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Camptothecin and its analog SN-38, the active metabolite of irinotecan, inhibit binding of the transcriptional regulator and oncoprotein FUBP1 to its DNA target sequence *FUSE*

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Abstract

The transcriptional regulator *FUSE* Binding Protein 1 (FUBP1) is overexpressed in more than 80% of all human hepatocellular carcinomas (HCCs) and other solid tumor entities including prostate and colorectal carcinoma. FUBP1 expression is required for HCC tumor cell expansion, and it functions as an important pro-proliferative and anti-apoptotic oncoprotein that binds to the single-stranded DNA sequence *FUSE* to regulate the transcription of a variety of target genes. In this study, we screened an FDA-approved drug library and discovered that the Topoisomerase I (TOP1) inhibitor camptothecin (CPT) and its derivative 7-ethyl-10-hydroxycamptothecin (SN-38), the active irinotecan metabolite that is used in the clinics in combination with other chemotherapeutics to treat carcinoma, inhibit FUBP1 activity. Both molecules prevent *in vitro* the binding of FUBP1 to its single-stranded target DNA *FUSE*, and they induce deregulation of FUBP1 target genes in HCC cells. Our results suggest the interference with the FUBP1/*FUSE* interaction as a further molecular mechanism that, in addition to the inactivation of TOP1, may contribute to the therapeutic potential of CPT/SN-38. Targeting of FUBP1 in HCC therapy with SN-38/irinotecan may be a particularly interesting option because of the high FUBP1 levels in HCC cells and their dependency on FUBP1 expression.

Keywords:

FUBP1; AlphaScreen; camptothecin; SN-38; HCC

Chemical compounds cited in this article

Camptothecin (PubChem CID: 24360); SN-38 (PubChem CID: 104842); irinotecan (PubChem CID: 60838), sorafenib (PubChem CID: 216239), topotecan (PubChem CID: 60700)

1. Introduction

Far Upstream Element Binding Protein 1 (FUBP1) is required for hepatocellular carcinoma (HCC) tumorigenesis, and our previous studies demonstrated that the shRNA-mediated downregulation of FUBP1 sensitizes HCC cells for apoptosis-inducing chemotherapeutic drugs like mitomycin C (1, 2). FUBP1 was originally found as a transcriptional activator of the proto-oncogene *c-myc* that increases transcription levels of *c-myc* upon binding to the single-stranded *FUSE* DNA sequence upstream of the promoter (3). The protein interacts with the *FUSE* element via its central DNA-binding domain, which consists of four tandem K homology (KH) repeats and forms a DNA-binding furrow. Besides *c-myc*, FUBP1 is known to directly repress the cell cycle inhibitor gene *p21*, and further target genes include *CCND2* (*Cyclin D2*), the pro-apoptotic BCL2 family member *BIK*, and *TCTP* (4, 5). FUBP1 is upregulated not only in HCC, but also in other solid tumor entities, such as prostate and colorectal cancer or clear cell renal cell carcinoma (6-8). Taken together, FUBP1 functions as a potent oncoprotein that controls sensitive regulators of cell fate at the transcriptional level. Therefore, FUBP1 with its characteristic DNA-binding cleft represents an attractive target for future molecular cancer therapies.

Recent attempts have led to the identification of two compound classes that interfere with FUBP1 functionality. Using a NMR high throughput screen (HTS) approach, Huth et al. demonstrated that benzoyl anthranilic acid interacts with the hydrophobic region of the FUBP1 KH3 domain (9). However, as FUBP1 inhibitors, this substance class is severely limited because of poor solubility and unacceptable high IC_{50} values. In our own work, we identified pyrazolo[1,5a]pyrimidines as a new class of FUBP1 inhibitors (10). However, when we tried to further optimize the most promising

pyrazolo[1,5a]pyrimidine derivative to achieve lower IC_{50} values, we started to lose biological activity.

In this study, we used a bead-based proximity assay (AlphaScreen) to screen a small FDA-approved drug library to find already approved drug substances that would prevent the binding of FUBP1 to *FUSE* DNA and thereby interfere with the ability of FUBP1 to regulate tumor-relevant gene expression. We describe the identification and validation of camptothecin (CPT) and its clinically used analog SN-38, the active metabolite of the prodrug irinotecan, as potent inhibitors of the FUBP1/*FUSE* interaction.

2. Materials and methods

2.1 Cell Lines and Materials

Cell lines used in this study included human embryonic kidney cells (HEK293T, DSMZ (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany; no. ACC-635)), and the human hepatocellular carcinoma cell lines Hep3B (ATCC no. HB-8064) and HepG2 (ATCC no. HB-8065). CPT was purchased from *Santa Cruz* (Dallas, USA), SN-38 and sorafenib from *Selleckchem* (Boston, USA), topotecan (TTN) from *Sigma-Aldrich Chemie GmbH* (Steinheim, Germany). Oligonucleotides for SPR and qRT-PCR experiments were synthesized by *Biospring GmbH* (Frankfurt am Main, Germany).

2.2 Cell culture and chemotherapeutic treatment

Hep3B cells were cultured in Advanced MEM and HepG2 in RPMI medium. Both media contained 10% FCS, 1% penicillin/streptomycin (10 µg/ml) and 1% L-glutamine (100 mM). CPT (*Santa Cruz*) and SN-38 (7-ethyl-10-hydroxycamptothecin; *Selleckchem*, Boston, MA, USA) were pre-diluted in 100% DMSO to a concentration of 10 mM and 1 mM, respectively. Sorafenib (*Selleckchem*) was dissolved in 100% DMSO at a stock concentration of 10 mM. The cell culture medium was replaced by fresh medium containing the defined compound concentrations.

2.3 FUBP1 protein expression from *E.coli* bacteria and human HEK293T cells

Human full-length *FUBP1* cDNA sequence (codon-optimized for expression in *E. coli*; *coFUBP1*) was purchased from *GenScript* (New Jersey, USA). The *coFUBP1* sequence was cloned into the bacterial expression vector *pET28b* with a C-terminal

hexahistidine tag (6xHis) under the control of a *T7* promoter. Expression of coFUBP1 in the *E. coli* bacteria strain *BL21(DE3)* was induced via a published auto-induction protocol (11). A freshly prepared overnight culture was diluted 1:100 to inoculate the main culture in a 10 l fermenter. Cells were grown to an optical density (OD_{600}) of 0.8. Afterwards, the temperature was reduced from 37°C to 22°C, and cells were grown for 35 hours before harvesting. To prevent excessive foam formation, 2 ml anti-foam were added daily. The ventilation of the system was ensured by a constant airflow between 4 and 12 l/min.

For cell lysis, the bacterial pellet from a 5 liter auto-induced bacterial cell suspension was resuspended in lysis buffer (50 mM MES pH 6.5, 50 mM NaCl), including *Complete* EDTA-free Protease Inhibitor Cocktail (*Roche*, Mannheim, Germany) and DNase I (*Sigman-Aldrich*). Subsequently, the cell suspension was processed in the Constant Cell Disruption System (*Constant System Limited*, Northants, United Kingdom) using a three-step protocol. The cell suspension was applied with a pressure of 1 kbar, followed by two runs at 2 kbar. All steps were performed at 4°C. The soluble protein fraction was separated from cell debris using a 50 min. centrifugation step (20,000 x g; 4°C).

For eukaryotic expression in HEK293T cells, the *huFUBP1* coding sequence with an additional 6xHis tag was cloned into the *pcDNA3.1(+)* vector (*Invitrogen*, Darmstadt, Germany). Cells were transfected using polyethylenimine (*Sigma-Adrich Chemie GmbH*, Steinheim, Germany), harvested after two days of protein production and lysed using the following lysis buffer: 50 mM Tris (pH 7.4), 0.26 M sucrose, 1 mM Na-ortho-vanadate, 1 mM EDTA, 1 mM EGTA, 10 mM Na- β -glycerolphosphate, 50 mM NaF, 5 mM Na-pyrophosphate and 1% Triton-X-100.

2.4 FUBP1 protein purification by IMAC, Heparin affinity chromatography and size exclusion chromatography (SEC)

The following purification steps were performed at 4°C, using the Äkta purifier[®] system (*GE Healthcare*, Munich, Germany). The cleared lysate was loaded onto a self-packed Omnifit[®] Chromatography Column (*Diba Industries*, Danbury, USA) with Ni-Sepharose High Performance affinity media for high-resolution (*GE Healthcare*) at a ratio of 25:1 for coFUBP1 and 140:1 for huFUBP1 and a flow rate of 2.5 ml/min. After absorption of the His-tagged protein by the column, it was equilibrated with 4 column volumes (CVs) of washing buffer (coFUBP1: 50 mM MES pH 6.5, 50 mM NaCl, 4 mM Imidazole; huFUBP1: 50 mM Tris pH 7.4, 0.26 M sucrose, 0.05% (v/v) β -mercaptoethanol, PMSF), using a flow rate of 4 ml/min. Elution of the protein was achieved with an imidazol gradient (2 ml/min), and the protein of interest eluted at approximately 50 - 60% of the final elution buffer (coFUBP1: 50 mM MES pH 6.5, 50 mM NaCl, 404 mM Imidazole; huFUBP1: 50 mM Tris pH 7.4, 0.26 M sucrose, 400 mM imidazole, 0.05% (v/v) β -mercaptoethanol, PMSF), corresponding to an imidazole concentration of 220 mM. Fractions displaying an elution peak at A_{280} absorption were analyzed by SDS-PAGE electrophoresis.

The FUBP1-containing fractions were pooled and applied to HiTrap heparin HP columns (*GE Healthcare*), 5 x 5 ml for coFUBP1 and 1 x 5 ml for huFUBP1, with a flow rate of 2.5 ml/min for purification of DNA-binding proteins. The column was washed with 10 CVs of heparin washing buffer (50 mM MES pH 6.5, 50 mM NaCl, 3 mM DTT), using a flow rate of 2 ml/min. For elution of the FUBP1 protein, a 3-step gradient of heparin elution buffer (50 mM MES pH 6.5, 2 M NaCl, 3 mM DTT) was applied: 1.: 0 - 17% of final heparin elution buffer for 51 CVs, 2.: 17 - 21% for 15 CVs; and 21 to 28% for 18 CVs.

For further purification by SEC, FUBP1-containing fractions were pooled, tenfold concentrated using an *Amicon* stirr cell system (first concentration step using a 10 kDa membrane and a subsequent step with a 30 kDa membrane; *Millipore GmbH*, Schwalbach/Ts., Germany) and applied to a HiLoad 16/60 Superdex 200 column (*GE Healthcare*; 0.5 ml/min). SEC fractions containing recombinant protein were pooled, and the identity of the recombinant FUBP1 protein was verified by Western Blot and mass spectrometry analyses.

2.5 Screening of potential FUBP1 inhibitors using AlphaScreen technology

To identify potential FUBP1 inhibitors, an AlphaScreen protocol was established to screen one of the Prestwick Chemical Libraries[®] (*Prestwick Chemical*, Illkirch, France) that contains 1,280 mostly approved drugs (FDA, EMA and other agencies; <http://www.prestwickchemical.com/prestwick-chemical-library.html>). Bacterially expressed recombinant coFUBP1 was coupled to protein A acceptor beads via anti-FUBP1 antiserum (N-15, *Santa Cruz Biotechnology*), and 5'-biotinylated *FUSE* oligonucleotide was bound to streptavidin donor beads included in the Protein A Acceptor Bead Kit by *Perkin Elmer* (Waltham MA, USA). Compounds from the library (diluted in DMSO) were pipetted into 384 well plates (final concentration 50 μ M). 10 μ l of a mixture containing 3 nM coFUBP1, 1.6 nM *FUSE*, 3 μ M poly dIdC, 10 pM anti-FUBP1 antibody (N-15) and acceptor beads (1:50 diluted) were then added. After one hour of incubation at room temperature, 5 μ l donor beads (1:50 diluted in AlphaScreen assay buffer (100 mM Tris pH 7.4, 50 mM NaCl, 0.1% BSA, 0.01% Surfact Amp, 4 mM DTT)) were included. Following an additional 20 hour incubation step at room temperature, the screening results were quantified using the Envision multilable plate reader SN 1040002 (*Perkin Elmer*). Potential hits were defined as

inhibitory molecules that diminished the signal intensity generated by donor and acceptor bead proximity to 30% or less.

2.6 Surface Plasmon Resonance (SPR)

SPR experiments were performed using a ProteOn™ XPR36 system (BIO-RAD, Munich, Germany). The *FUSEp21* DNA (representing the *FUSE* element 3.2 kb upstream of the human *p21* promoter (2)) was synthesized as a 53 nucleotide (nt) long single-stranded oligonucleotide (sequence: 5'-ctggcttttgttttcattttgtttttgttttgttttggagacaa-3') by *Biospring GmbH*. The single-stranded oligonucleotide *FUSEp39* (5'-tttgttttgtttttgagacaaagt -3'; (2)) served as a non-binding negative control. The oligonucleotides *FUSEp21* or *FUSEp39* were immobilized on NLC Sensor Chips (BIO-RAD) with final response units (RUs) of 20 to 60 and flushed with a dilution series of huFUBP1, coFUBP1 or Maltose-Binding Protein (MBP; nonbinding protein control) ranging in concentration from 10 to 200 nM for the dissociation constant determination of recombinant protein. In the reverse setting, the recombinant proteins were immobilized with final RUs of 1,500 (MBP) and 3,600 (coFUBP1) on GLM Sensor Chips (BIO-RAD) and flushed with single-stranded DNA oligonucleotides in the liquid phase. IC₅₀ values were generated with immobilized *FUSEp21* and 100 nM huFUBP1 in the liquid phase after prior incubation with a dilution series of 0.01 - 100 µM compound (e.g. CPT or SN-38). All data obtained were normalized to the *FUSEp39* channel (negative control), and analyses were performed in the equilibrium mode, fitting to a Langmuir 1:1 binding model.

2.7 Microscale Thermophoresis (MST)

For the MST measurements, the recombinant coFUBP1 protein was labelled using the NT.115 Protein Labeling Kit RED-NHS (*NanoTemper Technologies*, Munich, Germany) with the amine-reactive red fluorescent dye NT-647 according to the manufacturer's manual. 1 nM to 100 μ M of unlabelled single-stranded *FUSEp21* oligonucleotide were titrated to a constant concentration of 1 μ M coFUBP1 protein. The experiments were performed in a Monolith NT.115TM device using MonolithTM Standard Treated Capillaries, and fluorescence was excited with red LED light (100%) at an intensity of 15%.

2.8 Lentiviral knockdown of FUBP1 expression

Lentiviral knockdown of FUBP1 expression in Hep3B cells was performed as described in (2).

2.9 Western Blot Analysis

FUBP1 expression was detected via immunoblot using an anti-FUBP1 (1:1,000; Santa Cruz, clone N-15) or an anti-HIS antibody (1:2,000; Penta HIS, *Quiagen GmbH*, Hilden, Germany) and a secondary rabbit anti-goat antibody (1:10,000; Invitrogen, cat. no. 81-1620). β -Actin levels were assessed as a loading control using a goat-derived antiserum (1:2,000; clone C11, *Santa Cruz Biotechnology*) together with the same secondary anti-goat antibody used for FUBP1 detection. Western Blot signals were quantified using the *Fusion Fx* system (*Vilber Lourmat*, Eberhardzell, Germany), and FUBP1 protein levels were normalized relative to β -Actin expression.

2.10 RNA Isolation, cDNA Synthesis and qRT-PCR

RNA isolation from mammalian cells was performed using the RNeasy[®] Mini Kit (*Qiagen*) according to the manufacturer's manual with 1×10^6 cells per condition. Beforehand, cells were incubated for 6 hours with the indicated drugs or solvent control. 1.5 μ g isolated RNA was transcribed into cDNA using the Omniscript[®] Reverse Transcription Kit (*Qiagen*) according to the manufacturer's manual with additional on-column DNase digestion. mRNA expression levels were quantified using a LightCycler480 (*Roche*) with 4-titude 96-well plates and SYBR[®] Green real-time PCR Master Mix (*life technologies*, Darmstadt, Germany). qPCR reactions were performed in technical duplicates and biological triplicates in a total reaction volume of 25 μ l. mRNA levels were normalized to *GAPDH* expression and calculated according to the $2^{-\Delta\Delta C_t}$ method (12). The results were normalized to gene expression in DMSO-treated control cells.

2.11 Primer Sequences for qPCR analysis

GAPDH for: 5'- aatggaaatcccatcaccatct -3'

GAPDH rev: 5'- cgccccacttgattttgg -3'

p21 for: 5'- tggagactctcagggtcgaa -3'

p21 rev: 5'- ccggcgtttgagtgga -3'

BIK for: 5'- cttgatggagaccctcctgtatg -3'

BIK rev: 5'- aggtccaggtcctcttcaga -3'

TCTP for: 5'- gccatgtggatatcaggacaa -3'

TCTP rev: 5'- ccttctgtcttcttgggta -3'

CCND2 for: 5'- cagggtgggagaagaaatag -3'

CCND2 rev: 5'- ctacctccaattcccacacta -3'

2.12 Cell Expansion Assay

1×10^5 Hep3B cells were seeded into 6-well plates and treated with DMSO (as a control), camptothecin, or sorafenib in increasing concentrations up to 72 hours. After 24 hours, 48 hours and 72 hours, cells were collected, stained with crystal blue and counted in a Neubauer improved counting chamber.

2.13 Statistical Analysis

Statistical analysis was performed with GraphPad Prism software applying two-tailed t test and variable slope test (four parameters). p values < 0.05 (*), $p < 0.01$ (**), $p < 0.001$ (***) and $p < 0.0001$ (****) were considered statistically significant.

3. Results

3.1 Recombinantly expressed FUBP1 protein binds with high affinity to its single-stranded target DNA sequence FUSE

Codon-optimized human full length FUBP1 protein was expressed using an *E. coli* auto-induction system (11). Subsequently, the protein was purified via a 3-step protocol including immobilized metal ion affinity, heparin affinity and size exclusion chromatography, and its identity was confirmed by Western blot analysis (**Fig. 1A**) and by mass spectrometry. We then determined the specific binding capacity of the protein to its single-stranded target DNA sequence *FUSE* by surface plasmon resonance (SPR) measurements. For this purpose, we immobilized the recombinant FUBP1 protein to the surface of a GLM sensor chip, and a 53 nt-long single-stranded DNA oligonucleotide, which contained the *FUSE* sequence identified upstream of the human *p21* gene (*FUSEp21*; (2)), was added as the analyte in the liquid phase (see **Fig. 1B**). As shown in **Fig. 1C** (upper left panel), the recombinant protein tightly bound to its target *FUSEp21* DNA with a dissociation constant K_D of 11.8 nM, while it did not interact with the single-stranded DNA oligonucleotide *FUSEp39* (**Fig. 1C**, lower left panel). This negative control oligonucleotide consists of an unrelated non-*FUSE* DNA sequence and is not bound by FUBP1, as demonstrated previously in an Electrophoretic Mobility Shift Assay (EMSA; (2)). A preparation of recombinant Maltose Binding Protein (MBP) served as a negative control protein that did not interact with the *FUSEp21* and *FUSEp39* DNA oligonucleotides in our SPR experiments (**Fig. 1C**, right panels).

We wanted to employ an independent second method to confirm the functionality of our recombinant FUBP1 protein and its binding to the *FUSE* target DNA sequence.

Microscale thermophoresis (MST) represents such an orthogonal methodology to quantify the strength of interactions which allows both unbound binding partners the maximum degree of freedom in liquid phase. Our MST experiments presented in **Fig. 2** revealed a K_D of 12.3 nM for the binding of bacterially expressed FUBP1 to the single-stranded *FUSEp21* oligonucleotide, a result in excellent accordance to our SPR-derived K_D value.

To exclude the possibility that bacterially expressed FUBP1 protein is lacking important post-translational modifications that would influence its interaction with the *FUSE* DNA sequence in the subsequent AlphaScreen setup, we expressed recombinant human FUBP1 in the human embryonic kidney cell line HEK293T. The protein was purified with the same 3-step protocol that was used for purification of bacterially expressed FUBP1. We reversed the set-up of the previous SPR experiments and immobilized a biotinylated version of the single-stranded 53 nt-long *FUSEp21* DNA oligonucleotide on the surface of a NeutrAvidin-coupled Sensor Chip. The recombinant FUBP1 protein that was expressed in HEK293T cells was added as the analyte in the liquid phase (**Fig. 3A**). Again, FUBP1 protein bound tightly to its target *FUSEp21* DNA with a dissociation constant K_D of 10.4 nM (**Fig. 3B**), which is in the same range as the K_D value of 11.8 nM that we had obtained before in SPR experiments for the interaction of bacterially expressed recombinant FUBP1 with the *FUSEp21* DNA oligonucleotide.

3.2 Binding of recombinant FUBP1 to its target FUSE DNA sequence can be efficiently inhibited by camptothecin and SN-38

To identify small molecules that would bind to FUBP1 and inhibit its interaction with single-stranded *FUSE* DNA and its function as an oncoprotein, we coupled

recombinant FUBP1 protein and the biotinylated *FUSEp21* oligonucleotide to donor and acceptor beads to perform an *Amplified luminescent proximity homogeneous assay* (AlphaScreen) binding assay with a *Prestwick*® chemical library that consists of 1,280 small molecules most of which represent approved drugs. Cross titrations to determine the optimal FUBP1 and *FUSEp21* concentrations and a validation experiment with increasing concentrations of free *FUSEp21* oligonucleotide added to the beads were performed as previously described (10). With the optimized AlphaScreen set-up, we screened the drug library and identified the natural cytotoxic quinoline alkaloid camptothecin (CPT; (13)) as a potent inhibitor of the interaction between FUBP1 and *FUSE*. We quantified the inhibitory concentration of 50% (IC_{50}) of CPT for its capacity to prevent FUBP1/*FUSE* interactions in additional SPR experiments as 3.2 μ M (**Fig. 4A**).

Next, we tested whether the close CPT analog 7-ethyl-10-hydroxycamptothecin (SN-38), the active metabolite of irinotecan, which is used in clinical applications instead of CPT due to its low solubility (14), is also capable of interfering with the FUBP1/*FUSEp21* interaction. Indeed, incubation of recombinant FUBP1 with SN-38 reduced the binding of FUBP1 to the single-stranded *FUSEp21* oligonucleotide to a similar extent as CPT did, with an IC_{50} value of 1.9 μ M as quantified by SPR measurements (**Fig. 4B**) and an IC_{50} value of 0.78 μ M as determined by AlphaScreen experiments (**Fig. 4C**).

3.3 Inhibition of FUBP1 by SN-38/CPT leads to deregulated FUBP1 target gene expression and inhibits HCC cell expansion

We screened for a FUBP1 inhibitor based on its ability to prevent or disrupt the binding of the protein to its single stranded target DNA *FUSE*, which would ultimately

lead to the deregulation of FUBP1 target genes. The knockdown of *FUBP1* resulted in increased mRNA levels of *p21* and *BIK* as well as decreased *CCND2* (*Cyclin D2*) and *TCTP* (*transcriptionally controlled tumor protein*) expression levels in HCC cells (2, 4). Therefore, we performed qRT-PCR experiments to detect changes in the expression levels of these FUBP1-regulated genes as a molecular readout to confirm the inhibition of FUBP1 by SN-38 or CPT in HCC cells. Treatment of the HCC cell line Hep3B with SN-38 led to a significant increase in *p21* and *BIK* levels and a decrease in *CCND2* and *TCTP* mRNA expression levels, while FUBP1 protein levels remained unchanged (**Fig. 5A**). Similar results were obtained for *p21* and *BIK* in the HCC cell lines Hep3B and HepG2 upon incubation with CPT (**Fig. 5B, C**), suggesting that SN-38 and CPT interfere with FUBP1 functionality in cells. The increase of *p21* protein in Hep3B and HepG2 cells upon SN-38 treatment, which was expected from the increased *p21* mRNA levels, was confirmed by Western blot analysis (**Fig. 5D**).

Finally, we monitored the expansion of an established HCC cell line following incubation with CPT by counting cell numbers. The single treatment of Hep3B cells with 1 μ M CPT completely arrested cell expansion after 48 hours, while normal exponential cell growth was still observed in the presence of 1 μ M of the multi-kinase inhibitor sorafenib (**Fig. 6**), which represents the standard HCC chemotherapeutic (15). This CPT-induced expansion stop is in line with our previous finding of reduced proliferation in *FUBP1* knockdown HCC cells (2).

3.4 The TOP1 inhibitor and CPT derivative topotecan (TTN) does not interfere with the FUBP1/FUSE interaction and does not alter FUBP1 target gene expression

Since CPT and SN-38 not only prevent binding of FUBP1 to its target DNA sequence *FUSE*, but also represent established Topoisomerase I (TOP1) inhibitors (for review

see (14, 16, 17)), we used the potent TOP1 inhibitor and chemical CPT derivative TTN (18) for further studies to distinguish the cellular consequences of FUBP1 and TOP1 inhibition. The chemical structures of CPT, SN-38 and TTN, that are based on the identical planar pentacyclic ring structure, are very similar and closely related (see **Fig. 7A**). SN-38 and TTN induced DNA double strand breaks to a comparable extent as determined by γ -H2AX experiments, indicating a similar level of TOP1 inhibition by these substances in our assay (data not shown). However, in contrast to SN-38, TTN was not able to prevent the binding of FUBP1 to single-stranded *FUSE_{p21}* DNA as determined by SPR and AlphaScreen experiments (**Fig. 7B, C**). Of note, unlike SN-38 and CPT, TTN treatment of Hep3B cells did not lead to a significant upregulation of the FUBP1 target genes *p21* and *BIK* (**Fig. 7D**). These findings confirm the importance of FUBP1 (rather than TOP1) as a SN-38 target protein for the upregulation of *p21* and *BIK* expression in HCC cells. This conclusion is supported by our observation that the upregulation of *p21* upon *FUBP1* knockdown in Hep3B cells is not further increased when the FUBP1-deficient cells (expressing wildtype levels of TOP1) were subsequently treated with SN-38 (**Fig. 7E**).

4. Discussion

Large scale screenings to identify substances with biological activity of therapeutic interest represent a key step in modern drug development. Substance libraries that consist of already approved drugs provide the great advantage that if these drugs are tested for other diseases than the ones they are approved for (“repurposing”), usually the preclinical (safety) data required for clinical studies already exist. In such cases, the drugs can be tested in patients within a relatively short time frame.

We decided to screen a FDA-approved drug library for effective small molecule inhibitors of the transcriptional regulator FUBP1. The protein represents an attractive target molecule that is overexpressed in several solid tumor entities (6-8). For HCC, its pro-proliferative and anti-apoptotic oncogenic signaling properties were demonstrated (1, 2). The remarkable overexpression of FUBP1 in most of the HCCs and the increased apoptosis sensitivity of HCC tumor cells following the knockdown of *FUBP1* (2) suggested this molecule as a promising molecular target for chemotherapeutic treatment using a FUBP1 inhibitor, possibly in combination with a cytotoxic drug.

First, we set up an *in vitro* AlphaScreen system, and for this purpose, we expressed full-length recombinant FUBP1 both in bacteria and in human HEK 293T cells. After purification, we demonstrated the strong interaction between the recombinant protein and a single-stranded DNA oligonucleotide that represents the sequence of the *p21 FUSE* element (2). Both recombinant protein preparations from bacteria and human cells bound to the *FUSEp21* oligonucleotide with a K_D value in the low nanomolar range (**Fig. 1C, Fig. 2, Fig. 3B**), suggesting that no posttranslational modifications of

FUBP1 are required for the FUBP1/*FUSE* interaction that would only occur in mammalian cells.

We then coupled the recombinant protein and the single-stranded *FUSEp21* DNA oligonucleotide to donor and acceptor beads and performed an AlphaScreen with a FDA-approved drug library. Out of the 1,280 small molecule drugs, we identified CPT as a potent antagonist of FUBP1 that would prevent the interaction of recombinant FUBP1 with *FUSEp21* in AlphaScreen and SPR *in vitro* experiments (**Fig. 4**). CPT was originally discovered as an inhibitor of Topoisomerase 1, and its close homologue SN-38, the active metabolite of irinotecan, is currently used in the combination regimen FOLFIRI (infusional therapy with irinotecan, 5-fluorouracil (5-FU) and leucovorin (19)) for first line treatment of colon cancer patients (but not HCC patients) because of its superior solubility compared to CPT (20). Our cell culture experiments confirmed the deregulation of FUBP1 target gene expression upon incubation of HCC cells with SN-38/CPT (**Fig. 5**), supporting the notion that both substances interfere with the transcriptional control of FUBP1. In a first experiment to investigate the potential therapeutic value of CPT as an FUBP1 inhibitor in HCC cells, we incubated the HCC cell line Hep3B with the substance and observed that the cell numbers did not increase in a 72-hours expansion analysis. In comparison, the multi-kinase inhibitor and gold standard for HCC therapy, sorafenib, was not able to diminish cell expansion when used at the same concentration as CPT (**Fig. 6**).

Because CPT and SN-38 are well-established TOP1 inhibitors ((14, 16, 17) and references therein), we addressed the question whether in addition to inactivating TOP1 the inhibition of FUBP1 may be relevant for the cellular effects we observed upon treatment of HCC cells with CPT or SN-38. For this purpose, we analyzed the

TOP1 inhibitor TTN, which is closely related to CPT and SN-38. The results showed that, in contrast to SN-38/CPT, TTN does not interfere with the binding of FUBP1 to its target DNA sequence *FUSE*, despite its almost identical chemical structure. Consequently, and despite the inhibition of TOP1, upregulation of the FUBP1 target genes *p21* and *BIK* was not observed upon treatment of Hep3B cells with TTN (**Fig. 7A-D**). Similarly, the upregulation of *p21* in *FUBP1* knockdown cells could not be further increased upon concurrent treatment of the FUBP1-deficient cells with SN-38, despite inhibition of the established SN-38 target TOP1 in these cells (**Fig. 7E**).

The inactivation of transcriptional FUBP1 activity by CPT and SN-38 adds a further possible mode of action for these established chemotherapeutic drugs, which, until today, have only been linked to the inhibition of TOP1. Further studies will reveal whether both molecular mechanisms contribute to the therapeutic potential of SN-38. The identification and optimization of an FUBP1-specific inhibitor that does not interfere with TOP1 function would allow to separate the influence of both pathways. HCC represents a particular interesting tumor entity for the application of an FUBP1 inhibitor because of the high FUBP1 level in HCC cells and their dependence on FUBP1 expression as demonstrated in our *FUBP1* knockdown experiments.

Until today, TACE therapy of intermediate HCC patients lacks a standard in treatment panels and the choice of chemotherapeutic agents. We had previously published that interfering with FUBP1 activity, e.g. by *FUBP1* knockdown, sensitizes HCC cells for the cytotoxic activity of established chemotherapeutics (2). Our data support a rationale for a combinatorial treatment of HCCs with SN-38 and, for example, mitomycin c (MMC). MMC is regularly used for HCC TACE therapy, and both drugs, irinotecdan and MMC, represent approved and affordable

chemotherapeutics. It will be interesting to test this combination therapy in appropriate HCC mouse models and possibly in human HCC patients during the course of a clinical trial.

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Conflicts of interest

none

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Figure legends

Figure 1: Bacterially expressed recombinant FUBP1 binds tightly to the *FUSEp21* oligonucleotide.

A. Purified, recombinant His-tagged FUBP1 protein was detected using coomassie staining (left) and western blot analysis with an anti-His antibody (right). **B.** SPR measurements were performed with bacterially expressed recombinant FUBP1 protein immobilized via amine coupling to a GLM sensor chip for SPR analysis and the single-stranded *FUSEp21* DNA oligonucleotide as the analyte in the liquid phase. **C.** Top row, left panel: Immobilized recombinant FUBP1 and free single-stranded *FUSEp21* oligonucleotide interacted with a K_D of 11.8 nM. Top row, right panel: The unrelated recombinant protein maltose binding protein (MBP) did not bind to *FUSEp21*. Bottom row: Neither FUBP1 (left) nor MBP (right) interacted with the negative control single-stranded DNA oligonucleotide *FUSE39*.

Figure 2: MST experiments confirm binding of recombinant full length FUBP1 protein to the *FUSEp21* oligonucleotide in liquid phase.

Label-free microscale thermophoresis (MST) analysis with recombinant FUBP1 protein and the single-stranded *FUSE* oligonucleotide *FUSEp21* revealed a binding constant K_D of 12.3 nM for the interaction.

Figure 3: SPR analysis validates a strong interaction of recombinant FUBP1 protein expressed in human cells to the *FUSE* element.

A. SPR measurements were also performed with the *FUBP1p21* oligonucleotide immobilized to a NeutrAvidin-coupled NLC Sensor Chip and FUBP1 full length protein expressed in HEK293T cells as the analyte in the liquid phase. **B.** SPR

analysis in this set up again revealed a tight binding of FUBP1 protein to the *FUSEp21* oligonucleotide with a K_D of 10.4 nM.

Figure 4: Camptothecin and its close homologue SN-38 inhibit binding of recombinant FUBP1 to the single-stranded *FUSE* DNA sequence.

A dose-dependent interference with the FUBP1/*FUSEp21* interaction upon addition of CPT (**A**) or SN-38 (**B**, **C**) was observed in SPR (**A**, **B**) and AlphaScreen (**C**) measurements. The IC_{50} value of CPT was calculated as $3.2 \pm 0.6 \mu\text{M}$ (SPR, (**A**)), the IC_{50} value of SN-38 as $1.9 \pm 0.7 \mu\text{M}$ (SPR, (**B**)) and $0.78 \pm 0.2 \mu\text{M}$ (AlphaScreen, (**C**)). The curves in **A-C** show one representative experiment each, whereas the IC_{50} values were quantified from three independent measurements.

Figure 5: Treatment of HCC cell lines with SN-38 or CPT leads to deregulated FUBP1 target gene expression.

A. Following incubation of Hep3B cells with SN-38, mRNA expression levels of FUBP1 target genes (*p21*, *BIK*, *CCND2* and *TCTP*) were significantly deregulated, whereas FUBP1 protein levels were not influenced. **B**, **C.** mRNA expression levels of *p21* and *BIK* were significantly increased in Hep3B (**B**) and HepG2 (**C**) cells treated with $1 \mu\text{M}$ CPT, while the FUBP1 protein levels remained stable. **D.** p21 protein levels increase in Hep3B and HepG2 cells after treatment with $1 \mu\text{M}$ SN-38 for six hours. In **A-D**, representative examples of the Western blot analyses are shown, and the bar charts represent the quantification results for FUBP1 and p21 protein expression that were obtained with the *Fusion Fx* system. Three independent Western blot analyses were used for each quantification. qPCR data represent the mean \pm standard deviation of three biological replicates, each consisting of technical duplicates.

Figure 6: Incubation with CPT inhibits the expansion of the HCC cell line Hep3B.

72-hour cell expansion assays of Hep3B cells treated with 1 μ M CPT or 1 μ M sorafenib in comparison with untreated and DMSO solvent-treated control cells. The data represent the mean \pm standard deviation of three biological triplicates, each performed in technical duplicates.

Figure 7: The TOP1 inhibitor topotecan does not prevent FUBP1 binding to FUSE.

A. The structural formula of camptothecin, SN-38 and topotecan reveal the close relationship and high similarity of all three substances. **B, C.** SPR (**B**) and AlphaScreen (**C**) assays showed no inhibition of FUBP1 binding to *FUSE* DNA by TTN, in contrast to SN-38 that was included as a positive control. The graphs show one representative experiment out of three independent replicates. **D.** mRNA expression levels of the FUBP1 target genes *p21* and *BIK* are not affected in Hep3B cells following drug exposure to TTN. The experiments were performed in biological triplicates. **E.** Left panel: lentiviral transduction of Hep3B cells with *shFUBP1* leads to significant *FUBP1* downregulation. Right panel: Treatment with 1 μ M SN-38 for 6 hours significantly increases *p21* mRNA expression levels in control shRNA-transduced Hep3B cells, while SN-38 does not further increase the amount of *p21* in *FUBP1* knockdown cells. Data represent the mean \pm standard deviation of four replicates.

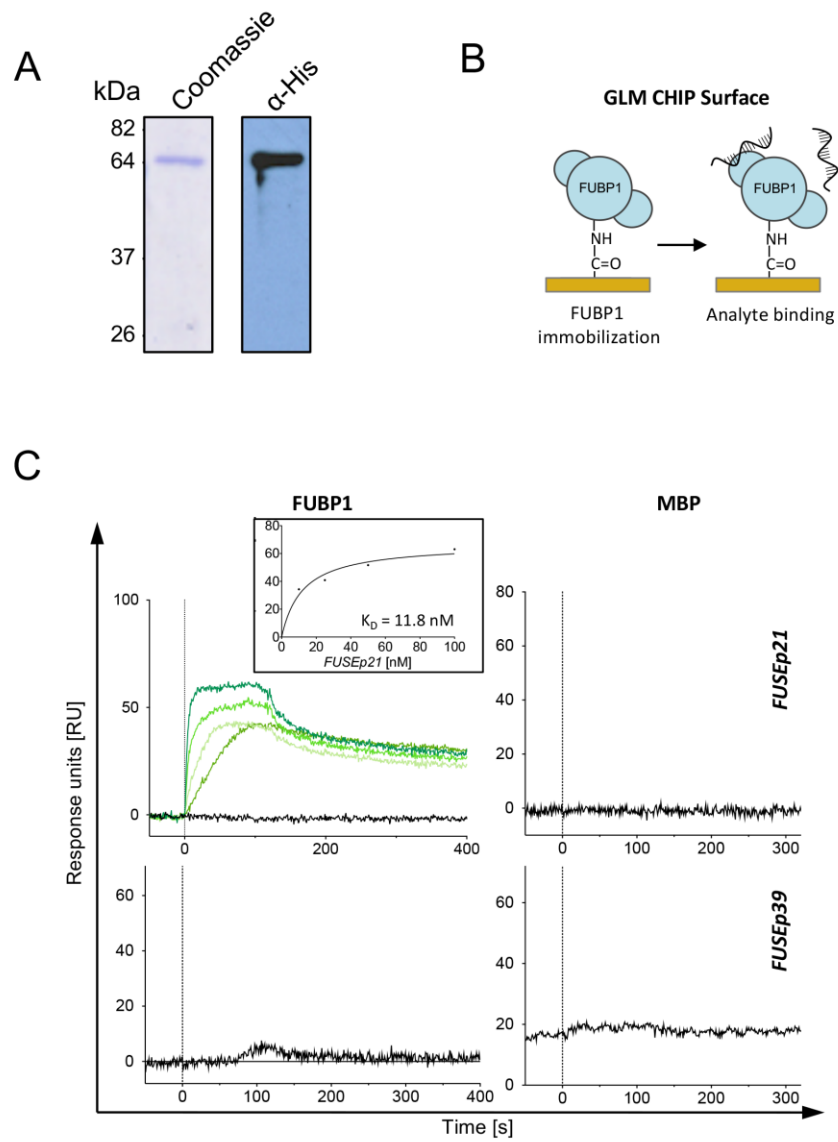


Figure 1

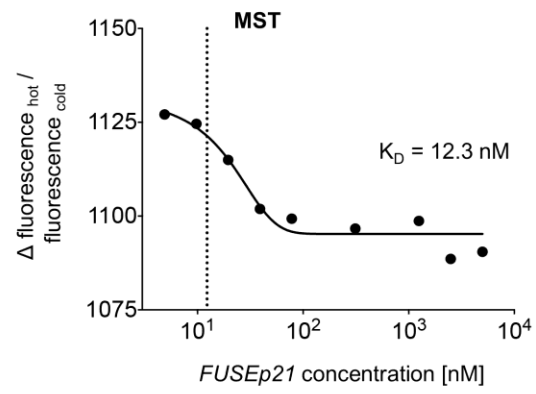


Figure 2

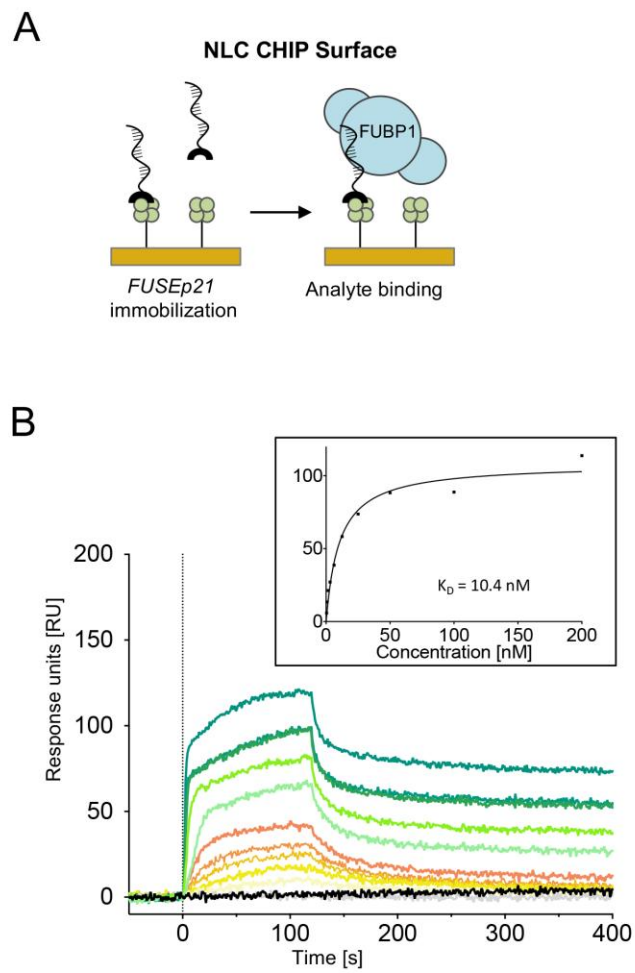


Figure 3

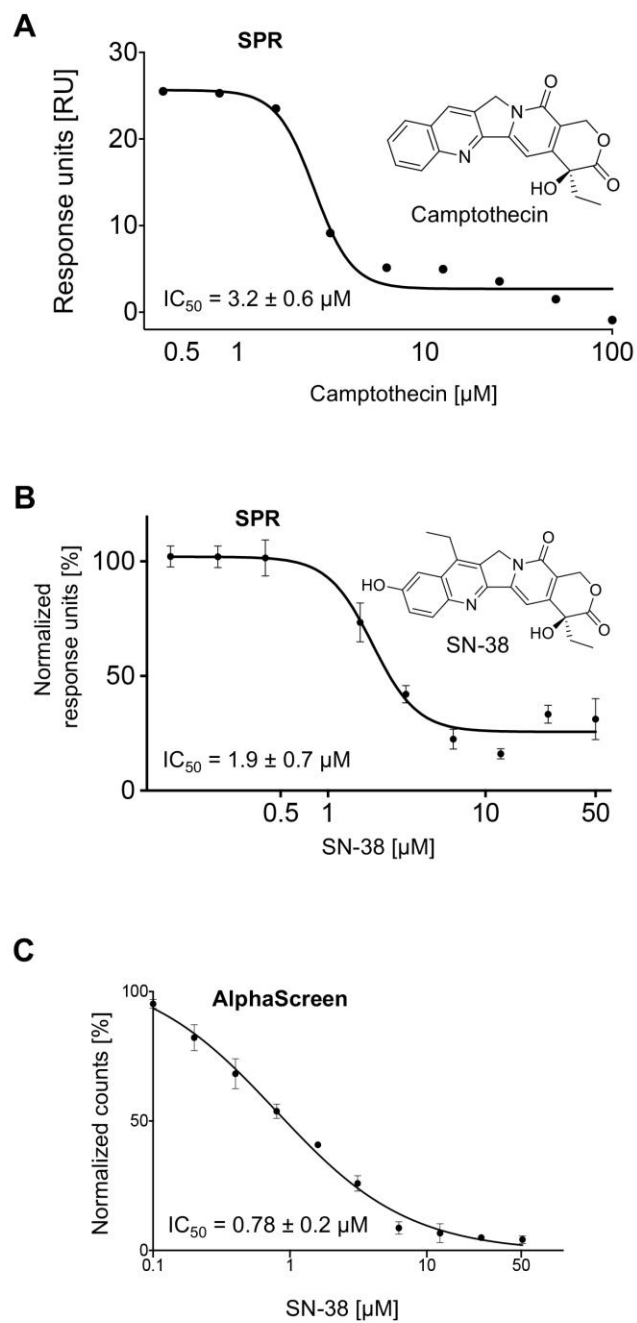


Figure 4

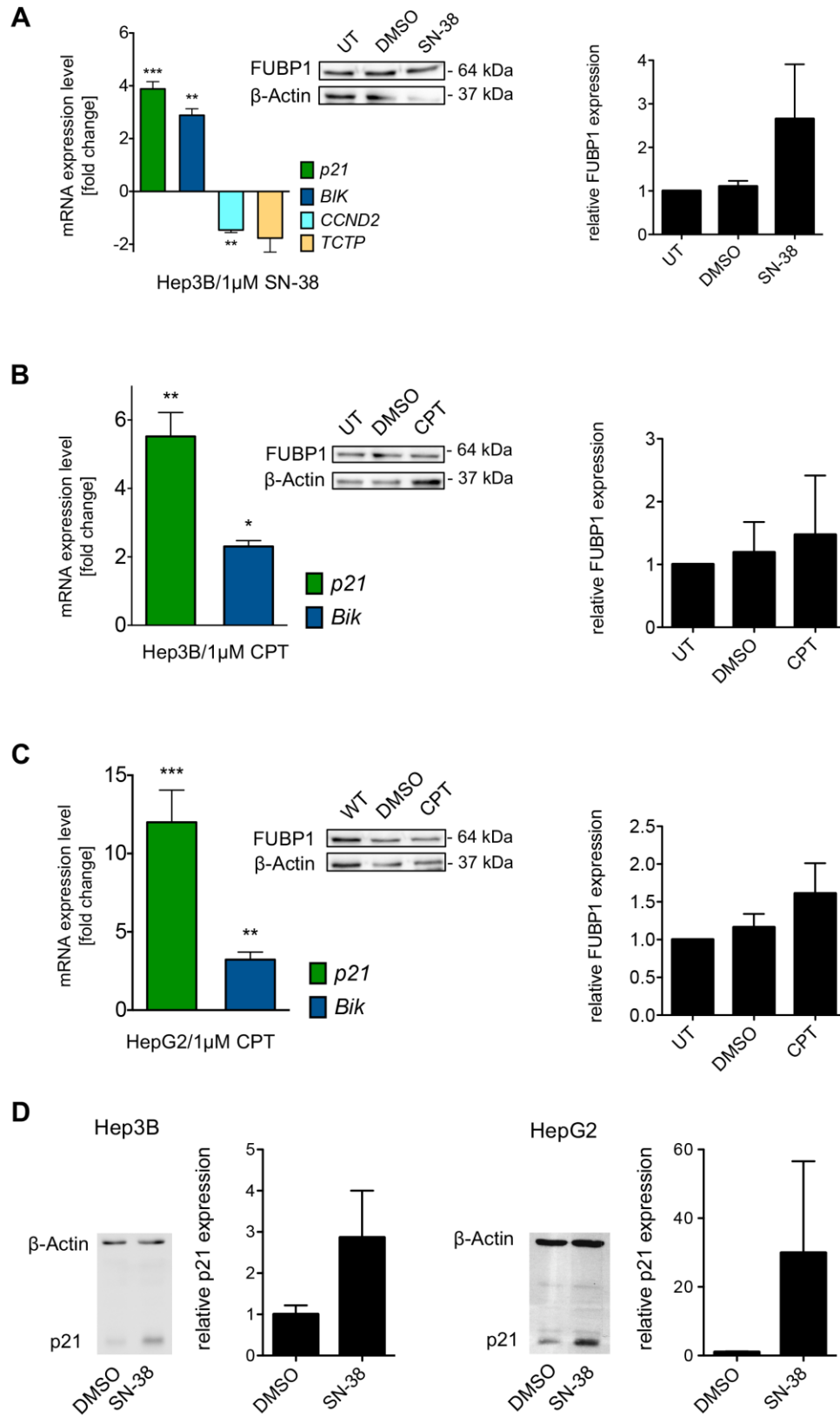


Figure 5

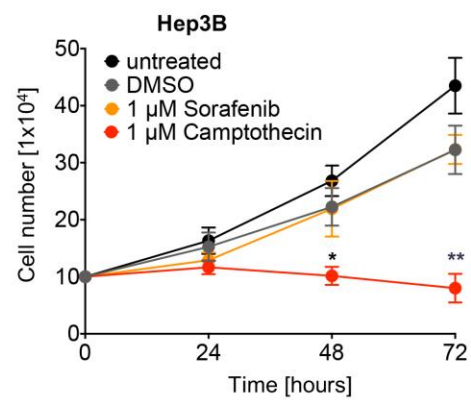


Figure 6

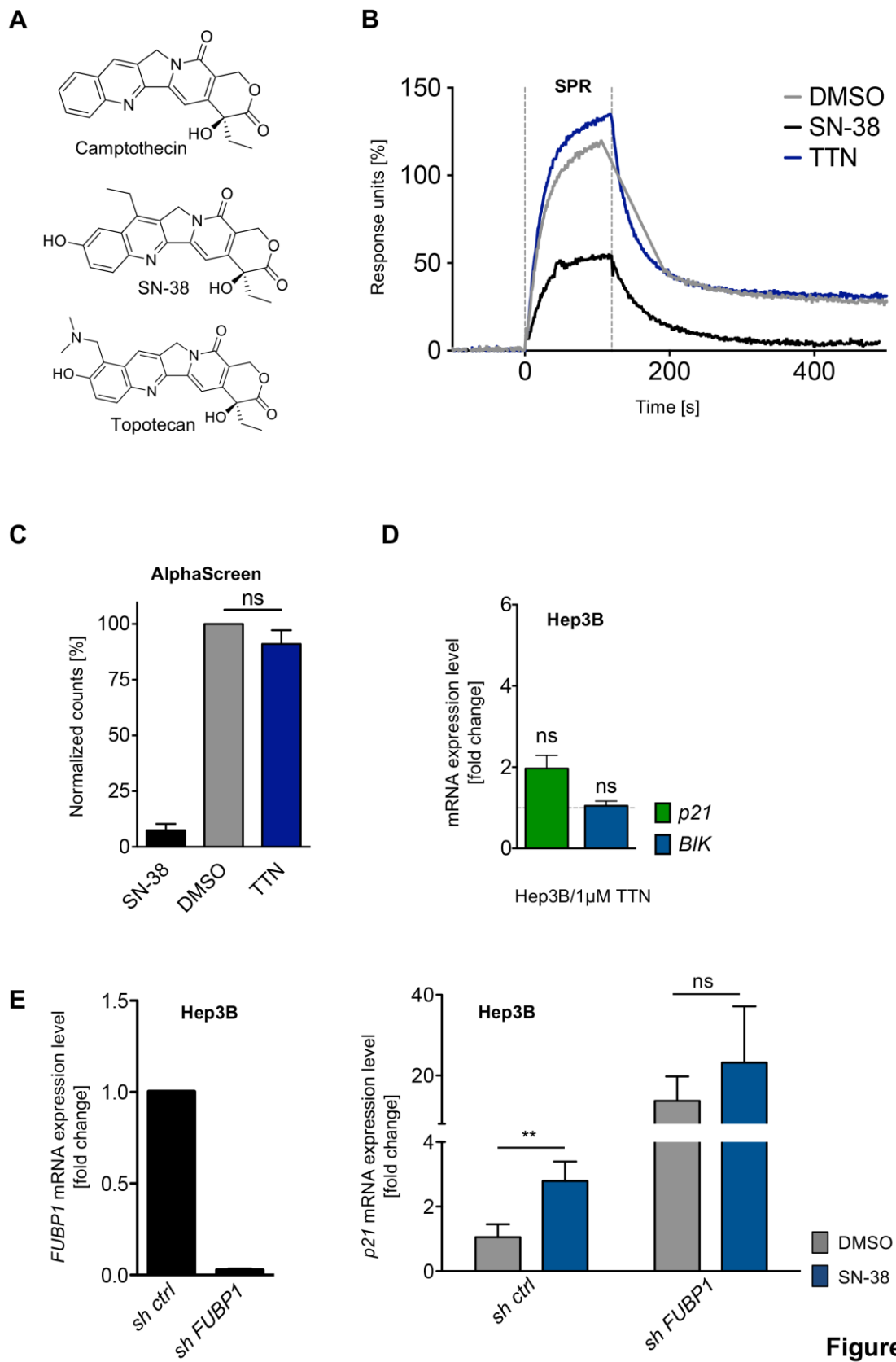
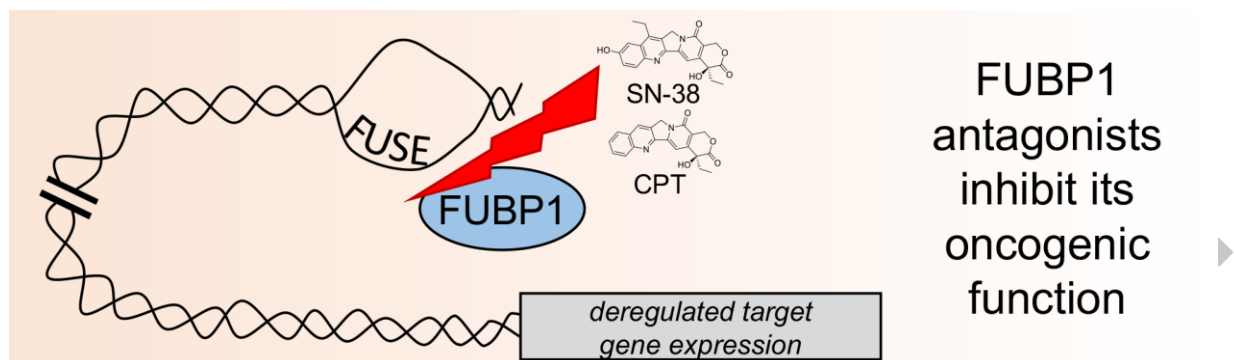


Figure 7



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