Transcription corepressor.

Group	Size (aa)	Location chromosome	Cellular distribution	Complex	Role
Class I					
HDAC1	483	1p34	N	Sin3, NURD	TC
HDAC2	488	6q21	N	Sin3, NURD	TC
HDAC3	428	5q31	N	NCOR1/NCOR2-GPS2-TBL1X	
HDAC8	377	Xq13	N		TC
Class IV					
HDAC11	347	3p25.2	N		
Class II					
HDAC4	1084	q37.2	N, C	NCOR1/NCOR2	TC
HDAC5	1122	17q21	N, C		TC
HDAC6	1215	Xp11.22–23	N, C		
HDAC7	855	12q13.1	N, C	Sin3, NCOR2	TC
HDAC9	1011	p21-p15	N, C		
HDAC10	669	22q13.31	N, C	NCOR2	TC

In 1996, a major breakthrough in the study of HDAC came with the purification and cloning of the first human HDAC enzyme, HDAC1 (originally called HD1) [2]. The predicted amino acid sequence derived from the complete cDNA sequence of HDAC1 revealed a high degree of similarity to the yeast transcriptional regulator RPD3 [3]. That same year, a transcriptional corepressor protein (later called HDAC2), also with high homology to yeast RPD3, was identified from a yeast two-hybrid experiment with the human YY1 transcription factor [4]. Similar to previous experiments used to characterize HDAC1, immunoprecipitation of HDAC2 from human cells followed by enzymatic assays showed that HDAC2 contained HDAC activity [5]. HDAC1 and HDAC2 exist together in multiprotein complexes, and many transcription factors target HDAC1 and HDAC2 to specific promoters to repress transcription.

Classes I, II and IV are zinc dependent metalloproteins and class III is NAD^+-dependent. [Table 1][6]. HDAC1, 2, 3 and 8 define the class I group, which is mostly nuclear, smaller in size (350–500 amino acids) and is more importantly implied in cancer progression. Class II has two subgroups. Class IIa is represented by HDAC4, 5, 7 and 9 while class IIb consists of the two HDAC6 and 10. Finally HDAC11 is in its own class IV. Class III HDAC are the NAD dependant group of the seven sirtuins SIRT1-7.

Class I HDAC

Taking advantage of the recent discovery of trapoxin (TPX), a potent inhibitor of HDAC activity [7,8], Taunton and colleagues [2] isolated HDAC1 from a human T-cell line using a trapoxin-based affinity matrix. HDAC1 was subsequently identified as a growth factor-inducible enzyme with HDAC activity in mouse T-cells [9]. Sequence analyses revealed that both the human and mouse HDAC1 proteins are highly homologous to the Saccharomyces cerevisiae RPD3 protein [9,10], a known transcriptional regulator [3]. Although mouse HDAC1 failed to complement the yeast rpd3Δ deletion [9], extensive investigations since then have clearly demonstrated the crucial role played by HDAC1 in the transcriptional repression of a variety of mammalian genes involved in cell cycle progression, proliferation, differentiation, development and cancer.

The mammalian HDAC1 protein belongs to an ancient family of enzymes highly conserved throughout eukaryotic and prokaryotic evolution. HDAC1, together with HDAC2, HDAC3, and HDAC8, belongs to the RPD3-like class I of HDAC. A phylogenetic analysis has revealed that class I can be further divided into an HDAC1/HDAC2 and an HDAC3 subclass [11]. The existence of an HDAC1/HDAC2 subclass within class I highlights the high degree of similarity that exists between these two enzymes: in mammals, HDAC1 and HDAC2 exhibit approx 82% identity, and their genomic organization is almost identical.

This indicates that HDAC1 and HDAC2 arose from a relatively recent gene duplication and suggests that they have probably undergone little functional divergence either from their common ancestor or from each other. Indeed, both HDAC1 and HDAC2 are widely expressed nuclear proteins, they are found in similar protein complexes, and they have been shown to heterodimerize [6,12,13]. Furthermore, HDAC1 appears to influence the expression of HDAC2, and vice versa. However, the observation that HDAC1 deletion leads to embryonic lethality in the mouse [14] indicates that, despite some functional overlap, HDAC1 and HDAC2 also have distinct and nonredundant biological functions.

HDAC1 is a metalloenzyme containing three important functional domains: an N-terminal HDAC association domain (HAD; residues 1–53), which is essential for HDAC1 homodimerization, association with HDAC2 as well as other proteins, and catalytic activity [6]; a central zinc-binding catalytic domain termed HDAC consensus motif (residues 25–303), which contains several conserved histidine and aspartate residues and forms the active site pocket of the enzyme [6,15]; and a C-terminal lysine-rich domain (residues 438–482) containing the core nuclear localization signal (NLS) KKAKRVKT and the IACEE motif involved in the interaction with the pocket proteins pRB, p107, and p130 [16]. Interestingly, a truncated HDAC1 protein lacking the NLS can still translocate into the nucleus through association with an intact HDAC1 protein , and Taplick and colleagues [6] have therefore proposed that homodimerization plays a pivotal role in the activity of the enzyme.

HDAC are believed to repress transcription mainly through deacetylation of the histone tails that protrude from the nucleosomes, resulting in local modification of chromatin structure [17]. HDAC1 has been shown to deacetylate all four core histones in vitro and appears to preferentially deacetylate specific lysine residues on histone H4 [18]. The enzyme has also been found to deacetylate a subset of histones H3 and H4 in vivo [14]. In

addition to histones, HDAC1 can also deacetylate nonhistone proteins, such as the tumor suppressor p53 [18,19], the transcription factors E2F1 [20,21] and YY1 [22], and proliferating cell nuclear antigen (PCNA) [23].

HDAC1 was originally identified in the mouse as a growth factor inducible protein [9] and its expression has been found to correlate with proliferation in various tissues, embryonic stem (ES) cells, and several transformed cell lines [9,14], thereby suggesting a link between HDAC1 and regulation of cellular proliferation. Indeed, both over expression of HDAC1 in mouse fibroblasts and disruption of the gene in mouse embryos and ES cells have been shown to severely perturb proliferation and cell cycle progression [9,14], indicating that maintenance of cell type-specific deacetylase levels is crucial for unrestricted proliferation. In line with this idea, HDAC2 and HDAC3 protein levels have been found to be upregulated in the absence of HDAC1 [14], and loss of [24]. It is, however, important to note that the effects of HDAC1-mediated transcriptional repression on the regulation of cell cycle progression, proliferation, and differentiation are diverse and seem to be dependent on HDAC1 molecular partners.

The tumor suppressor p53 is known to activate p21 in response to DNA damages, thereby inducing cell cycle arrest and apoptosis [25]. Interestingly, p53 is also recruited to the p21 promoter through direct interaction with SP1 [26,27] and has been shown to compete with HDAC1 for the regulation of p21 expression following DNA damage [26]. Taken together, these findings indicate that HDAC1 is a crucial negative regulator of p21 transcriptional activity, although the possibility cannot be excluded that other HDAC may also be involved in the repression of p21 [28]. In addition to inhibition of p21, HDAC1 has also been shown to repress p53 function by direct deacetylation of the protein [18,19]. Acetylation of p53 stabilizes and activates the protein in response to genotoxic stress, while deacetylation

appears to provide a rapid mechanism to inhibit p53-mediated cell cycle arrest and apoptosis and to restore normal cell growth once DNA repair is completed [29].

Transcriptional repression mediated by HDAC1 clearly plays a crucial role in regulation of cell cycle progression, proliferation, and differentiation. However, increasing evidence indicates that HDAC1 can also influence various other biological processes by mechanisms that do not necessarily involve transcriptional repression.

In recent years, evidence has emerged that HDAC1 is involved in cellular defense against viral infection. For example, viral transcription mediated by open reading frame 50 (ORF50), an activator of early and late genes in the lytic cycle of the Kaposi's sarcoma-associated herpesvirus, is repressed upon association with HDAC1 [30,31]. Furthermore, inhibition of HDAC activity appears to be important for viral infection. For example, Gam1, an early gene product essential for the replication of the avian adenovirus CELO, can interact with HDAC1 and inhibit its enzymatic activity [32]. Viral transforming proteins such as the human papillomavirus oncoprotein E7 are also known to interfere with the binding of HDAC1 to pRB, thereby promoting cell cycle progression [16,33,34]. Although blocking of HDAC activity appears to be important for viral activity, it may also have negative consequences for the virus, as HDAC inhibition has been reported to activate cellular mechanisms to fight against the viral infection [35]. In line with this idea, a recent clinical study by Lerhman and colleagues [36] has suggested that inhibition of HDAC1 in resting CD4+ T cells could contribute to elimination of human immunodeficiency virus (HIV) infection in human patients. Chromatin-associated repression is one mechanism that maintains HIV-1 latency. HDACi reverses this repression resulting in viral expression from quiescently infected cells. Clinical studies with the HDAC inhibitor valproic acid (VPA) failed to substantially decrease the latent pool within resting CD4(+) cells. At clinically relevant concentrations, Givinostat (ITF2357 Italfarmaco S.p.A. Italy) increased p24 by 15-

fold in ACH2 cells and by 9-fold in U1 cells, whereas VPA increased expression less than 2-fold [37].

Extensive studies have shown that HDAC inhibitors induce cell cycle arrest, differentiation, and/or apoptosis in a variety of transformed cell lines, as well as in tumor-bearing animals. Consequently, these inhibitory compounds have FDA approvement for antitumor therapy [38,39]. Several mechanisms can be proposed to explain the antitumor effects of HDACi. The general view is that accumulation of acetylated histones and nonhistone proteins leads to activation or repression of a specific subset of genes and molecular pathways crucial for repression of tumor cell growth. This mechanism is consistent with the finding that the expression of only a limited number of genes is affected by HDACi [38]. With increasing evidence for HDAC involvement in molecular and cellular processes other than transcriptional regulation, it is also reasonable to hypothesize that HDAC inhibitors exert their effects by directly interfering with DNA replication or mitotic division. Finally, based on the observation that tumor cells (which by definition exhibit alterations in various molecular pathways involved in cell growth and differentiation) are much more sensitive to the effects of HDAC inhibitors than normal cells [40], it was proposed that activation or repression of specific genes and pathways by HDAC inhibitors may generate conflicting signals in tumor cells, leading to cell death or apoptosis. The antitumor effects of HDAC inhibitors clearly suggest that HDAC play a major role in cancer development. However, as most HDAC inhibitors affect the activity of several enzymes, it is difficult to identify the particular HDAC involved in tumor formation. One way to evaluate the importance of specific HDAC in cancer is through analysis of their expression levels in tumors. Recent reports have indicated that HDAC1 is upregulated in gastric and prostate cancers [41-44], and in both cases, its overexpression has been shown to correlate with downregulation of gelsolin [43,44], a known target of HDAC inhibitors [38]. Furthermore, HDAC1 knockdown in

human cervical carcinoma cells has been found to induce changes in cellular morphology and to inhibit proliferation, thereby Meunier and Seiser suggesting that HDAC1 is essential for tumor cell survival [45]. Interestingly, downregulation of HDAC1 expression has been shown to be associated with cellular differentiation in a variety of human breast tumor cell lines [46].

One mechanism by which HDAC inhibitors appear to repress tumor cell growth is through the activation of the tumor suppressor p21, which is required for inhibition of cell cycle progression [40].

A report has shown that transcriptional activation of p21 by the HDAC inhibitor suberoylanilide hydroxamic acid (SAHA) is accompanied by a marked reduction in HDAC1 bound at the p21 promoter [28]. Furthermore, displacement of HDAC1 from the p21 promoter by the pRB binding protein Che-1 [47,48] has been shown to activate p21 and consequently inhibit proliferation in human colon carcinoma cell lines [49]. Taken together, these findings strongly suggest that HDAC1 could promote tumor formation through selective repression of p21. Another mechanism by which HDAC1 could promote tumorigenesis is via suppression of ER-α, whose loss is known to be critical for breast cancer progression [50]. Finally, it has been reported that HDAC1 expression can be induced by hypoxia in a lung carcinoma cell line, leading to downregulation of the tumor suppressors p53 and von Hippel-Lindau factor and stimulation of hypoxia-induced angiogenesis [51]. These findings suggest that HDAC1 could influence tumor progression by promoting angiogenesis.

In most cell types, HDAC3 is located both in the nucleus and cytoplasm, and its subcellular distribution is regulated by competing nuclear import and export signals. Although a sequence present at position 29 to 41 of HDAC3 (LALTHSLVLHYGL) resembles the

canonical nuclear. However, it is known that immunoprecipitated HDAC3 complexes contain many other proteins with HDAC activity [52,53].

One of the biggest challenges in the study of HDAC3, as in the study of other HDAC, is to understand the exact mechanisms by which HDAC3 changes gene expression and how it affects the functions of the transcriptional machinery. The purified endogenous HDAC3 complex from HeLa cells contained nuclear receptor corepressor (NCoR) and silencing mediator for retinoid and thyroid receptors (SMRT) proteins. it is reasonable to predict that HDAC3 is the main component recruited by DNA binding factors that utilize NCoR or SMRT to repress transcription.

As expected for an enzyme with important cellular functions, the activity of HDAC3, like that of all other HDAC, is highly regulated by multiple mechanisms. Expression of the human *HDAC3* gene can be induced by treatment of peripheral blood mononuclear cells (PBMCs) with PHA, PMA, and α-CD3 [54]. In addition, HDAC3 is expressed at higher levels in the embryonic form of biliary atresia compared with the perinatal form [55]. In nude mice bearing human ovarian carcinoma xenografts, treatment with Taxol (paclitaxel) increases expression of HDAC3 [56]. Furthermore, the expression of mouse HDAC3 is induced in HDAC1-deficient cells [14]. In contrast, treatment of PBMCs with granulocyte/macrophage colony-stimulating factor (GM-CSF) downregulated the levels of human HDAC3 mRNA [54].

In the human virology arena, HDAC3 has been shown to regulate cytomegalovirus infection by repression of the viral major immediate early promoter in nonpermissive cells [57]. In yeast, loss of HOS2 affects acetylation at ribosomal protein gene promoter regions, implying that HDAC3 might be important in the control of ribosome biogenesis [58].

Unfortunately, no HDAC3 animal knockout models are currently available, and our knowledge of the biological function of HDAC3 in vertebrates is extremely limited.

Overexpression of HDAC3 in THP-1 or HeLa cells led to increased cell size, aberrant nuclear morphology, and G2/M cell cycle arrest, suggesting the involvement of HDAC3 in cell cycle control [54,59]. In HeLa cells, siRNA-mediated HDAC3 knockdown also caused significant morphological changes and inhibited cell proliferation [45]. From these studies, it is clear that a critical concentration of HDAC3 is required for normal cell survival.

HDAC8 is a class I member of the histone deacetylases family, although lying phylogenetically close to the evolutionary boundary between class I and class II HDAC. After a comprehensive review of the current understanding of the biology of HDAC8 and its gene, this HDAC is selectively expressed by cells showing smooth muscle cell differentiation, including smooth muscle, myofibroblastic, and myoepithelial cells.

In 2000, database searches of expressed sequence tags showing high similarity with class I human histone deacetylases (HDAC; HDAC1–3) led three independent groups to clone a cDNA encoding a novel human histone deacetylase [60,61]. This fourth identified class I HDAC was called HDAC8 because at that time seven human HDAC had already been discovered, falling into class I (HDAC1–3) and class II (HDAC4–7).

Amino acid sequence comparisons have indicated that HDAC8 is most similar to HDAC3, with 34% amino acid identity and 54% similarity, when one considers conservative amino acid substitutions [60,61].

It has been suggested that HDAC8 mRNA is ubiquitously expressed since this transcript could be detected in all normal human tissues examined [60,61]. Intriguingly, different authors have observed HDAC8 transcript to be expressed at the highest levels in different organs, including normal human brain [60,61], pancreas [60,62], kidney [61,62], prostate and liver [61]. Nevertheless, the expression profile of HDAC8 mRNA has been found to be distinctly different from that of HDAC1 to 3 transcripts [61].

HDAC8, rather than being detected in all cell types, is exclusively expressed by some prostate stromal cells as well as by cells present in vascular walls [63]. Further extensive investigations of HDAC8 expression by immunohistochemistry in normal human tissues have demonstrated that HDAC8 is exclusively expressed by normal human cells showing smooth muscle differentiation in vivo, including vascular and visceral smooth muscle cells, myoepithelial cells, and myofibroblasts [64].

Unexpectedly, the enzyme has been found to be predominantly cytosolic, both in human tissues and in in vitro grown primary human vascular smooth muscle cells (HSMCs), where it displays a cytoskeleton-like pattern of distribution reminiscent of actin stress fibers [64]. These latter cells exhibit substantially higher amounts of HDAC8 than primary human fibroblasts and HeLa cervix epithelial cells [64].

CLASS II HDAC

Class IIa HDAC, which include HDAC 4, 5, 7, and 9, are defined by a large, functionally important noncatalytic N-terminal domain. This domain mediates both the recruitment of class IIa HDAC to specific promoters and the signal-dependent shuttling of the class IIa HDAC between the nucleus and the cytoplasm. The combination of these two functionalities in the N-terminal domain of class IIa HDAC defines them as signal-dependent repressors of specific sets of genes. Thus they have important roles in the developmentally regulated expression of genes that are involved in the differentiation and function of muscle, immune, and neural cells. Because of the extensive functional characterization of their shared N-terminal domain and also the rapidly emerging phenotypic data from mutant animals, class IIa HDAC are perhaps the best understood of the HDAC.

Class II HDAC exist in both the nucleus and cytoplasm, and the shuttling of class II HDAC out of the nucleus, which is regulated by 14-3-3 proteins, is a major mechanism by which their activity is regulated [65]. Class II HDAC can be further separated into class IIa (HDAC 4, 5, 7 and 9) and IIb (HDAC 6 and 10) based upon the existence of tandem deacetylase domains in HDAC 6 and 10 [1]. Class I and II HDAC share significant homology at the deacetylase domain but differ in their N-terminal sequence. HDAC11, which shares some but not sufficient homology to both class I and II HDAC is assigned to its own class, class IV [66].

HDAC 4, 5, and 9 are particularly well expressed in brain, heart, and skeletal muscle, whereas HDAC7 is highly expressed in heart, thymus, and lung. HDAC4 also appears to be very highly expressed in ovary and intestine [Table 2].

Studies on the expression patterns of HDAC7 in the thymus (by fluorescence-activated cell sorting/reverse transcriptase polymerase chain reaction) and of HDAC9 in the developing mouse embryo (by *in situ* RNA hybridization) revealed that they are expressed within defined subsets of the cells within these tissues. In thymus, HDAC7 appears to be expressed predominantly in the cd4/cd8 double-positive subset of thymocytes [67].

The budding yeast *Hda1* is the enzyme that defines this family [33]. In humans, class II HDAC are subdivided into classes IIa and IIb. As with the class I HDAC, the enzymes of this family perform a wide variety [68] of highly regulated functions. These enzymes also do not contain DNA binding activity, suggesting interactions with other proteins in order to repress transcription [68,69].

Table 2 Tissue Distribution of Class IIa HDAC mRNA Expression. Abbreviations: HDAC, histone deacetylase; MITR, myocyte enhancer factor 2-interacting transcription repressor; PBL, peripheral blood lymphocytes. +++, and ++, highly expressed; +, detectably expressed, –, not detected; ND, no data. Conflicting findings are separated by slashes (e.g., +/–).

Tissue	HDAC4	HDAC5	HDAC7	HDAC9/MITR
Brain	+++	++	–	+++
Colon	++	ND	+	+
Gall bladder	ND	ND	ND	+
Heart	++	+++	+++	+++
Kidney	+	+	–	+
Liver	+/–	++	+	+
Lung	+/–	+	++	–/+
Ovary	+++	ND	+	ND
Pancreas	+ +	+	+	+
PBL	+/–	ND	+	ND
Placenta	+	+	+	+
Prostate	+++	ND	+	ND
Skeletal	++	++	+	++/–
muscle	+	ND	+	+
Small intestine	+	–	–/+	+
Spleen	++	+	–	ND
Testis	ND	ND	+++	ND

Class IIa

Class IIa HDAC consist of HDAC4, -5, -7, -9, and a splice variant of HDAC9 that contains only the N-terminus region of the protein, designated myocyte enhancer factor 2 (MEF2) interacting transcription repressor (MITR) [68]. Many interactions regulate the ability of class IIa HDAC to repress transcription. A striking feature of class IIa HDAC, however, is their ability to shuttle between the nucleus and cytoplasm [69,70]. The cytoplasmic chaperon protein 14-3-3 is responsible for cytoplasmic sequestration of class IIa enzymes [71]. 14-3-3 binds to the HDAC that is phosphorylated on one or two of the three N terminal serines by calcium calmodulin-dependent protein kinase (CaMK), which is

activated after Ca^{2+} release [72]. Once the HDAC reaches the cytoplasm and is thus phosphorylated, complex formation with 14-3-3 sequesters the enzyme in the cytoplasm, thwarting its ability to repress transcription.

Many of the interactions that allow the different class IIa HDAC to repress transcription have been discovered[68-70]. Class IIa enzymes bind to the corepressor SMRT/NCoR complex described earlier (which includes *HDAC3*) [73,74]. *Bcl6* is a BTB/POZ zinc finger transcriptional repressor that, upon overexpression, protects Bcell lines from apoptosis induced by DNA damage. In keeping with this activity, recent studies have shown that overexpression of *Bcl6* suppresses *p53* expression [75]. However, it has also been reported that *Bcl6* binds to the N-terminus of class IIa HDAC [76]. This suggests that *Bcl6* can recruit either the SMRT/NCoR complex, which can then recruit a class IIa HDAC, or the class IIa HDAC directly. Class IIa HDAC do not exhibit enzymatic activity in isolation [68,70] but only in complex with the SMRT/NCoR corepressor complex [53,77].

Class IIb

Class IIb consists of *HDAC6* and *-10*. *HDAC6* was found to be α*-tubulin* deacetylase [77,78]*;* it also binds polyubiquitin chains on misfolded proteins. Recently, a link with Parkinson's disease was revealed when *HDAC6* was found to interact with cytoplasmic *dynein*, a microtubule minus end-directed motor protein necessary for the transport of misfolded proteins [79]. This evidence suggests that *HDAC6* is an adapter protein that allows aggregated, misfolded, and polyubiquitinated proteins [80] to come together with dynein, with subsequent transport to aggresomes. Tubulin hyperacetylation is correlated with more stable microtubules [68]. Thus acetylation and deacetylation of tubulin may be important for the movement of such misfolded proteins along the microtubules, highlighting the role of *HDAC6* in this transport. Moreover, *HDAC6* colocalizes with ubiquitin

conjugates and α-*synuclein* in structures resembling neuronal inclusion bodies, i.e., Lewy bodies, a defining feature of Parkinson's disease [81].

CLASS III HDAC

Class III HDAC are related to the yeast NAD_+ dependent HDAC silent information regulator 2 *(Sir2p)*, which is involved in gene silencing through the generation of heterochromatin-like compacted chromatin that is hypoacetylated in histone H3 and H4 tails. Yeast has four SIR silent information regulator (SIR) proteins, all involved in the formation of specialized repressed chromatin, but only *Sir2p* possesses enzymatic activity on its own [82,83].

Class III HDAC include homologs of *Sir2p* in all higher organisms, including 7 homologs in humans *(SirT1–7) [84,85]*. In addition, proteins with some similarity to *Sir2p* have been found in bacteria [85]. In yeast, a family of four proteins with similarity to *Sir2p* called homologs of Sir2p *(Hst)1–4p* has also been defined, although not much is known about their function [85]. Interestingly, Hstps are probably the true orthologs of the class III members of higher organisms because the SIR machinery is absent in higher eukaryotes. Other evidence supporting this idea is the cellular localization and specificity of these proteins. For instance, *Hst1p* might be the ortholog of *SirT1* and *Hst2p* the ortholog of *SirT2* and *SirT3 [83,85]*.

Class IV HDAC

HDAC11 is the sole class IV HDAC. Expression of HDAC11 is enriched in the brain, heart, muscle, kidney and testis, but little is known about its function. It is composed of a deacetylase domain that shows homology to class I and II HDAC domains, with small N- and C-terminal extensions.

HISTONE DEACETYLASE INHIBITORS (HDACi)

Natural and synthetic inhibitors of HDAC have not only contributed to the discovery of HDAC enzyme molecules and the elucidation of their functions but have also developed as attractive therapeutic agents for diseases including cancer. After the disclosure of the crystal structure of the HDAC-like protein bound to the inhibitor, the momentum of research on HDAC inhibitors increased, and several inhibitors are currently under clinical trials. This chapter focuses on the current knowledge of all classes of HDAC inhibitors and the most recent progress in their clinical development.

One of the most important developments in this field of research was the isolation of specific HDAC inhibitors from natural sources. In the early 1990s, trichostatin A (TSA) and other fungal antibiotics such as trapoxin (TPX) were shown to inhibit HDAC at nanomolar concentrations and to allow the induction of histone hyperacetylation in living cells [7,86]. These HDAC inhibitors played important roles not only in biochemical analyses of the role of histone acetylation but also in cloning the HDAC cDNA [2]. In addition, it is becoming increasingly clear that reversible acetylation of proteins other than histones is one of the key posttranslational modifications controlling the activity of proteins. The HDACi have been widely used as powerful tools for identification and functional analysis of the acetylated nonhistone proteins. The clinical importance of the HDAC inhibitors has been emphasized, in particular for cancer treatment.

These agents act selectively in altering the transcription of relatively few of the expressed genes (generally 2% to 10% of expressed genes are increased or decreased in their rate of transcription) [87-90].

The first compound described as an inhibitor of HDAC activity was sodium butyrate [91]. The effect of butyrate as a noncompetitive inhibitor was shown with a partially purified

enzyme [92]. However, its effective concentration exceeds millimolar levels, which raised the possibility that it affects other enzymes, the cytoskeleton, and membranes. Therefore, butyrate should be used with caution. TPX, which had been discovered to induce morphological reversion of transformed phenotype to normal in several oncogene-transformed cells, was also shown to inhibit HDAC [7]. In contrast to TSA, TPX was an irreversible inhibitor of HDAC. Cocrystallization of the bacterial histone deacetylase-like protein (HDLP) with TSA demonstrated that TSA mimics the substrate and that TSA binds by inserting its long aliphatic chain into the tube-like HDLP pocket and inhibits the enzyme activity by interacting with the zinc and active-site residues through its hydroxamic acid at one end of the aliphatic chain [93]. Hydroxamic acid, a functional group of the inhibitor, coordinates the zinc through its carbonyl and hydroxyl groups, resulting in the formation of a penta-coordinated zinc.

The classes of compounds that have been identified as strong HDAC inhibitors have basic structures mimicking that of TSA, which possesses an aromatic group as a cap, an aliphatic chain for a spacer, and a hydroxamic acid as a functional group interacting with the active-site zinc. The functional groups include carboxylates, hydroxamic acids, electrophilic ketones containing epoxyketones, anilides, and thiols.

The class I/II HDAC active site structure may have features of both metallo- and serine proteases, and the proposed catalytic mechanism for deacetylation is analogous to that of the zinc proteases such as thermolysin and matrix metalloproteinases. Since the zinc protease inhibitors have a zinc-chelating group such as a hydroxamic acid and thiol [94], it is reasonable that these functional groups have great potential to inhibit HDAC activity.

In contrast to the zinc proteases, however, HDAC has a tube-like tunnel in the active-site pocket, which forms van der Waals interactions between the residues lining the pocket and

the aliphatic chain of acetylated lysine. It is therefore likely that a long spacer of the inhibitor is necessary for access of the functional group to the active site zinc. The aliphatic chain length of 5 that corresponds to the length between the carbonyl group and the carbon in acetylated lysine was proposed as the best spacer for inhibition [95]. The cap group may be necessary for packing the inhibitor at the rim of the tube-like active-site pocket. Some synthetic inhibitors have been developed based on this structure-activity relationship information.

Carboxylates

The carboxylate class [96], which is defined as possessing a carboxylate in the metal binding moiety, has generally poor HDAC inhibitory activity in comparison with other inhibitors . Butyrate was the first identified HDAC Inhibitor of this family [97]. Related compounds such as phenyl butyrate and the anticonvulsant valproic acid were later found to be HDAC inhibitors [98].

If the metal chelating functional group is a carboxylic acid, it seems that the activity is lower than the other functional groups such as hydroxamic acid. This may be owing to the weak coordination with zinc ions in comparison with other functional groups. It has been shown that butyrate possesses some selectivity, which poorly inhibits class IIb HDAC such as HDAC6 and -10 [99]. Pivaloyloxymethyl butyrate (AN-9) is an acyloxyalkyl ester prodrug of butyrate. Owing to better cell permeability in comparison with butyrate, AN-9 was shown to be greater than butyrate at inducing inhibition of cancer cell proliferation and malignant cell differentiation [100,101].

Hydroxamic Acids

Hydroxamic acid is one of the well-studied ligands for the zinc present at the bottom of the narrow binding pocket of HDAC. They are still the most popular patented class of HDACi.

Givinostat belong to this family of HDACi. The hydroxamic acid inhibitors have been divided into four categories according to the linker and ZBG: aryl hydroxamates, cinnamyl hydroxamates, non-aryl hydroxamates, and ZBG and any other hydroxamates especially with functional CAP group.

TSA, which was originally isolated from a Streptomyces as an antifungal antibiotic against Trichophyton [102], was rediscovered as a strong differentiation inducer in murine erythroleukemia (MEL) cells [103]. Another biological character of TSA was to induce cell cycle arrest at both the G1 and G2 phases [104].

During analysis of the target molecule for TSA, it was accidentally found that TSA induced hyperacetylation of core histones in cells. Furthermore, TSA blocked partially purified mouse HDAC activity with a low nanomolar inhibition constant in vitro, and HDAC activity from a mutant cell line resistant to TSA was resistant to TSA [86]. This genetic evidence clearly showed that HDAC is the molecular target for TSA. The chemical structure of TSA is unique as a natural product in that it contains a hydroxamic acid at one end of the aliphatic chain. Suberoylanilide hydroxamic acid (SAHA, vorinostat, Zolinza®, Merck&Co., White House Station, NJ, USA), the first HDACi approved by the FDA in 2006 for the treatment of cutaneous T-cell lymphoma, validates HDAC inhibition as a strategy for cancer therapy. SAHA was developed as a strong differentiation inducer during the designed synthesis of the hybrid polar compounds containing a hydroxamic acid and was found to be a potent inhibitor of partially purified HDAC [105]. SAHA has been shown to suppress cell growth in prostate cancer cell lines [87] and human breast cancer cells [106] and to inhibit the growth of the human prostate CWR22 xenograft in nude mice [87]. Furthermore, SAHA inhibited the incidence and growth of carcinogen-induced mammary tumors in rats without toxicity [107] and induced remission in a transgenic model mouse of therapy resistant acute promyelocytic leukemia [108]. The phase I trial of SAHA has been

completed, and the results showed that it could be administered safely and had antitumor activity in solid and hematologic tumors [109].

Pyroxamide (suberoyl-3-aminopyridineamide hydroxamic acid) is a new member of the hydroxamic acid-based hybrid polar compounds and was designed to increase solubility in aqueous solution compared with SAHA. Pyroxamide was shown to induce growth inhibition of tumor cells [110]. LAQ-824 is a cinnamyl hydroxamic acid derivative.

LAQ-824 selectively inhibited growth of cancer cells at submicromolar concentration, induced apoptosis in tumor cells only but not in normal cells, and exhibited antitumor activity in a xenograft animal model [111].

Of the cinnamyl hydroxamic acid derivatives, LAQ-824 exhibited a high maximum tolerated dose with low gross toxicity [112]. Cyclic hydroxamic acid containing peptide (CHAP) is an analog of TPX, in which the epoxyketone is replaced with the hydroxamic acid .CHAP has the ability to inhibit HDAC1 at a low nanomolar concentration [95,113]. Based on the structure of TSA and SAHA associated with HDLP, a large number of inhibitors with the hydroxamic acids have been designed and synthesized.

Electrophilic Ketones

TPX is a cyclic tetrapeptide containing a unique amino acid, 2-amino-8-oxo-9, 10-epoxydecanoic acid (Aoe), and was originally identified as a fungal metabolite inducing morphological reversion of v-*sis*-transformed NIH-3T3 cells [114]. A number of structurally related cyclic tetrapeptides with Aoe have been isolated from the natural source, some of which were toxins produced by phytopathogens. Since the biological activity of TPX is similar to that of TSA, which can induce morphology reversion of the transformed cells, it was speculated that the target molecule for TPX is HDAC or its functionally related proteins. Indeed, TPX caused hyperacetylation of histones in a variety of mammalian cells.

An in vitro experiment using partially purified HDAC revealed that TPX inhibited HDAC activity at low nanomolar concentrations. The epoxy ketone group in Aoe seems likely to play a role in forming a covalent bond between TPX and the active-site residues of enzymes, since the chemical reduction of the epoxide group impaired the inhibitory activity [7]. Taking advantage of the ability of TPX to bind covalently to HDAC, Taunton et al. [2] succeeded in isolating HDAC1 by means of a synthesized affinity probe using TPX.

Although the cyclic tetrapeptide compounds containing Aoe are potent inhibitors for HDAC in vitro, they exhibit very weak activity in animal models, probably owing to the chemical instability of the epoxy ketone group in blood. Apicidin, a cyclic tetrapeptide isolated from *Fusarium pallodoroseum* as a potent and broad-spectrum antiprotozoal agent, exerts its biological activity by reversibly inhibiting HDAC activity [115]. Apicidin contains an ethyl ketone moiety in its side chain instead of the epoxy ketone. The high potency of HDAC inhibitory activity of apicidin further confirmed the importance of the cyclic tetrapeptide scaffold for the inhibitor design. SAHA-based straight-chain trifluoromethyl ketones have also been reported as HDAC inhibitors [116]. Trifluoromethyl ketones are known to be readily hydrated, resulting in zinc chelation and inhibition of zinc proteases [117]. We have synthesized cyclic tetrapeptide containing trifluoromethyl ketones as the zinc binding functionality and these compounds exhibited excellent inhibitory activity [118].

Anilides

The anilide class of inhibitors includes bezamides with a phenylene diamine as the functional group such as MS-275, which is suggested to interact with the HDAC active-site zinc through the 2-substituted anilide moiety. It has been shown that MS-275 inhibits HDAC purified from human leukemia cells and induces accumulation of hyperacetylation at submicromolar concentrations [119]. In addition, MS-275 has been shown to inhibit cell growth of diverse solid tumor cell lines [120]. This class exhibits generally weak inhibitory

activity against HDAC in comparison with the hydroxamic acids. However, MS-275 strongly inhibited growth in various types of human tumor xenografts [119]. CI-994, originally identified as an angiogenesis inhibitor, has been shown to possess poor inhibitory activity against HDAC in vitro. CI-994 increased the amount of acetylated histones and delayed tumor growth in mouse xenograft models [17].

Thiols

FK228 (also known as FR901228 and depsipeptide; isolated as the bacterial metabolite is an atypical HDAC inhibitor as it does not contain a visible functional group that interacts with the zinc ion in the HDAC binding pocket, although FK228 has strong HDAC-inhibitory activity [121]. Recently, it has been shown that FK228 was activated by chemical reduction, yielding two free thiol groups, one of which is accessible to the catalytic zinc as a functional group. Blocking the thiol groups by methylation completely abolished the inhibitory activity. Since glutathione is present at millimolar concentrations in the cell, FK228 can be converted to its active and reduced form (RedFK) by cellular reducing activity. Consistent with this idea, mutations in the glutathione synthesis pathway conferred FK228 resistance on yeast cells [122]. FK228 exhibited a potent antitumor activity in xenograft models [123].

Psammaplins isolated from the sponge *Pseudoceratina purpurea* are structurally unique inhibitors of HDAC [124]. These compounds contain a disulphide linkage, and therefore the inhibitory activity may be owing to chelation of the zinc ion with sulfhydryl in the active-site pocket. Studies on the mechanism of action of psammaplin and its synthetic derivatives also supported the possibility of a cellular disulphide cleavage-type mechanism of inhibition [125,126]. Recently, we synthesized a series of cyclic tetrapeptides containing a functional thiol group named sulfur containing cyclic peptide (SCOP). SCOP inhibited HDAC1 and HDAC4 at nanomolar concentrations but was weak in inhibiting HDAC6 and

HDAC8 [127]. By disulfide formation with SCOP itself or other mercaptans, SCOP can be converted to a “homodimer” or a “heterodimer”, acting as a prodrug like FK228. Once the homodimer is incorporated into the cells, it becomes reduced and activated by cellular reducing activity, giving two active molecules. In the case of hybrid-type inhibitors, other functions such as better solubility, drug delivery, and other enzyme inhibitory activity can be added into the partner compounds.

Inhibitors of Class III HDAC

The first compounds that were demonstrated to inhibit in vitro deacetylase activity of sirtuins were nonhydrolyzable NAD analogs such as Carba-NAD [128]. However, such compounds do not appear to be suitable for in vivo studies since they are not cell permeable and they inhibit other NAD dependent enzymes. To solve these problems, high-throughput cell-based screens were conducted to identify cell-permeable small-molecule inhibitors of NAD-dependent deacetylase activity of sirtuin [129,130]. All small-molecule inhibitors identified by these screens were analogs of α substituted α-naphthol, such as sirtinol, M15, and splitomicin, and they inhibited transcriptional silencing by Sir2 in yeast. Sirtinol and M15 were shown to inhibit yeast Sir2 and human SIRT2 in vitro, whereas splitomicin demonstrated some selectivity against inhibition of deacetylase activity between yeast sirtuins. These compounds were used to elucidate cellular functions of sirtuin in plant or mammalian cells [131-133].

NAD Analog

Based on the requirement of NAD for the deacetylation reaction by Sir2, an uncleavable NAD analog was designed as a Sir2 inhibitor. Carba-NAD is a carbocyclic analog of NAD in which a 2,3-di-hydroxycylopentane methanol replaces the β-D-ribonucleotide ring of the nicotinamide ribonucleoside moiety of NAD [134]. It inhibits HST2 deacetylase activity in a

competitive manner in vitro [128]. This result is also consistent with the requirement for NAD cleavage during the enzymatic reaction.

Nicotinamide

Nicotinamide is a byproduct of the Sir2 deacetylase reaction and is a natural noncompetitive inhibitor for Sir2. Nicotinamide abolishes silencing at the rDNA, telomeres, and mating-type loci and shortens life span, indicating that nicotinamide creates a phenocopy of the *sir2* mutation in yeast [135]. Nicotinamide also suppresses transcriptional repression of Hst1 [136]. Indeed, the deacetylase activity of several Sir2 family members, including yeast Sir2 and Hst2 [135,137] and human SIRT1, -2, and -3 [138-140], can be inhibited by nicotinamide. Nicotinamide inhibits the enzyme activity by intercepting an ADP-ribosyl-enzyme-acetyl peptide intermediate with regeneration of NAD [141]. Since nicotinamide inhibition is uniquely tied to the catalytic mechanism of the Sir2 deacetylation reaction, nicotinamide is most likely a general inhibitor for all Sir2 family members. Physiological concentrations of nicotinamide inhibit yeast Sir2 [128] and human SIRT1 in vitro [135]. In addition, reduction of nicotinamide by Pnc1, which encodes a nicotinamidase that converts nicotinamide to nicotinic acid, enhanced Sir2-mediated silencing and longevity in yeast [136]. Thus, nicotinamide may function as an in vivo negative regulator for Sir2 [142].

β-Naphthol Analogs

Small-molecule inhibitors were identified by cell-based chemical screening for compounds that suppress Sir2-mediated silencing using yeast strains in which either URA3 or TRP1 is inserted within Sir2-silenced loci such as the telomere or homeodomain regulator (HMR). All these inhibitors are structurally related analogs of β-substituted β-naphthol. Sirtinol and M15 were first identified as small-molecule inhibitors for sirtuinsby Schreiber and co-

workers [129]. Sirtinol inhibits in vivo silencing activity of yeast Sir2 as well as in vitro deacetylase activity of recombinant yeast Sir2 and human SIRT2 but not HDAC1, indicating that sirtinol is a specific inhibitor for sirtuins. Since sirtinol and M15 share 2-hydroxy-1-naphthaldehyde within their structure, the 2-hydroxy-1-naphthaldehyde moiety could be responsible for the inhibitory activity of these compounds.

Indeed, 2-hydroxy-1-naphthaldehyde alone can partially inhibit SIRT2 activity in vitro. Recently, sirtinol was used to identify SIR1 in *Arabidopsis*, a regulator of many auxin-inducible genes, by a genetic screen for mutants that were resistant to the effects of sirtinol [131]. Furthermore, sirtinol and M15 were used to delineate a role of Sir2 in regulating skeletal muscle differentiation [132].

Splitomicin, identified by Simon and co-workers [129], is structurally related to sirtinol. It can create a phenocopy of a *sir2* mutant in yeast and can inhibit in vitro deacetylase activity of Sir2 against a histone H4 peptide [130]. A screen for the alleles of *SIR2* that are resistant to the antisilencing effects of splitomicin revealed a small helical module of Sir2 that creates a putative substrate binding site [143-145] as a possible site where splitomicin acts [130]. Thus, splitomicin probably inhibits deacetylase activity of Sir2 by interfering with the access of an acetylated substrate to this region. Among Sir2 and four Sir2 homologs (Hst1–4) in *S. cerevisiae*, the effects of splitomicin on transcription correlated most highly with a *sir2* mutation, less with an *hst1* mutation, and not at all with other *sir2* homologs mutations [146], suggesting that splitomicin is a selective inhibitor for Sir2 in *S. cerevisiae*.

In contrast to splitomicin, dehydrosplitomicin, an analog of splitomicin unsaturated at the 1,2-position of splitomicin, has been shown to repress Hst1-regulated genes but not to perturb the silencing by Sir2 [146], indicating that an inhibitory activity of dehydrosplitomicin is selective for Hst1. The chimeric and mutagenetic approaches revealed that leucine-244 within the small helical module of Hst1, which corresponds to

tyrosine-298 of Sir2, is an essential residue for the relative resistance of Hst1 toward splitomicin [146]. These observations suggest that splitomicin and dehydrosplitomicin may be useful tools for investigating individual roles of Sir2 and Hst1 in *S. cerevisiae*.

IMMUNOMODULATORY EFFECTS OF HDACi

Several studies in vitro and in vivo support the concept that inhibition of HDAC is a strategy to reduce the production and activity of pro-inflammatory cytokines. The anti-inflammatory activities of the HDAC inhibitor, SAHA, ITF2357 and LAQ824 have been described [147-150]. Other studies have increased the scope of the antiinflammatory activity of HDAC inhibitors. For example, in the lpr/lpr mouse, which spontaneously develops lethal nephritis similar to that of lupus erythematosus, administration of the HDAC inhibitor trichostatin A (TSA) reduced the severity of the disease [151]. In a similar study, daily treatment with SAHA resulted in decreased proteinuria, reduced cytokine and nitric oxide production as well as less damage to the renal tissues [152].

Several recent experimental studies have demonstrated that HDAC inhibitors can modulate immune responses at concentrations much lower than those needed for antitumor effects [147,151-154]. SAHA and ITF2357 [38,149,150].

Different Authors have demonstrated that administration of SAHA at the time of allogeneic bone marrow transplantation (BMT) suppresses proinflammatory cytokine production and reduces systemic acute graft-versus-host disease (GVHD), the major toxicity of allogeneic BMT [154,155]. HDAC inhibitors have also been shown to regulate several other

inflammatory and immune-mediated diseases [147,149,151,153,156]. Inhibitors of HDAC represent a new class of targeted antinflammatory agents.

Acetylation can promote the activation, nuclear translocation and DNA binding of transcription factors such as STAT3, NF-κB and RUNX1, and thereby promote expression of multiple genes, including pro-inflammatory cytokines and other mediators of inflammation and immunity.

The evidence of the anti-inflammatory effects of HDACi has been accruing for many years, but has not led to their development for immuno-inflammatory disorders by pharmaceutical companies. Despite their potency, existing HDACi drugs have toxicities or other limitations that have largely restricted their development to the potential treatment of patients with malignancies. However, this assessment is changing as new insights into the roles of individual HDAC enzymes are emerging and new cellular targets are identified.

Over the past decade, the recognition and characterization of T_{regs} that express FOXP3 in maintaining host homeostasis has captured the attention of many investigators. $FOXP3^+$ T_{regs} play a key part in limiting autoimmunity and maintaining peripheral tolerance, and mutations of FOXP3 lead to lethal autoimmunity in humans and mice [157-160]. From a therapeutic perspective, some groups are testing expansion of small numbers of T_{regs} before adoptive transfer back into an individual. However, repeated stimulation of expanded T_{regs} has been found to lead to loss of FOXP3 expression, especially when using T_{regs} generated in vitro [161,162]. An alternative approach has arisen from recent insights into the epigenetic regulation of FOXP3 [163-166]. Therapeutic manipulation of FOXP3 acetylation using HDACi can promote the development and suppressive functions of $FOXP3^+$ T_{regs}, with beneficial consequences in models of transplant rejection, colitis and arthritis [164,167].

The role of cytokines and other mediators in the development and persistence of inflammatory diseases is well established. More recently, recognition of a requirement for chromatin remodelling to promote transcription factor binding and activation of cytokine and other genes has led researchers to consider HDACI use as a new approach to treat immuno-inflammatory disorders.

With regard to acute inflammation, treatment of mice with SAHA [147] or NVP-LAQ824 [148], two hydroxamic acid pan-HDACi, suppressed lipopolysaccaride-induced production of the cytokines TNFα, IL-1β, IL-6 and IFNγ in dendritic cells both in vitro and in vivo [147,148,168]. Consistent with these findings, HDACI therapy is known to impair activation of NF-κB in dendritic cells and macrophages, although the mechanisms remain controversial [169], and to impair their differentiation and maturation [170,171]. Recently, pan-HDACi exposure was shown to promote STAT3 acetylation [172] and indoleamine 2,3-dioxygenase (IDO) production by dendritic cells [173]. As IDO catabolizes tryptophan, an amino acid that is essential for T cell activation, induction of IDO blocks T cell activation and the development of murine graft-versus-host disease post-bone marrow transplantation [173]. In vivo use of TSA or SAHA also inhibited T cell cytokine production and proliferation [174-176] and promoted T cell anergy [177], in conjunction with altered chromatin remodelling at the IL-2 promoter and acetylation of key transcription factors, including NF-κB [175,176].

With regard to chronic inflammation, TSA or SAHA decreased fibroblast proliferation and extracellular matrix production in murine models of fibrosis [178,179], and both in vitro and in vivo TSA or SAHA blocked TGFβ-induced differentiation of fibroblasts into myofibroblasts and impaired epithelial–mesenchymal transformation [179,180]. Selective HDAC targeting might also be a useful strategy to limit inflammation, as in cultured epithelial cells, HDAC6 promotes TGFβ-induced epithelial–mesenchymal transformation

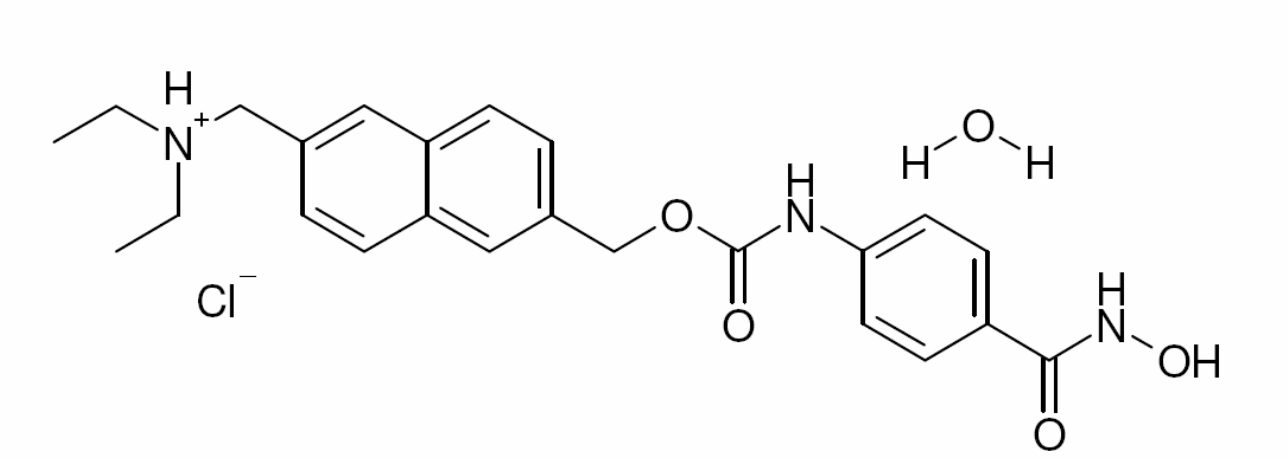
by deacetylating SMAD3 or SMAD3-interacting proteins [181], and in vitro, HDAC4 can inhibit expression of a TGFβ repressor, TGIF [182].

In murine models of T cell-dependent disease, therapy with TSA or SAHA decreased the severity of ConA-hepatitis [183], T_H2-associated lung airway hypersensitivity responses; experimental allergic encephalomyelitis [184]; renal disease in MRL/lpr mice [151]; colitis [156]; arthritis [185,186]; graft-versus-host disease post-bone marrow transplantation [187] and the 'cytokine storm' induced by the CD3 monoclonal antibody therapy used in a bone marrow transplant conditioning regimen [188]. The underlying mechanisms responsible for the beneficial effects of pan-HDACi therapy in these models were usually attributed, by extrapolation from the cancer literature, to effects on effector T cell apoptosis [189] and no specific HDAC were identified as key targets for therapy.

At least seven companies are developing HDACi for treatment of inflammation and/or autoimmunity, with most attention being given to identification of hydroxamic acid derivatives. Interestingly, even within this class of compounds, some pan-HDACi and class I HDACi have been identified, as well as agents with individual HDAC selectivity. The latter include hydroxamates selective for HDAC6 (class IIb) or HDAC8 (class I), indicating the robust range of potential inhibitory actions of this broad class of compounds. One such hydroxamate, ITF2357 has shown clinical benefit and safety in Phase II trials in children with active systemic onset juvenile idiopathic arthritis [190]. Givinostat significantly reduced the systemic feature score and number of painful joints.

ITF2357 (GIVINOSTAT)

PHYSICO-CHEMICAL CHARACTERISTICS
Molecular formula: $C_{24}H_{30}ClN_3O_5$
Molecular weight: 475.98
Family: hydroxamates
Structural formula:



In preclinical studies ITF2357 has been demonstrated to fully inhibit class I and II HDAC at concentration of $1x10^{-6}$ M. In contrast, ITF2357 had no effect on the activity of class III HDAC when tested at concentrations up to 1 M.

The inhibitory effect of ITF2357 on HDAC was also evaluated in living cells using the total protein acetylation as the read-out. Human peripheral blood mononuclear cells (PBMCs) or cell lines were treated with increasing doses of ITF2357 and the resulting protein acetylation determined by western blotting. ITF2357 at doses as low as 10-100 nM induced histone hyperacetylation indicating intracellular inhibition of HDAC [149]. There was no evidence of cell death in LPS-stimulated PBMCs at 100 nM ITF2357, using assays for DNA degradation, annexin V, and caspase-3/7. By Northern blotting of PBMCs, there was a 50% to 90% reduction in LPS-induced steady-state levels of TNFα and IFNγ mRNA but no effect on IL-1β or IL-8 levels. Real-time PCR confirmed the reduction in TNFα RNA

by ITF2357. Oral administration of 1.0 to 10 mg/kg ITF2357 to mice reduced LPS-induced serum TNFα and IFNγ by more than 50%. Anti-CD3-induced cytokines were not suppressed by ITF2357 in PBMCs either in vitro or in the circulation in mice. In concanavalin-A-induced hepatitis, 1 or 5 mg/kg of oral ITF2357 significantly reduced liver damage. Thus, low, nonapoptotic concentrations of the HDAC inhibitor ITF2357 reduce pro-inflammatory cytokine production in primary cells in vitro and exhibit anti-inflammatory effects in vivo [149] and influences both proliferation and inflammatory pathways in HL-60 cells [191].

As reported recently by Lewis et al. ITF2357 reduces proinflammatory cytokine and nitric oxide production in mouse peritoneal macrophages and cultured mouse splenocytes [192]. The same Authors have recently demonstrated that ITF2357 prevents cytokine-induced cell death in insulin-secreting rat INS-1 cells.

The drug has potent anti-neoplastic activity in vitro and in vivo through direct induction of leukemic cell apoptosis. Furthermore, the drug inhibits production of growth and angiogenic factors by bone marrow stromal cells, in particular IL-6 and VEGF[193].

In a phase II A study was conducted to evaluate the safety and efficacy of Givinostat in patients with Polycythaemia Vera (PV), Essential Thrombocythaemia (ET) and Myelofibrosis (MF), bearing the JAK2V617F mutation. Three major responses were registered among 16 MF patients. Pruritus disappeared in most patients and reduction of splenomegaly was observed in 75% of PV/ET and 38% of MF patients. Reverse transcription polymerase chain reaction identified a trend to reduction of the JAK2V617F allele burden. Givinostat was well tolerated and induced haematological response in most PV and some MF patients [194].

Chromatin-associated repression is one mechanism that maintains HIV-1 latency. Inhibition of histone deacetylases (HDAC) reverses this repression resulting in viral

expression from quiescently infected cells. At clinically relevant concentrations, ITF2357 increased p24 by 15-fold in ACH2 cells and by 9-fold in U1 cells, whereas VPA increased expression less than 2-fold. Analogues of ITF2357 primarily targeting HDAC-1 increased p24 up to 30-fold. In CD4(+) T cells treated with ITF2357, CXCR4 expression decreased [37].

When given at a dose of 100 mg twice daily alone or combined with dexamethasone, ITF2357 proved tolerable but showed a modest clinical benefit in advanced MM [195]. It was also showed efficacy in a colitis-associated cancer in mice [189].

PURPOSE OF THE STUDY

At concentrations lower than those used for antitumor effects, HDACi can modulate inflammation primarily by reducing cytokine production as well as inhibiting immune responses [147,151-154]. HDAC inhibitors have also been shown to reduce the severity of disease in mouse models of inflammatory bowel disease bowel disease [156,189]. In other inflammatory and immune-mediated diseases, such as models of lupus [151,152,196], inhibitors of HDAC represent a new class of therapeutic options. In the present study, we investigated the HDACi ITF2357 for pharmacokinetics profile, safety and effects on ex vivo cytokine production in a Phase I trial of healthy subjects.

PATIENTS AND METHODS

Human Subjects and Eligibility.

This open-label Phase I study (Protocol DM/00/2357/01) was approved by the Ethical Commission of the Bavarian Physician's Chamber (Munich, Germany). All subjects provided written informed consent in accordance with the Declaration of Helsinki before enrollment. Subjects were deemed healthy based on clinical history, physical examination, ECG, and laboratory tests of blood and urine. Body weight was between 60 and 95 kg and a body mass index between 18 and 28 kg/m^2. Subjects were excluded due to evidence of hepatitis B and C and HIV-1 infection. In addition, drug or alcohol abuse, a donation of greater than 400 mL of blood during previous 3 months and the use of prescribed medication during previous 30 days were exclusion criteria. Over-the-counter medicine in previous 14 days or a history of severe allergic disease also served as exclusion criteria. Subjects were allowed to withdraw from the study at any time. Subjects were hospitalized from 24 hours prior to dosing until 48 hours after dosing and were followed for seven days after dosing. Table 3 and 4 summarize the subjects and the treatment schedules.

Table 3 - Pharmacokinetics Studies design and volunteers characteristics. o.d., every day; b.i. d., twice a day

Study Design	Treatment Doses	No. Subj. (*)	Age Range (years)	M/F	Duration of Treatment (and FU)
Single rising doses Double blind, placebo controlled, randomized	50 mg PO, 100 mg PO, 200 mg PO, 400 mg PO	64	21-39	M	Single dose (1 week FU)
Repeat rising doses Double blind, placebo controlled, randomized	50 mg PO o.d., 100 mg PO o.d., 200 mg PO o.d., 100 mg PO b.i.d.	41	21-40	M	7 days (4 weeks FU)

Table 4 - Extent of exposure for healthy volunteers by studies and treatment. o.d., every day; b.i. d., twice a day

Treatment	Dose	N	Completed study (per protocol)	Time on treatment (weeks)	Time on observation (weeks)
Single dose	50 mg	9	9	---	6
	100 mg	9	9		6
	200 mg	9	9		6
	400 mg	9	9		6
	Placebo	10	10		10
	Total	64	64		34
Repeat dose	50 mg o.d.	8	8	8	40
	100 mg o.d.	9	8	9	45
	200 mg o.d.	8	8	8	40
	100 mg b.i.d.	8	8	8	40
	Placebo	8	8	8	40
	Total	41	40	41	205

Study Design and Treatment plan.

Three studies were performed. The first study was a single dose escalation study. The second study was a multiple dose study in which each subject received a dose of ITF2357 each morning for seven consecutive days. In the third study, a safety study, the volunteers received ITF2357 once or twice a day for eight weeks and observed for 40 weeks. The three studies were each double-blinded, randomized and placebo-controlled. There was a random placebo control group in each dose group. The doses of ITF2357 were

administered as capsules in the morning after a 12 hour fast. Placebo capsules were administered in equal numbers.

Pharmacokinetics.

Pharmacokinetics were determined with single or repeat doses of ITF2357. Venous blood samples (5 mL) were collected into heparinized tubes according to a predetermined schedule up to 48 hours after dosing. Plasma concentrations of ITF2357 and its main metabolites, ITF2374 and ITF2375, were assayed using a specific and validated LC-MS/MS method. Pharmacokinetic parameters were derived from plasma concentrations at 0, 0.25, 0.5, 1, 2, 4, 6, 8, 10, 12, 16, 24 and 48 hours after oral dosing.

Safety and Tolerability.

A total of 105 subjects were enrolled, 64 subjects involved in single dose studies and 41 subjects in the repeated dose study (Tables 1 and 2). ITF2357 was studied at daily doses ranging from 50 mg to 400 mg in single dose and 50 mg to 200 mg in repeat-dose study, with administration frequencies of once or twice daily. All clinical adverse events and laboratory abnormalities were evaluated by the investigators for potential relationship to ITF2357. Those considered possibly, probably, or definitely related to ITF2357 were classified as drug-related. Tolerability was monitored throughout the study periods and at follow-ups. Blood pressure and heart rate were monitored before dosing and at the times of blood sampling for pharmacokinetics. ECG was recorded before, 24 and 48 hours and 7 days after dosing. Complete blood counts, blood chemistries and urinalysis were performed before, 12 (only hematology), 24, 48 hours and 7 days after the last dosing.

Ex vivo whole blood cultures for cytokine production.

Blood was drawn after oral dosing at various times in order to measure ex vivo unstimulated and endotoxin (lipopolysaccharide, LPS)-induced cytokine production. One milliliter of blood was directly collected in sterile heparinized culture tubes containing a premeasured (1 mL) amount of RPMI 1640 (Cellgro Mediatech, Inc., Manassas, VA) tissue culture fluid or 1 mL of RPMI 1640 containing 20 ng of LPS (*Escherichia coli* serotype 0127: B8, Sigma). Therefore, the final concentration of LPS was 10 ng/mL as the blood was diluted one part in two. After 24 hours at 37ºC, the supernatant was removed and frozen at -80 °C until assayed for cytokines. Mature 17 kiloDalton IL-1β was measured. Also, TNFα, IL-6, IL-10, IL-1Ra and IFNγ were assayed by ElectroChemiLuminescence (ECL) method (BioVeris, Gaithersburg, MD). Antibodies for these assays were purchased (R&D Systems, Minneapolis, MN). biotinylated and ruthenylated according to the manufacturer's instructions (BioVeris). The cytokine standards were obtained from Peprotech (Rocky Hill, NJ, USA). The lowest limit of detection of the ECL assay was 20 pg/mL for each cytokine.

White blood cell (WBC) counts were obtained for each experimental condition. The relative concentrations of cytokines in picograms per mL were converted to cytokine (in pg/ml) per million cells.

RESULTS

Safety and tolerability of ITF2357.

One hundred and five male subjects gave informed consent. There were 40 adverse events (AEs). AEs were classified as mild (31/40, 77%) or moderate (9/40, 22%). No AEs were classified as severe. In the single dose study, 9 moderate AEs were reported by 6 (15%) subjects, but these occurred only in the groups treated with 200 mg or higher. Seven of the 9 moderate AEs were judged as possibly related to the study drug by the investigators. The most frequently reported AE was headache (n=4) of mild to moderate intensity.

In the week-long repeat dose trial, AEs were reported by 12 (29%) subjects out of 41 treated; ten subjects treated with ITF2357 and 2 with placebo. Subjects receiving ITF2357 at 50 mg or 100 mg reported no AEs. The most frequently reported AEs were headache (n=6 by 4 subjects), palpitations (n=8 by 2 subjects), dysuria (n=5 by 1 subject), nausea (n=2 by 2 subjects with 1 in placebo) and abdominal pain (n=3 by 2 subjects with 1 in placebo). Twenty-six AEs (26/40, 65%) were assessed as possibly related to study drug by the investigators.

Hematologic changes.

Non-specific changes in WBC counts were observed at doses of 200 mg/day or higher. A reduction in platelets counts was observed across all ITF2357-treated groups. The magnitude of the decrease was dose-dependent. The effect was evident from day 5 onward, reaching the lowest value on day 9, i.e. 48 hours after last dosing. On day 9 the mean platelet counts decreased by 17%, 25% and 35% in groups receiving ITF2357 50 mg, 100 mg and 200 mg each day, respectively (see Figure 10). For the most part, the effect on platelets recovered on day 14 (7 days after last dosing) and was fully reversed 4 weeks after last dosing (see Figure 10). The pattern of platelet decrease in the group receiving ITF2357 100 mg twice day was comparable to that observed in the 200 mg each day group. Additional hematological evaluation performed in the 100 mg twice a day group revealed that the effect was more pronounced on day 11; the trend reversed towards normality on day 14 and full recovery was observed on day 21 onwards.

Routine Laboratory Findings.

There were no changes in liver or renal function tests at any dose or dosing schedule.

Pharmacokinetics of single oral dose ITF2357.

Mean plasma concentration time profiles after a single oral administration of ITF2357 at each dose level are shown in Figure 1. Plasma concentrations of ITF2357 reached peak levels approximately 2 hours after dosing for all dose levels and displayed bi-phasic elimination profiles with a mean apparent terminal elimination phase (half-life) ranging from 5.3 to 6.9 hrs. Dose-proportionality of the drug was assessed over the 50 to 400 mg dose range. Results suggest that there is a proportional increase in area under the curve (time from zero) and Cmax of the drug with increasing dose levels of ITF2357.

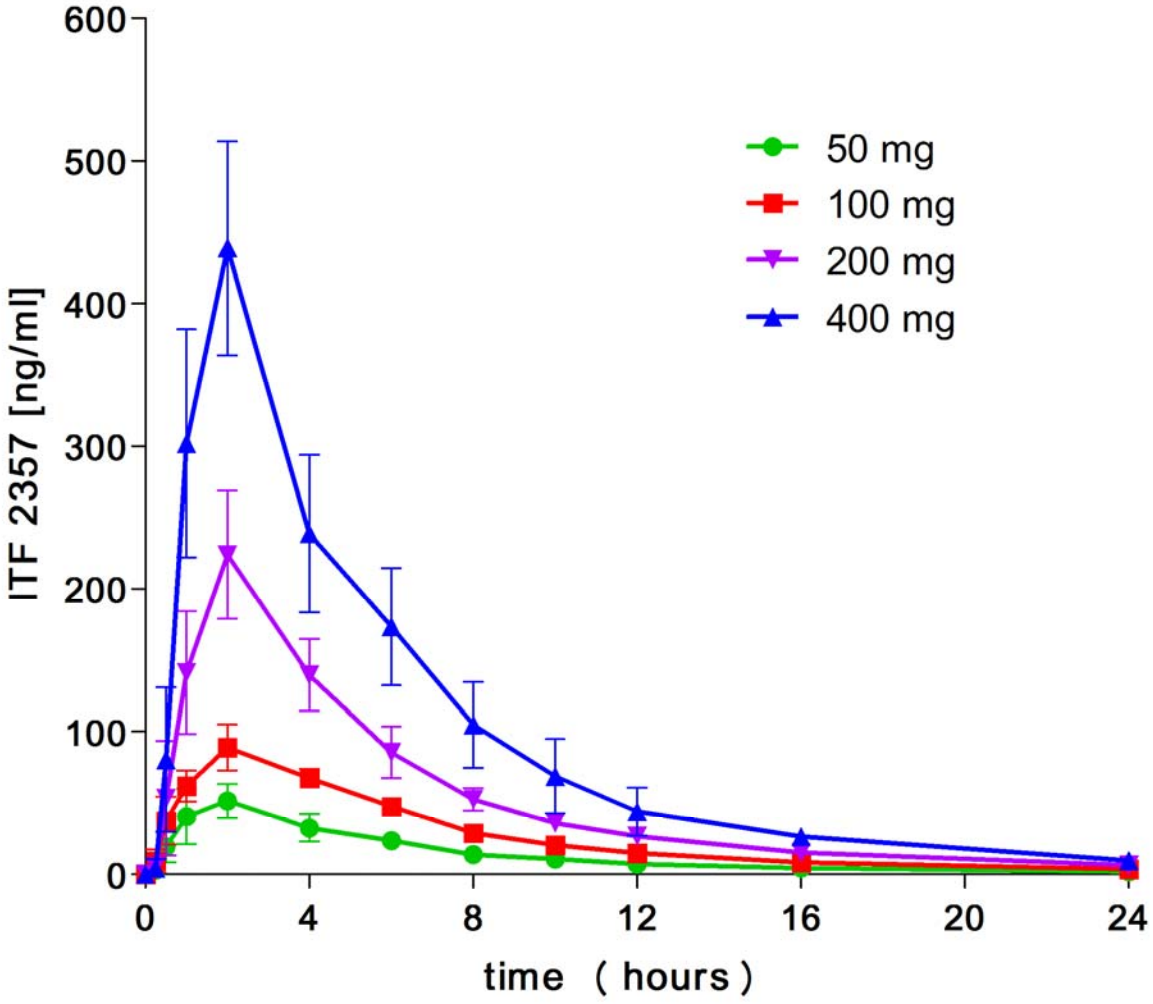


Figure 1. Pharmacokinetics of ITF2357 in healthy male subjects following administration of single oral dose. Mean (± SD) plasma concentrations of ITF 2357. N= 9 in each group.

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Repeated oral doses.

Oral repeated dose studies with ITF2357 have been conducted in rats, dogs and monkeys. Based on these studies, we calculated a safe single oral dose in humans to be 100 mg and a highest dose of 200 mg/day for the 7 days repeat administration study. Mean plasma concentrations measured on day 1 and 7 after oral administration of ITF2357 at 50, 100 and 200 mg each day and 100 mg twice a day for 7 consecutive days are presented in Figure 2. For the each day treatment, maximal concentrations ranged

between 2.4 to 2.6 hours on Day 1 and between 2.1 to 2.8 hours on Day 7. The elimination half-life of ITF2357 was fairly comparable between the 50 mg, 100 mg and 200 mg doses, ranging between 5.73 and 6.15 hours on Day 1 and 6.47 and 7.31 hours on Day 7. Based on the pre-dose concentration values of ITF2357, steady-state appears to have been achieved within 5 to 7 days.

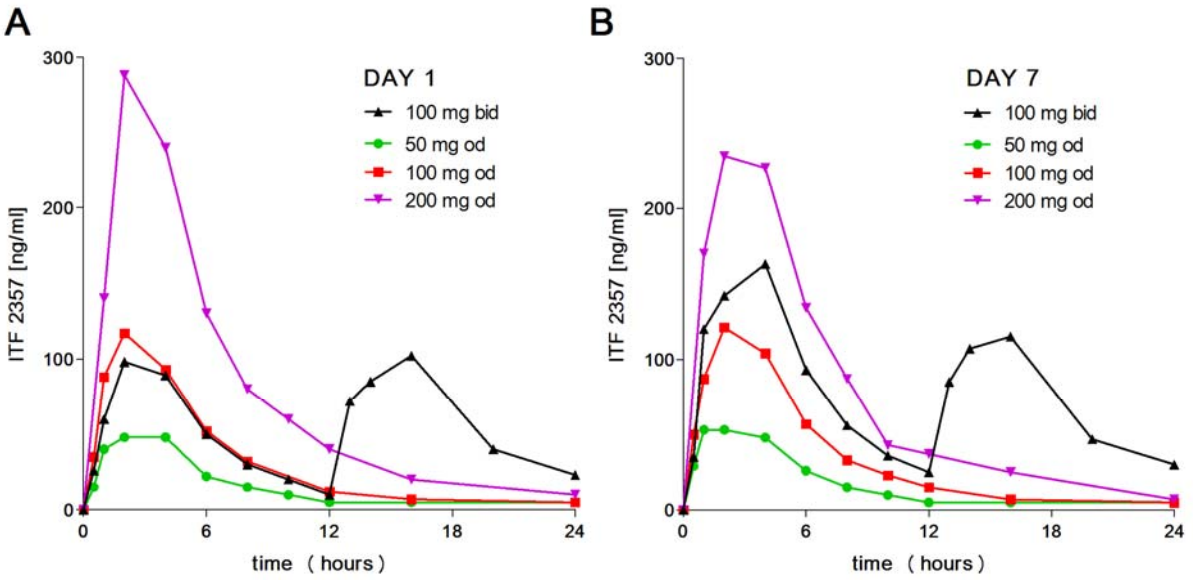

Figure 2. Pharmacokinetics of ITF2357 in healthy male subjects. (A) Mean (± SD) plasma concentrations of ITF 2357 after a single oral dose. (B) Mean (± SD) plasma ITF 2357 on day 7 after 7 daily doses. N= 8 in each group.

Cytokine production in ex vivo whole blood cultures.

Whole heparinized blood taken at various time points following oral dosing was cultured for 24 hours with endotoxin (10 ng/mL) and the supernatants were assayed for IL-1β, TNFα, IL-6, IL-1Ra, IL-10 and IFNγ. As shown in Figure 3A, the maximal decrease in TNFα production occurred at 4 hours at all doses and the 4 hour time point was also the time of maximal decrease in IL-1β (Figure 3B). At a dose of 100mg, the mean maximal concentration of ITF2357 of approximately 100 ng/ml (200 nM) occurs at 2 hours. However, the mean decrease in TNFα of about 37% (non-significant) of baseline occurs at

4 hours. After an oral dose of 50mg, the maximal concentration at 2 hours is about 100 nM. At 4 hours after dosing, the mean reduction in LPS-induced TNFα was 51% (p=0.02). The amount of ITF2357 carried over in the plasma of the subjects into the culture is 50% lower than that shown in Figure 1 since the blood is diluted in the culture one part in 2. For example, using a single dose of 50mg, the concentration of ITF2357 at 4 hours is approximately 40nM and the concentration carried-over into the culture would be 20 nM. At 20 nM, LPS-induced cytokines in PBMCS is significantly reduced [149]. A similar reduction in IL-1β was also observed (Figure 3B), particularly at the 100 mg dose (-77%, p=0.001).

By 12 and 24 hours following a single dose of ITF2357, there is no significant reduction of either TNFα or IL-1β in whole blood cultures from subjects treated with 50 or 100mg, although in the 200mg and 400mg dose group, reductions at 12 but not 24 hours were observed. We next examined the production of TNFα and IL-1β at 12 and 24 hours calculating the cytokines per million WBC. As shown in Figure 4A and 4B, there is no suppression at 12 and 24 hours when the amount of cytokine is examined per million WBC in the 50 and 100mg dose group, although suppression was present in the 200 and 400 mg group. By 24 hours, all dosing groups produced cytokines comparable to that of the baseline level. From these data, we conclude that the ITF2357-associated reduction of LPS-induced TNFα and IL-1β production in whole blood cultures is transient and returns to pre-drug levels by 12 hours after dosing.

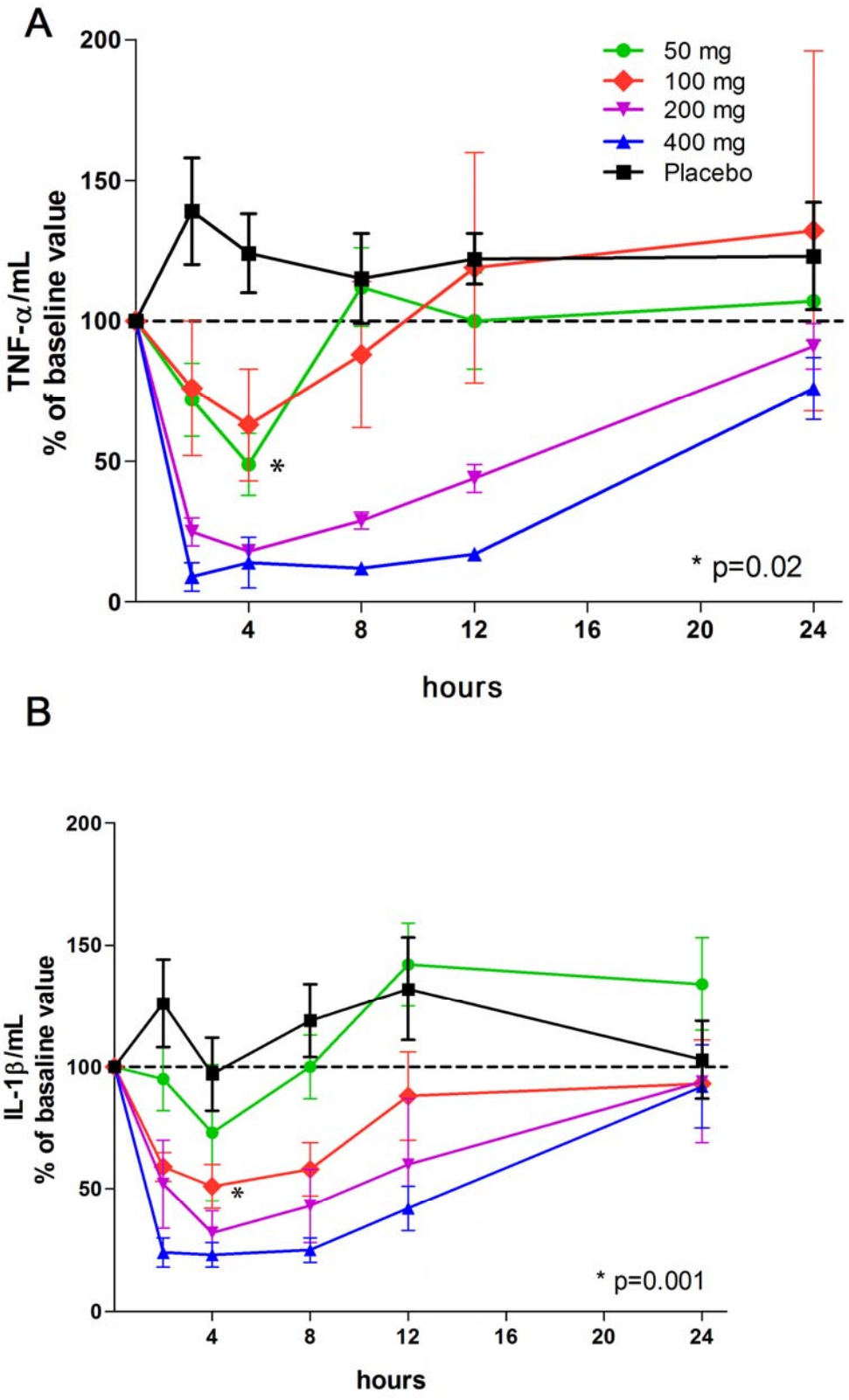

Figure 3. LPS-induced TNFα and IL-1β in whole blood cultures. (A) Mean ± SEM of TNFα per mL expressed as percent change of basal production just prior to the oral dosing. The hours after the dose are indicated under the horizontal axis. (B) IL-1β in the same samples shown in A. N=9 for each group.

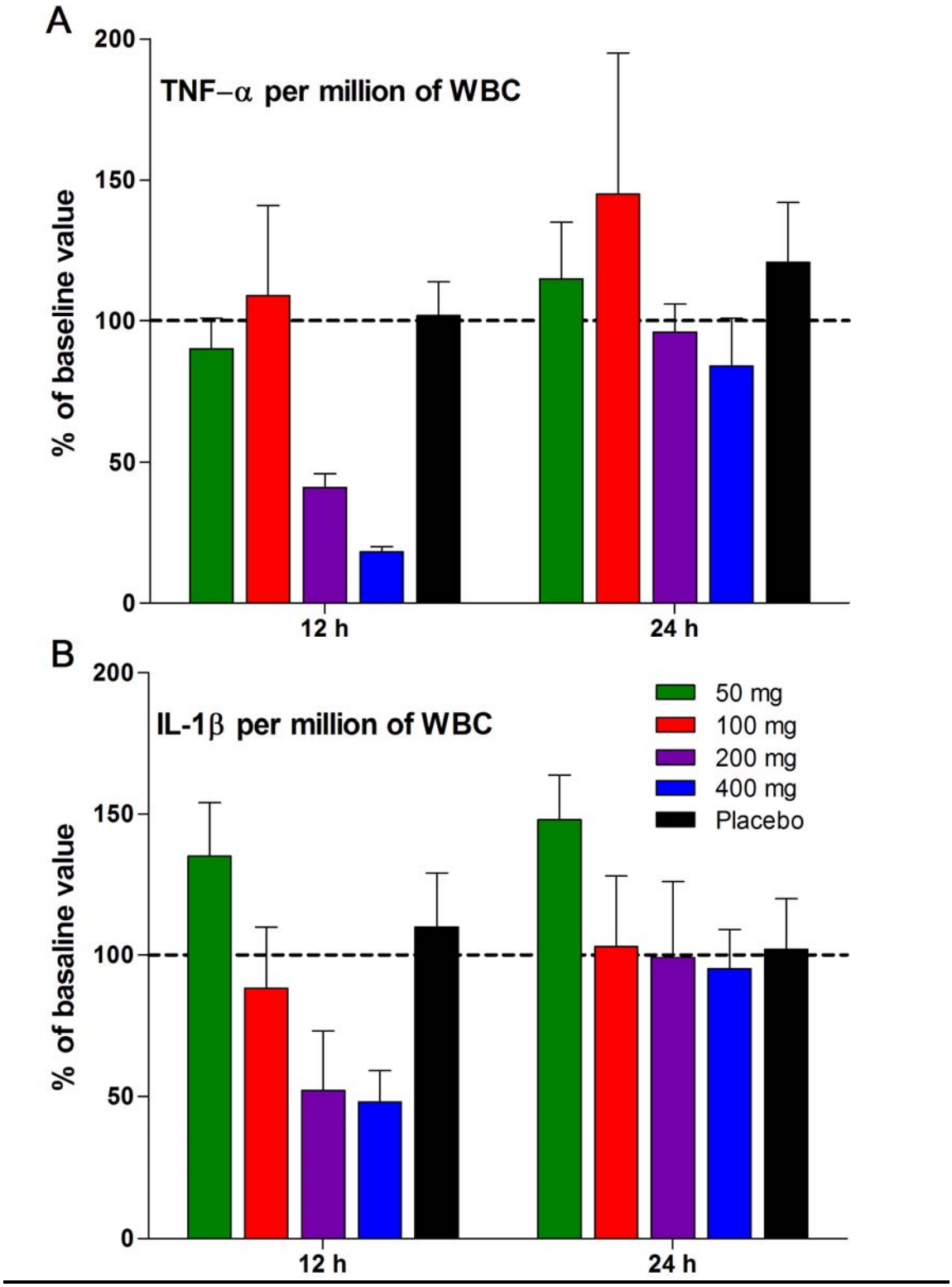

Figure 4. Production of LPS-induced TNFα and IL-1β per white blood cell. (A) Mean ±SEM of TNFα expressed as percent change from baseline per million total WBC 12 and 24 hours after a single dose. Complete blood counts were obtained at baseline, 12 and 24 hours following oral dosing of ITF2357. (B) IL-1β in the same samples shown in A. N= 9 in each group. Effect of 7 daily doses of ITF2357 on cytokine production.

Daily dosing regimen was carried out in order to ascertain whether a sustained course of ITF2357 would affect cytokine production different from that observed following a single dose (Figure 5A and 5B). As shown in Figure 5B, the reduction in IL-1β following 7 daily doses of 200mg is nearly the same as that observed following the first day. Nearly the same pattern as shown in Figure 5 was observed on day 7 for TNFα, IL-6 and IFNγ (data not shown).

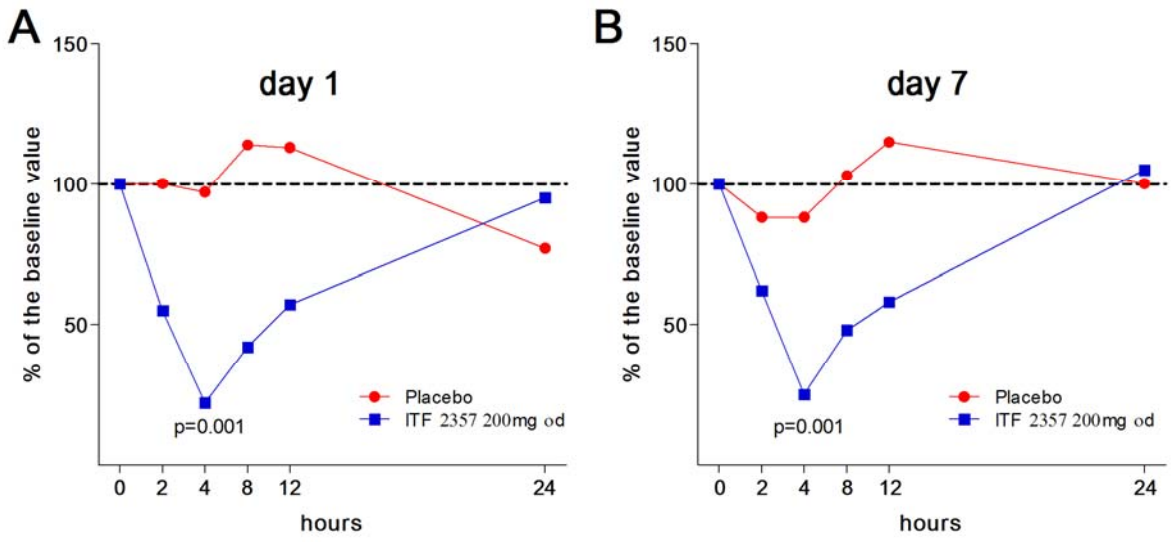

Figure 5. Comparison of a single dose to the 7th dose of ITF2357 on LPS-induced IL-1β production. (A) Mean±SEM of IL-1β per mL expressed as percent change of basal production just prior to the single oral administration. The hours after the dose are indicated under the horizontal axis. (B) Mean±SEM on day 7 of IL-1β following the 7th daily dose of 200mg. N=9 for each group.

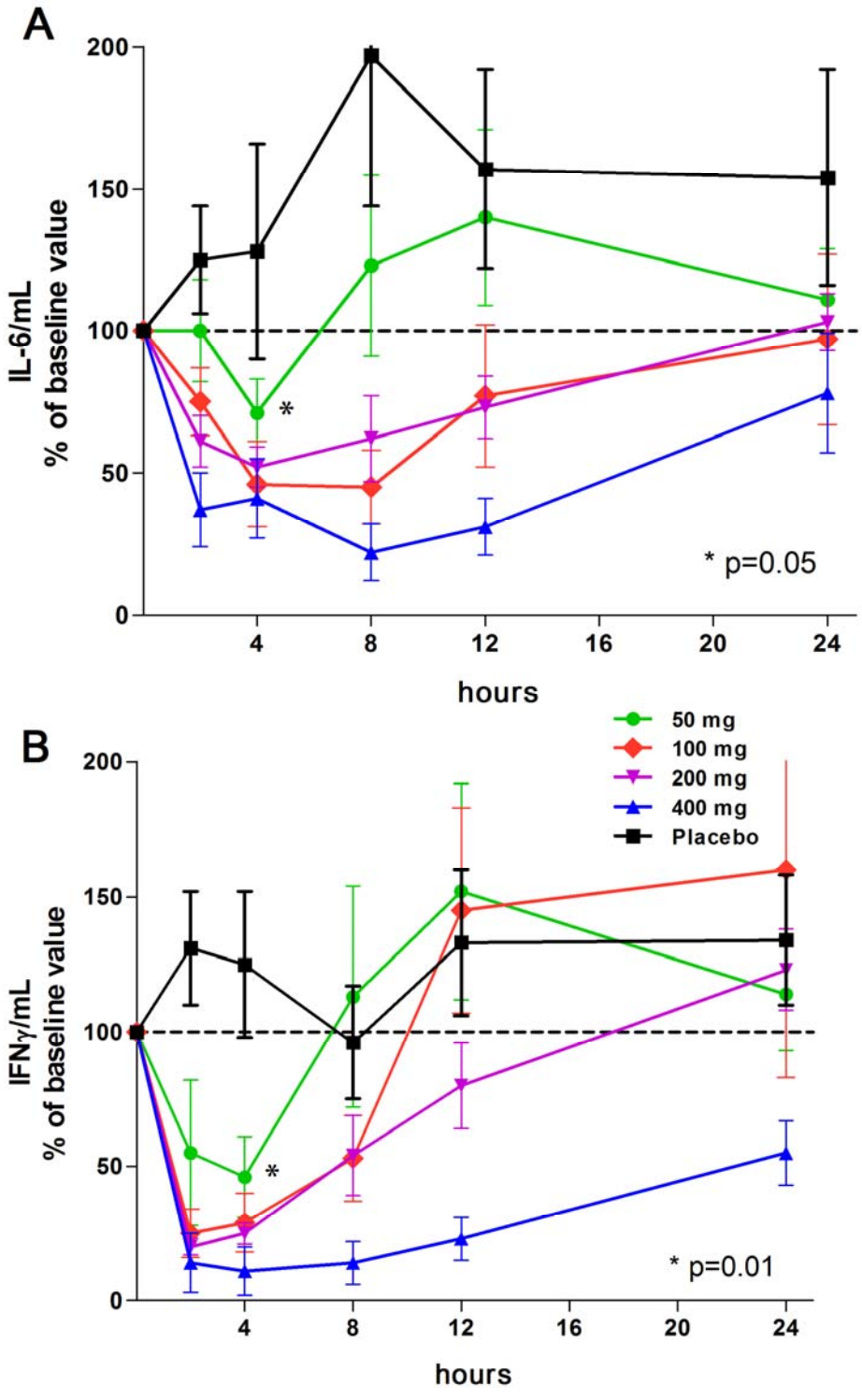

Figure 6. LPS- induced IL-6 and IFNγ in whole blood cultures. (A) Mean ± SEM of IL-6 per mL expressed as percent change of basal production just prior to the oral dosing. The hours after the dose are indicated under the horizontal axis. (B) IFN-γ in the same samples shown in A. N=9 for each group. See Methods for details.

IL-6 and IFNγ production.

In the same samples of data shown in Figure 3, we measured levels of IL-6 and IFNγ induced by LPS. These data are shown in Figures 6A and 6B. A single dose of 50mg resulted in a statistically significant (p=0.01) reduction in IFNγ of 63% compared to that of the placebo group at the 4 hour time point (Figure 6B). The reduction was of 77% at a dose of 100mg. IL-6 was also reduced at the 4 hour time point (-45% p=0.05). However, 12 hours after dosing, production of IFNγ returns and exceeds that of basal levels (not significant). A similar pattern was observed for IL-6 (Figure 6A).

The amount of IFNγ was also calculated per million lymphocytes since in the LPS model used in the present study, LPS induces IL-12 and IL-18, which in turn, induce T-cells to produce IFNγ. As shown in Figure 8, the production of IFNγ at 12 and 24 hours based on per million lymphocyte is no different from that observed per million WBC except that the elevated production 12 hours (Figure 7B) after 50 or 100mg is no longer observed per million lymphocytes (Figure 8).

No decrease in IL-1Ra.

As shown in Figures 9, there were no significant decreases at 4 hours at all doses including the Placebo. Of note, at the 50 mg dose, there was an increase production of IL-1Ra at 4 and sustained after 80 hours before returning to baseline levels after 24 hours. These samples in which IL-1Ra was determined are the same as those used to measure changes in TNFα and IL-1β production (Figures 3A and 3B). There was no statistically significant changes in IL-10 production at doses of 50 or 100mg each day (data not shown).

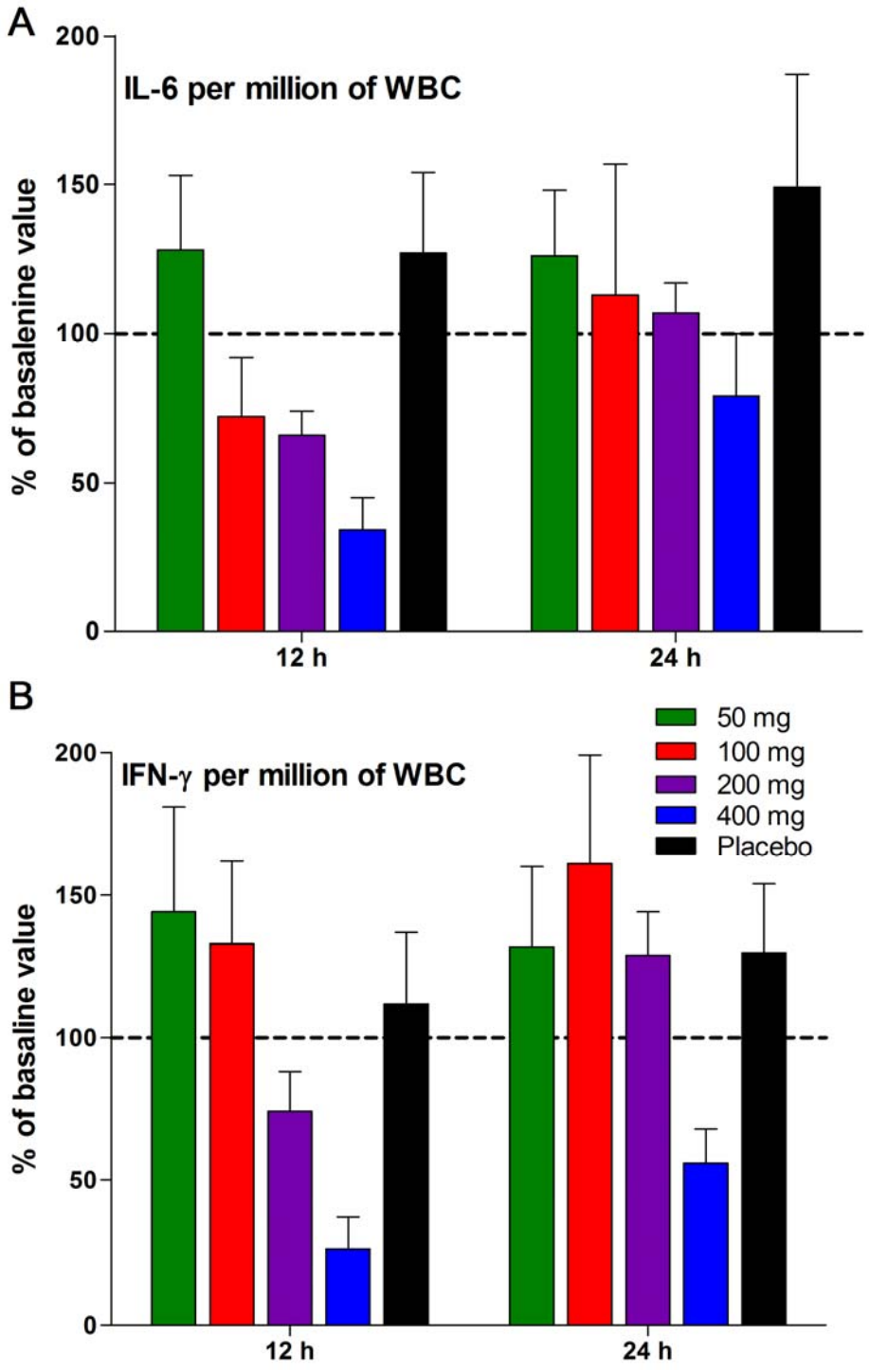

Figure 7. LPS- induced IL-6 and IFNγ in whole blood cultures. (A) Mean ± SEM of IL-6 per mL expressed as percent change of basal production just prior to the oral dosing. The hours after the dose are indicated under the horizontal axis. (B) IFNγ in the same samples shown in A. N=9 for each group.

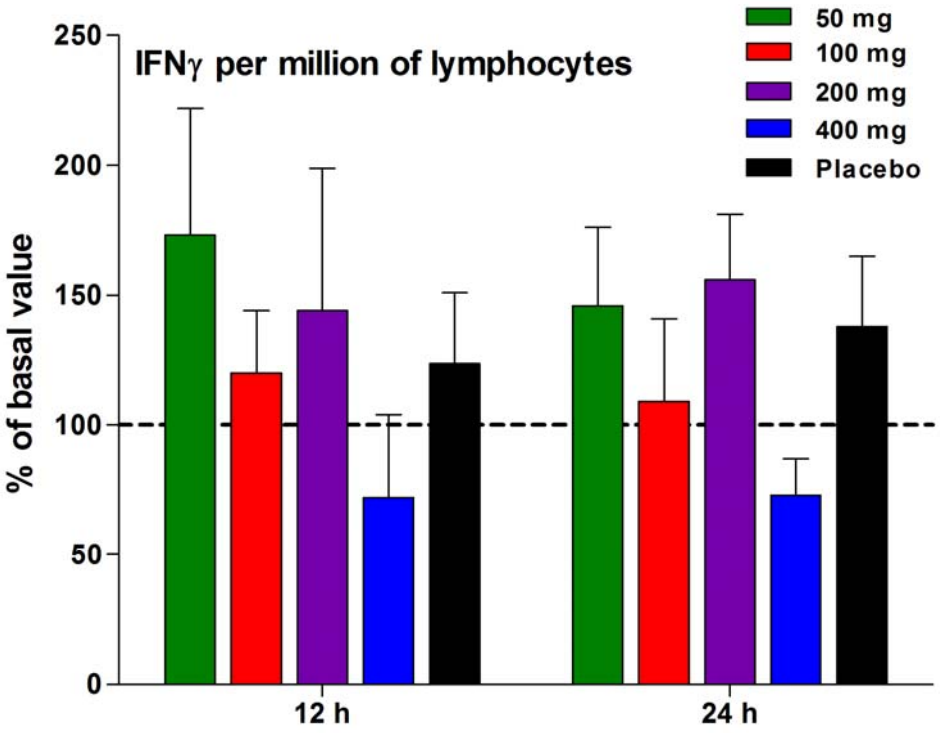

Figure 8. Production of LPS-induced IFNγ per lymphocyte. (A) Mean ±SEM of IFNγ expressed as percent change from baseline per million lymphocytes 12 and 24 hours after a single dose. Total lymphocyte counts were measured at baseline, 12 and 24 hours following oral dosing of ITF2357. N= 9 in each group.

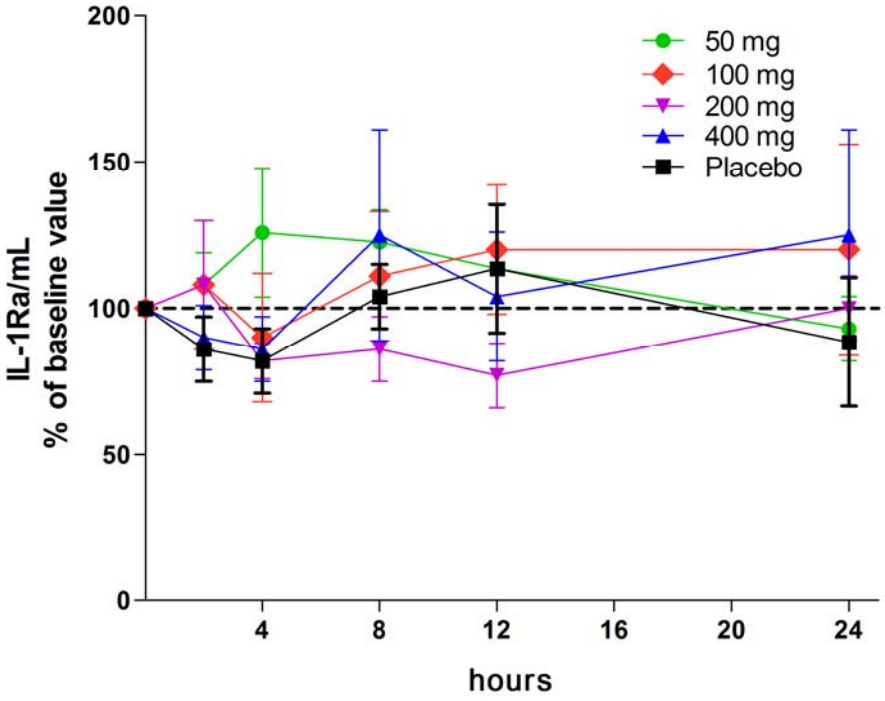

Figure 9. LPS- induced IL-1Ra in whole blood cultures. Mean ± SEM of IL-1Ra per mL expressed as percent change of basal production just prior to the oral dosing. The hours after the dose are indicated under the horizontal axis. N=9 for each group.

Effect of ITF2357 on platelet counts.

Subjects were treated with daily dosing for 7 days and platelet counts were determined. A reduction in platelets counts was observed across all ITF2357-treated groups. As shown in Figure 10, the magnitude of the decrease was clearly dose-dependent. The effect was evident from day 5 onward, reaching the lowest value on day 9, i.e. 48 hours after last dosing. On day 9 the mean platelet counts decreased by 17%, 25% and 35% in groups receiving ITF2357 50 mg, 100 mg and 200 mg each day, respectively (Figure 10). For the most part, the effect on platelets recovered on day 14 (7 days after last dosing) and was fully reversed 4 weeks after last dosing (Figure 10). The pattern of platelet decrease in the group receiving ITF2357 100 mg twice day was comparable to that observed in the 200 mg each day group. Additional hematological evaluation performed in the 100 mg twice a day group revealed that the effect was more pronounced on day 11; the trend reversed towards normality on day 14 and full recovery was observed on day 21 onwards.

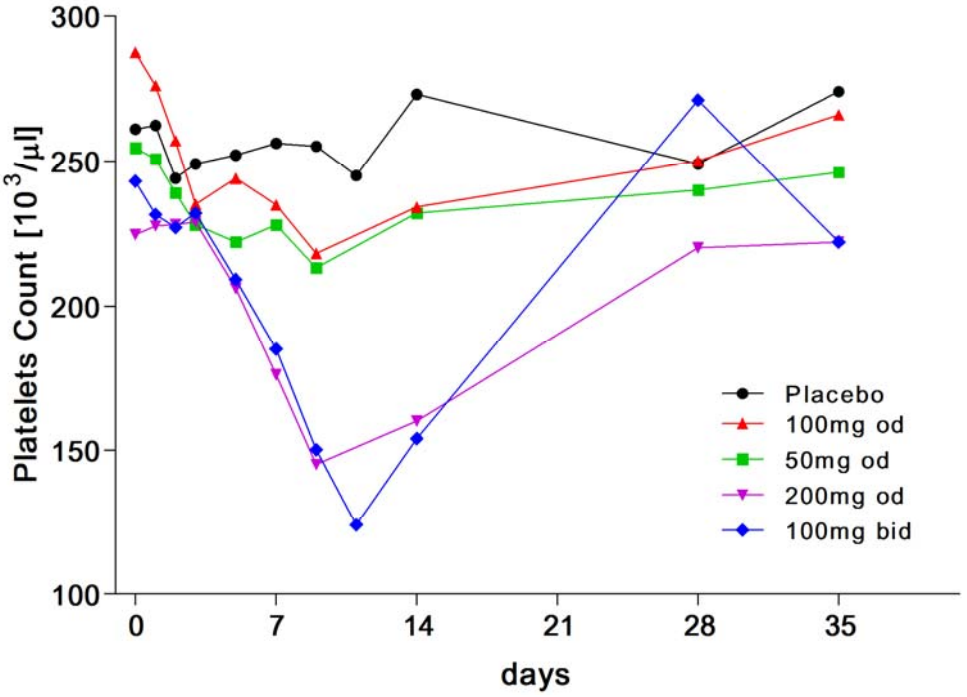

Figure 10. Platelet counts in healthy volunteers receiving 7-day oral doses of ITF2357 or placebo. Mean platelet count in ITF2357-treated groups receiving ITF2357 50 mg, 100 mg and 200 mg each day, respectively.

DISCUSSION

These studies described the first evaluation of the pharmacokinetics, safety, and tolerability of the oral histone deacetylase inhibitor ITF2357 (givinostat) in humans following the administration of increasing single and multiple doses as well as long term use. Similar to the short term use, there were no serious adverse effects of ITF2357 in the long term studies. ITF2357 was found to be orally bioavailable, eliminated slowly and displayed dose-proportional pharmacokinetics up to a total dose of 400 mg per day. No further increases in concentrations in plasma were observed with the multiple doses after 7 days, suggesting that there was no accumulation of the drug. Absence of accumulation, expressed as the ratio of the AUC on day 7 to the AUC on day 1, was also confirmed. Following the administration of all dose levels in both studies, ITF2357 was quickly metabolized with a short half-life. This implies that twice-daily dosing would be an appropriate clinical dosage regimen for the treatment of inflammatory conditions.

The results from these studies of the pharmacokinetics of ITF2357 in humans corroborate findings from preclinical investigations with animals, in which dose-related increases in exposure were observed in others species. In rats, the drug was administered consecutively up to 26 weeks. A dose of 50 mg/kg/day administered for four consecutive

weeks was determined to be safe. In other two studies (of 13 and 26 weeks) there was no adverse effect level) of the drug at 10 mg/kg/day. The data in the 13-week study in monkeys showed no adverse effects at the dose of 10 mg/kg/day.

In humans, ITF2357 was well tolerated in both the single-dose and the multiple-dose trials. The most commonly reported adverse events were mild to moderate and nonspecific and included headache, nausea, palpitations and dysuria. There also was no suggestion that ITF2357 caused cardiac adverse events, including clinically significant ECG findings or QT prolongation. Although there were clearly reduced platelets in subjects receiving the higher doses of ITF2357, the rates of adverse events did not appear to be dose-dependent over the range of doses studied (i.e., 50- to 400mg single doses and repeated for 7 days). Inhibition of histone deacetylase enzymes and reducing synthesis and release of several cytokines can explain the effect on platelets reduction. Thrombocytopenia is also reported for other HDAC inhibitors. The platelet count never dropped to critical values, even with higher doses, and consistently showed a rapid recovery after the discontinuation of the drug.

Safety is always a fundamental consideration when evaluating a drug especially when used in the treatment of diseases with no immediate danger to the patient. Although the single doses of 200 and 400 mg resulted in marked decrease in cytokines, these doses were also associated with greater lowering of platelets as well as side effects. Therefore, the likely therapeutic dosing will be 50 or 100 mg per day in twice divided doses. A dose of 1.5 mg/kg divided in two doses (or 100 mg total per day) was used to treat children Systemic Onset Juvenile Idiopathic Arthritis. In that study, ITF2357 was safe and effective after 12 weeks of therapy [197].

These studies also established that ITF2357 as an anti-inflammatory agent during in ex vivo culture of whole blood. The present work expands the finding that HDAC

inhibition reduces cytokine production, particularly cytokines relevant to autoimmune/inflammatory diseases. The implications of these studies are that inhibition of HDAC by low oral doses of ITF2357 may be effective in the treatment of certain autoimmune and autoinflammatory diseases, particularly those currently treated with TNFα or IL-1β blockade. Inhibitors of HDAC increase as well as decrease an equal number of genes in the same cells [198]. Although the general property of inhibitors of HDAC is one of increasing gene expression of pro-apoptotic genes in cancer cells [199], this property requires micromolar concentrations in vitro and comparable concentrations in vivo. At nanomolar concentrations, ITF2357 suppresses cytokines in LPS-stimulated PBMCS [149]. Indeed, the concentrations of ITF2357 in carried over into the whole blood cultures were in the low nanomolar range.

The acetylation state of the subunit p65 plays a critical role in the regulation of NFκB [200]. Such studies may explain the anti-cytokine effect of ITF2357 especially on TNFα production [149]. Thus, an inhibition of NFκB-dependent transcription by the HDAC inhibitors could possibly contribute to suppression of inflammation [201,202]. Whatever the mechanism of ITF2357 suppression of LPS-induced cytokines in the whole blood culture, the maximal reduction was consistently observed 4 hours after the 50 and 100 mg doses, and not two hours after dosing, this latter time point being the maximal plasma concentration. These data suggest that the circulating cytokine-producing cells have been affected by exposure to ITF2357 in vivo and exhibit a reduced ability to produce LPS-induced cytokine hours later. Thus, the data do not support the concept that the suppression was due to the carry over into the culture; rather suppression appears to be via a mechanism that required at least two hours following maximal exposure to ITF2357.

The reduction in secretion of LPS-induced IL-1β is not inconsequential from a clinical perspective. Indeed, severe systemic inflammatory diseases are due to greater

release of active IL-1β from cultured PBMCs in vitro compared with PBMCs from healthy subjects. These diseases include systemic onset juvenile idiopathic arthritis [203], Muckle-Wells syndrome [204,205], familial cold-induced auto-inflammatory syndrome [206], neonatal-onset multisystem inflammatory disease [207], hyper IgD syndrome [208], and Schnitzler's syndrome [209]. The increased secretion of IL-1β in these diseases is due to a single point mutation in the NOD-like receptor family gene termed cryopyrin [206] (now nucleotide-binding domain and leucine-rich repeat containing protein 3, NLRP3), which controls the activation of procaspase-1 [205]. Alternatively, ITF2357 may affect the ability to secrete IL-1β via the specialized secretory lysosomal pathway and activation of phosphatidylcholine-specific phospholipase C [210]. Another pathway in the secretion of IL-1β is the P2x7 receptor [211,212], which may also be affected by mechanisms of hyperacetylation.

Although this study was a Phase I trial on the safety and tolerability of ITF2357, the pharmacokinetic data are helpful in future trials, particularly using the twice daily dosing schedules. The transient reduction in inducible cytokines at 50 and 100mg in whole blood cultures can be viewed as both a mechanism of action at clinically achievable doses as well as a safety margin. Cytokine blockade such as the widespread use of anti-TNFα monoclonal antibodies are associated with increased opportunistic infections and possibly lymphoma. There appears to be a greater incidence of infections associated with long lasting monoclonal antibodies such as infliximab and adalimumab compared to etanercept [213]. One explanation for these differences affecting host responses to infection is the differences in half-lives of these agents. Infliximab has the longest duration in the circulation (weeks) whereas etanercept has the shortest (days). Therefore, the short duration of cytokine inhibition may provide a greater parameter of safety compared to long-acting cytokine inhibitors.

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