



Inhibition of Insulin-Like Growth Factor 2 mRNA-Binding Protein 1 Sensitizes Hepatic Tumor Cells to Chemotherapeutic Drugs by Modulating ATP-Binding Cassette Transporters

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Abstract

Multidrug resistance limits systemic therapy for hepatocellular carcinoma. This study evaluated whether insulin-like growth factor 2 mRNA-binding protein 1 (IGF2BP1) regulates multidrug resistance protein 1 (MDR1) and multidrug resistance-associated protein 3 (MRP3) in HepG2/C3A hepatic tumor cells. Three specific small interfering RNAs reduced IGF2BP1 protein levels to $39 \pm 6\%$, $41 \pm 13\%$, and $37 \pm 2\%$ of control; in parallel, MDR1 protein levels decreased to $40 \pm 10\%$, $41 \pm 17\%$, and $42 \pm 7\%$, and MRP3 protein levels decreased to $58 \pm 10\%$, $50 \pm 9\%$, and $57 \pm 5\%$. Using the selected small interfering RNA, MDR1 and MRP3 transcript levels decreased to $55 \pm 4\%$ and $55 \pm 6\%$ of control, respectively. *In silico* analyses predicted interaction of IGF2BP1 with both transcripts, particularly in the 3' untranslated regions. Sorafenib showed a half-maximal inhibitory concentration of $8.25 \pm 0.3 \mu\text{M}$ at 72 h; treatment with $2 \mu\text{M}$ sorafenib for 48 h increased IGF2BP1, MDR1, and MRP3 protein levels to $128 \pm 4\%$, $122 \pm 3\%$, and $142 \pm 1\%$ of control, respectively. IGF2BP1 knockdown prevented sorafenib-induced MDR1 transcript upregulation and reduced MRP3 transcript levels. Pharmacological inhibition of IGF2BP1 with BTYNB decreased half-maximal inhibitory concentration values from 54 ± 4 to $40 \pm 1 \mu\text{M}$ for sorafenib and from 48 ± 1 to $30 \pm 12 \mu\text{M}$ for doxorubicin. These findings identify IGF2BP1 as a regulator of drug resistance-associated phenotypes in hepatic tumor cells.

Keywords: IGF2BP1, MDR1, MRP3, Sorafenib, Chemosensitization, HepG2/C3A

List of Abbreviations:

ABC: ATP-binding cassette; ActD: actinomycin D; BTYNB: 2-[(5-bromo-2-thienyl)methylene]amino benzamide; CDS: coding sequence; DMEM: Dulbecco's modified Eagle's medium; DMSO: dimethyl sulfoxide; DOX: doxorubicin; EX: EX-527; FBS: fetal bovine serum; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; HCC: hepatocellular carcinoma; IGF2BP1: insulin-like growth factor 2 mRNA-binding protein 1; MDR: multidrug resistance; MRP3: multidrug resistance-associated protein 3; MTT: 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide; P-gp: P-glycoprotein; RBP: RNA-binding protein; RT-qPCR: reverse transcription quantitative polymerase chain reaction; SFB: sorafenib; siRNA: small interfering RNA; SIRT1: sirtuin 1; TCGA-LIHC The Cancer Genome Atlas-Liver Hepatocellular Carcinoma; UTR: untranslated region.

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Introduction

Hepatocellular carcinoma (HCC) is the most common primary liver malignancy and ranks among the leading causes of cancer-related mortality worldwide. Despite advances in surveillance and treatment, the prognosis remains dismal, with most patients diagnosed at advanced stages and a five-year survival rate below 20% [1, 2]. Systemic therapies have modestly improved outcomes, yet their efficacy is consistently undermined by the rapid development of multidrug resistance (MDR). Sorafenib (SFB), a multikinase inhibitor introduced in 2007 as the first systemic therapy for advanced HCC, exemplifies this challenge. Although SFB remains widely prescribed, its clinical benefit is limited by adaptive resistance mechanisms that emerge within months of treatment initiation [3, 4]. Understanding the molecular basis of MDR in HCC is therefore essential to identify new therapeutic vulnerabilities and improve patient outcomes.

One of the most critical and well-characterized mechanisms of MDR involves the overexpression of ATP-binding cassette (ABC) efflux transporters in cancer cells, such as MDR1 (also known as P-glycoprotein; P-gp/ABCB1) and MRP3 (multidrug resistance-associated protein 3/ABCC3). These transporters pump chemotherapeutic drugs out of the cell, thus reducing intracellular drug accumulation and attenuating cytotoxicity [3, 5]. Their upregulation has been repeatedly associated with poor response to SFB and doxorubicin (DOX), two agents commonly used in HCC therapy [6]. Attempts to directly inhibit ABC transporters have largely failed in the clinic due to toxicity, compensatory induction of alternative pumps, and lack of tumor selectivity [5, 7]. These limitations have shifted attention toward upstream regulatory pathways that coordinate transporter expression, offering the possibility of modulating multiple efflux pumps through a single molecular node.

Post-transcriptional regulation of gene expression has emerged as a critical determinant of cancer progression and therapy resistance. RNA-binding proteins (RBPs) play central roles in stabilizing, localizing, and translating specific mRNAs, thereby shaping oncogenic programs and adaptive responses to therapy. Insulin-like growth factor 2 mRNA-binding protein 1 (IGF2BP1) is an oncofetal RBP normally silenced in adult tissues but aberrantly re-expressed in several cancers, including HCC [8]. IGF2BP1 stabilizes transcripts of oncogenes such as MYC and KRAS, and its overexpression correlates with aggressive tumor behavior [9]. Importantly, IGF2BP1 has been implicated in MDR in other malignancies: in ovarian cancer, it stabilizes MDR1 mRNA [10], while in colorectal cancer, its silencing reduces MDR1 and ABCG2 expression [11]. In HCC, IGF2BP1 has been shown to enhance MRP2 translation [12], but whether it regulates other ABC transporters such as MDR1 or MRP3,

and whether it contributes to SFB resistance, remains unknown.

This gap is clinically significant. If IGF2BP1 stabilizes MDR-related transcripts in HCC, it could represent a pharmacologically tractable upstream regulator of drug efflux. Unlike direct transporter inhibitors, targeting IGF2BP1 may attenuate resistance by destabilizing multiple efflux pump mRNAs simultaneously, thereby reducing redundancy and minimizing toxicity. Furthermore, the possibility that SFB itself induces IGF2BP1 expression introduces an additional layer of complexity. SFB has not previously been linked to RBP regulation, yet adaptive induction of IGF2BP1 could provide a mechanism by which HCC cells rapidly acquire resistance. The involvement of SIRT1, a deacetylase frequently overexpressed in HCC and known to modulate MDR1 expression [13], is particularly intriguing. Whether SIRT1 connects SFB exposure to IGF2BP1 induction has not been investigated, but such a pathway would establish a novel regulatory axis linking drug treatment to post-transcriptional remodeling of transporter expression.

Taken together, these observations raise a central hypothesis: IGF2BP1 contributes to the regulation of MDR-associated ABC transporters in hepatic tumor cells, with a particularly relevant post-transcriptional role in MDR1 expression. In addition, SFB may induce IGF2BP1 expression, SIRT1 activity may participate in this response, and pharmacological inhibition of IGF2BP1 may sensitize hepatic tumor cells to SFB and doxorubicin. Testing this hypothesis is pharmacologically relevant, as it may uncover a druggable regulatory node associated with efflux transporter expression and adaptive resistance. By defining the IGF2BP1-ABC transporter axis, this study aims to provide mechanistic insight into drug resistance-associated phenotypes and establish a framework for therapeutic strategies that exploit RBP inhibition to attenuate MDR in hepatic tumor models.

Materials and Methods

Chemicals

SFB was purchased from Cayman Chemical (Ann Arbor, MI, USA). EX-527 (EX), Actinomycin D (ActD), phenyl-methylsulfonyl fluoride (PMSF), pepstatin A, leupeptin and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO, USA). DOX was purchased from ChemCruz (Santa Cruz, CA, USA). DMSO was purchased from Merck (Darmstadt, Germany). All other chemicals were of analytical grade purity and used as supplied.

Cell lines and treatments

The human HepG2/C3A cell line was obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). HepG2/C3A is a clonal derivative of HepG2. Although

HepG2 has been widely used as a hepatocellular carcinoma-like hepatic tumor model, its hepatoblastoma origin has also been recognized. Therefore, the present findings should be interpreted within the limitations of this model and require validation in additional HCC-derived cell lines and/or primary HCC samples. Cells were used between passages 20 and 30 and maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco, Life Technologies, Grand Island, NY, USA) supplemented with L-glutamine, 10% fetal bovine serum (FBS; Internegocios S.A., Buenos Aires, Argentina), 100 U/mL penicillin, and 100 µg/mL streptomycin at 37°C in a humidified atmosphere of 95% air and 5% CO₂. Cells were routinely tested for mycoplasma contamination every 15 days by polymerase chain reaction (PCR) directly on cell culture supernatants.

For SFB concentration selection, HepG2/C3A cells were seeded in 96-well plates at a density of 10,000 cells/well. The next day, cells were treated with different concentrations of SFB (0-32 µM) for 72 h, and cell viability was assessed by MTT assay as described in Section 2.6. The IC₅₀ value was calculated as the concentration inhibiting 50% of cell growth. For subsequent treatments, cells were seeded in 6-well plates at a density of 5 × 10⁵ cells/well. After 24 h of attachment, cells were treated with 0.75 or 2 µM SFB for 48 or 72 h for protein expression analysis, or with 2 µM SFB for 48 or 72 h for mRNA expression analysis. Total RNA was isolated for RT-qPCR, or cells were harvested for western blotting. In all experiments, control cells were incubated with vehicle (DMSO). The final concentration of DMSO in the culture medium was always below 0.1% v/v.

The SIRT1 deacetylase activity inhibitor EX was used to evaluate the participation of SIRT1 in the induction of IGF2BP1 produced by SFB. For this study, HepG2/C3A cells were seeded in 6-well plates at a density of 5 × 10⁵ cells/well. After 24 h, the culture medium was replaced with fresh medium containing the following treatments: 1) Control (DMSO), 2) SFB 2 µM, 3) EX 1 µM, and 4) SFB 2 µM and EX 1 µM (combined treatment). After 72 h treatment, cells were harvested and protein samples were used for western blot studies.

Prediction of IGF2BP1-ABC transporters interaction

To globally assess the interaction probability between IGF2BP1 and the ABC transporters of interest, *MDR1* and *MRP3*, different bioinformatics tools were used. We first evaluated the RNA-binding potential of IGF2BP1 to *MDR1* and *MRP3* mRNA using the RPI-Seq software from Iowa State University (<http://pridb.gdcb.iastate.edu/RPISeq>) [14, 15]. The software requires two query fields in plain-text format, the amino acid sequence of the protein and a nucleic acid sequence of the target mRNA. The software uses existing interaction databases to predict whether domains within the

primary amino acid sequence can bind to the target nucleic acid string. The input of the amino acid sequence of IGF2BP1 was selected from the National Library of Medicine's NCBI website and was inserted into the protein sequence field of the RPI-Seq website. RNA transcripts were also sourced from the NLM NCBI database. The mRNA regions (5'UTR; CDS and 3'UTR) were identified using the UCSC Genome Browser program (<http://genome.ucsc.edu/index.html>) and also entered in plain text format into the RNA query field.

For identification of potential m⁶A sites in *MDR1* and *MRP3* transcripts, SRAMP software was used to generate a graphical prediction score of m⁶A sites in full transcript sequences. This software analyses the position pattern of nucleotides in comparison with already known structures found in databases using a machine-learning framework [16, 17]. Thus, input information consisted of *MDR1* transcript variant 1 FASTA sequence and *MRP3* transcript variant 1 FASTA sequence and results are shown as a two-axis graph of combined score based on nucleotide position.

For the identification of RNA-protein binding regions we generated an interaction profile and interaction matrix by using catRAPID fragments module, an algorithm based on individual interaction propensities of polypeptide and nucleotide sequence fragments, especially used to evaluate interactions when protein (or RNA) is larger than 750 aa (1200 nt) [18]. This algorithm calculates and assigns an interaction score to each region of the input RNA sequence. Additionally, we identified a possible IGF2BP1 binding motif in the *MDR1* and *MRP3* mRNA sequences using the web-based RBP-binding prediction tool RBPmap (<http://rbpmap.technion.ac.il>) [15, 19], that compares the input sequences to known consensus binding sites of human and mouse proteins and ranks hits based on their fit to the consensus motif. This tool assigns a z-score and a p-value to each region in which a consensus binding motif was identified.

siRNA transfection

Three different and specific IGF2BP1 siRNAs and scramble siRNA were synthesized using the Silencer siRNA construction kit (Ambion, Life Technology, Carlsbad, CA, USA). The sequences of template oligonucleotides (DNA) used were designed following the guidelines described by Elbashir et al. [20]. Template oligonucleotides were 29 nucleotides in length. The eight nucleotides at the 3' end of each oligonucleotide were 5'-CCTGTCTC-3'. Template sequences for IGF2BP1 were siRNA 1, sense: 5'-AAATTTGACCAAGAACTGGCCCCTGTCTC-3',
a n t i s e n s e :
5'-AAGGCCAGTTCTTGGTCAAATCCTGTCTC-3';
siRNA 2, sense:
5'-AATTGCCTGGTCTGCTCCCGGCCTGTCTC-3',
a n t i s e n s e :

5'-AACCGGGAGCAGACCAGGCAACCTGTCTC-3';
siRNA 3: sense:
5'-AATATATTAGGAGGAGAGGCCCTGTCTC-3',
a n t i s e n s e :
5'-AAGGCCTCTCCTCCTAATATACCTGTCTC-3';
control siRNA, sense:
5'-AATTAGGTCACGGTGGCATAGCCTGTCTC-3',
a n t i s e n s e :
5'-AACTATGCCACCGTGACCTAACCTGTCTC-3'.
Synthesis conditions were as suggested by the manufacturer.

For siRNA transient transfection, HepG2/C3A cells were seeded at a density of 3×10^5 cells/well in a 6-well plate in antibiotic free DMEM. After 24 h of attachment, cells were exposed to a transfection mixture of 100 nM of the corresponding siRNA with Dharmafect 4 siRNA Transfection Reagent (Dharmacon, Lafayette, CO, USA), both dissolved in OPTIMEM culture medium for 48 h according to manufacturer's instructions. For treatments with SFB, fresh medium containing SFB or DMSO was added 24 h after transfection was initiated and the cells were incubated for another 48 h. At the end of the treatment, total RNA was isolated for RT-qPCR or cells were harvested and protein samples were used in western blot studies.

Transcription inhibition with Actinomycin D

Actinomycin D (ActD) was used to inhibit transcription and evaluate the post-transcriptional contribution to the regulation of IGF2BP1 and ABC transporter mRNA levels. To assess the effect of SFB under transcriptional blockade, HepG2/C3A cells were pretreated with 5 µg/mL ActD or vehicle for 30 min. Then, SFB or vehicle was added, and cells were further incubated for 48 h. Total RNA was isolated at the end of the treatment. In a separate set of experiments aimed at evaluating ABC transporter mRNA stability after IGF2BP1 depletion, HepG2/C3A cells were transfected with IGF2BP1 siRNA or control siRNA for 24 h and then treated with 5 µg/mL ActD or vehicle for 6 h. Total RNA was isolated at the end of the treatment and used for RT-qPCR analysis.

IGF2BP1 inhibition assay

In order to evaluate the effect of IGF2BP1 inhibition against the cytotoxicity exerted by SFB, HepG2/C3A cells were seeded in 96-well plates at a density of 1.2×10^4 cells/well. After 24 h of culture, fresh medium containing 5 µM of the IGF2BP1 small inhibitor 2-[(5-bromo-2-thienyl)methylene]amino benzamide (BTYNB, Axon Medchem, Groningen, the Netherlands) was added and cells were further incubated for 72 h. Cells were then rinsed and incubated with different concentrations of SFB (0–100 µM), or DMSO, for 16 h. Cell viability was then assessed through MTT assay.

The MTT reagent was added to the culture medium at a final concentration of 0.5 mg/mL and incubated at 37°C. After

2 h, cells were lysed and formazan crystals were solubilized by the addition of 200 µL of DMSO and the absorbance of the metabolite produced by viable cells was quantified at 540 nm (with reference filter at 650 nm) on a microplate reader (DTX 880 multimode detector; Beckman Coulter Inc., Fullerton, CA, USA). From the absorbance data obtained, firstly, SFB or DOX concentration-response curves were generated in order to obtain the IC_{50} of these compounds, using the GraphPad software (GraphPad Software, La Jolla, CA, USA) and, secondly, viability percentages were obtained as a function of SFB or DOX (100 % viability was considered for untreated cells).

Western Blotting

Western blotting was performed using total cell lysates. Preparations containing 20 µg of total protein were loaded onto 10% SDS-polyacrylamide gel and subjected to electrophoresis. After electrotransfer onto PVDF membranes (GE Healthcare Life Sciences, Chicago, IL, USA), the blots were blocked overnight at 4 °C with PBS containing 0.3 % Tween 20 and 5 % non-fat dry milk, and then incubated overnight with primary antibodies: anti-IGF2BP1 (1:1000, ab82968, Abcam, Cambridge, UK), anti-MDR1 (1:1000, C219, Calbiochem, Darmstadt, Germany), or anti-MRP3 (1:1000, #14182, Cell Signaling, Danvers, MA, USA or M0318, Sigma-Aldrich Corp, St. Louis, MO, USA for 72 h SFB treatment, Fig. 3b). Equal loading was systematically checked by detection of GAPDH (1:1000, FL-335, Santa Cruz Biotechnology, Santa Cruz, CA, USA). Immune complexes were detected by incubation with horseradish peroxidase-linked secondary antibodies (1:5000) for 1 h. Immunoreactive bands were detected by chemiluminescence (Pierce™ ECL Western Blotting Substrate, Thermo Scientific) and analyzed using Gel-Pro Analyzer software (Media Cybernetics, Silver Spring, MD, USA) [21].

RNA isolation and RT-qPCR analysis

After cell treatments, total RNA was isolated using TRIzol® reagent (Invitrogen, Carlsbad, CA, USA). cDNA was synthesized from 2 µg of total RNA with the Superscript First-Strand Synthesis System for RT-qPCR (Invitrogen, Carlsbad, CA, USA) using random hexamers according to manufacturer's instructions. Quantitative real time PCR (qPCR) was performed on an Applied Biosystems™ StepOne™ Real-Time PCR System using the $5 \times$ HOT FIREPol® EvaGreen® qPCR Mix Plus (ROX) (Solis Biodyne, Tartu, Estonia). All the primers were used at a final concentration of 1 µM. Forward and reverse primers were 5'-CCTGCTGGCTCAGTATGGT-3' and 5'-GACATTCACCACTGCCGTCTC-3' for IGF2BP1, 5'-TACTTGGTGGCACATAAAC-3' and 5'-CCAAAGACAACAGCTGAAA-3' for MDR1, 5'-GTCCGCAGAATGGACTTGAT-3'

and 5'-TCACCACTTGGGGATCATTT-3' for *MRP3*, 5'-CAACCTTCTTGCAGCTCCTC-3' and 5'-TTCTGACCCATACCCACCAT-3' for *GAPDH*. *IGF2BP1*, *MDR1* and *MRP3* mRNAs were normalized to *GAPDH* mRNA as housekeeping gene.

Statistical analysis

Results were expressed as mean $\pm$ S.E.M. Statistical analysis was performed using the Student's t-test (for two experimental groups), one-way ANOVA followed by Newman-Keuls test or two-way ANOVA with repeated measures followed by Tukey's test (for more than two experimental groups). Significance was set at $p < 0.05$. Analyses were performed using GraphPad Prism 6 software.

Results

IGF2BP1 knockdown reduces *MDR1* and *MRP3* expression

To investigate the role of IGF2BP1 in regulating *MDR1* and *MRP3* expression, we designed three siRNAs targeting different regions of human *IGF2BP1* mRNA (siRNA 1,

2, and 3). We observed a significant decrease in IGF2BP1 protein expression in HepG2/C3A cells transfected with all siRNAs compared to cells transfected with control siRNA ($39 \pm 6\%$, $41 \pm 13\%$, and $37 \pm 2\%$ vs. control siRNA $100 \pm 12\%$, respectively; Fig. 1a). Next, we analyzed the protein expression of *MDR1* and *MRP3* in cells with silencing of IGF2BP1 to evaluate if IGF2BP1 was capable of modulating ABC transporters under constitutive conditions. Transient silencing of IGF2BP1 using any of the three siRNAs led to a significant decrease in *MDR1* ($40 \pm 10\%$, $41 \pm 17\%$, and $42 \pm 7\%$ vs. control siRNA $100 \pm 3\%$, respectively) and *MRP3* ($58 \pm 10\%$, $50 \pm 9\%$, and $57 \pm 5\%$ vs. control siRNA $100 \pm 1\%$, respectively) protein levels (Fig. 1a). Although all three siRNAs showed efficient knockdown of IGF2BP1 in HepG2/C3A cells, siRNA 1, hereafter referred to as IGF2BP1 siRNA, was chosen for subsequent experiments.

We also measured the mRNA levels of *MDR1* and *MRP3* in HepG2/C3A with IGF2BP1 knockdown. We observed that *MDR1* and *MRP3* levels were significantly decreased after IGF2BP1 silencing (*MDR1*: $55 \pm 4\%$ vs. control $100 \pm 12\%$ and *MRP3*: $55 \pm 6\%$ vs. control $100 \pm 1\%$; Fig. 1 b). Together,

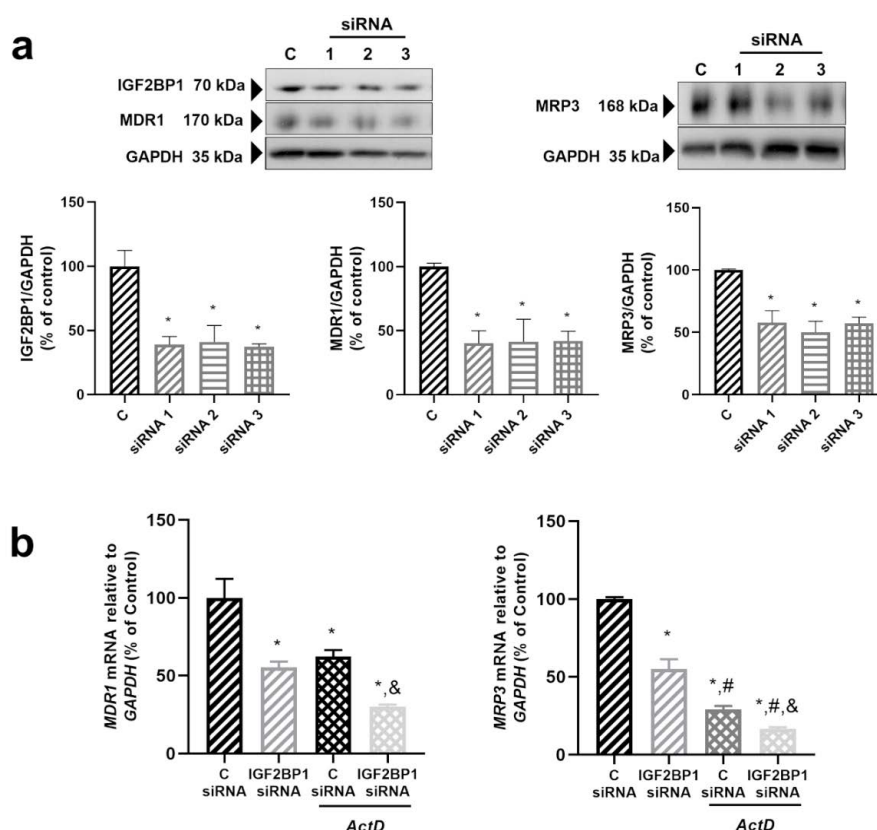


Figure 1: Effect of IGF2BP1 depletion on ABC transporter expression. (a) Protein levels of IGF2BP1, *MDR1* and *MRP3* detected by western blotting after 48 h of transfection with control siRNA (C siRNA) or three different IGF2BP1-specific siRNAs (1, 2 and 3). (b) mRNA levels of *MDR1* (left) and *MRP3* (right) after siRNA transfection for 24 h and 6 h of incubation with 5 μ g/mL actinomycin D (ActD). * different from transfection control (C siRNA); # different from IGF2BP1- specific siRNA (IGF2BP1-siRNA), & different from transfection control+ActD (C siRNA+ActD). $p < 0.05$, $n = 3$.

these results indicate that IGF2BP1 depletion reduces both MDR1 and MRP3 expression under basal conditions. The ActD experiments support a post-transcriptional contribution of IGF2BP1 to MDR1 regulation. For MRP3, the decrease observed after IGF2BP1 silencing suggests that IGF2BP1 participates in its regulation, although the underlying mechanism appears less straightforward and may involve additional transcriptional, post-transcriptional, or indirect regulatory pathways.

In silico analyses predict potential IGF2BP1-binding regions in MDR1 and MRP3 transcripts

Due to the regulatory role of IGF2BP1, which involves regulating most of its target mRNAs through interaction with the 3'UTR, we performed an *in silico* analysis to assess the probability of interaction between the IGF2BP1 protein sequence and the mRNA sequences of *MDR1* and *MRP3*. These analyses were performed to generate testable hypotheses regarding potential IGF2BP1-mRNA interactions rather than to establish direct binding events. First, the predicted interaction between IGF2BP1 and the mRNA from both ABC transporters was evaluated at the level of the complete ABC transporter sequence and by evaluating individual regions (5'UTR - CDS - 3'UTR). This was done using the RPISeq algorithm, a family of machine learning classifiers (Random Forest:RF or Support Vector Machine:SVM) for predicting RNA-protein interactions using sequence information only. From Table 1, it can be seen that for both transporters in their full sequence the classifier values are higher than 0.5 indicating a positive interaction (RF:0.75 and SVM:0.959 for *MDR1* and RF:0.8 and SVM:0.513 for *MRP3*) with IGF2BP1. When the regions of the mRNA are analyzed, it is observed that for *MDR1* the high interaction probability is mainly due to the interaction probability of IGF2BP1 with the 3'UTR region of *MDR1* (RF:0.75 and SVM:0.97). For the case of

MRP3, although at least one of the values of both classifiers is higher than 0.5 in the different regions of the mRNA, only for the 3'UTR region (RF and SVM: 0.75) the interaction can be considered as positive.

Subsequently, we also predicted, using catRAPID fragment module, the probable regions of interaction of IGF2BP1 with ABC transporters. As can be seen in the interaction plot (Fig. 2a), the interaction score between IGF2BP1-*MDR1* is high at the end of CDS and at the beginning of the 3'UTR of the nucleotide sequence. More specifically, the nucleotides between positions 3664-3887 in the *MDR1* sequence show a high probability of interaction with the amino acid residues at positions 51-103, 226-277 and 302-353 of IGF2BP1. For *MRP3*, two potential interaction regions were detected between nucleotide positions 3843-4070 and 2261-2488, although with a lower probability of binding. Additionally, several shorter secondary interaction zones with lower interaction scores were identified at positions 156-383, 905-1132, and 1399-1626. As observed in the *MRP3*-IGF2BP1 interaction profile (Fig. 2a), similar to the case of *MDR1*, the IGF2BP1 amino acid residues most involved in the binding are situated between positions 51-103 and 302-353. Unlike *MDR1*, an interaction region is also detected in residues at positions 226-277.

Moreover, we used the software SRAMP to predict potential m⁶A modifications in *MDR1* and *MRP3* transcripts, since IGF2BP1 has been reported as m⁶A reader. Consistent with the aforementioned results, *MDR1* showed with a very high confidence score two possible m⁶A sites in positions 3663 and 3682 (Fig. 2b). For its part, *MRP3* showed a more diffuse m⁶A pattern, consistent with catRAPID fragments results, finding a very high confidence score in position 2401. Finally, we utilized the RBPmap motifs database to map the consensus IGF2BP1 binding site in the RNA sequence

Table 1: Predicted interaction between IGF2BP1 and MDR1 and MRP3. A summary of the predicted probabilities for interaction between IGF2BP1 and target ABC transporter mRNAs, based on RNA-Protein interaction RPISeq, is shown. The predicted values of the classifiers RF (Random Forest) and Support Vector machine (SVM), 0.0 - 1.0, suggested > 0.5 to be statistically significant for potential interaction between the protein and target site.

mRNA SEQUENCE NAME	RBP PROTEIN	Region (nt position)	RF Classifier	SVM Classifier
>NM_001348945.2 Homo sapiens ATP binding cassette subfamily B member 1 (MDR1), transcript variant 1, mRNA	IGF2BP1	Complete sequence (1...5586)	0.75	0.959
		5'UTR (1...517)	0.6	0.35
		3'UTR (4360...5586)	0.75	0.97
		CDS (518...4359)	0.75	0.92
>NM_003786.4 Homo sapiens ATP binding cassette subfamily C member 3 (MRP3), transcript variant 1, mRNA		Complete sequence (1...5693)	0.8	0.513
		5'UTR (1...56)	0.5	0.46
		3'UTR (4641...5693)	0.75	0.75
		CDS (57...4640)	0.85	0.47

of *MDR1*, since the previous analysis done using catRAPID showed an interaction-matrix more focused for *MDR1* than for *MRP3*. These sites consist of nucleotide sequences (with their possible variations) for which there is experimental evidence of binding to a specific protein, in this case, IGF2BP1 (Fig. 2c).

The p-values, consensus binding motifs, and motif variations are provided in Supplementary Tables S1 and S2. Consistent with the previous analyses, a greater number of consensus IGF2BP1-binding motifs was observed in the *MDR1* sequence than in the *MRP3* sequence. In both cases, most predicted motifs were located within the 3'UTR regions of the transcripts.

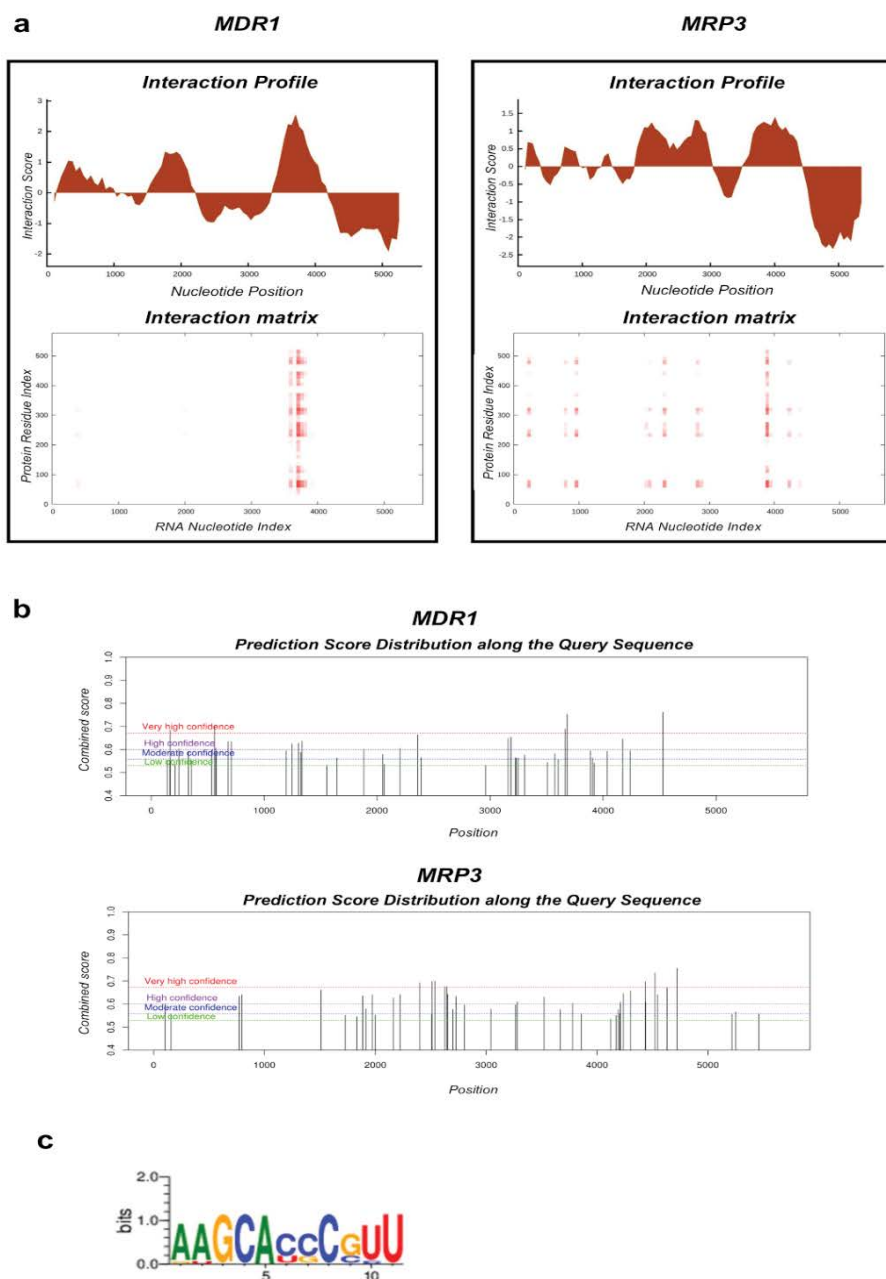


Figure 2: *In silico* prediction of potential IGF2BP1-binding regions. (a) Predicted interaction sites between IGF2BP1 and *MDR1* (left) and *MRP3* (right) mRNAs. The interaction profile (top) represents the interaction score of the protein (y-axis) along the RNA sequence (x-axis), giving information about the transcript regions that are most likely to be bound by the protein. The interaction matrix (bottom) shows, as a heat-map, the protein (y-axis) and RNA (x-axis) regions predicted to interact. The shades of red of the heat-map indicate the interaction score of the individual amino acid and nucleotide pairs. (b) Prediction score distribution along *MDR1* and *MRP3* transcripts sequences. The graph shows confidence thresholds corresponding to $\alpha < 0.15$ (low confidence), $\alpha < 0.1$ (moderate confidence), $\alpha < 0.05$ (high confidence) and $\alpha < 0.01$ (very high confidence). (c) Probable mRNA binding region predicted by RBPmap tool.

Sorafenib induces IGF2BP1, MDR1 and MRP3 expression

To select the SFB concentration for subsequent experiments, HepG2/C3A cells were exposed to increasing concentrations of SFB for 72 h, and cell viability was assessed by MTT assay. The calculated IC_{50} value was $8.25 \pm 0.3 \mu M$ ($n=4$); therefore, sub-cytotoxic concentrations were selected for further studies. Treatment with $2 \mu M$ SFB increased the protein levels of IGF2BP1, MDR1 and MRP3 after 48 h of incubation (IGF2BP1: $128 \pm 4\%$ vs. control $100 \pm 3\%$; MDR1: $122 \pm 3\%$ vs. control $100 \pm 2\%$; MRP3: $142 \pm 1\%$ vs. control $100 \pm 2\%$; Fig. 3a). This induction was also observed after 72 h of treatment with $2 \mu M$ SFB (IGF2BP1: $176 \pm 3\%$

vs. control $100 \pm 2\%$; MDR1: $139 \pm 2\%$ vs. control $100 \pm 1\%$; MRP3: $183 \pm 9\%$ vs. control $100 \pm 1\%$; Fig. 3b). Since no significant changes were observed with $0.75 \mu M$ SFB at 48 h, subsequent experiments were performed using $2 \mu M$ SFB. To determine whether the increase in protein expression was associated with changes in transcript levels, IGF2BP1, MDR1 and MRP3 mRNA levels were measured by qPCR. SFB treatment significantly increased IGF2BP1, MDR1 and MRP3 mRNA levels after 48 h (IGF2BP1: $160 \pm 10\%$ vs. control $100 \pm 3\%$; MDR1: $136 \pm 7\%$ vs. control $100 \pm 4\%$; MRP3: $142 \pm 4\%$ vs. control $100 \pm 8\%$; Fig. 3c) and 72 h (IGF2BP1: $184 \pm 12\%$ vs. control $100 \pm 7\%$; MDR1: $193 \pm 18\%$ vs. control $100 \pm 5\%$; MRP3: $142 \pm 12\%$ vs. control $100 \pm 9\%$; Fig. 3d).

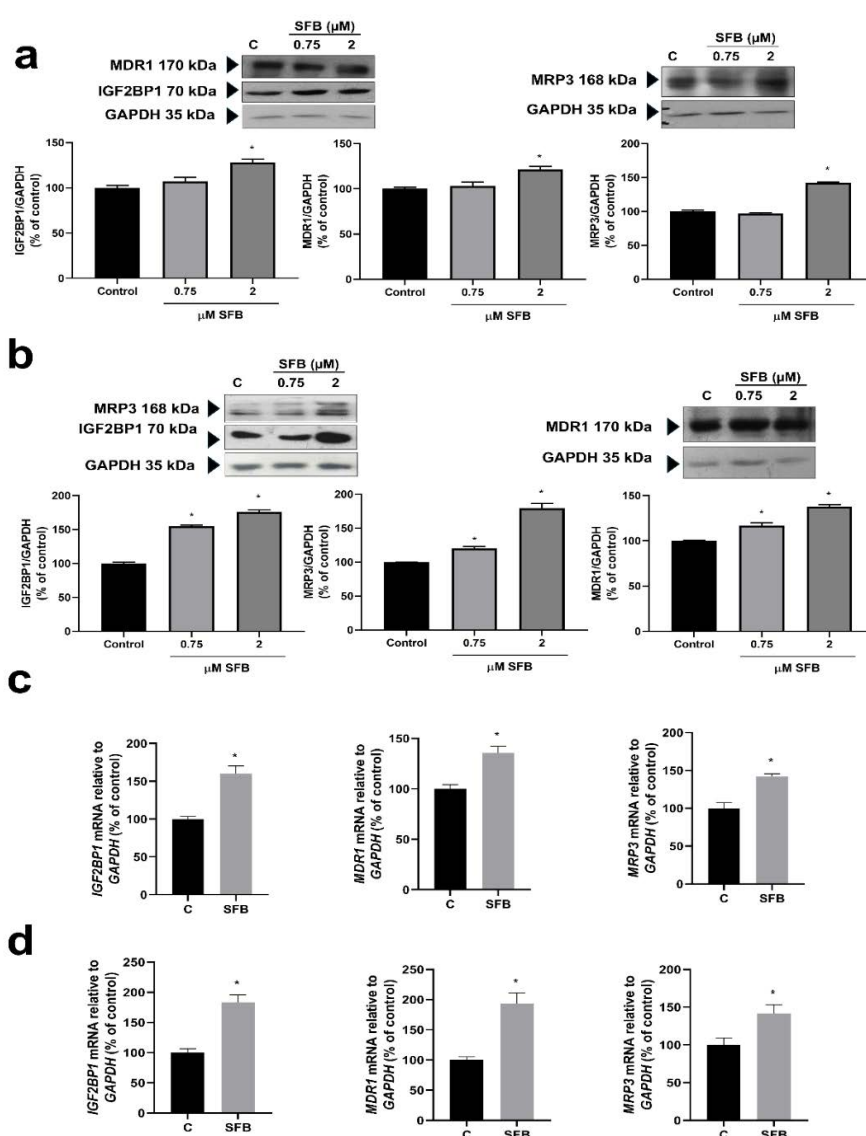


Figure 3: Effect of SFB on IGF2BP1, MDR1 and MRP3 expression in HepG2/C3A cells. (a, b) Protein levels of IGF2BP1, MDR1, and MRP3 after 48 h and 72 h of incubation with SFB (0.75 or $2 \mu M$). (c, d) Corresponding mRNA levels after 48 h and 72 h of SFB ($2 \mu M$) treatment. * different from control (C), $p < 0.05$, $n = 3-6$.

Involvement of IGF2BP1 in MDR1 and MRP3 modulation by SFB

To investigate whether IGF2BP1 participates in the induction of MDR1 and MRP3 by SFB, IGF2BP1 expression was knocked down in HepG2/C3A cells followed by SFB treatment. Protein expression analysis showed that IGF2BP1 depletion prevented SFB-induced MDR1 upregulation (C siRNA + SFB: $123.5 \pm 2.5\%$ vs. IGF2BP1 siRNA + SFB: $89 \pm 7\%$; Fig. 4a). In contrast, IGF2BP1 knockdown did not prevent SFB-induced MRP3 protein expression (Fig. 4a).

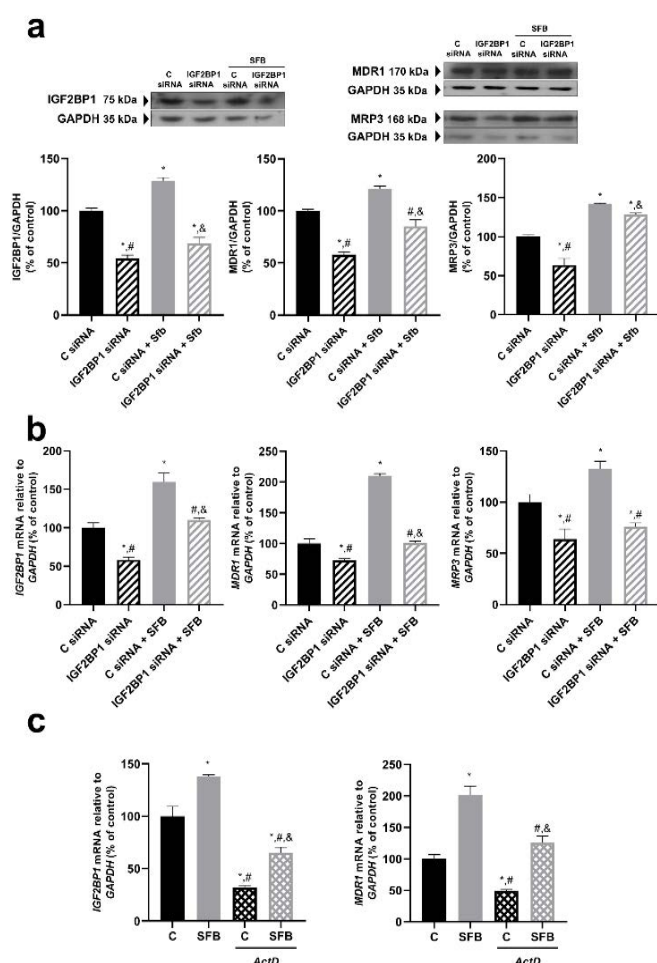


Figure 4: Involvement of IGF2BP1 in MDR1 and MRP3 modulation by SFB. (a) Protein levels of IGF2BP1, MDR1 and MRP3 in HepG2/C3A cell line after siRNA transfection for 24 h and 48 h of incubation with SFB (2 μ M). (b) Corresponding mRNA levels after siRNA transfection and SFB (2 μ M) treatment. * different from transfection control (C siRNA); # different from treated control (C siRNA + SFB), & different from IGF2BP1-specific siRNA (IGF2BP1 siRNA), $p < 0.05$, $n = 3-4$. (c) IGF2BP1 (left) and MDR1 (right) mRNA levels in HepG2/C3A cells incubated with SFB (2 μ M) for 48 h, post pre-treatment with 5 μ g/mL actinomycin D (ActD) or its vehicle. * different from control (C); # different from SFB (2 μ M), & different from control+ActD (C+ActD). $p < 0.05$, $n = 3$.

We next evaluated *MDR1* and *MRP3* mRNA levels under the same experimental conditions. IGF2BP1 depletion prevented the SFB-induced increase in *MDR1* mRNA levels (C siRNA + SFB: $209.5 \pm 4.4\%$ vs. IGF2BP1 siRNA + SFB: $101.0 \pm 2.4\%$) and also reduced *MRP3* mRNA levels in SFB-treated cells (C siRNA + SFB: $132.4 \pm 7.6\%$ vs. IGF2BP1 siRNA + SFB: $76.0 \pm 3.5\%$; Fig. 4b). Based on these results, we further evaluated whether SFB-induced IGF2BP1 and *MDR1* mRNA upregulation persisted under transcriptional blockade. HepG2/C3A cells were pretreated with ActD before and during SFB exposure. IGF2BP1 and *MDR1* mRNA levels remained higher after SFB treatment even in the presence of ActD, suggesting that SFB-induced upregulation of these genes occurs, at least in part, at the post-transcriptional level (Fig. 4c). Together, these results indicate that IGF2BP1 depletion prevents SFB-induced MDR1 upregulation at both mRNA and protein levels. In contrast, MRP3 showed a divergent response: IGF2BP1 knockdown reduced SFB-induced *MRP3* mRNA levels but did not prevent MRP3 protein induction. This transcript–protein dissociation suggests that, under SFB exposure, MRP3 expression may be shaped by additional regulatory mechanisms beyond IGF2BP1-dependent mRNA control, including differences in translation efficiency, protein stability, degradation rate, or transporter trafficking.

SIRT1 participation in IGF2BP1 induction by SFB.

To evaluate whether SIRT1 could be involved in SFB-induced IGF2BP1 upregulation, HepG2/C3A cells were treated with the SIRT1 activity inhibitor EX-527. Treatment with EX-527 (1 μ M) reduced IGF2BP1 protein levels compared with control cells ($77 \pm 8\%$ vs. control $100 \pm 2\%$). Moreover, the combined treatment with EX-527 and SFB prevented the SFB-induced increase in IGF2BP1 protein expression, resulting in significantly lower IGF2BP1 levels than those observed in control cells ($21 \pm 5\%$ vs. control $100 \pm 2\%$). Consistent with previous findings reported by Ceballos et al. in HepG2 cells [22], EX-527 reduced MDR1 protein levels both in the absence and presence of SFB ($69 \pm 5\%$ and $63 \pm 7\%$, respectively). For MRP3, EX-527 reduced basal protein levels ($70 \pm 4\%$) and partially prevented the induction produced by SFB, although MRP3 protein expression remained higher in the combined treatment than in EX-527-treated cells (Fig. 5).

Effect of the IGF2BP1 inhibition by BTYNB on cell sensitivity to chemotherapeutic drugs.

Treatment with the IGF2BP1 inhibitor BTYNB did not markedly affect HepG2/C3A cell viability at concentrations ranging from 0 to 50 μ M. A modest reduction in cell viability of approximately 15% was observed only at 100 μ M (Fig. 6a). To confirm the inhibitory effect of BTYNB on IGF2BP1 activity, we measured the mRNA levels of

E2F1, a well-known IGF2BP1 target. BTYNB significantly reduced E2F1 expression at 5 μ M (Fig. 6b), consistent with previously published findings [23]. We next evaluated whether pharmacological inhibition of IGF2BP1 modified the sensitivity of HepG2/C3A cells to chemotherapeutic drugs. BTYNB pretreatment enhanced the cytotoxic effect

of both SFB and DOX, as shown by a significant reduction in IC_{50} values. For SFB, the IC_{50} decreased from 54 ± 4 μ M in control cells to 40 ± 1 μ M in BTYNB-treated cells ($p = 0.024$; Fig. 6c,d). For DOX, the IC_{50} decreased from 48 ± 1 μ M in control cells to 30 ± 12 μ M in BTYNB-treated cells ($p < 0.0001$; Fig. 6e,f).

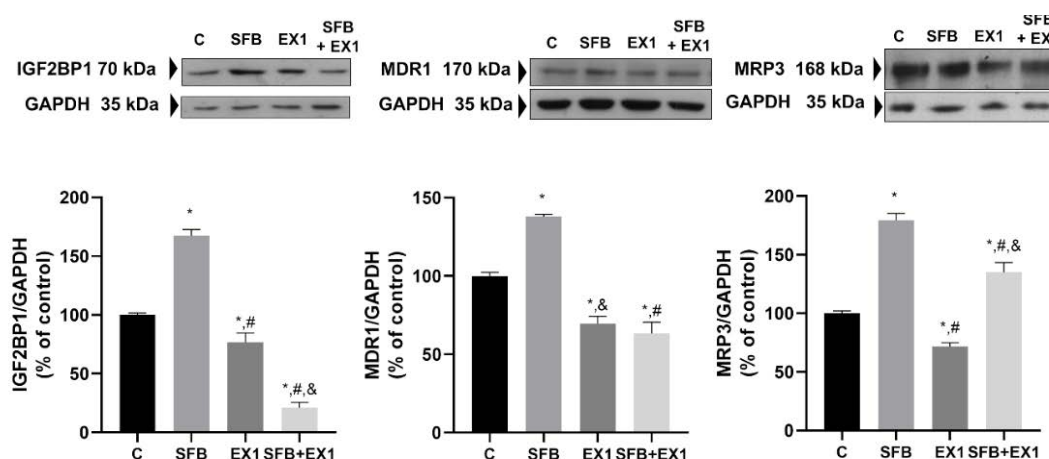


Figure 5: Participation of SIRT1 in the modulation of IGF2BP1, MDR1 and MRP3 by SFB. (a) Protein levels of IGF2BP1, MDR1 and MRP3 after 72 h of treatment with SFB (2 μ M), EX (1 μ M), SFB (2 μ M) + EX (1 μ M) or DMSO (control). * different from control (C), # different from SFB (2 μ M), & different from combined treatment (SFB + EX). $p < 0.05$, $n = 4$.

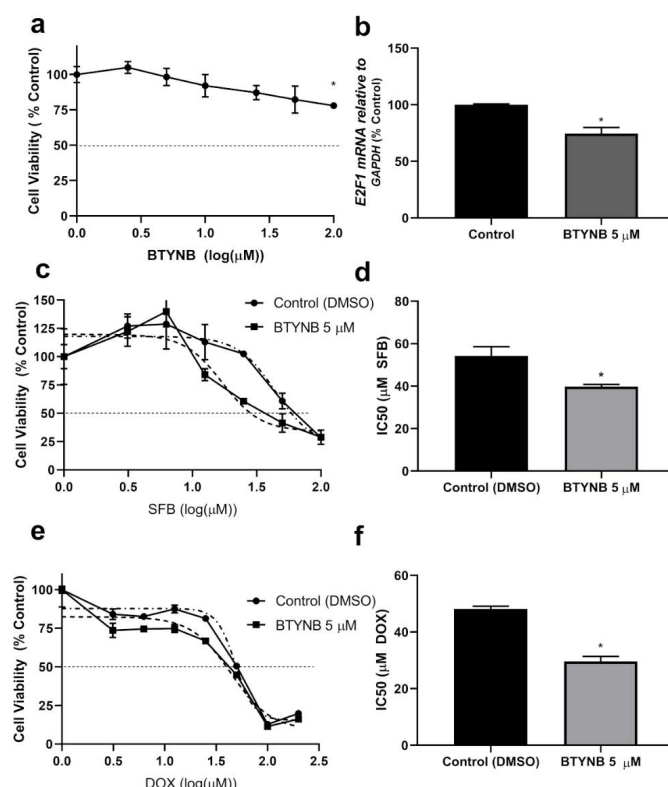


Figure 6: Cytotoxicity assay with SFB and DOX in HepG2/C3A after IGF2BP1 inhibition by BTYNB. a) HepG2/C3A cells incubated with different concentrations of BTYNB. Viability percentage of HepG2/C3A cells as a function of BTYNB concentration. (b) Effect of BTYNB on a known target of IGF2BP1. *E2F1* mRNA expression levels in HepG2/C3A cells incubated during 72 h with BTYNB 5 μ M or vehicle (Control). *: $p < 0.05$ vs. Control (0 μ M BTYNB). Viability percentage of HepG2/C3A cells as a function of SFB (c) and DOX (e) concentration. IC_{50} values for SFB (d) and DOX (f) were obtained through nonlinear regression. * different from control (C), $p < 0.05$, $n = 3$.

Discussion

Among the mechanisms involved in MDR development in hepatic tumors, one of the most relevant is the overexpression of drug efflux pumps such as MDR1 and MRP3 [5]. The limited success of direct ABC transporter inhibitors in clinical oncology has increased interest in upstream regulatory mechanisms that control transporter expression and may indirectly attenuate drug resistance phenotypes. In this context, RNA-binding proteins (RBPs) have emerged as relevant post-transcriptional regulators of cancer progression and therapy response. In a microarray analysis of 60 human HCC samples, IGF2BP1 was the most strongly upregulated RBP among the 116 RBPs analyzed [24]. Although IGF2BP1 has been implicated in chemoresistance through *MDR1* mRNA stabilization in ovarian cancer and in the modulation of MDR-associated transporters in colorectal cancer [10, 11], its relationship with ABC transporter regulation in hepatic tumor models remains poorly characterized.

RBPs regulate gene expression by interacting with specific sequence motifs or structural elements within the CDS, 5'UTR or 3'UTR of target RNAs [8]. These interactions can influence RNA localization, stability, and translation. IGF2BPs recruit target transcripts to cytoplasmic messenger ribonucleoprotein particles, which may condense into RNA granules such as processing bodies and stress granules, thereby protecting mRNAs from decay and modulating translation-related events [9]. More recently, IGF2BPs have also been described as reader proteins that regulate RNA fate in an m6A-dependent manner [25]. By silencing IGF2BP1 in HepG2/C3A cells, we observed a reduction in MDR1 and MRP3 protein expression, accompanied by impaired transporter activity, as shown by increased intracellular accumulation of Rh123 and CF, respectively (Supplementary Material S1). IGF2BP1 depletion also reduced the mRNA levels of both ABC transporters. The 6 h ActD assay further supported a post-transcriptional contribution of IGF2BP1 to MDR1 regulation, consistent with a role in *MDR1* mRNA stabilization. In the case of MRP3, the decrease observed after IGF2BP1 silencing indicates that IGF2BP1 contributes to its regulation. However, because MRP3 protein induction persisted under SFB treatment despite reduced *MRP3* mRNA levels, and because the predicted IGF2BP1-MRP3 interaction profile was weaker than that observed for MDR1, MRP3 regulation appears to involve additional or indirect mechanisms.

In line with our findings, Sparanese and Lee demonstrated that, in ovarian cancer cells, IGF2BP1 binds to the mRNA encoding MDR1 protecting it from endoribonuclease cleavage [26]. In the present study, the potential interaction between IGF2BP1 and *MDR1* and *MRP3* transcripts was explored through bioinformatics analysis. Using the RPISeq algorithm, positive interaction probabilities were predicted

between IGF2BP1 and the complete mRNA sequences of both transporters, with particularly high classifier values for the 3'UTR region of *MDR1*. RBPmap also predicted potential IGF2BP1-binding motifs within the *MDR1* transcript. Together, these findings support the involvement of IGF2BP1 in the post-transcriptional regulation of MDR1 in HepG2/C3A cells. Conversely, the evidence for MRP3 appears less straightforward. Although RPISeq and RBPmap predicted potential IGF2BP1-binding sites within the *MRP3* transcript, the interaction profile was weaker and more diffuse than that observed for *MDR1*. Moreover, the relatively low interaction scores and the less defined IGF2BP1-MRP3 interaction regions predicted by catRAPID raise uncertainty about a direct binding mechanism. Post-transcriptional regulation of MRP3 remains poorly explored. An et al., demonstrated that the long non coding RNA (lncRNA) FOXD2AS1 induced MRP3 through downregulation of miRNA-143 [27], and IGF2BP1 silencing has been reported to decrease FOXD2-AS1 levels in HCC-derived cells [28]. Thus, an indirect regulatory mechanism involving FOXD2-AS1 or related pathways could contribute to the effect of IGF2BP1 on MRP3 expression. Direct biochemical validation, such as RNA immunoprecipitation or CLIP-based assays, will be required to conclusively demonstrate IGF2BP1 binding to these transcripts and to define the precise molecular determinants of these interactions. Therefore, the current data should be interpreted as supportive evidence for IGF2BP1-mediated post-transcriptional regulation, particularly for MDR1, rather than as definitive proof of direct binding to both transporter mRNAs.

Another relevant finding of this study is that SFB increased IGF2BP1 expression in HepG2/C3A cells. This observation suggests that exposure to SFB may trigger an adaptive post-transcriptional response involving IGF2BP1. Because SFB also increased MDR1 and MRP3 expression under the same experimental conditions, we evaluated whether IGF2BP1 contributed to the induction of these transporters. IGF2BP1 silencing prevented the SFB-induced increase in MDR1 at both mRNA and protein levels, supporting MDR1 as the most consistent IGF2BP1-dependent ABC transporter identified in this model. In contrast, MRP3 showed a partial and more complex dependence on IGF2BP1. IGF2BP1 knockdown reduced SFB-induced MRP3 mRNA levels but did not abolish the increase in MRP3 protein expression. This transcript-protein dissociation may reflect a temporal lag between mRNA and protein changes, but it may also indicate that MRP3 protein abundance under SFB exposure is sustained by additional mechanisms, such as enhanced translation, increased protein stability, reduced degradation, or altered transporter trafficking. This interpretation is consistent with the *in silico* analyses, which predicted a stronger and more focused interaction profile for MDR1

than for MRP3. Therefore, while our data support a role for IGF2BP1 in MRP3 regulation, the direct contribution of IGF2BP1 to *MRP3* mRNA control remains less certain than that observed for *MDR1*.

A well described phenomenon in HCC is the upregulation of SIRT1 [13]. SIRT1 has also been reported to exert context-dependent functions according to tissue and cancer type, although most evidence in HCC supports an oncogenic role [13]. In addition, SIRT1 has been linked to MDR-associated pathways. For instance, SIRT1 mediates MDR1 overexpression in breast tumor cells through the regulation of β -catenin signaling and NF- κ B-specific transcriptional activity [29], and SIRT1 overexpression increased MDR1 expression in HepG2 cells [30]. Wen et al. showed that EX-527 decreased MRP3 expression in nude mice bearing HCC xenografts [31], while SIRT1 knockdown in HepG2 cells reduced MDR1 and MRP3 protein levels [22]. In the present study, pharmacological inhibition of SIRT1 with EX-527 prevented the SFB-induced increase in IGF2BP1 and MDR1 protein levels and partially modified the response of MRP3. These findings suggest that SIRT1 activity may contribute to the adaptive induction of IGF2BP1 and selected ABC transporters in response to SFB. However, because SIRT1 expression or activity was not directly manipulated by genetic approaches, and because EX-527 may influence additional SIRT1-dependent cellular programs, the proposed SIRT1-IGF2BP1 link should be interpreted as a working hypothesis rather than as a fully established mechanistic pathway. Further studies using SIRT1 knockdown/overexpression and downstream pathway analysis will be required to determine whether SIRT1 directly mediates SFB-dependent IGF2BP1 induction.

BTYNB is the first reported allosteric inhibitor of IGF2BP1 [32]. BTYNB was found to prevent IGF2BP1 binding to *E2F1* mRNA in leukemic cancer cells, diminishing the transcription factor E2F1 expression [23]. We confirmed the inhibitory effect of BTYNB on IGF2BP1 by evaluating *E2F1*, a target gene of IGF2BP1. For cytotoxicity studies we chose DOX as a known MDR1 substrate [33] and SFB as an MRP3 reported substrate [34]. Our results show that the inhibition of IGF2BP1 by BTYNB enhanced the cytotoxic effect of both drugs in HepG2/C3A cells, as evidenced by the lower IC_{50} value in the BTYNB group compared to the control group. Consistent with established substrate relationships [33, 34], and the accepted use of surrogate fluorescent substrates (Rh 123 and CF, shown in S1), our data suggest transporter-mediated effects on drug sensitivity. Drug-specific efflux of SFB and DOX was not measured; therefore, while ABC transporter modulation is implicated, the chemosensitization observed may also reflect IGF2BP1 effects on additional targets. Further studies are needed to prove whether BTYNB is increasing SFB or DOX cytotoxicity by diminishing their

efflux out of cells through an ABC transporter. While our results consistently support a role for IGF2BP1 in modulating drug sensitivity, BTYNB specificity is not fully established and potential off-target effects cannot be fully excluded.

In addition, *in silico* survival analysis using the TCGA-LIHC dataset showed that high combined expression of IGF2BP1, MDR1 and MRP3 was associated with poorer survival in the HCC cohort analyzed (Supplementary Table S3 and Figure S2). Although this analysis does not establish causality, it supports the clinical relevance of evaluating IGF2BP1 together with MDR-associated transporters in the context of therapeutic response.

Conclusions

In conclusion, this study identifies IGF2BP1 as a regulator of MDR1 and a contributor to MRP3 modulation in HepG2/C3A hepatic tumor cells. Transient silencing of IGF2BP1 reduced MDR1 protein expression to approximately 40-42% of control values and MRP3 protein expression to approximately 50-58% of control values, while decreasing *MDR1* and *MRP3* transcript levels to $55 \pm 4\%$ and $55 \pm 6\%$ of control, respectively. SFB at 2 μ M increased IGF2BP1, MDR1, and MRP3 protein levels after 48 h to $128 \pm 4\%$, $122 \pm 3\%$, and $142 \pm 1\%$ of control, respectively. Under SFB exposure, IGF2BP1 knockdown prevented MDR1 upregulation at both protein and transcript levels and reduced *MRP3* transcript levels, although it did not abolish MRP3 protein induction. In addition, pharmacological inhibition of IGF2BP1 with BTYNB sensitized cells to SFB and DOX, reducing half-maximal inhibitory concentration values from 54 ± 4 to 40 ± 1 μ M and from 48 ± 1 to 30 ± 12 μ M, respectively. Overall, these findings support IGF2BP1 as a potential upstream modulator of drug resistance-associated phenotypes in hepatic tumor cells.

Conflicts of Interest

The authors declare that they have no competing interests.

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Author Contributions

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María Bucci-Muñoz: Investigation, Formal analysis, Methodology, Writing – original draft.

Verónica Livore: Methodology.

Luciano Anselmino: Methodology, Formal analysis.

Claudia Banchio: Resources.

Fernando Crocenzi: Conceptualization, Writing – review & editing.

Ariel Quiroga: Conceptualization, Writing – review & editing.

Juan Pablo Rigalli: Conceptualization, Writing – review & editing.

María Paula Ceballos: Conceptualization, Funding acquisition, Writing – review & editing.

María Laura Ruiz: Investigation, Conceptualization, Resources, Funding acquisition, Project administration, Writing – review & editing.

All authors reviewed and approved the final version of the manuscript.

Availability of Data and Materials

The datasets generated and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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