Phenotypic Screen Identifies JAK2 as a Major Regulator of FAT10 Expression

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Abstract

FAT10 is a ubiquitin-like protein known to target proteins for proteasomal degradation in a similar manner to ubiquitin. It is highly upregulated upon pro-inflammatory cytokines namely TNFα, IFNγ and IL6, and is also found to be highly expressed in different cancer types. Accumulating evidence suggests FAT10 is involved in cancer development and may even have a pro-tumorigenic role. However, its biological role is still not clear and regulatory pathways controlling its expression are still lacking. Thus, there is a need to better understand the factors involved in FAT10 regulation, especially under inflammatory conditions which characterize the cancerous environment where FAT10 is expressed. To do so, we set a phenotypic screen using a library of compounds with annotated cellular targets. We identify AZ960, a potent JAK2 inhibitor, which significantly downregulates FAT10 expression under pro-inflammatory cytokines induction, in an NFκB-independent manner. This points out JAK2 as a major regulator of FAT10 expression. We further corroborate previous reports of STAT1 and STAT3 mediating regulation of FAT10 expression and suggest STAT5 as an additional potential mediator. Overall, we elucidated the pathway regulating FAT10 transcription and discovered a potential tool compound to chemically downregulate FAT10 expression, and further study its biology.

Introduction

FAT10, is an 18-kDa ubiquitin-like protein (UBL). It consists of two tandem ubiquitin-like domains, each with ~30% identity to ubiquitin [1]; also known as diubiquitin or ubiquitin D (UBD). FAT10 was originally identified by genomic sequencing of the human major histocompatibility complex (MHC) locus, hence its name- HLA-**F** **A**djacent **t**ranscript number **10** [2,3]. Since it is mapped to MHC class I genomic region it is thought to have an immune function, however its biological role is not yet clear.

Like other UBLs, FAT10 covalently modifies target proteins. Interestingly, FAT10 is the only UBL, other than ubiquitin, known to target proteins for proteasomal degradation [4]. The conjugation of FAT10 to its substrates is presumed to be performed by a three-step enzymatic cascade, in a similar manner to ubiquitin, with UBA6 and UBE2Z as its E1 and E2 enzymes respectively, acting in a bi-specific manner for both FAT10 and ubiquitin[5–8]. No E3 ligase has been shown thus far to be FAT10 specific.

Unlike ubiquitin, that is removed from its substrates by de-ubiquitinating enzymes (DUBs) to be reused, currently there is no known DUB for FAT10 and it is not recycled but rather degraded along with its substrate. In accordance, the half-life of FAT10 is ~1h [9], in comparison to ~9h of ubiquitin [10,11].

FAT10 exists only in vertebrates, linking it to a late evolutionary development, correlated with the adaptive immune system. FAT10 is mainly expressed in immune cells and tissues [1,12–14]; However, its expression may be induced in non-immune cells by pro-inflammatory cytokines such as IFNγ, TNFα and IL6 [15,16]. Accordingly, FAT10 is found to be highly expressed in many cancer types [17–19].

In recent years there have been accumulating evidence suggesting that FAT10 is up-regulated in various epithelial cancers and that it may be involved in cancer development. Over-expression of FAT10 was shown to confer malignant properties to non-tumorigenic and tumorigenic cells *in vitro* [20]. Further, cells over expressing FAT10 promoted massive tumor formation when injected to mice, in comparison to control injected cells [16,20]. Several links of FAT10 to cancer-related proteins were made, for example, FAT10 was suggested to inhibit p53 [16], which therefore may promote cell proliferation. FAT10 was also reported to stabilize β-catenin, a central factor in Wnt signaling, thus activating the pathway leading to known tumorigenic properties[21]. Finally, FAT10 was found to interact with MAD2, a mitotic checkpoint protein, and to have a role in control of mitotic regulation [22,23].

Despite of its involvement in cancer, there are still gaps regarding what mediates the up-regulation of FAT10 expression in the tumorigenic inflammatory environment. Nevertheless, Choi et al. extensity described the binding sites of the transcription factors STAT3 and NF-kB on FAT10 promotor, activating gene expression in a synergistic manner upon IL-6 and IFNγ treatment[16]. Furthermore, Gao et al. have presented a compound, Silibinin, that downregulates FAT10 expression under inflammatory conditions and leads to smaller tumors formed by subcutaneous injection of cancer cells to nude mice[24]. Silibinin is a natural compound used to treat liver and gallbladder disorders[25] and was shown to demonstrate anti-cancer activities. However, it is suggested to work through different pathways including MAPK, NFκB and STATs signaling[26–28], thus, may support the finding that STAT3 and NF-kB control FAT10 transcription but due to its broad range of targets, might not suggest of a specific pathway of regulation. Moreover, for therapeutic purposes, Silibinin displays relatively poor EC_{50} (~50µM) for FAT10 inhibition, at these high doses, many off-targets are likely hit as well.

During the past two decades, the paradigm for cancer treatment has evolved dramatically from relatively nonspecific cytotoxic agents to selective, mechanism-based therapeutics. Although our understanding of cancer pathogenesis has tremendously evolved, resistance to chemotherapy and molecularly targeted therapies is still a major problem in current cancer research and clinical practice. Thus, deciphering mechanistic regulation of FAT10, along with identifying a chemical tool to manipulate its expression could serve both for research and therapeutic purposes applying for different cancer types characterized by high FAT10 expression.

Here, screening a library of compounds with validated activity and annotated cellular targets, we identified a small molecule, AZ960, known to inhibit Janus Kinase 2 (JAK2), that also dramatically downregulates FAT10 expression upon pro-inflammatory cytokine induction. Further, we elucidated the downstream effectors of JAK2 that mediate FAT10 transcription, validated STAT1 and STAT3 as crucial regulators of FAT10 and identified STAT5 as a possible regulator of FAT10 independently of NFκB.

Material and methods

Cloning, plasmids and transfection

FAT10 promoter region was extracted from genomic DNA of HEK293 and cloned into pLEX vector. All cloning procedures were done using restriction-free (RF) method. Inducible shRNA knock-down HEK293 cell line was generated by lentiviral infection using TRIPZ inducible shRNA system (Dharmacon).

Cell culture

HEK 293 and A549 cells were grown in DMEM supplemented with 10% fetal bovine serum, CaCo-2 cells were grown in DMEM supplemented with 20% fetal bovine serum and Caki-1 cells were grown in RPMI supplemented with 10% fetal bovine serum. Media of all cell line was also supplemented with 1% Penicillin/streptomycin and L-glutamine (2mmole/liter) (Biological industries), grown at 37°C with 5% CO_2. NFκB reporter A375 cell line was kindly provided by Ravid Strausmann.

Cytokines and compounds

Cells were treated with cytokines at indicated time points using TNFα (400 U/ml, 20ng/ml), IFNγ (200 U/ml, 10ng/ml) and IL6 (100 U/ml, 10ng/ml) (Peprotech). AZ960 was purchased from ArkPharm (AK471620) and added at indicated time points using 250nM (or 1µM where indicated specifically).

Real time PCR

RNA was extracted from cells using Direct-Zol kit (Zymo Research) and cDNA was synthesized using High-Capacity cDNA kit (Thermo Fisher Scientific). Quantitative real-time PCR was performed using SYBR Green (Applied Biosystems) with primers as outlined:

Gene	F 5' - 3'	R 5' - 3'
FAT10	CCGTTCCGAGGAATGGGATTT	GCCATAAGATGAGAGGCTTCTCC
Actin	CATGTACGTTGCTATCCAGGC	CTCCTTAATGTCACGCACGAT

GAPDH	CAACGGATTTGGTCGTATTG	GATGACAAGCTTCCCGTTCT
GFP	AAGCTGACCCTGAAGTTCATCTGC	CTTGTAGTTGCCGTCGTCCTTGAA
NFKBIA	ATGCTCAGGAGCCCTGTAATG	CCCCACACTTCAACAGGAGT
IRF1	AAGTCCAGCCGAGATGCTAA	TAGCTGCTGTGGTCATCAGG
TNFAIP3	GCGTTCAGGACACAGACTTG	TTCATCATTCCAGTTCCGAGTATC
IL8	TCTGGCAACCCTAGTCTGCT	AAACCAAGGCACAGTGGAAC
TNF	TGCTGCAGGACTTGAGAAGA	GAGGAAGGCCTAAGGTCCAC
JAK2	TCTGGGGAGTATGTTGCAGAA	AGACATGGTTGGGTGGATACC

High-throughput bioactive library screen

Libraries screened: LOPAC1289 (Sigma), Spectrum Collection (MicroSource), Natural Product Library (Selleck Chemicals), Bioactive Screening Libraries (Selleck Chemicals) and Prestwick Chemical Library (Prestwick Chemicals). Screen was performed in 1536 well plate format, cells were incubated with compounds (6.6 µM) 2h prior to induction with cytokines. 24h later plates were imaged using laser scanning imaging cytometer (Acumen). 1h prior to imaging, Hoechst 33342 (2.5 µg/ml) was added to cells for live nuclear staining. Compounds that were evaluated in a dose response manner were tested for viability using CellTiter-Glo luminescent assay (Promega).

Western Blot analysis and Antibodies

Cells were lysed using NP-40 lysis buffer (50mM Tris pH7.5, 150 mMNaCl, 5mM EDTA pH8, 0.5% NP-40 with protease and phosphatase inhibitors). Total lysates were separated using 9% or 12% SDS-polyacrylamide gels. Antibodies used: Rabbit anti FAT10 (Sc-67293), Mouse anti STAT1 (Sc-464), mouse anti pSTAT1 (Sc-8394), Rabbit anti STAT3 (Cst-9132), Rabbit anti pSTAT3 (Cst-9145) and Rabbit anti pSTAT5 (Cst-9351).

Results

To identify changes in FAT10 expression we generated a HEK293 based reporter cell line expressing GFP downstream to FAT10 promoter (Figure 1A). Under basal conditions HEK293 cells do not express FAT10 (Figure 1B). This allowed for a wide dynamic range of GFP transcription in response to activation of the FAT10 promoter by the stimulatory cytokines TNFα and IFNγ (indicated TI). 24h induction with these two cytokines induced a robust and measurable GFP readout. FAT10 mRNA levels upon induction displayed similar kinetics for both endogenous and exogenous FAT10 promoter activity (Figure 1C,D).

Using this reporter cell line, we screened a library of 7065 bio-active small molecules with annotated biological and pharmacological activities. Cells were pre-incubated with the compounds for two hours, then stimulated with TNFα and IFNγ for 24 hours and imaged using a laser scanning imaging cytometer (Figure 1E). Since we were interested in downregulation of FAT10 promoter activity, we had to exclude any toxicity effects that may lead to decreased fluorescent signal. To assess cell viability, we performed live cells staining using Hoechst, one hour before imaging (Figure 1F).

The screen yielded 204 hits with greater than 60% signal reduction at 6.6μM, and viability greater than 60% (Figure 2A). Further manual analysis excluded 79 of the compounds due to known annotated toxic effects. The remaining hit compounds were evaluated based on their annotated cellular targets (Figure 2B). Among the most targeted protein families were HDACs, HSP90, CDKs, JAKs and ErbB members. Pathway analysis of the protein targets of these compounds, revealed several key signaling pathways that may be involved in the regulation of FAT10. The JAK/STAT signaling pathway, being the one with the highest fold enrichment score (Figure 2C, Table S1). Not surprisingly, some of the hits are inhibitors of STAT3 and NFκB pathway components, which were previously reported to positively regulate FAT10 expression [16].

Positive hits were further triaged based on potency of signal inhibition as well as their target protein identities, to maximize the variety of possible targets with a putative role in FAT10 regulation. 68 compounds were evaluated in a full dose response manner. This enabled to exclude compounds that effected cell viability in any of the tested concentrations. Of those, 21 compounds had an EC_{50} < 1μM (Figure 2D, Table S2). Seeing that FAT10 inhibition was evaluated upon pro-inflammatory cytokines induction, in which NFκB is a general master regulator, we wanted to avoid non-

selective transcription inhibition mediated via NFκB. Thus, we counter-screened against an NFκB reporter cell line to exclude compounds that significantly inhibit this pathway (Figure S1).

Eventually, we selected five compounds for further validation (Figure 2E). These compounds were then tested for their effect on endogenous FAT10 mRNA levels using qPCR analysis (Figure 3A). All five compounds exhibited an inhibitory effect on FAT10 mRNA expression, of those, two compounds (Afatinib and XMD8-92) showed only about 50% reduction while the other three (AZ960, Panobinostat and JQ1) resulted in complete downregulation of expression.

Since cancer-related inflammation has been recently described in many types of epithelial cancers[31], we hypothesized that FAT10 expression may be naturally upregulated in the cancerous tissue, as was previously reported[17,32]. Therefore, we wished to evaluate the identified compounds under inflammatory conditions to test whether FAT10 may be downregulated when it was already highly expressed. To that end, cells were treated with TNFα and IFNγ for six hours prior to administration of the compounds (Figure 3B). Again, we observed the same trend with regards to downregulation of FAT10 expression.

In order to better understand the pathway by which the compounds exert their inhibitory effect on FAT10 transcription, we analyzed the mRNA expression of NFKBIA (IκB), a representative downstream target genes of TNFα NFκB-mediated response, and IRF1 as representative target gene of IFNγ response (Figure S2A,B). All five compounds inhibited at least one of the genes to different extent, suggesting that TNFα/IFNγ-mediated pathways are required for FAT10 with no indication of another independent pathway controlling this process.

Considering that two of the compounds tested are inhibitors of major transcription regulators – HDACs (Panobinostat) and BRD4 (JQ1), we decided at this point to exclude them from further analysis as they may significantly alter various cellular transcriptional programs.

We next asked whether the remaining 3 compounds are able to cause downregulation of FAT10 even after prolonged pro-inflammatory conditions, mimicking the physiological state of the cancer-related inflammation. Thus, cells were induced with cytokines for 24 hours, then compounds were added for another 24 hours and harvested for qPCR analysis. Before adding the compounds, the medium containing the cytokines was either replaced (change media = C.M.) with cytokine-free fresh medium (Figure 3C) or the compounds were added without replacing the medium

(Figure 3D). Under these condition, only one compound managed to significantly and drastically reduce FAT10 mRNA levels: AZ960, a low-nM JAK2 inhibitor. Further qPCR analysis confirmed that the compound inhibits the IFNγ-mediated transcriptional response, where JAK family members play a key role (Figure S3A,B).

AZ960 inhibits FAT10 expression with an average (n=3) EC_{50} of 41 ± 7 nM (Figure 4A). To avoid off-targets follow-up experiments were performed with 250nM compound. It should be noted that in a kinase selectivity profile done by Gozgit et al. 100nM of AZ960 selectively inhibit JAK2 over other kinases[33]. To confirm AZ960 exerts its effect on FAT10 in an NFκB-independent manner we analyzed mRNA expression of four different NFκB target genes and found that indeed none is effected by AZ960 (Figure S4).

To further verify that downregulation of FAT10 using AZ960 is mediated through JAK2 inhibition and not any off-targets, we established an inducible shRNA JAK2 HEK293 cells line which enables to establish a JAK2 knock-down using Doxycycline (Dox). Cells were treated with cytokines for 24 hours prior to Dox application (Figure 4B). 72 hours after adding Dox, total 96h after cytokines treatment, we established 44% knock-down of JAK2 mRNA levels and observed a 73% reduction in FAT10 mRNA expression compared to induced cells transfected with control vector.

In order to establish that the effect of JAK2 inhibition on FAT10 expression is not cell type specific we examined three additional cell lines A549, Caki-1 and Caco-2 representing different tissue origins- lung, renal and colon respectively (Figure 4C). All cell lines responded similarly to the compound with 82%, 73% and 71% reduction of FAT10 mRNA levels respectively. Since two of the examined cell lines, Caki-1 and Caco-2, express relatively high basal levels of FAT10 (Figure S5) we wondered whether JAK2 inhibition through AZ960 could downregulate FAT10 expression independently of TNFα and IFNγ induction (Figure S6). No reduction of FAT10 expression was seen upon AZ960 treatment in the absence of cytokine stimulation in either of the cell lines. This suggests that JAK2 is a context specific FAT10 regulator that mediates predominantly FAT10 upregulation in response to TNFα/IFNγ induction.

IL6 is also known to upregulate FAT10 in combination with TNFα. Like IFNγ, IL6 also activates JAK-STAT signaling pathway. We compared the extent of reduction in FAT10 expression upon AZ960 treatment following induction of 24 hours using either combination of IFNγ and TNFα or IL6 and TNFα (Figure 4D). AZ960 showed inhibitory activity on FAT10 also using IL6 and TNFα induction, albeit reduced inhibition of 68% compared to 93% upon IFNγ and TNFα of FAT10 transcripts. This suggest that JAK2

is a general regulator of FAT10 expression, a main mediator in the case of IL6 and TNFα, and almost exclusive in the case of lFNγ and TNFα induction.

Upon activation, JAK2 phosphorylates downstream STAT proteins which then dimerize and translocate to the nucleus as transcription factors. JAK2 is known to phosphorylate STAT1, STAT3 and STAT5[34–36]. STAT3 was reported as a FAT10 regulator as well as STAT1 in a compensatory manner[16]. Therefore, we set out to determine which of the STAT proteins mediate the effect of JAK2 on FAT10 transcription using western blot analysis (Figure 4E). AZ960 treatment upon cytokine induction led to downregulation of total STAT1 levels while total STAT3 levels were not effected by the compound. Phosphorylation of all STAT proteins was inhibited upon treatment with the compounds. This suggest that STAT5 might also serve as a transcription factor for FAT10 expression, much like STAT1 and STAT3. However, this requires further examination. Total STAT5 levels could not be detected due to technical issues.

In summary, we have identified a compound, AZ960, known as a selective JAK2 inhibitor, that under pro-inflammatory cytokines significantly downregulates FAT10 expression. This points to JAK2 as a key mediator of FAT10 regulation in a process relevant to different cell types from various epithelial origin. We further elucidate the molecular mechanism of FAT10 transcriptional activation by validating STAT3 and STAT1 as the transcription factors controlling its expression, in an NF-kB independent manner, and suggesting in addition STAT5 as a possible regulator of FAT10.

Discussion

Using a phenotypic screen, we have identified a compound that completely abrogates FAT10 expression under pro-inflammatory conditions. This compound, AZ960, is a potent JAK2 inhibitor, which brings to the fore the major role of JAK2 in FAT10 regulation. We have shown that the effect of AZ960 is not cell type specific and further elucidate the downstream mediators STAT1, STAT3 and possibly STAT5, controlling FAT10 expression.

The initial screen for FAT10 inhibitors yielded some anticipated results from several aspects. On the one hand, seeing that our aim was to identify regulators of FAT10 transcription, many of the compounds that came up as hits were inhibitors of general transcriptional regulators such as members of HDAC and BET protein families. On the other hand, as FAT10 was previously reported to be regulated by NFκB and STAT3[16], we also identified inhibitors of these pathways, thus confirming the validity of our results.

Nevertheless, all the identified hits could possibly reflect assay limitations which allows detection of downregulation of FAT10 only under pro-inflammatory conditions. With that in mind, in the process of hits elimination, we have tried to exclude compounds which effect NFκB pathway seeing that NFκB serves as a major player in a variety of inflammatory conditions. This led to eventually identifying AZ960, a known JAK2 inhibitor, as a negative regulator of FAT10 expression under pro-inflammatory cytokines induction.

JAK2 has an essential role in hematopoietic development[39]. In the clinic, AZ960, as other JAK2 inhibitors are mainly used in cases of myeloproliferative neoplasms (MPNs) which include a group of hematopoietic disorders characterized by aberrant production of myeloid cells [40–42]. Many MPN patients carry a mutant form of JAK2, V617F [43], characterized by a gain-of-function, causing constitutive activation of kinase activity. Cells expressing this mutant exhibit un-regulated proliferation and growth rates.

Considering FAT10 was shown to be mainly upregulated in non-hematopoietic neoplasms such as colon, liver, kidney etc. there was no obvious link between JAK2 and FAT10 in cancer. However, a previously suggested link between JAK2 and FAT10 was reported by Choi et al. using AG490, a JAK2 inhibitor, where 12 hours of pre-treatment with 100μM AG490 led to inhibition of FAT10 expression upon three hours of IL6 induction[16]. Here, we show AZ960 to almost completely downregulate FAT10

expression after 24 hours of TNFα and IFNγ induction using only 250nM compound concentration.

Choi et al. also identified STAT3 as a major positive regulator of FAT10 with STAT1 working in its absence. This was corroborated by our study and shown to be JAK2 dependent and NFκB independent. Seeing that STAT5 is also known to be phosphorylated by JAK2, we indeed observed downregulation of STAT5 phosphorylated form upon AZ960 treatment, which may suggest STAT5 also has a role in FAT10 regulation.

Combining the evidence allows to draw a more complete picture of the pathway controlling FAT10 expression, depicting JAK2 activation upon binding of either IFNγ or IL6, then STAT1/3 recruitment and phosphorylation which leads to its dimerization and consequent translocation to the nucleus to promote FAT10 transcription together with NFκB (Figure 5). Of note, seeing that IFNγ and IL6 also activate other JAK family members like JAK1 and TYK2, we cannot rule out that an additional JAK member protein is involved in FAT10 regulation, and indeed in the initial screen there was also JAK1 inhibitor that came up as a possible hit but was later excluded due to more stringent criteria for FAT10 inhibition.

While we focused here on an established JAK2 inhibitor, which works through inhibiting IFNγ/IL6 downstream responses, the question remains whether other regulatory pathways exist for FAT10 besides the mentioned IFNγ/IL6 and NFκB pathways. As stated above, the phenotypic screening was limited to begin with due to the requirement of FAT10 expression only under pro-inflammatory conditions, thus resulting in compounds mainly inhibiting inflammatory pathway components. However, this is not necessarily an artifact of the assay, but could actually represent true FAT10 biology. Nevertheless, besides the reported regulators of FAT10, NFκB and STAT3, the primary screen results suggest other possible regulators which might take part in FAT10 regulation, though these probably are not the major ones. Since we were looking for the most efficient inhibitor, excluding in the process less potent compounds, we possibly missed other components regulating FAT10 to some extent. One could further investigate these compounds for novel players in this biological process.

We hypothesize that cancer cells being in an inflammatory environment in the body develop an addiction to the high levels of FAT10, thus depriving FAT10 in this context may lead to attenuation of the tumorigenic processes or even elimination. Elucidating JAK2 as a regulatory pivot in the pathway leading to FAT10 expression might suggest using JAK2 inhibition to target FAT10. In order to address any therapeutic potential,

future experiments evaluating the anti-cancer activity of AZ960 on FAT10 dependent cancer models should be conducted both *in-vitro* and *in-vivo*. Fortunately, seeing that AZ960 is an advanced drug candidate, pharmacological properties of the compound are not an issue. Furthermore, a reasonable approach is to use an approved JAK2 inhibitor such as, Ruxolitinib, which was already proven effective in the clinics and repurposing it for FAT10 over-expressing cancer cases. Of course, using an indirect inhibitor of FAT10 such as this could raise concerns regarding potential side effects of JAK2 inhibition rather than FAT10, but when time comes this could be properly controlled.

In summary, we explored JAK2 as a major regulator of FAT10 expression upon pro-inflammatory cytokines induction. Our work identified AZ960 as a potential tool to chemically downregulate FAT10 levels using sub-µM concentrations. This inhibitor may be used for research purposes at the moment while its anti-cancer activity mediated by FAT10 inhibition needs to be further examined as a potential cancer therapy.

Figures

<u>Figure 1–</u> ***Establishment of phenotypic screen to identify inhibitor of FAT10 expression. (A)*** Reporter cell line design. ***(B)*** GFP reporter protein is expressed upon TNFα and IFNγ treatment. ***(C)***, ***(D)*** Similar kinetics observed for endogenous and exogenous FAT10 promoter. qPCR analysis of GFP and FAT10 mRNA levels in both WT HEK293 and reporter cell line treated with TNFα and IFNγ over time. ***(E)*** Schematic representation of screen protocol. ***(F)*** Representative images of screen readout. Signal intensity of GFP objects was normalized to induced (TNFα and IFNγ) and non-induced DMSO control cells. Number of Hoechst stained objected was normalized to live DMSO controls and dead cells controls treated with 10% DMSO.

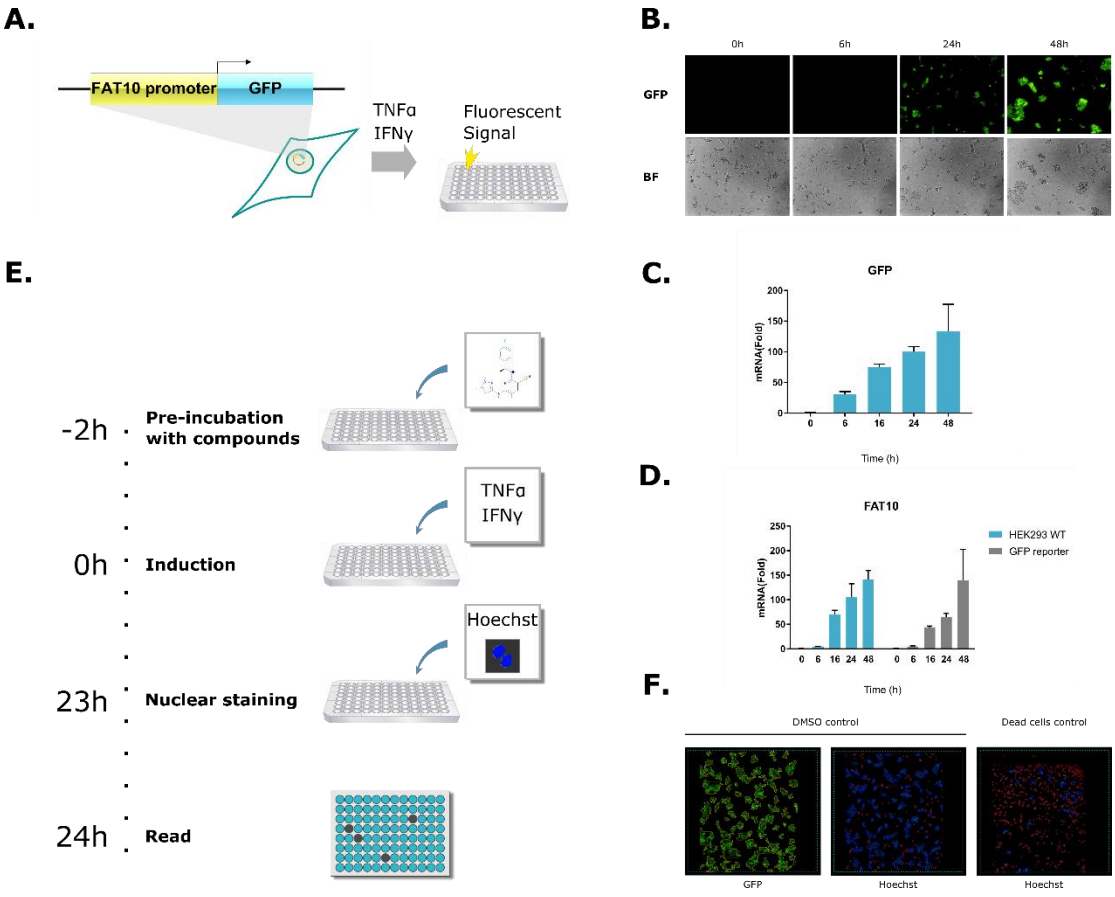
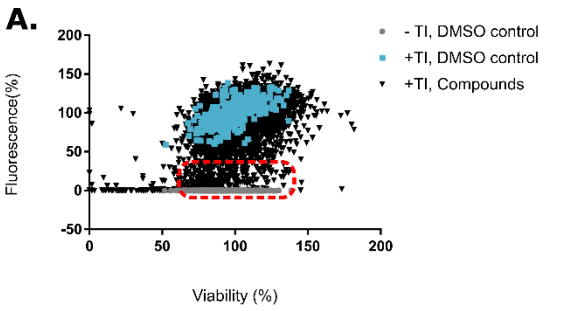
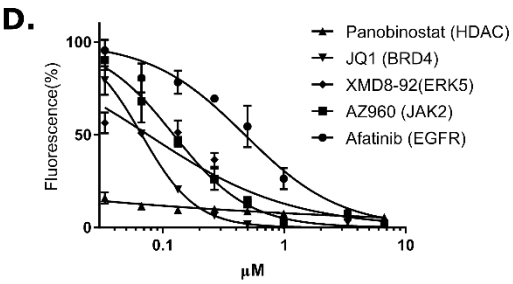
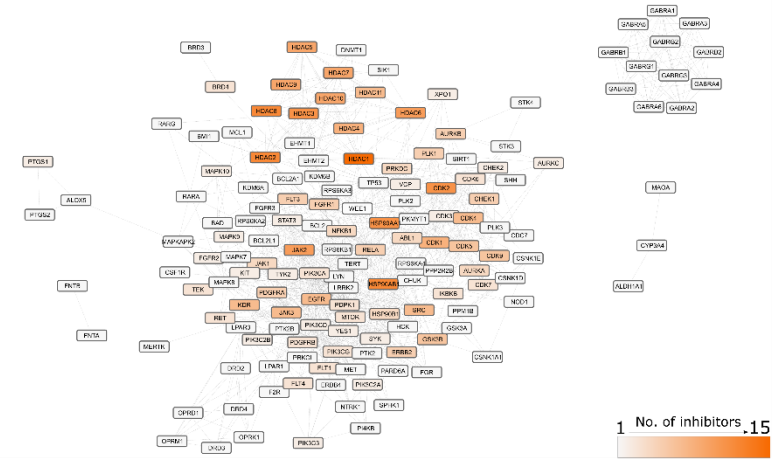


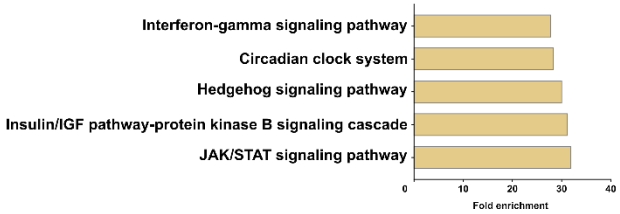
Figure 2 – *Characterization of screen results.* *(A)* Compound library screened sorted based on percentage of fluorescent signal and cell viability. Red rectangle represents selected hits. ***(B)*** Target characterization of hit compounds. Darker color represents multiple inhibitors. ***(C)*** Hit compounds target proteins involved in different signaling pathways. Top 5 enriched pathways presented. ***(D)*** EC_{50} of representative compounds. Cellular targets of the compounds denoted in parentheses. ***(E)*** Schematic representation of hits processing.





Compound	EC50
Panobinostat (HDAC)	<0.03
JQ1 (BRD4)	0.067
XMD8-92 (ERK5)	0.075
AZ960 (JAK2)	0.123
Afatinib (EGFR)	0.473





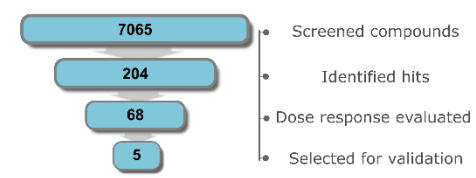


Figure 3 –***qPCR analysis of FAT10 mRNA levels identifies AZ960 as lead compound in downregulation of FAT10 expression.*** Afat. = Afatinib, Pano. = Panobinostat and XMD. = XMD8-92. ***(A)*** HEK293 cells were pre-incubated for 2h with compounds, then induced with TNFα and IFNγ for 24h and harvested for qPCR analysis of FAT10 mRNA levels. ***(B)*** HEK293 cells were induced with TNFα and IFNγ for 6h, then treated with compounds for additional 18h for a total of 24h induction with cytokines, then harvested for qPCR analysis of FAT10 mRNA levels. ***(C)*** HEK293 cells were induced with TNFα and IFNγ for 24h, then medium was replaced to cytokine-free medium and cells were treated with compounds for additional 24h, then harvested for qPCR analysis of FAT10 mRNA levels. Changed Medium = C.M. ***(D)*** HEK293 cells were induced with TNFα and IFNγ for 24h, then treated with compounds for additional 24h without replacing the medium, then harvested for qPCR analysis of FAT10 mRNA levels.

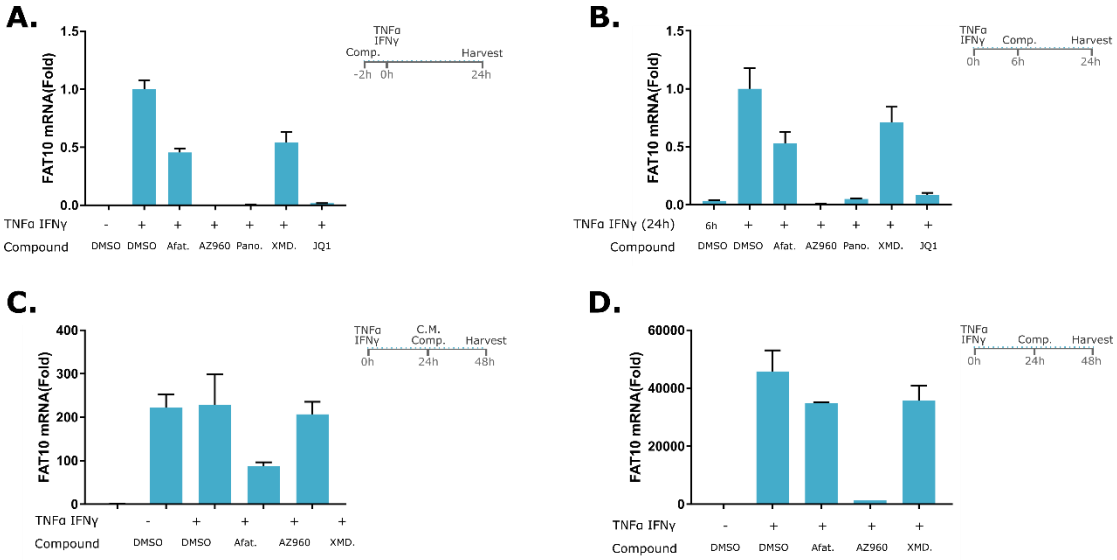


Figure 4 – ***Characterization of AZ960 and its effect on FAT10 expression upon induction. (A)*** AZ960 exhibits sub-µM EC_{50} for downregulation of FAT10 mRNA levels upon induction. HEK293 cells were induced with TNFα and IFNγ for 24h, then treated with AZ960 in indicated concentrations for additional 24h and harvested for qPCR analysis. ***(B)*** Genetically inhibiting JAK2 using shRNA cause downregulation of FAT10 upon pro-inflammatory cytokine induction. HEK293 cells were induced with TNFα and IFNγ for 24h, then transfected with shRNA vector and harvested 24h after transfection for qPCR analysis. ***(C)*** The effect of AZ960 on FAT10 expression is not cell type specific found also in cancer cells with lung, renal and colon origins. Cells were induced with TNFα and IFNγ for 24h, then treated with AZ960 (250nM) for additional 24h and harvested for qPCR analysis of FAT10 mRNA levels. ***(D)*** AZ960 also downregulates FAT10 expression upon IL6-TNFα induction, albeit to a lesser extent. HEK293 cells were induced with TNFα, IFNγ and IL6 as indicated for 24h, then treated with AZ960 (250nM) for additional 24h and harvested for qPCR analysis of FAT10 mRNA levels. ***(E)*** AZ960 cause reduced phosphorylation of STAT1, STAT3 and STAT5 and downregulation of total STAT1 protein levels upon induction with TNFα and IFNγ. HEK293 cells were induced with TNFα and IFNγ for 24h, then treated with AZ960 (250nM) for additional 24h and harvested for western blot analysis.

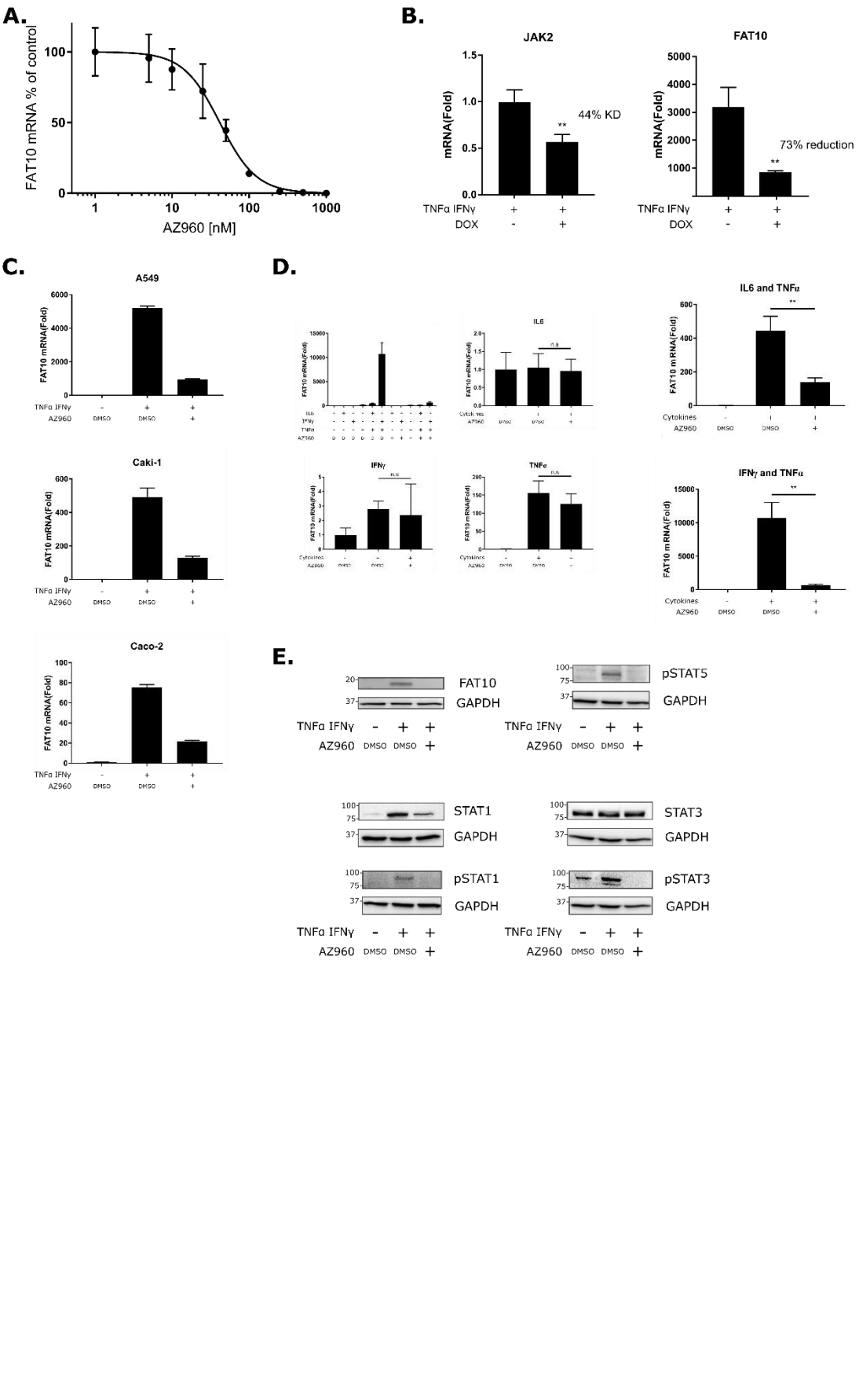
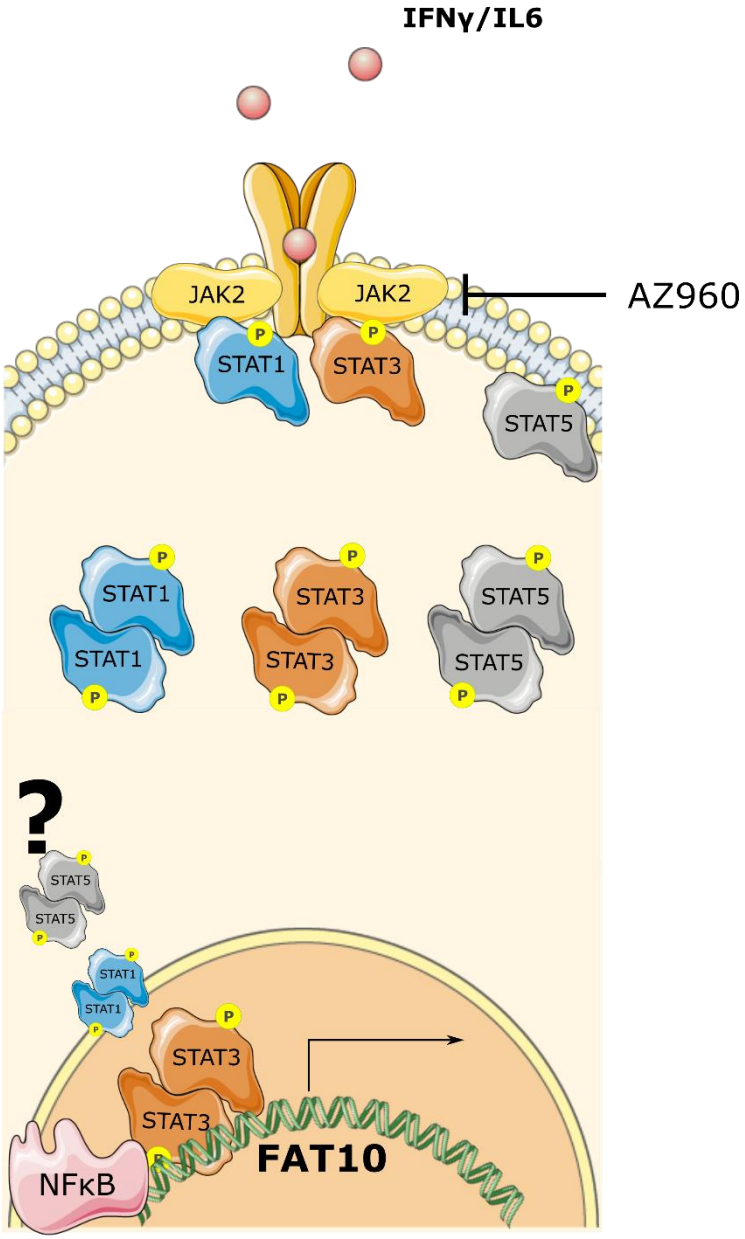

A.
FAT10 mRNA % of control
AZ960 [nM]
B.
JAK2
mRNA(Fold)
44% KD
FAT10
73% reduction
TNFα IFNγ
DOX
C.
A549
Caki-1
Caco-2
FAT10 mRNA(Fold)
AZ960
DMSO
D.
IL6
IL6 and TNFα
IFNγ
TNFα
IFNγ and TNFα
Cytokines
E.
FAT10
GAPDH
pSTAT5
STAT1
STAT3
pSTAT1
pSTAT3

Figure 5 – ***Model of FAT10 transcription activation upon IL6/IFNγ***



Supplementary tables and figures

Table S1 – Pathway enrichment analysis of all targets of identified hit compounds (Panther database).

PANTHER Pathways	Fold Enrichment	Raw P-value
JAK/STAT signaling pathway	31.88	1.55E-05
Insulin/IGF pathway-protein kinase B signaling cascade	31.1	6.00E-12
Hedgehog signaling pathway	30.01	1.91E-05
Circadian clock system	28.34	3.16E-03
Interferon-gamma signaling pathway	27.72	2.28E-06
Hypoxia response via HIF activation	27.05	2.28E-08
Ras Pathway	25.15	4.01E-15
VEGF signaling pathway	23.54	7.80E-13
p53 pathway feedback loops 2	22.5	8.83E-10
PDGF signaling pathway	22.14	5.56E-25
p53 pathway	21.74	2.75E-15
B cell activation	21.58	1.67E-11
Toll receptor signaling pathway	20	1.79E-08
CCKR signaling map	18.89	1.50E-22
Interleukin signaling pathway	18.44	1.03E-11
p53 pathway by glucose deprivation	18.22	8.30E-04
Axon guidance mediated by netrin	18.22	1.42E-05

Apoptosis signaling pathway	18.22	5.05E-16
Angiogenesis	18.22	2.53E-21
Insulin/IGF pathway-mitogen activated protein kinase kinase/MAP kinase cascade	15.94	1.71E-04
Parkinson disease	15.62	5.99E-11
EGF receptor signaling pathway	15.11	5.34E-14
T cell activation	15	3.54E-09
FGF signaling pathway	14.88	2.60E-12
Endothelin signaling pathway	12.91	3.93E-07
PI3 kinase pathway	11.39	1.12E-04
FAS signaling pathway	11.25	2.96E-03
Integrin signalling pathway	10.23	1.18E-09
Alzheimer disease-amyloid secretase pathway	9.96	2.02E-04
Oxidative stress response	8.79	1.39E-03
Inflammation mediated by chemokine and cytokine signaling pathway	7.76	7.59E-09
Dopamine receptor mediated signaling pathway	7.65	8.15E-03
Cadherin signaling pathway	6.03	2.15E-04
Gonadotropin-releasing hormone receptor pathway	5.62	1.73E-05
Heterotrimeric G-protein signaling pathway-Gq alpha and Go alpha mediated pathway	5.5	2.58E-03
Heterotrimeric G-protein signaling pathway-Gi alpha and Gs alpha mediated pathway	5.39	1.07E-03

Wnt signaling pathway	4.28	1.55E-04

Table S2 – EC_{50} of compounds that had no effect on cell viability tested in a dose response manner.

Compound	EC50	R^2
Panobinostat	<0.03	0.789
KX2-391	0.035	0.940
Selinexor (KPT-330)	0.049	0.998
BI 2536	0.052	0.911
(+)-JQ1	0.067	0.993
XMD8-92	0.075	0.885
AZ 960	0.123	0.977
TPCA-1	0.203	0.980
Ruxolitinib (INCB018424)	0.256	0.780
CEP33779	0.299	0.984
TG101348 (SAR302503)	0.357	0.988
Tivantinib (ARQ 197)	0.388	0.925
Cyt387	0.465	0.983
Afatinib (BIBW2992)	0.473	0.964
SKI II	0.507	0.963
Rebastinib (DCC-2036)	0.564	0.554
BAY 61-3606	0.656	0.929
Ki16198	0.661	0.913
BIX 01294	0.738	0.903
Neratinib (HKI-272)	0.743	0.930
Tofacitinib (CP-690550)	0.745	0.812
NVP-BEP800	>1	0.895
Pifithrin-μ	>1	0.626
Aurothioglucose	>1	0.234
Fenoprofen Calcium	>1	0.007

Figure S1 – *NFκB reporter cell line expresses mStrawberry fluorophore upon TNFα treatment.* Both cell lines were treated with the indicated cytokines for 24h then imaged.

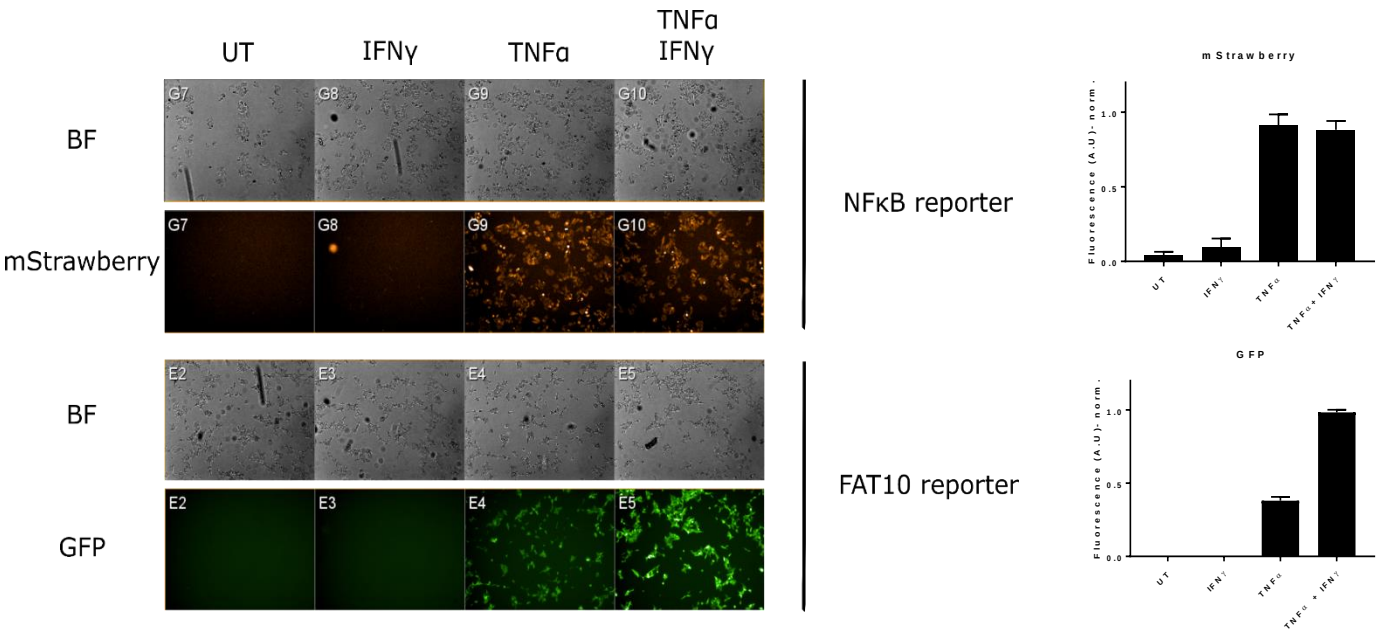
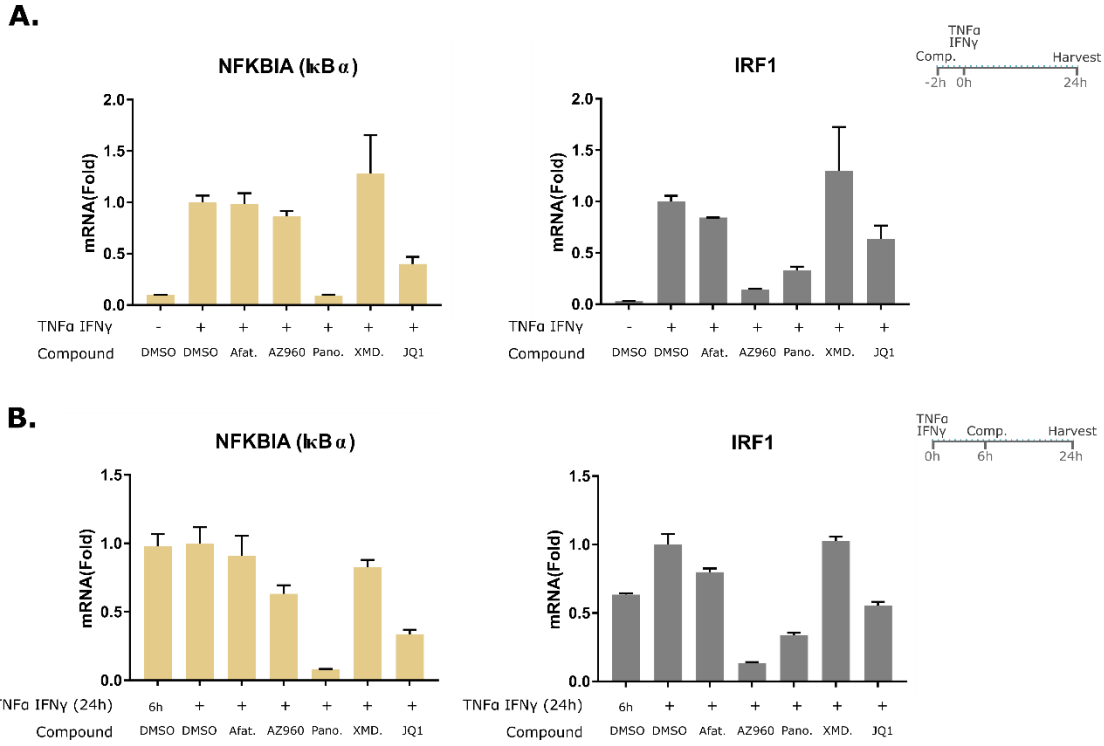


Figure S2 – *qPCR analysis reveals that all compounds inhibit at least one signaling pathway TNFα or IFNγ.* Afat. = Afatinib, Pano. = Panobinostat and XMD. = XMD8-92. ***(A)*** HEK293 cells were pre-incubated for 2h with compounds, then induced with TNFα and IFNγ for 24h and harvested for qPCR analysis of NFKBIA (IκB) and IRF1 mRNA levels. ***(B)*** HEK293 cells were induced with TNFα and IFNγ for 6h, then treated with compounds for additional 18h for a total of 24h induction with cytokines, then harvested for qPCR analysis of NFKBIA (IκB) and IRF1 mRNA levels.



<u>Figure S3 –</u> ***AZ960 inhibits IFNγ-mediated transcriptional response***. ***(A)*** HEK293 cells were induced with TNFα and IFNγ for 24h, then medium was replaced to cytokine-free medium and cells were treated with compounds for additional 24h, then harvested for qPCR analysis of NFKBIA (IκB) and IRF1 mRNA levels. Changed Medium = C.M. ***(B)*** HEK293 cells were induced with TNFα and IFNγ for 24h, then treated with compounds for additional 24h without replacing the medium, then harvested for qPCR analysis of NFKBIA (IκB) and IRF1 mRNA levels.

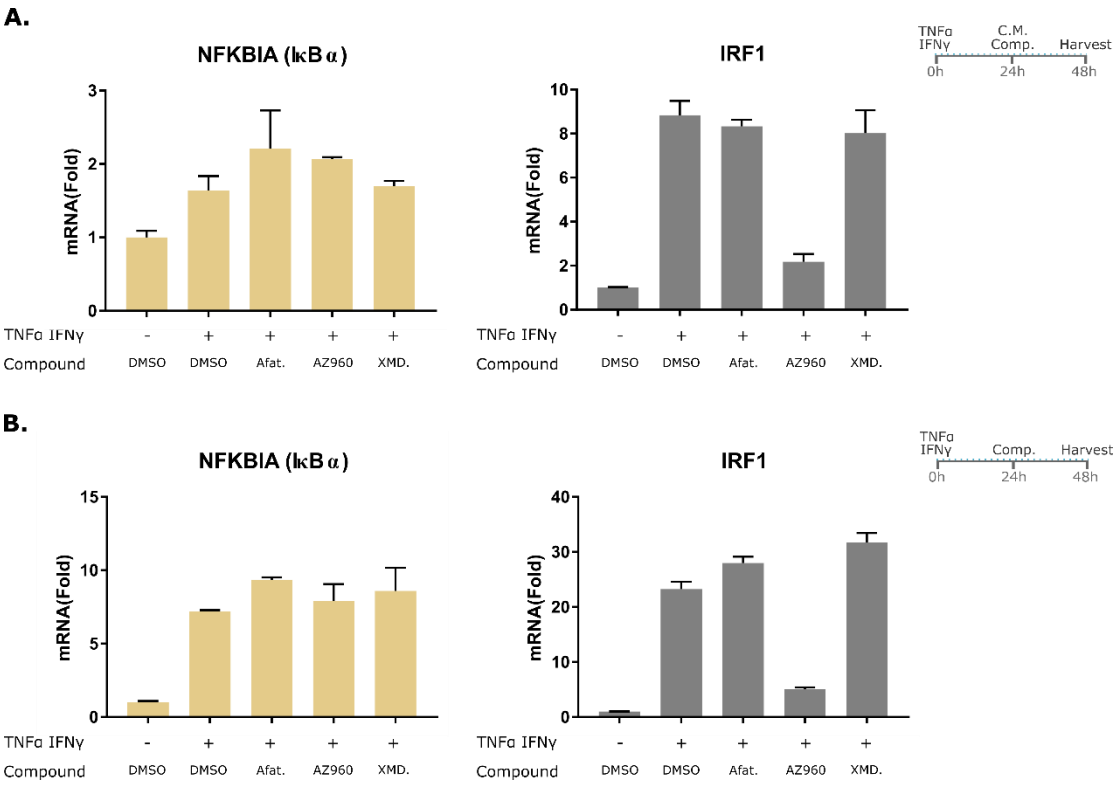


Figure S4 – ***AZ960 does not inhibit NFκB pathway but does inhibit IFNγ signaling.*** NFκB target genes TNFAIP3 (A20), NFKBIA (IκB), IL8 and TNF. IRF1 is upregulated in response to IFNγ signaling. HEK293 cells were induced with TNFα and IFNγ for 24h, then treated with AZ960 (250nM) for additional 24h and harvested for qPCR analysis.

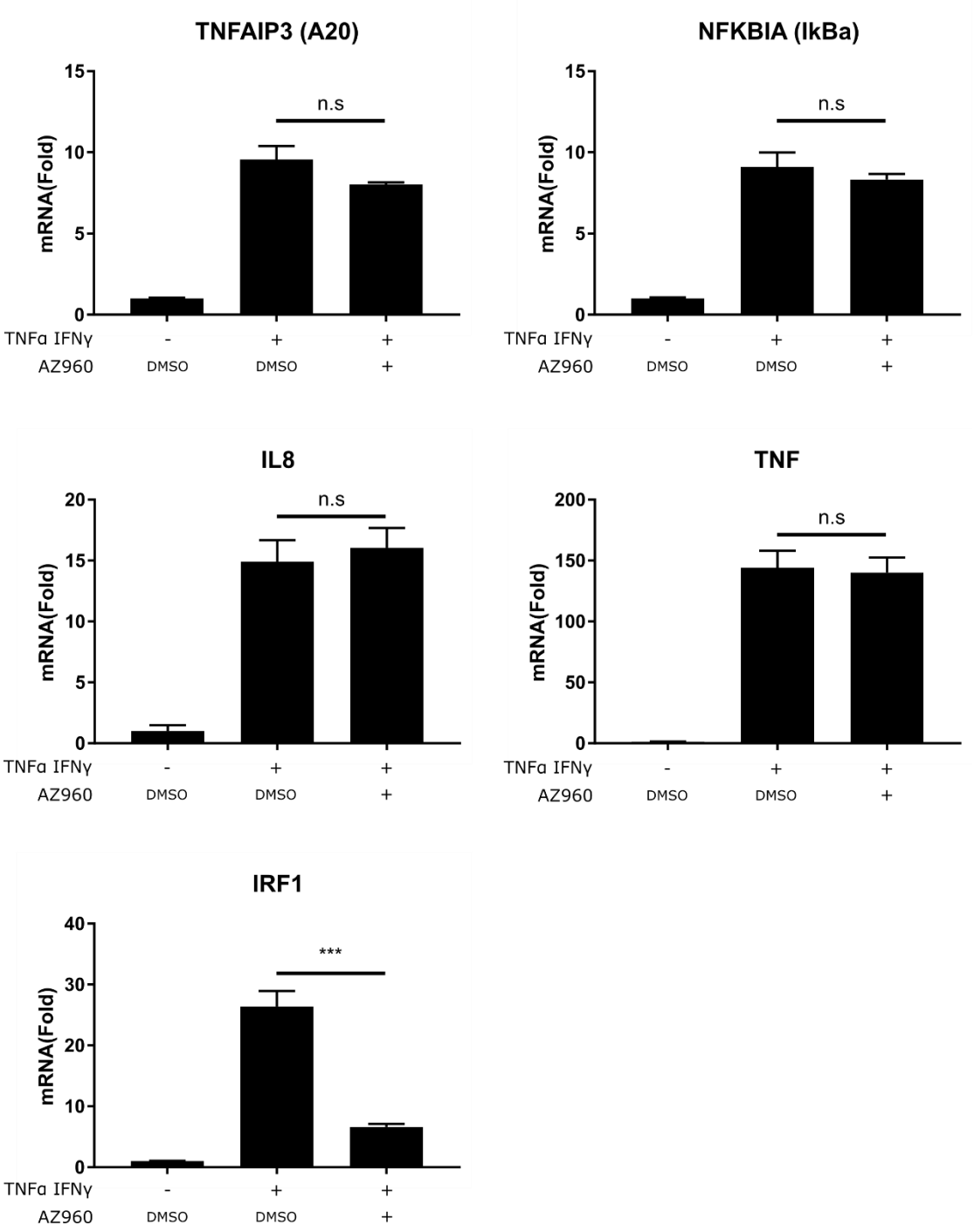


Figure S5 – *Basal FAT10 mRNA levels in different cell lines*. qPCR analysis of FAT10 mRNA levels of indicated cell lines.

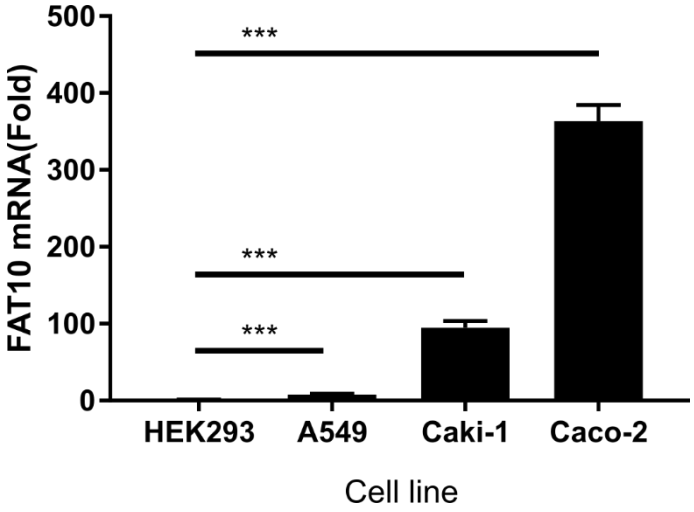
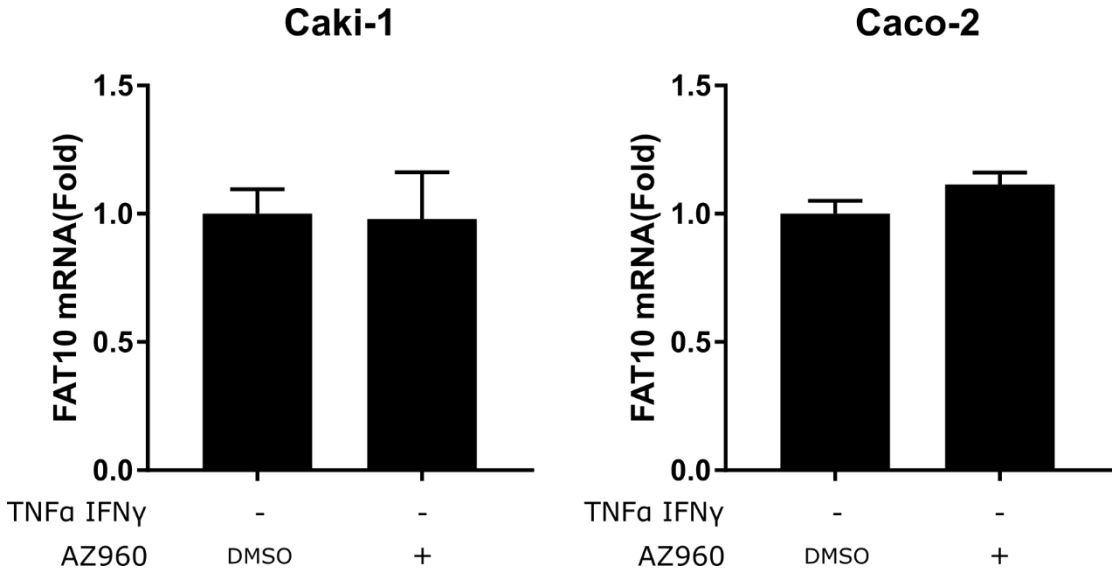


Figure *S6* – *AZ960 does not effect basal FAT10 levels.* Cells were treated with AZ960 (250nM) for 24h then harvested for qPCR analysis of FAT10 mRNA levels.



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