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Role of TNF-α and the NF-κB pathway in drug-induced organ injuries

Benedetti, G.O.E.

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A siRNA screen for drug and TNF–α cytotoxicity synergy response

Giulia Benedetti*, Lisa Fredriksson*, Bram Herpers*, Marjo de Graauw and Bob van de Waterw

* Equal contribution

Division of Toxicology, Leiden/Amsterdam Center for Drug Research, Leiden University, The Netherlands

Manuscript in preparation

Abstract

Drug-induced liver injury (DILI) is a major clinical problem that involves crosstalk between drug toxicity and the immune system, and particularly the pro-inflammatory cytokine tumor necrosis factor α (TNF-α). We have previously shown that the hepatotoxicant diclofenac suppresses TNF-α-induced NF-κB-dependent survival signalling routes and sensitizes HepG2 cells to apoptosis through c-Jun N-terminal kinase (JNK) and caspase 8 activation. To better understand the contribution of individual signalling components to TNF-α-mediated aggravation of diclofenac-induced cell death, we carried out a targeted small interfering RNA (siRNA) screen using HepG2 cells exposured to diclofenac, TNF-α or diclofenac in combination with TNF-α as a model system. In total, 1567 siRNAs were used targeting individual kinases, (de)ubiquitinases and immune components. This led to the identification of 12 genes that essentially control the apoptotic response under these specific conditions. 6 of these genes evidently were known to regulate the previously identified NF-κB and JNK signalling pathways, whereas others were shown to control the p53 and Wnt signalling pathways. Moreover, gene expression of some of these genes was differentially regulated by drug/cytokine treatment, indicating that we identified toxicity mechanisms that may lead to the identification of novel toxicity biomarkers.

1. Introduction

The liver is the central organ for xenobiotic metabolism and is therefore one of the primary target organs for many toxic insults. Drug-induced liver injury (DILI) is the leading cause of acute liver failure (1, 2). This hepatotoxicity leads to morbidity and mortality in humans and is therefore one of the major reasons for drug withdrawal post-marketing.

Several mechanisms have been implicated in the onset and progression of DILI, including disruption of the intracellular calcium homeostasis, induction of oxidative stress, activation of apoptosis, interruption of bile canalicular transport, inhibition of fatty acid oxidation and inhibition of mitochondrial function (3, 4). Furthermore, increasing evidence has indicated that inflammatory processes are intimately involved in chemical-induced hepatotoxic processes and are responsible for producing mediators that can affect liver damage or repair (5). Following the initial toxic injury of liver cells, adjacent cells, as well as liver-resident macrophages (Kupffer cells) are activated to secrete inflammatory products, of which the pro-inflammatory cytokine tumor necrosis factor α (TNF-α) is predominant (5, 6).

TNF-α severely enhances liver damage caused by different xenobiotics, including the widely prescribed non-steroidal anti-inflammatory drug (NSAID) diclofenac (5, 7-11). We previously showed in an *in vitro* human liver cell model (HepG2) that diclofenac-induced toxicity was enhanced by the presence of TNF-α. The enhanced apoptosis by TNF-α was due to perturbation of the pro-survival NF-κB signalling pathway upon drug exposure, leading to an increase in c-Jun N-terminal kinase (JNK)-mediated pro-apoptotic signalling and caspase-8 activation (9). Although a first draft of the molecular mechanism of diclofenac and TNF-α interaction was drawn, the contribution of the individual signalling components in these and other signalling pathways had not yet been explored in detail.

To better understand the contribution of individual signalling components to TNF-α-mediated aggravation of diclofenac cell death, we carried out a targeted small interfering RNA (siRNA) screen to identify genes involved in regulating apoptosis of HepG2 cells induced by diclofenac, TNF-α or diclofenac in combination with TNF-α. Knockdown of 1567 individual kinases, (de)ubiquitinases and immune components with siRNAs led to the identification of 12 genes involved in HepG2 apoptotic cell death induced by either diclofenac, TNF-α or a combination of diclofenac and TNF-α. These genes included ZNRF3, PRPF19, and SOCS5 respectively. Most of these genes were known to regulate mainly 3 different signalling pathways in other cell types: the NF-κB, JNK and p53 signalling pathways.

2. Material and methods

2.1. Materials

Human recombinant TNF-α was acquired from R&D Systems (Abingdon, UK). Diclofenac sodium was from Sigma-Aldrich (Zwijndrecht, The Netherlands). AnnexinV-Alexa633 was made as described (12).

2.2. Cell culture

Human hepatoma HepG2 cells were obtained from American Type Culture Collection (clone HB-8065, ATCC, Wesel, Germany). HepG2 cells stably expressing GFP-p65 (NF-κB subunit) were created by 400 µg/ml G418 selection upon pEGFP-C1-p65 transfection using Lipofectamine™ 2000 (Invitrogen, Breda, The Netherlands). For all experiments the cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS), 25 U/ml penicillin, and 25 µg/ml streptomycin

between passages 5 and 20.

2.3. RNA interference

Transient knockdown of individual target genes was achieved using siGENOME SMARTpool siRNA reagents in the primary screen or single siRNA sequences in the secondary deconvolution screen (50 nM; Dharmacon ThermoFisher Scientific, Landsmeer, The Netherlands). HepG2 cells were transfected using INTERFERin siRNA transfection reagent according to the manufacturer's procedures (Polyplus transfection, Leusden, The Netherlands) and left for 72 hours to achieve maximal knockdown before treatment. The negative controls were GFP and mock (INTERFERin only) transfection and caspase-8 transfection was used as positive control.

2.4. Exposures

Prior to imaging, nuclei were stained with 100 ng/ml Hoechst 33342 in complete DMEM for 45 minutes. The cells were then exposed to diclofenac (500 μM) or DMSO (0.2%) for 8 hours followed by challenge with human TNF-α (10 ng/ml).

2.5. Apoptosis measurements

Apoptosis was determined by the live cell apoptosis assay previously described (12). Briefly, binding of Annexin V-Alexa633 conjugate to phosphatidyl serine present on the membranes of apoptotic cells was imaged after drug exposure with a BD Pathway 855 imager (Becton Dickinson, Erembodegem, Belgium). The relative Annexin V fluorescence intensity per image was quantified using Image Pro (Media Cybernetics, Bethesda, MD) and normalized to the number of nuclei or cell area. Apoptosis was measured at 8 and 24 hours for the primary screen and was followed overtime for the deconvolution screen. For the primary screen, each siRNA plate was imaged separately with both DMSO and diclofenac treatments and the screen was performed once. For the deconvolution screen, 5 different siRNA plates were imaged at once with both DMSO and diclofenac treatments using the Twister®II microplate handler (CaliperLife Sciences, Teralfene, Belgium) directly connected to both the BD pathway imager and an incubator (LiCONiC Instruments) and controlled via the iLink Pro 1.1 SP4 software. The deconvolution screen was performed in triplicates and the area under the curve was used to determine the fold-changes.

2.6. Statistical procedures

All numerical results are expressed as mean ± standard error of the mean (S.E.M.). Statistical significance was determined by GraphPad Prism using an unpaired t-test. The level of confidence is represented by P-values indicated in the figures. Heatmap representations were performed using the MultiArray Viewer software.

3. Results

3.1. Design of a siRNA screen setup to identify novel regulators of drug-induced liver cell apoptosis

Previously we have shown that the pro-inflammatory cytokine TNF-α enhanced diclofenac-induced apoptosis of HepG2-GFP-p65 cells (9). To identify genes functional involved in the regulation of the synergistic apoptosis induced by diclofenac and TNF-α, we set-up an siRNA screen using these HepG2-p65-GFP cells in a live-cell apoptosis imaging assay. To validate the siRNA-mediated knock-down, cells were transfected with a non-targeting siRNA (siCtrl) and siRNA targeting caspase 8 (siCasp8), which essentially controls diclofenac-induced apoptosis (9). siRNA transfected cells were exposed to 500 μM diclofenac or 0.2% DMSO for 8 hours and thereafter challenged with 10 ng/ml TNF-α for an additional 16 hours. Cell death was determined at 8 hours and at 24 hours by imaging Annexin-V coupled to alexa-633 binding to apoptotic cells (Fig. 1A). No significant difference was observed between non-transfected and siCtrl transfected cells, while a significant separation between the positive control siCasp8 and negative control siCtrl was observed (Fig. 1B). The average Z' factor (13) of diclofenac/TNF-α treated plates based on siCtrl and siCasp8 was ~0.87, indicating a strong signal to noise ratio (Fig. 1B), thereby allowing identification of genes of interest with relatively high confidence.

3.2. Primary siRNA screen identifies 91 potential genes involved in drug-induced HepG2 cell death

Next, dedicated libraries consisting of 780 siRNAs targeting kinases, 621 siRNAs targeting (de)ubiquitinases and 166 siRNA targeting components of the immune system involved in TNF receptor (TNFR) and toll-like receptor (TLR) signalling were used to identify genes involved in the regulation of the synergistic apoptosis of HepG2-GFP-p65 cells induced by diclofenac and TNF-α. Cells were transfected, exposed and

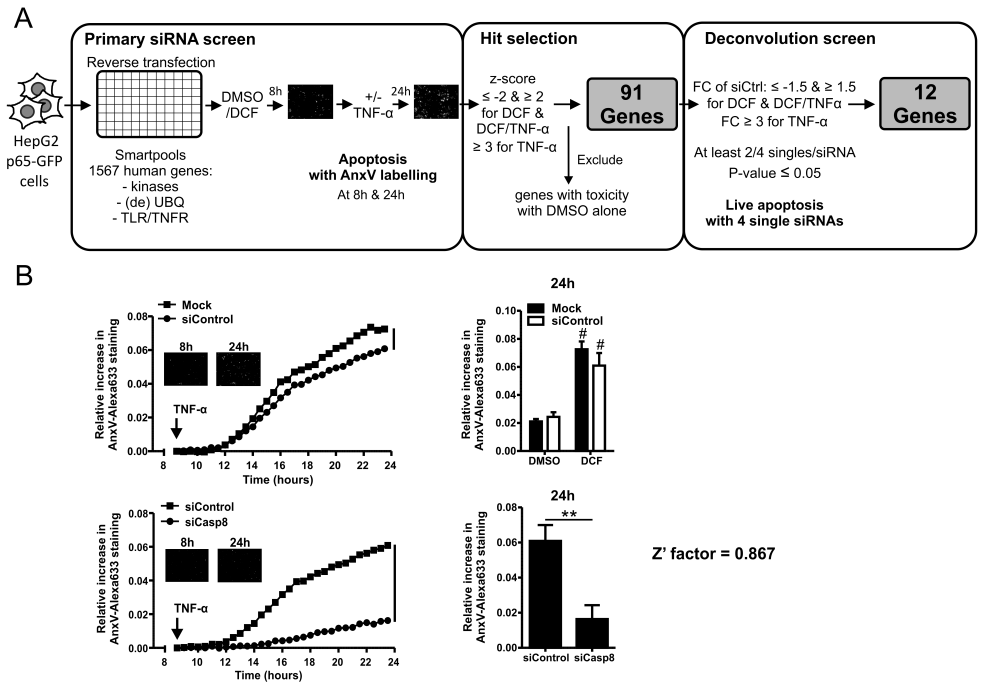

Figure 1. Design of a siRNA screen setup to identify novel regulators of drug-induced liver cell apoptosis. HepG2-GFP-p65 cells were transfected with targeted siRNA libraries, exposed to diclofenac or DMSO for 8 hours followed by stimulation with TNF-α for the remaining 16 hours. Apoptosis was determined using an Annexin V-Alexa633 live apoptosis assay. 91 hits were selected from the primary screen and 12 were further confirmed in a secondary deconvolution screen (A). siRNA control (siCtrl) and siRNA against caspase 8 (siCasp8) were used as negative and positive controls respectively which allowed calculation of the Z' factor of the screen (B). The data are represented as means of more than three independent experiments ± S.E.M. ** P ≤ 0.01 and # P ≤ 0 .05 compared to vehicle-treated cells.

cell death was determined as described above (Fig. 1A). For hit selection, siRNAs were ranked against the average values of siRNA control and mock using z-scores (14). siRNAs affecting general survival under DMSO conditions (z-score ≥ 3.0) were excluded (Fig. 1A and Fig. 2A). Four different classes were defined as siRNAs affecting 1) diclofenac-induced apoptosis at 8h, 2) TNF-α-induced apoptosis at 8 hours, 3) diclofenac-induced apoptosis at 8 hours as well as diclofenac/TNF-α-induced apoptosis at 24 hours and 4) diclofenac/TNF-α-induced apoptosis at 24 hours without affecting diclofenac-induced apoptosis at 8 hours. Using a z-score ≥ 2.0 or ≤ -2.0 for diclofenac and diclofenac/TNF-α treatments and a z-score ≥ 3.0 for TNF-α treatment, a total of 91 hits were identified in our primary screen (Fig. 2B and Table 1). Of these 91 hits, 51 were involved in diclofenac/TNF-α-induced apoptosis, 28 hits regulated the apoptosis induced by both diclofenac

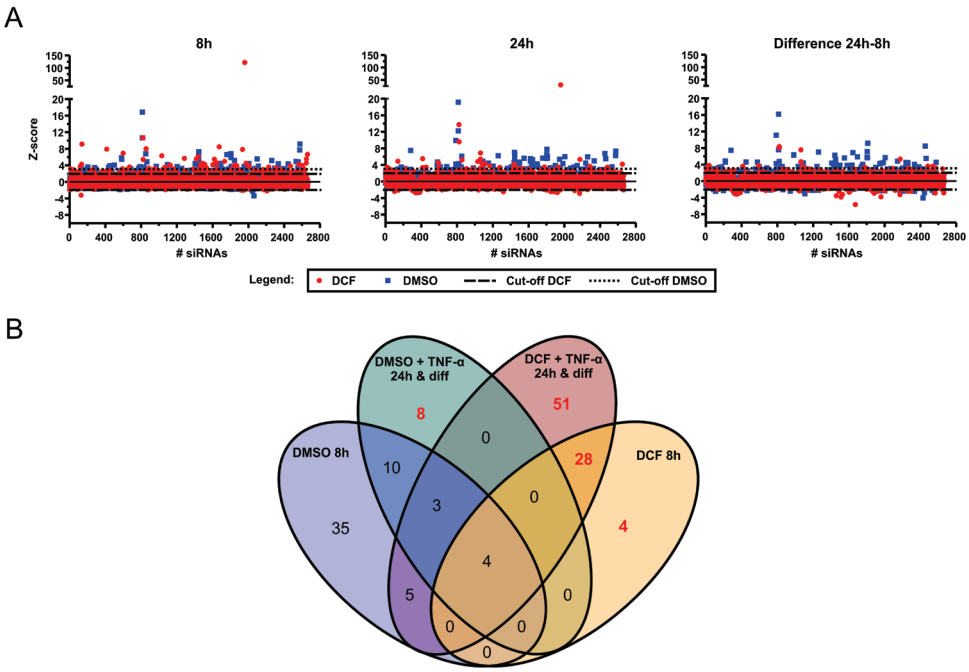

Figure 2. Primary siRNA screen identifies 91 potential genes involved in drug-induced HepG2 cell death. The hits were ranked according to their z-scores for both DMSO and diclofenac treatments at 8 and 24 hours as well as for the difference between 24 and 8 hours (A) and the repartition of the hits within the different treatments was represented in a Venn diagram. The numbers represented in bold and red indicates the hits selected for the secondary screen (B).

as well as diclofenac/TNF-α, 4 only affected diclofenac treatment and 8 were regulators of TNF-α-induced apoptosis (Fig. 2B and Table 1). It is important to note that the hits identified as diclofenac/TNF-α apoptosis regulators are only those ones that did not sensitize for diclofenac alone at 8h, since apoptosis at 24h for diclofenac alone was not determined.

3.3. Hit validation by single siRNA deconvolution

To validate the 91 hits identified in our primary screen, a secondary deconvolution screen was performed in triplicate to test the specific targeting of each of the four different siRNA duplexes present in the SMARTpool. We considered the primary hits validated when at least 2 out 4 individual siRNAs had the same effect as the SMARTpool with a fold-change of ≥ 1.5 and ≤ -1.5 compared to siCtrl for diclofenac and diclofenac/

Table 1. Hits identified in the primary screen.

Gene	Description	z-score 8h	z-score 24h	z-score diff
	Diclofenac hits only			
MINK	misshapen-like kinase 1	2.45	0.54	-1.57
WEE1	WEE1	2.13	1.99	1.57
ERBB4	v-erb-a erythroblasticleukemia viral oncogene homolog 4	2.13	0.41	-1.64
BUB1	budding uninhibited by benzimidazoles 1	2.02	1.87	1.52
	Diclofenac and diclofenac+TNF-α hits			
RNF165	ring finger protein 165	122.25	30.15	-3.77
ANKRD3	receptor-interacting serine-threonine kinase 4	9.14	4.92	2.83
CDC2	cell division cycle 2, G1 to S and G2 to M	7.91	5.53	3.42
ZNRF3	zinc and ring finger 3	6.69	-0.72	-2.83
ZNF364	ring finger protein 115	5.54	4.20	2.98
RNF180	ring finger protein 180	4.51	-0.61	-2.00
UBR1	ubiquitin protein ligase E3 component n-recognin 1	4.23	-1.48	-2.85
TYK2	tyrosine kinase 2	3.85	3.16	2.22
AKT2	v-akt murine thymoma viral oncogene homolog 2	3.73	2.27	1.51
RNF26	ring finger protein 26	3.69	2.39	-2.34
NFKB2	nuclear factor of kappa light polypeptide gene enhancer in B-cells 2 (p49/p100)	3.44	0.52	-3.50
CSNK2B	casein kinase 2, beta polypeptide	3.18	4.55	3.73
KIAA0317	KIAA0317	2.84	2.42	1.68
RIPK2	receptor-interacting serine-threonine kinase 2	2.76	2.80	2.35
NCOR2	nuclear receptor co-repressor 2	2.73	3.49	2.48
PTPN5	protein tyrosine phosphatase, non-receptor type 5	2.72	2.69	2.46
IBRDC2	ring finger protein 144B	2.66	3.08	2.22
PRKD1	protein kinase D1	2.62	3.54	3.40
MERTK	c-mer proto-oncogene tyrosine kinase	2.60	2.65	1.55
CRKL	v-crk sarcoma virus CT10 oncogene homolog (avian)-like	2.53	2.52	1.91
UBE2A	ubiquitin-conjugating enzyme E2A	2.45	3.45	3.67
NFKBIE	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, epsilon	2.43	3.17	2.33
GRK4	G protein-coupled receptor kinase 4	2.37	3.08	2.79
MEFV	Mediterranean fever	2.35	2.32	1.75
TNFRSF7	CD27 molecule	2.33	3.67	2.77
HK1	hexokinase 1	2.15	2.63	2.31
KDM5C	lysine (K)-specific demethylase 5C	2.03	2.50	2.24
CDADC1	cytidine and dCMPdeaminase domain containing 1	2.03	3.37	3.62
	Diclofenac+TNF-α hits only			
JHDM1D	jumonji C domain containing histone demethylase 1 D	-0.20	5.41	4.71
LOC283116	tripartite motif-containing protein LOC642612-like	-0.17	4.29	3.21

Gene	Description	z-score 8h	z-score 24h	z-score diff
MDM2	murine double minute 2	0.69	3.41	2.71
FBXW8	F-box and WD repeat domain containing 8		2.88	2.88
PRKAG3	protein kinase, AMP-activated, gamma 3 non-catalytic subunit	1.67	2.80	2.92
FLJ10761	ethanolamine kinase 2	1.26	2.66	2.90
UBE2T	ubiquitin-conjugating enzyme E2T	0.97	2.62	3.65
TLR4	toll-like receptor 4	0.88	2.60	2.74
MAK	male germ cell-associated kinase	1.77	2.60	2.58
RNF17	ring finger protein 17	1.69	2.58	2.61
LOC120824	tripartite motif-containing protein ENSP00000309378-like	0.08	2.57	2.88
CASP5	caspase 5	1.76	2.50	2.69
TRIM	T cell receptor associated transmembrane adaptor 1	1.54	2.45	2.57
PRPS2	phosphoribosyl pyrophosphate synthetase 2	1.68	2.41	2.46
SMURF2	SMAD specific E3 ubiquitin protein ligase 2	0.62	2.33	3.04
RNF40	ring finger protein 40	1.23	2.33	2.05
UBE2QL1	ubiquitin-conjugating enzyme E2Q family-like 1	1.96	2.25	2.02
UBE2G2	ubiquitin-conjugating enzyme E2G 2	1.19	2.25	2.53
RASGRF2	Ras protein-specific guanine nucleotide-releasing factor 2	0.98	2.19	2.43
SEPHS1	selenophosphatesynthetase 1	1.99	2.17	2.05
RNFT1	ring finger protein, transmembrane 1	-1.27	2.15	2.51
PRKD3	protein kinase D3	-0.44	2.12	3.19
KRTAP5-9	keratin associated protein 5-9	-0.60	1.97	2.15
RNF2	ring finger protein 2	-1.35	1.96	2.38
TRIM45	tripartite motif-containing 45	-0.90	1.77	2.05
UBE2B	ubiquitin-conjugating enzyme E2B	0.76	1.76	2.11
MGRN1	mahogunin, ring finger 1	0.12	1.75	2.49
TRIM73	tripartite motif-containing 73	-0.19	1.70	2.03
BCR	breakpoint cluster region	-0.21	1.64	2.41
MARCH11	membrane-associated ring finger (C3HC4) 11	-0.57	1.59	2.08
UHMK1	U2AF homology motif (UHM) kinase 1	-0.34	1.54	3.21
TNFRSF25	tumor necrosis factor receptor superfamily, member 25	-1.53	1.54	2.02
STK32C	serine/threonine kinase 32C	-0.20	1.49	2.19
UBR5	ubiquitin protein ligase E3 component n-recognin 5	0.54	1.43	2.02
ADRBK1	adrenergic, beta, receptor kinase 1	-0.31	1.41	2.42
MAPK9	mitogen-activated protein kinase 9	-0.41	1.10	2.14
PRICKLE4	prickle 4	-1.00	1.04	2.52
RPS6KA4	ribosomal protein S6 kinase, 90kDa, polypeptide 4	-0.26	1.02	2.07
DCUN1D5	DCN1, defective in cullinneddylation 1, domain containing 5	-0.90	0.83	2.36
LRRK1	leucine-rich repeat kinase 1	-0.94	0.67	2.17
GAK	cyclin G associated kinase	-0.98	0.57	2.05

Gene	Description	z-score 8h	z-score 24h	z-score diff
TNIK	TRAF2 and NCK interacting kinase	1.08	-0.65	-2.42
TNFRSF13B	tumor necrosis factor receptor superfamily, member 13B	1.35	-0.82	-2.43
DUSP5	dual specificity phosphatase 5	-0.97	-1.86	-2.00
TRIM64C	tripartite motif containing 64C	0.77	-1.98	-2.17
DGKA	diacylglycerol kinase, alpha 80kDa	-1.26	-2.01	-2.01
PFKFB1	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 1	-1.27	-2.11	-2.10
SOCS5	suppressor of cytokine signalling 5		-2.19	-2.19
RC3H1	ring finger and CCCH-type zinc finger domains 1	-0.62	-2.46	-2.09
RIPK1	receptor (TNFRSF)-interacting serine-threonine kinase 1	-0.91	-2.49	-2.90
STUB1	STIP1 homology and U-box containing protein 1	-0.94	-2.85	-3.22
	TNF-α hits only			
CALCOCO2	calcium binding and coiled-coil domain 2	1.82	3.57	4.36
PLK4	polo-like kinase 4	1.35	4.27	5.90
BIRC2	baculoviral IAP repeat-containing 2	1.20	3.14	4.87
CSNK2A2	casein kinase 2, alpha prime polypeptide	0.91	4.05	4.63
PRPF19	pre-mRNA processing factor 19	0.84	3.12	4.06
RNF39	ring finger protein 39	0.77	3.28	4.34
FBXO30	F-box protein 30	-0.14	4.19	4.26
IKBKG	inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase gamma	-0.52	3.28	6.31

TNF-α treatments and ≥ 3.0 for TNF-α treatment with a p-value ≤ 0.05 (Fig. 1A). Because of variable transfection efficiency in some of the triplicate plates, only the best 2 out of 3 plates were used for hit validation. In total 12 hits out of the 91 could be confirmed as controllers of apoptosis in HepG2-GFP-p65 cells (Fig. 3A, 4 and 5). Most of the hits mediated diclofenac and diclofenac/TNF-α-induced apoptosis, and only one, PRPF19, regulated TNF-α-induced apoptosis (Fig. 3B, 4 and 5). These hits comprised an equal proportion of protectors and inducers (knock-down sensitizes or protects against apoptosis, respectively) (Fig. 3B, 4 and 5) and had several different molecular functions (Fig. 3C). Out of the 6 hits involved in the regulation of diclofenac/TNF-α-induced apoptosis, 5 were inducers, being CALCOCO2, SOCS5, UBE2G2, NFKB2 and TNIK (Fig. 3 and 5). All the hits regulating diclofenac-induced cell death were protectors and these hits included TRIM45, ZNRF3, KIAA0317, CASP5 and RIPK1 (Fig. 4). An additional 11 primary screen hits were identified with 2 out 4 individual siRNAs having the same

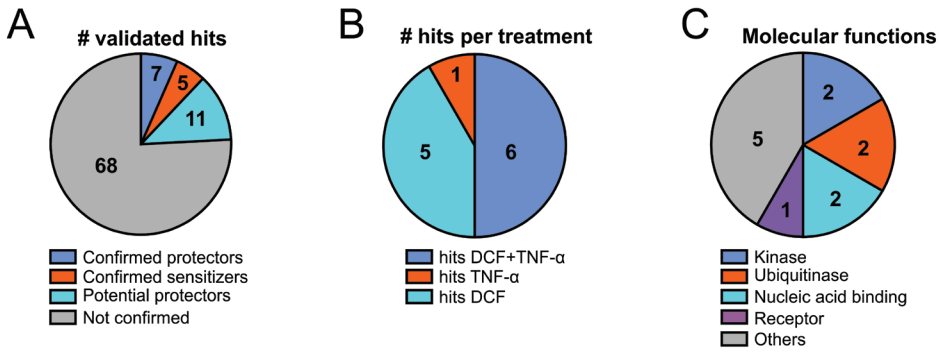

Figure 3. Hit validation by single siRNA deconvolution. The primary hits were validated in a secondary deconvolution screen. The number of primary hits validated and the number of protectors and inducers are indicated (A), as well as the number of hits per treatment (B) and their molecular functions (C).

effect as the SMARTpool, but just short of statistical significancy. Therefore these hits were termed "potential hits", as future experiments might confirm these as significant hits from three high-confident experiments. All of these potential hits were protectors in the primary screen (Fig. 3A, 4 and 5).

3.4. Network analysis revealed involvement of the hits in liver and kidney toxicity

To identify which signalling networks are regulated by the 12 identified target genes, an interaction-enriched network from these 12 hits was realized using the Ingenuity Pathway Analysis (IPA) software. The hits involved in diclofenac/TNF-α-induced apoptosis and in the apoptosis induced by diclofenac alone were separated and two networks were generated; the unique TNF-α hit PRPF19 was not included in the networks (Fig. 6 A and C). These networks revealed that the hits were interacting with different molecules and could not directly be linked to one-another. Since we are interested in the potential involvement of the identified hits in liver toxicity responses, we made an overlay of toxicity pathways related to liver injury with IPA®. This overlay showed that mainly hits regulating the diclofenac/TNF-α-induced apoptosis similarly control hepatotoxicity (Fig. 6 B and D). This observation indicates that the hits identified with the diclofenac/TNF-α treatment are likely to be involved in liver injury *in vivo*.

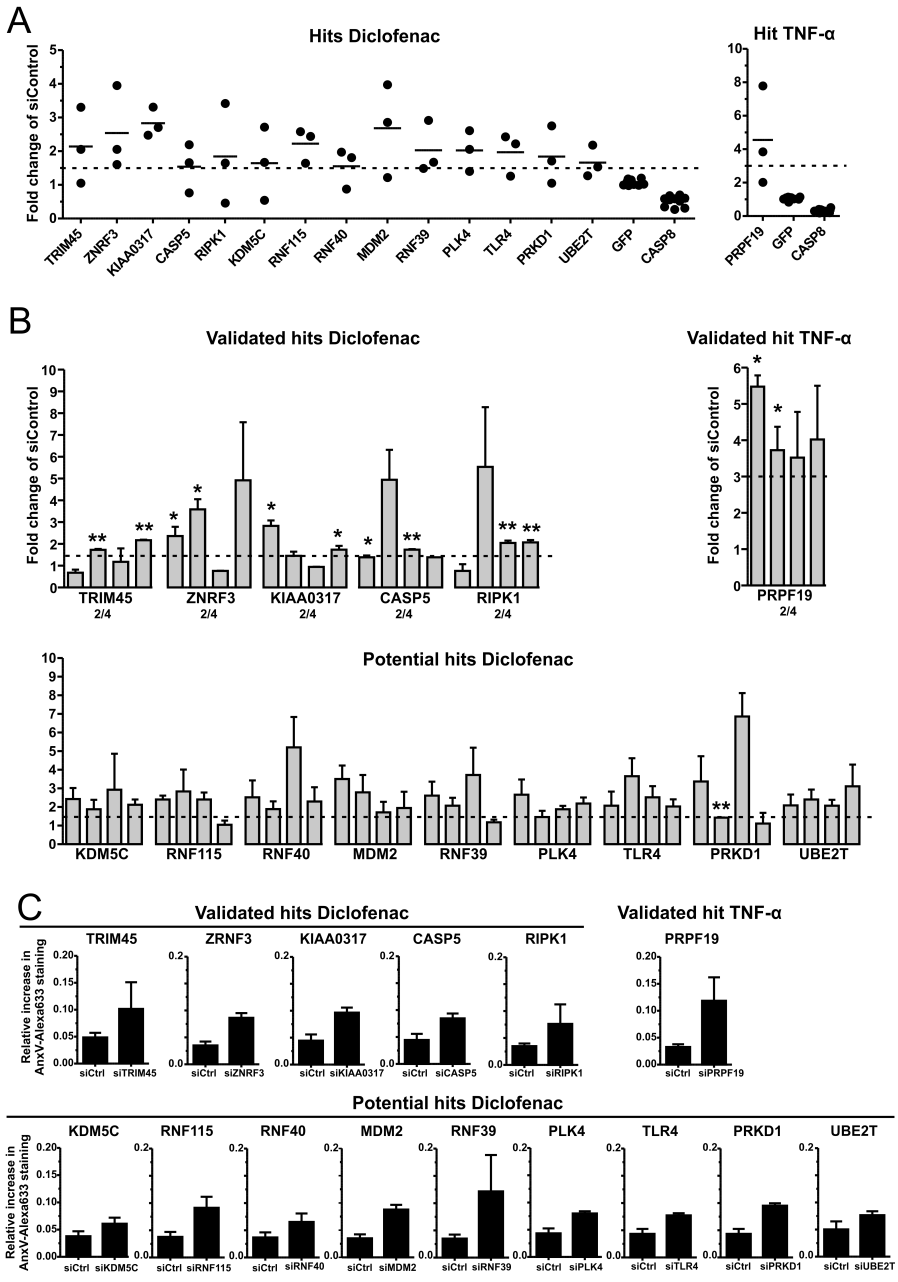

Figure 4. Identification of 6 protectors against diclofenac or TNF-α treatment. The 6 hits identified with both diclofenac and TNF-α treatments had at least 2 out of 3 replicates of the SMARTpools above the threshold (A) and 2 out of 4 single siRNAs having the same effect as the SMARTpool and were statistically significant (B). The hits for which 2 out of 4 single siRNAs had the same effect as the SMARTpools but were not statistically significant were termed potential hits. The relative Annexin-V values measured for each hit are also represented (C). The data are represented as means of two or three independent experiments ± S.E.M. * $P \leq 0.05$ and ** $P \leq 0.01$.

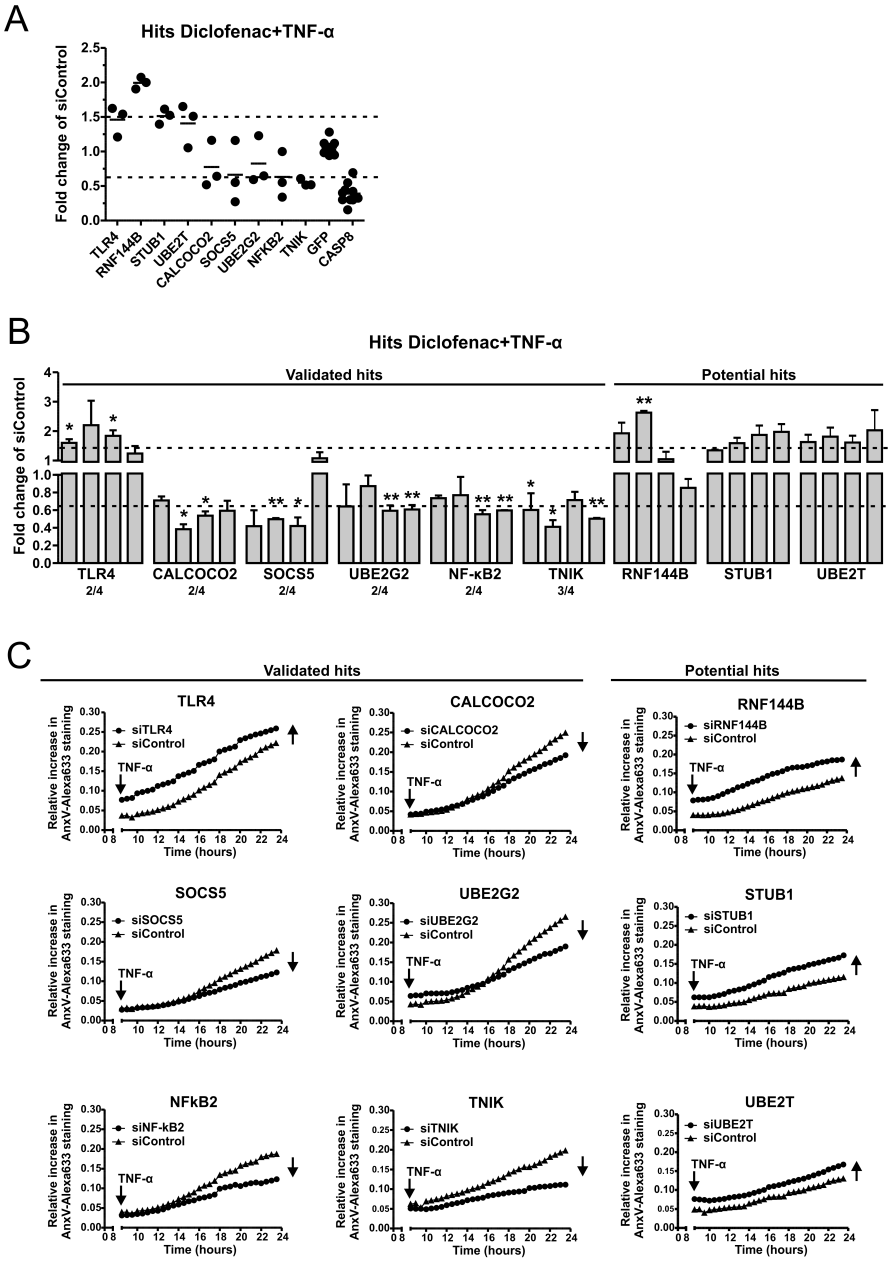

Figure 5. One protector and 5 inducers were identified for diclofenac/TNF-α treatment. The 6 hits identified with the combined diclofenac/TNF-α treatment had at least 2 out of 3 replicates of the SMARTpools above the threshold (A) and 2 out of 4 single siRNAs having the same effect as the SMARTpool and statistically significant (B). The hits for which 2 out of 4 single siRNAs had the same effect as the SMARTpools but were not statistically significant were termed potential hits. For the diclofenac/TNF-α treatment, live apoptosis was followed over time (C) and the area under the curve was used to determine the fold-changes. The data are represented as means of two or three independent experiments ± S.E.M. * $P \leq 0.05$ and ** $P \leq 0.01$.

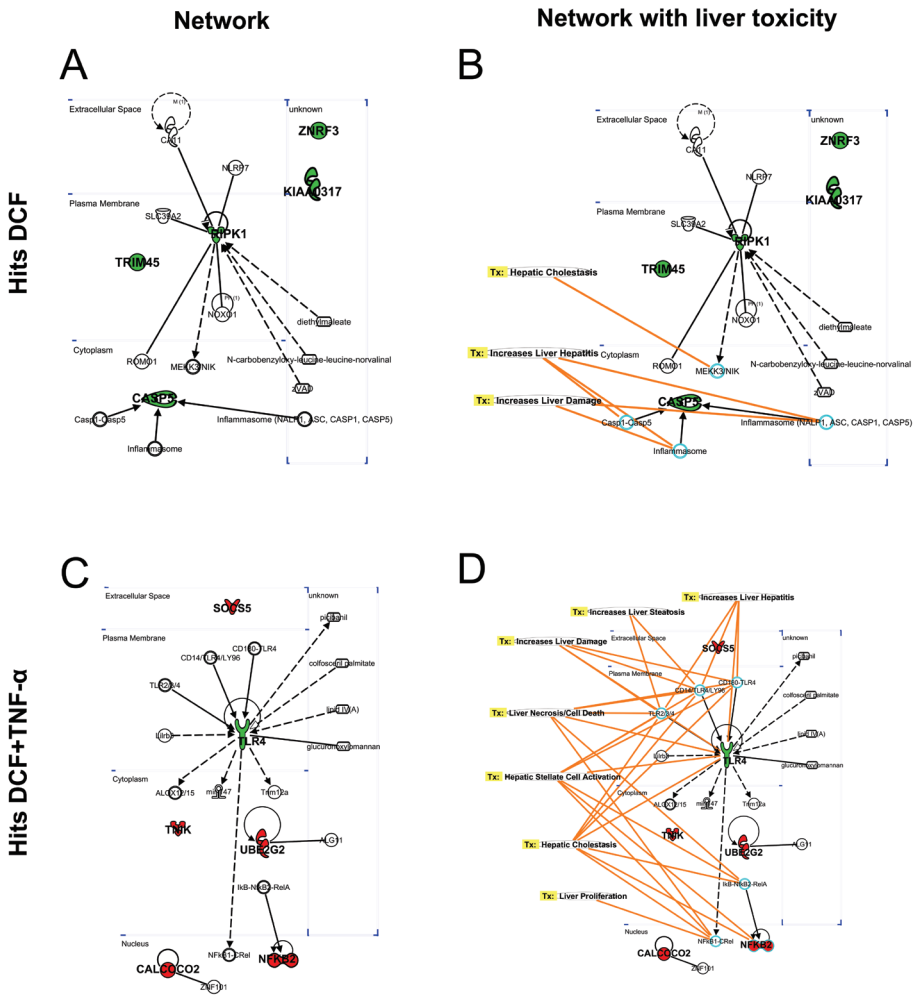

Figure 6. Network analysis revealed involvement of the hits in liver toxicity. Interaction-enriched networks were generated with IPA with connection of the hits together followed by enrichment of the network with 15 other molecules for hits with diclofenac treatment (DCF) (A) and hits with diclofenac/TNF-α treatment (DCF+TNF-α) (C). Association of the hits with liver toxicity and diseases related injuries (B and D) was performed. Sensitizers are represented in red and protectors in green.

3.5. Gene expression analysis reveals differential regulation of some of the hits upon drug/TNF-α co-treatment

In a parallel study we identified genes that are differentially expressed in HepG2

cells upon diclofenac or diclofenac/TNF-α treatment. In order to see whether our target genes' roles in diclofenac-induced apoptosis of HepG2-GFP-p65 cells could be correlated to gene expression changes, the fold-change in gene expression of our validated hits was calculated. Of the 12 confirmed hits, 4 showed a significant change (fold-change ≥ 1.5 or ≤ -1.5) in gene expression after either diclofenac and/or diclofenac/TNF-α treatment (Fig. 7A). For CALCOCO2 and ZNRF3 the gene expression changes were in line with the effect of the siRNA-mediated knock-down of these genes: increased expression of CALCOCO2 upon diclofenac/TNF-α treatment (Fig. 7A) correlated with a protection against diclofenac/TNF-α-induced apoptosis upon knock down of CALCOCO2 (Fig. 5); decreased ZNRF3 expression upon diclofenac treatment (Fig. 7A) correlated with increased diclofenac-induced apoptosis upon knockdown (Fig. 4). These observations indicate that CALCOCO2 and ZNRF3 could be potential biomarkers for hepatotoxicity.

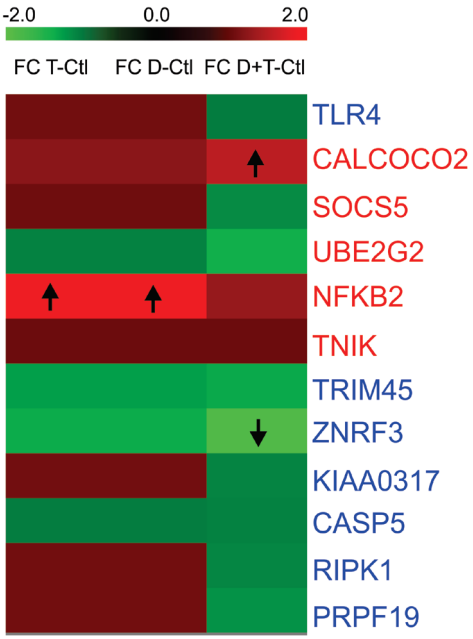

Figure 7. Gene expression analysis reveals differential regulation of some of the hits upon drug/TNF-α co-treatment. The gene expression changes (fold change, FC) of the 12 validated hits after exposing HepG2 cells to TNF-α for 4 hours (T), diclofenac for 8 hours (D) or diclofenac followed by 4 hours TNF-α stimulation (D+T) compared to vehicle (DMSO, Ctl) treatment are represented in a heatmap. Arrows indicate up-regulated or down-regulated genes with at least a 1.5 fold-change for the corresponding treatment. Sensitizers are represented in red and protectors in blue

3.6. The identified hits are mostly regulators of the NF-κB, JNK and p53 signalling pathways

In order to gain insight into the potential role of the identified hits, a literature survey

was realized. This survey indicated that 5 out of the 12 hits are linked to regulation of the NF-κB pathway, 4 to the regulation of JNK activation and 2 to the regulation of the p53 signalling pathway in other cell types (Table 2). This indicates that the majority of the hits that affected the death-promoting synergy between diclofenac and TNF-α play a role in the previously identified NF-κB and JNK pathways (9). However, we also identified novel pathways such as the p53, interleukin 6 (IL-6), and Wnt signalling pathways, as well as pathways related to the endoplasmic reticulum (ER) and the regulation of c-myc, indicating that these stress and survival pathways likely co-determine the outcome of diclofenac/TNF-induced apoptosis in liver cells.

4. Discussion

Since drug-induced liver injury is a major concern in a clinical setting, as well as for drug development, it is of outmost importance to understand the underlying molecular mechanisms of DILI. In the current study, we have investigated the role of kinases, (de)ubiquitinases and immune components in the regulation of apoptosis in HepG2-GFP-p65 cells by combinations of siRNA-mediated knockdown followed by exposure to the hepatotoxic drug diclofenac, the pro-inflammatory cytokine TNF-α and the combination diclofenac/TNF-α. In total, 91 genes were identified in the primary screen, of which 12 were confirmed in the re-screen. Within this set of 12 hits, one specifically regulated TNF-α-induced apoptosis, 5 controlled diclofenac-only-induced apoptosis and 6 mediated the TNF-α-enhanced diclofenac-induced apoptosis. To the best of our knowledge, these 12 genes were not previously identified as regulators of drug-induced apoptosis in liver cells.

Most of the validated hits have previously been associated with activation of the NF-κB and/or the JNK signalling pathways, which are two important signalling pathways regulating TNF-α-mediated diclofenac-induced HepG2 apoptosis (9). TRIM45 was shown to be a repressor of both NF-κB and JNK activation (15), and its knock-down sensitizes for diclofenac-induced apoptosis in this study is likely due to deregulation of caspase cascade (16, 17). Since its knock-down enhanced diclofenac-induced apoptosis in HepG2-GFP-p65 cells, it could be that its primary role in these cells is the activation of the NF-κB pathway and therefore the regulation of the expression of anti-apoptotic genes. RIPK1 plays a dual role in controlling cell death and cell survival. It controls TRAIL-induced necroptosis in HepG2 cells (18) and it is required for the activation of JNK1 in DNA damage-induced PARP1-mediated cell death (19). The primary role of

Table 2.Literature based survey on the involvement of the identified hits in apoptosis.

Gene	Type	Identified function	Regulated signalling pathway	Ref
		Hits Diclofenac+TNF-α		
TLR4	Receptor	It activates NF-κB via MyD88 and plays a role in innate immunity. It has a deleterious role in both liver and kidney injury.	Regulation of the NF-κB pathway	(22,38)
CALCOCO2	Calcium binding protein	It is a subunit of nuclear domain 10 (ND10) bodies that are believed to be associated with the nuclear matrix. CALCOCO2 was identified as a novel poly-ubiquitin binder in the NF-κB pathway.	Regulation of the NF-κB pathway	(21,39)
SOCS5	Suppressor of cytokine signalling	Overexpression of SOCS5 partially inhibits interleukin-6 (IL-6) signalling *in vitro*.	Regulation of the IL-6 signalling	(40)
UBE2G2	E2 ubiquitin-conjugating enzyme	It constitutes an essential component of the endoplasmic reticulum-associated degradation (ERAD) pathway in both fibrosarcoma HT1080 and HEK293 cells. The ERAD pathway targets misfolded, unassembled or tightly regulated proteins of the endoplasmic reticulum (ER) for K48-linked poly-ubiquitination and ultimately proteasomal degradation.	Regulation of the degradation of some anti-apoptotic proteins involved in ER stress	(29)
NFKB2	Transcription factor	It is a NF-κB member, also known as p52. After DNA damage p52 dimers are recruited on multiple, p53 dependent and independent, target genes associated with promoting cell cycle arrest and cell death. It was also shown to repress c-myc.	Regulation of p53-dependent apoptosis and/or repression of c-myc	(34)
TNIK	Kinase	The activation of both JNK1 and JNK2 by TNF-α could be blocked through TNIK knockdown, which dampened the AP1 luciferase activity accordingly. In addition, adenovirus mediated the down-regulation of TNIK-triggered intrinsic apoptosis in SW480 cells by activating caspase-9 and PARP-1.	Regulation of JNK activation	(23)

Gene	Type	Identified function	Regulated signalling pathway	Ref
		Hits Diclofenac		
TRIM45	Member of the tripartite motif family	Overexpression of TRIM45 in COS-7 cells inhibits the transcriptional activities of Elk-1 and AP-1. The encoded protein may function as a transcriptional repressor of the mitogen-activated protein kinase pathway. TRIM45 also negatively regulates TNF-α-induced NF-κB-mediated transcription and suppresses cell growth.	Regulation of the NF-κB pathway and JNK activation	(15)
ZNRF3	Cell-surface transmembrane E3 ubiquitin ligase	It is associated with the Wnt receptor complex, and inhibits Wnt signalling by promoting the turnover of frizzled and LRP6.	Regulation of Wnt signalling	(35)
KIAA0317		Nothing known.		
CASP5	Inflammatory caspase	Caspase-5 is expressed only in a restricted manner with its detection in placenta, lung, liver, spleen, small intestine, colon, and peripheral blood lymphocytes. The overexpression of caspase-5 in HeLa and COS cells induces apoptotic cell death. Caspase-5 appears to interplay with caspase-1 in inflammatory responses in human retinal pigment epithelial cells.	Regulation of the caspase cascade in apoptosis and regulation of the NF-κB pathway	(16,17)
RIPK1	Kinase	It promotes cell survival in TNF-α stimulated cells via stabilizing TRAF2 and cIAP1.	Regulation of NF-κB signalling pathway	(20)
		Hit TNF-α		
PRPF19	Pre-mRNA-processing factor	PRPF19 forms a complex with other proteins found at the core of catalytically activated spliceosome and plays a critical role in activation of the spliceosome responsible for pre-mRNA splicing. PRPF19 is involved in selective regulation of p21Cip1 mRNA splicing and counteracts p53-mediated apoptosis.	Regulation of p53-dependent apoptosis	(30,31)

RIPK1, however, is to promote cell survival upon TNF-α stimulation by stabilizing TRAF2 and c-IAP1 (20) to activate the downstream NF-κB response. Since RIPK1 knock-down in HepG2-GFP-p65 cells increases diclofenac-induced cell death, we therefore speculate that RIPK1 importantly regulates the activation of the NF-κB survival pathway rather than that it contributes to the diclofenac-induced apoptotic response. CALCOCO2, identified as a novel poly-ubiquitin binder in the NF-κB pathway (21), was revealed to be an inducer of diclofenac/TNF-α apoptosis in HepG2-GFP-p65 cells. Furthermore, its gene expression was increased upon diclofenac/TNF-α treatment. Even though its role in the regulation of NF-κB activation in HepG2 cells is not yet studied, we hypothesize that CALCOCO2 is involved in the termination of NF-κB activation and thereby potentiates diclofenac/TNF-α synergistic cell death. TLR4, identified as a protector of apoptosis in HepG2-p65-GFP cells, is known to activate the NF-κB pathway. However, activation of TLR4 has been linked to deleterious effects in liver injury (22), which seems in contrast with our results. Such studies were performed *in vivo* and indicated that an up-regulation of the expression of TLR4 was necessary to elicit a weak response in hepatocytes (22). Since we did not observed any gene expression changes in HepG2 cells after TNF-α, diclofenac or diclofenac/TNF-α treatment, it could be that the response in our HepG2-p65-GFP cells only mildly activated the pro-survival NF-κB pathway without affecting other transcription factors known to be activated by TLR4 such as AP-1. The kinase TNIK was shown to regulate the activation of both JNK1 and JNK2 (23) which explains why its knock-down protected the HepG2-GFP-p65 cells against diclofenac/TNF-α-induced apoptosis.

In addition to the NF-κB and JNK pathways, other signalling pathways were identified to regulate HepG2 apoptosis, such as the p53 signalling pathway. TNF-α has been shown to activate p53 in several cancer cells (24-28). The uniquely identified gene controlling TNF-α-induced apoptosis, PRPF19, was shown to be part of the spliceosome complex that regulates pre-mRNA splicing (29, 30) and in particular, PRPF19 was shown to be involved in selective regulation of p21Cip1 mRNA splicing and to counteract p53-mediated apoptosis in cancer cells (31). p21 plays an important role in regulating the hepatocyte cell cycle, differentiation and liver development (32) and the p21 gene was shown to be constitutively expressed in HepG2 cells (33). Since the knock-down of PRPF19 in HepG2 cells induced apoptosis via TNF-α, we speculate that PRPF19 controls the inhibition of p53-dependent apoptosis in HepG2 cells. The NF-κB member NF-κB2 was also shown to regulate the p53 signalling pathway by promoting p53-dependent apoptosis after DNA damage (34). Therefore, protection against diclofenac/TNF-α synergistic apoptosis by knock-down of NF-κB2 in HepG2-GFP-p65

cells is possibly due to inhibition of p53-dependent apoptosis. The Wnt signalling pathway was also identified as a pathway regulating diclofenac-induced apoptosis of HepG2 cells. ZNRF3 is known to associate with the Wnt receptor complex leading to its inhibition (35). It was previously shown that the Wnt/β-catenin signalling transduction pathway is activated with aberrant expression of Wnt1 in HepG2 cells (36) and it is well known that the Wnt signalling can control apoptosis (37). Since knock-down of ZNRF3 resulted in increased diclofenac-induced apoptosis and its gene expression was down-regulated with diclofenac treatment in HepG2 cells, we propose that this protein is an important regulator of Wnt signalling activation thereby controlling the apoptosis of HepG2 cells. Other identified signalling pathways include the regulation of proteasomal degradation in the ER and the regulation of the IL-6 signalling.

In summary, by using a targeted siRNA approach in combination with fluorescence-based apoptosis measurements, we were able to identify 12 novel regulators of TNF-α, diclofenac and diclofenac/TNF-α-induced apoptosis in HepG2 cells. A plausible role of those hits in the regulation of HepG2 apoptosis was drawn from literature knowledge, however, further validation studies of the hits need to be done in order to confirm this.

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