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Effect of benzylglucosinolate on signaling pathways associated with Type 2 diabetes prevention

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List of abreviations

4E-BP1	eIF4E binding protein 1
ACC	Acetyl-CoA carboxylase-a
AKT/PKB	Protein Kinase B
AMPKα	Adenosine monophosphate-activated protein kinase
AREs	Antioxidant response elements
ATG	Autophagy-related gene
ATG12	Autophagy related protein 12
ATG5	Autophagy related protein 5
BAD	BCL2-Associated Agonist Of Cell Death
Bcl-2	B-cell lymphoma 2
BIM-1	B-cell lymphoma 2 interacting mediator of cell death
BITC	Benzyl isothiocyanate
BSA	Bovine serum albumin
BSP	Broccoli sprouts powder
cAMP	Cyclic adenosine monophosphate
CBP80	80 kDa nuclear cap-binding protein
CCNG2	Cyclin G2
Cdc25B	Cyclin G2 and M-phase inducer phosphatase 2
CDK1	Cyclin dependent kinase 1
CDK2	Cyclin-dependent kinase 2
CREB	cAMP response element binding protein
CREBH	cAMP-responsive element binding protein hepatocyte specific
CRT2	CREB regulated transcription co-activator 2
DAPI	4',6-diamidino-2-phenylindole
DAX-1/SHP	Dosage-sensitive sex reversal, adrenal hypoplasia critical region, on chromosome X, gene 1/ Small heterodimer partner
DDB1	DNA damage-binding protein 1
DMEM	Dulbecos Modified Eagle Medium
DYRK1	Dual specificity tyrosine-phosphorylation regulated kinase 1
EC50	Median efective concentration
ECACC	European Collection of Cell Cultures
eIF4E	Eukaryotic translation initiation factor 4E
EMEM	Eagle’s Minimum Essential Medium
ER	Endoplasmic reticulum

ERK	Extracellular signal-regulated kinase
ERRg	Estrogen receptor related gamma
FASL	Fas ligand
FASN	Fatty acid synthase
FCS	Fetal calf serum
FOXO	Forkhead box O transcription factor (human protein)
	Foxo (murine homolog)
FOXO1/3a	Forkhead box O transcription factor 1
G6Pase	Glucose-6-phosphatase
GABARAPL-1	GABA(A) Receptor-Associated Protein Like 1
GADD45	Growth Arrest / DNA Damage Repair
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GFP	Green fluorescent protein
GK	Glucokinase TLKO
GLS	Glucosinolates
GPX-2	Glutathione peroxidase 2
GSH	Glutathione
GSK-3α	Glycogen synthase kinase 3
GSSG	Oxidated GSH
H_2O_2	Hydrogen peroxide
HepG2	Human hepatoma
HNF4α	Hepatocyte nuclear factor 4 alpha
HO.	Hydroxy radical
HSA	Human serum albumin
IGF-1	Insulin-like growth factor 1
IGF-R	Insulin-like growth factor receptor
IR	Insulin receptor
IREs	Insulin response elements
IRS	Insulin responsive sequences
ITCs	Isothiocyanates
JNK	c-Jun N-terminal kinase
kDa	kilo Daltons (atomic mass)
KEAP1	Kelch-like ECH-associated protein 1
LC3	Microtubule-associated protein 1A/1B-light chain 3
LDL	Low density lipoprotein
MAF	V-maf musculoaponeurotic fibrosarcoma oncogene homolog

MAPK	Mitogen-activated protein kinase
MnSOD	manganese superoxide dismutase
mRNA	messenger RNA
MST-1	mammalian Ste20-like kinase
mTOR	Mammalian target of rapamycin
mTORC1	mammalian target of rapamycin complex 1
NAC	N-acetyl cysteine
NAD	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NEAA	Non-Essential Aminoacids
NO	Nitric oxid
NQO1	NAD(P)H dehydrogenase (quinone 1)
NRF2	Nuclear factor (erythroid derived)-like2
Nuc/Cyt	Nucleus / cytoplasm
O_2^-	Superoxide
OD	Optical density
p21CIP	Cyclin-dependent kinase inhibitor 1A
p27 KIP	Cyclin-Dependent Kinase Inhibitor 1B
p300/CBP	CREB-binding protein/E1A binding protein p300
p38α	α mitogen-activated protein
p70S6K	70 kDa ribosomal S6 kinase
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCAF	P300/CBP-associated factor
PDK	Phosphonositide dependent kinase
PDPK1	3-phosphoinositide dependent protein kinase-1
PEITC	Phenethyl isothiocyanate
PEPCK	Phosphoenolpyruvate carboxikinase
PGC-1 α	Peroxisome proliferator activated receptor gamma co-activator 1 alpha
PI3P	Phosphatidylinositol 3-phosphate (PI3P) phosphatase
PIP3	Phosphatidylinositol 3-phosphate
PIK3C3	PI3-kinase catalytic subunit type 3
PIP2	Phosphatidylinositol 4,5-bisphosphate
PKA	Protein Kinase A
PMSF	Phenylmethylsulfonyl fluoride
PCR	Polymerase chain reaction

PP2B	Protein phosphatase 2 B
PRAS40	Proline-rich Akt substrate of 40 kDa
PRMT1	Protein arginine N-methyltransferase 1
Prx	Peroxidase
PTEN	Phosphatase and tensin homolog
PTM	Post-translational modifications
qPCR	Quantitative real time PCR
Ral	Ras-related protein
RAPTOR	Regulatory-associated protein of mTOR
Rheb	GTP-binding protein
RICTOR	Rapamycin-insensitive companion of mTOR
ROS	Reactive oxygen species
RPL32	60S ribosomal protein L32
RT	Reverse transcription
RT	Room temperature
S6	Ribosomal Protein S6
S6K1	Ribosomal protein S6 kinase 1
SDS	Sodium Dodecyl Sulfate Salt
SDS	Rodium dodecyl sulfate
SEM	Error of the mean
SGK	Serum and glucocorticoid inducible kinase
SIK	Salt-inducible kinase
siRNA	Small interfering RNA
SIRT1	NAD-dependent deacetylase sirtuin-1
SKAR	S6K1 Aly/REF-like target
SKP2	S-phase kinase-associated protein 2
SMEK/PP4C	serine/threonine phosphatases suppressor of MEK null 1/ protein phosphatase 4
SQSTM1	Sequestosome-1
SREBP-1c	sterol regulatory element binding protein
SRXN1	Sulfiredoxin-1
T2D	Type 2 diabetes
TBST	Tris Buffered saline, supplemented with 0.1% Tween
TCF7L2	Transcription factor 7-like 2
TLKO	Triple knockout
TSC	Tuberous sclerosis complex

U-2OS	Human osteosarcoma cells
ULK1	UNC51-like kinase 1
UVRAG	Ultraviolet radiation resistance-associated gene
VPS34	Phosphatidylinositol 3-kinase catalytic subunit type 3

Summary

Type 2 diabetes (T2D) is a health problem throughout the world. In 2010, there were nearly 230 million individuals with diabetes worldwide and it is estimated that in the economically advanced countries the cases will increase about 50% in the next twenty years. Insulin resistance is one of major features in T2D, which is also a risk factor for metabolic and cardiovascular complications. Epidemiological and animal studies have shown that the consumption of vegetables and fruits can delay or prevent the development of the disease, although the underlying mechanisms of these effects are still unclear.

Brassica species such as broccoli (Brassica oleracea var. italica) and nasturtium (*Tropaeolum majus*) possess high content of bioactive phytochemicals, e.g. nitrogen sulfur compounds (glucosinolates and isothiocyanates) and polyphenols largely associated with the prevention of cancer. Isothiocyanates (ITCs) display their anti-carcinogenic potential by inducing detoxicating phase II enzymes and increasing glutathione (GSH) levels in tissues.

In T2D diabetes an increase in gluconeogenesis and triglyceride synthesis, and a reduction in fatty acid oxidation accompanied by the presence of reactive oxygen species (ROS) are observed; altogether is the result of an inappropriate response to insulin. Forkhead box O (FOXO) transcription factors play a crucial role in the regulation of insulin effects on gene expression and metabolism, and alterations in FOXO function could contribute to metabolic disorders in diabetes.

In this study using stably transfected human osteosarcoma cells (U-2 OS) with constitutive expression of FOXO1 protein labeled with GFP (green fluorescent protein) and human hepatoma cells HepG2 cell cultures, the ability of benzylisothiocyanate (BITC) deriving from benzylglucosinolate, extracted from nasturtium to modulate, i) the insulin-signaling pathway, ii) the intracellular localization of FOXO1 and iii) the expression of proteins involved in glucose metabolism, ROS detoxification, cell cycle arrest and DNA repair was evaluated.

BITC promoted oxidative stress and in response to that induced FOXO1 translocation from cytoplasm into the nucleus antagonizing the insulin effect. BITC stimulus was able to down-regulate gluconeogenic enzymes, which can be considered as an anti-diabetic effect; to promote antioxidant resistance expressed by the up-regulation in manganese superoxide dismutase (MnSOD) and detoxification enzymes; to modulate autophagy by induction of BECLIN1 and down-regulation of the mammalian target of rapamycin complex 1 (mTORC1) pathway; and to promote cell cycle arrest and DNA damage repair by up-regulation of the cyclin-dependent kinase inhibitor (p21CIP) and Growth Arrest / DNA Damage Repair (GADD45). Except for the nuclear factor (erythroid derived)-like2 (NRF2) and its influence in the detoxification enzymes gene expression, all the observed effects were independent from FOXO1, protein kinase B (AKT/PKB) and NAD-dependent deacetylase sirtuin-1 (SIRT1).

The current study provides evidence that besides of the anticarcinogenic potential, isothiocyanates might have a role in T2D prevention. BITC stimulus mimics the fasting state, in which insulin signaling is not triggered and FOXO proteins remain in the nucleus modulating gene expression of their target genes, with the advantage of a down-regulation of gluconeogenesis instead of its increase. These effects suggest that BITC might be considered as a promising substance in the prevention or treatment of T2D, therefore the factors behind of its modulatory effects need further investigation.

Zusammenfassung

Diabetes mellitus Typ 2 stellt auf der ganzen Welt ein Gesundheitsproblem dar. Im Jahr 2010 waren annähernd 230 Millionen Personen weltweit an Diabetes erkrankt und innerhalb der nächsten 20 Jahre wird in industrialisierten Ländern eine Steigerung der Fälle um 50% erwartet. Eines der Hauptmerkmale des Typ 2 Diabetes ist die Insulinresistenz, die auch als Risikofaktor für metabolische und kardio-vaskuläre Komplikationen gilt. Epidemiologische Studien und Tierversuche haben ergeben, dass durch Verzehr von Gemüse und Obst eine Prävention oder Verzögerung der Entwicklung dieser Krankheit erreicht werden kann, jedoch sind die zugrunde liegenden Mechanismen dieser Effekte noch nicht aufgeklärt.

Brassica Spezies wie Broccoli (*Brassica oleracea var. italica*) und Nasturtium (*Tropaeolum majus*) enthalten einen hohen Anteil an bioaktiven Pflanzeninhaltsstoffen, wie z. B. stickstoff- und schwefelhaltige Verbindungen (Glukosinolate und Isothiocyanate) und Polyphenole, die bisher hauptsächlich mit der Prävention von Krebs assoziiert wurden. Isothiocyanate (ITCs) erreichen ihr antikanzerogenes Potential durch die Induktion von entgiftenden Phase II Enzymen und eine Anhebung der Glutathion (GSH)-Spiegel im Gewebe.

Diabetes Typ2 geht einher mit einem Anstieg der Glukoneogenese und Triglycerid-Synthese, sowie einer Reduktion der Fettsäure-Oxidation in Verbindung mit erhöhten Spiegeln an reaktiven Sauerstoffspezies (ROS) insgesamt als Resultat einer unangemessenen Insulinantwort. Forkhead box O (FOXO) Transkriptionsfaktoren spielen eine wesentliche Rolle in der Regulation der Insulineffekte in Bezug auf die vermittelte Genexpression und den Metabolismus, wobei Veränderungen in der Funktion von FOXO zu metabolischen Entgleisungen im Diabetes beitragen können.

In dieser Studie wurde unter Verwendung von stabil transfizierten humanen Osteosarkoma-Zellen (U-2 OS) mit konstitutiver Expression von GFP (grün fluoreszierendes Protein)-markiertem FOXO1 und humanen Hepatoma-Zellen (HepG2) die Wirkung von Benzylisothiocyanat (BITC), dessen Vorstufe Benzylglukosinolat aus Nasturtium isoliert wurde, in Zellkulturen evaluiert wie Modulationen der i) Insulin-Signal-Kaskade, ii) intrazellulären Lokalisation von

FOXO1 und iii) Expression beteiligter Proteine am Glucose Metabolismus, der ROS Detoxifikation, Zellzyklus-Fixierung und DNA-Reparatur.

BITC erzeugte oxidativen Stress und induzierte als Antwort darauf eine Translokation von FOXO1 aus dem Zytoplasma in den Zellkern antagonisierend zum Insulin-Effekt. Eine Stimultion mit BITC war in der Lage, die Expression von Enzymen der Gluconeogenese herunter zu regulieren, was als antidiabetogener Effekt betrachtet werden kann, eine antioxidative Resistenz durch Induktion der Mangan-Superoxid-Dismutase (MnSOD) und entgiftender Enzyme zu erzeugen, Autophagie zu modulieren durch Induktion von BECLIN1 und Herunterregulation des „mammalian target of rapamycin complex1 (mTORC1)-Stoffwechselwegs, den Zellzyklus zu fixieren und DNA-Reparatur zu induzieren durch Hochregulation des Cyclin-abhängigen Kinase- Inhibitors p21CIP und GADD45 (growth arrest and DNA damage repair). Mit Ausnahme des nuklearen Faktors (erythroid derived)-like2 (NRF2) und dessen Einfluss auf die Genexpression von Entgiftungsenzymen waren alle beobachteten Effekte unabhängig von FOXO1, Proteinkinase B (PKB/AKT) und der NAD-abhängigen Deacetylase Sirtuin-1 (SIRT1).

Die gegenwärtige Studie liefert Anhaltspunkte dafür, dass Isothiocayanate neben dem antikanzerogenen Potential eine Rolle bei der Prävention von Typ 2 Diabetes spielen könnten. BITC-Stimulationen ahmen einen Fastenzustand nach, in dem kein Insulin-Signal ausgelöst wird, FOXO Proteine im Zellkern verbleiben und die Expression von Target-Genen modulieren, mit dem Vorteil einer Herunterregulation der Glukoneogenese anstelle seiner Zunahme. Diese Effekte legen nahe, dass BITC als vielversprechende Substanz zur Prävention und Behandlung von Typ 2 Diabetes angesehen werden könnte. Deshalb benötigen die Faktoren, die dessen modulatorische Effekte hervorrufen, weitere Untersuchungen.

Chapter 1

1. Introduction

1.1 Diabetes problem

Type 2 diabetes (T2D) is a chronic disease that has become a leading health problem throughout the world [1]. In 2010, there were nearly 230 million individuals with diabetes worldwide and is estimated that in the economically advanced countries the cases will increase about 50% in until 2030 [2]. T2D Diabetes is characterized by elevated blood sugar glucose levels resulting mainly from resistance to insulin, which is also a risk factor for metabolic and cardiovascular complications and has severe health consequences associated with numerous diabetic complications including retinopathy, neuropathy, and nephropathy [2,3].

Many anabolic functions depend on an appropriate insulin response, functions such as protein and glycogen synthesis, lipid synthesis and storage and the inhibition of fatty acid oxidation, glycogenolysis, gluconeogenesis and autophagy are insulin dependent [4]. Insulin resistance leads to hyperglycemia, the main feature in T2D, owing at least in part to the impaired ability of insulin to suppress expression or activity of gluconeogenic enzymes. Besides of the increase in gluconeogenesis, a reduction in fatty acid oxidation, which is intertwined with glucose oxidation, is observed under insulin resistant conditions [5], while triglyceride synthesis is maintained and does not become insulin resistant.

In T2D an increase in the production of free radicals with a subsequent induction of oxidative stress is also present. Under oxidative stress conditions the insulin signaling is reduced, which may contribute to insulin resistance, and to the progression of diabetes and related complications [6-8].

The presence of reactive oxygen species (ROS) activate the forkhead box O (FOXO) transcription factors. They mediate the effects of ROS through the modulation of gene transcription of factors involved in several cellular processes including glucose and lipid metabolism, cell cycle arrest and apoptosis [2]. FOXO proteins have a crucial role in the regulation of insulin effects on gene expression and metabolism, and alterations in FOXO functions could contribute to metabolic disorders in diabetes

[9]. The understanding of FOXO biology and the identification of modulators of its activity would provide new opportunities for developing effective strategies for the prevention and treatment of diabetes and the metabolic syndrome [9,10].

1.2 Forkhead box class O transcription factors

The superfamily of FOXO proteins are transcriptional regulators, characterized by a conserved 110 amino acid DNA-binding domain referred to the forkhead box or winged helix domain [11]. FOXO proteins are the vertebrate orthologs of the *Caenorhabditis elegans daf16* [11], a factor that is required for formation of a long-lived dormant form of the organism called the dauer larval stage [12]. FOXO1 in vertebrates is one component of highly conserved signaling pathways that connects growth and stress signals to transcriptional control, preventing cellular proliferation, inducing antioxidant and stress response genes, and modifying insulin sensitivity [12].

In humans, the forkhead family is comprised of 39 distinct members, which have been divided into 19 subgroups. Among the forkhead family, FOXO subgroup contains four members: FOXO1, FOXO3a (sometimes called just FOXO3), FOXO4 and FOXO6 [6]. FOXO proteins are widely expressed in all tissues. FOXO1 messenger RNA (mRNA) is abundant in adipose tissue, FOXO3 mRNA is highly expressed in the brain, FOXO4 mRNA in heart and FOXO6 mRNA is predominantly expressed in the developing brain [6]. There is evidence showing that loss of one out of three FOXO proteins (FOXO1, FOXO3 and FOXO4) can be compensated up to some degree by the absence of the others, since the triple conditional knockouts resulted in lymphomas, hemagiomas, and angiosarcomas, which is not observed with double knockout combinations [13]. Some exceptions occurs with FOXO1, which plays the major role in the regulation of insulin sensitivity, and FOXO3, which plays an important role modulating longevity in invertebrates, mice, and humans [12].

As FOXO1 is the primary transcription factor mediating insulin effects in hepatocytes, adipocytes and β cells [14], this study is focused on the effects of glucosinolates on the modulation of this protein.

1.2.1 Regulation of FOXO1 in response to insulin and growth factors

Since FOXO1 has a variety of cellular functions, in some cases antagonistic, it is tightly regulated by external stimuli. Environmental signals, including insulin, growth factors, nutrients, cytokines and oxidative stress induce post-translational modifications, mainly phosphorylation, acetylation, mono- and poly-ubiquitination which regulate the levels, subcellular localization and transcriptional activity of FOXO1 [15].

1.2.1.1 Phosphorylation of FOXO1 and nuclear cytoplasmic shuttling

FOXO1 intracellular mobility is regulated in two ways: i) factors that negatively modulate its transcriptional activity, induce FOXO1 nuclear export, and ii) factors that stimulate FOXO1 import from cytoplasm into the nucleus and promote FOXO1 activation and transcription of FOXO1 target genes.

FOXO1 negative regulation. The main pathway of FOXO1 regulation is that described for the phosphoinositide kinase PI3K-AKT (=PKB protein kinase B) signaling pathway [16], although under different conditions the extracellular signal-regulated kinase (ERK) [17], and cyclin-dependent kinase 2 (CDK2) [12] induce FOXO1 nuclear export as well. Binding of growth factors or insulin to their tyrosine kinase receptors induce recruitment of insulin receptor substrate (IRS) and activation of PI3K, which phosphorylates the phosphoinositide dependent kinase (PDK) and activates AKT at the phosphorylation sites T308, S473 and SGK (serum and glucocorticoid inducible kinase) family of protein kinases [6,18].

The activated AKT and SGK phosphorylate FOXO1 at three PKB/AKT consensus sites T24, S256 and S319, and promote its cytoplasmic sequestration and degradation preventing FOXO1 transcriptional activity [16]. Phosphorylation of FOXO1 by AKT and SGK also creates binding sites for the chaperone protein 14-3-3 [19], which promotes FOXO1 nuclear export, probably by exposing FOXO nuclear export sequence [20]. In addition, phosphorylation of the second site (S256) introduces a negative charge in the nuclear localization signal, preventing FOXO1 re-entry into the nucleus [21]. Thus, sequestration of FOXO1 transcription factor in the cytoplasm results from the enhanced nuclear export and decreased nuclear entry [16] (Fig. 1 and Table 1).

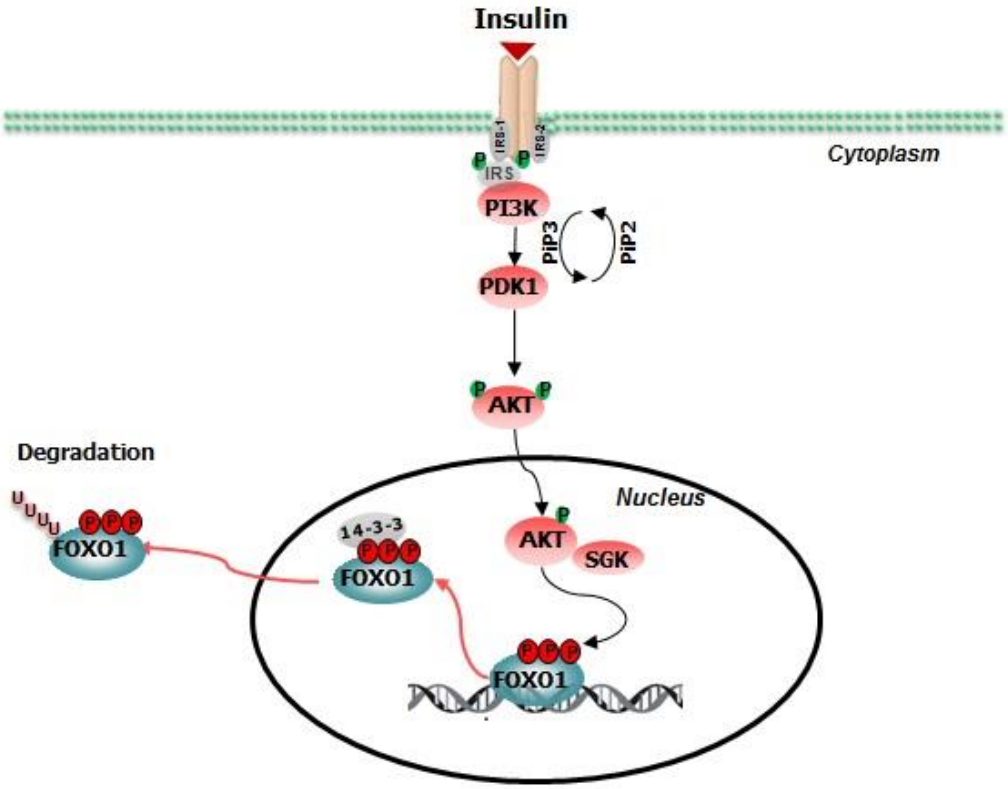


Figure 1. AKT/PI3K pathway modulation. In the presence of insulin or growth factors, following binding to the insulin receptor (IR) or (IGF-R) and autophosphorylation IRS activates the class 1A phosphotidylinositol 3-kinase (PI3K) to generate phosphatidylinositol (3,4,5)-triphosphate (PIP3), which recruits 3-phosphoinositide dependent protein kinase-1 (PDPK1) and AKT to the plasma membrane, where AKT is activated by PDPK1-mediated phosphorylation [18]. Following PI3K/AKT pathway activation, AKT and SGK translocate to the nucleus where they directly phosphorylate FOXO1 on three conserved residues. Phosphorylated FOXO1 binds to 14-3-3 proteins, which result in the export of FOXO from the nucleus into the cytoplasm. The sequestration of FOXO into the cytoplasm inhibits FOXO-dependent transcription.

Insulin stimulation inhibits FOXO-dependent transcription not only by sequestering FOXO factors in the cytoplasm. It also suppresses FOXO transcriptional activity, inhibits FOXO binding to DNA, promotes FOXO nuclear export, sequesters FOXO in the cytoplasm and induces FOXO degradation.

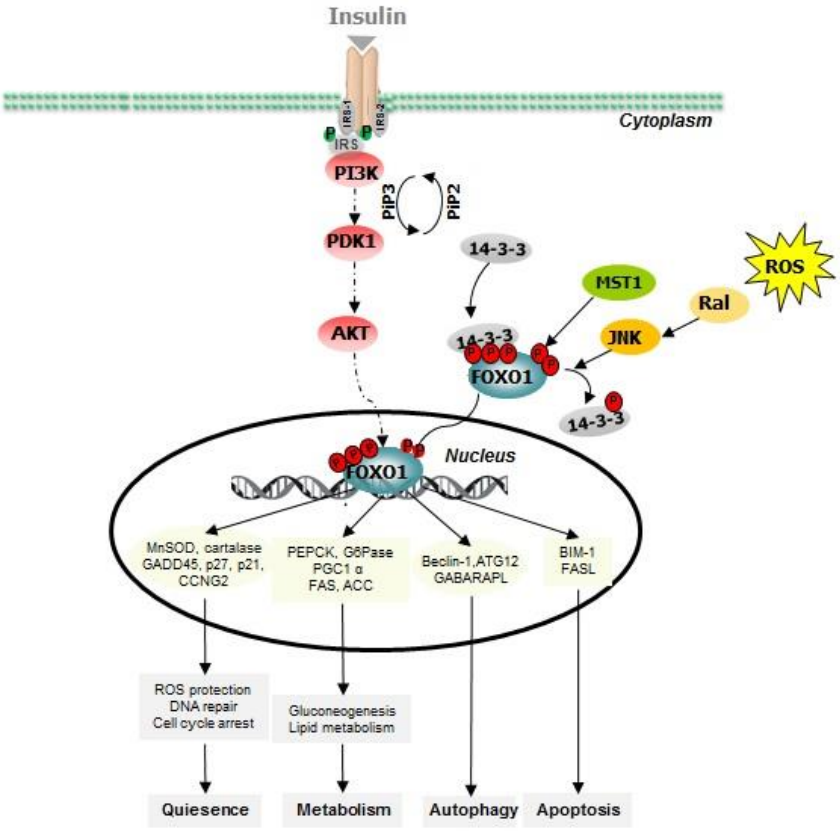


Figure 2. FOXO1 nuclear exclusion. Fasting conditions or oxidative stress promote FOXO1 nuclear translocation and accumulation. ROS activate MST1 and JNK, which induce FOXO1 nuclear translocation by disrupting the complex of FOXO and 14-3-3. Although FOXO phosphorylation by JNK does not directly inhibit the binding of 14-3-3 proteins, JNK can phosphorylate 14-3-3 directly, thus releasing 14-3-3 substrates. JNK activity is sufficient to overcome Akt inhibition of FOXO factors and causes FOXO factors to transcribe a program of genes involved in stress resistance, glucose and lipid metabolism, autophagy and apoptosis.

FOXO1 positive regulation. An impaired insulin signaling, phosphorylation by c-Jun N-terminal kinase (JNK) [22-24], α mitogen-activated protein (p38) [17] and Cyclin dependent kinase 1 (CDK1) [25] results in FOXO1 nuclear localization, promoting glucose production, lipid metabolism, cell cycle arrest, stress resistance, and cell death [9] (Fig 2). The production of glucose and triglycerides in excess leading to hypertriglyceridemia accounts in part for the concurrent pathogenesis of fasting hyperglycemia in insulin-resistant subjects with type 2 diabetes [9,26].

JNK, a (Mitogen-activated protein kinase) MAPK family member activated by stress stimuli, is responsible for FOXO1 regulation in humans [22,23]. Phosphorylation of FOXO1 by JNK in mammalian cells results in the translocation of FOXO from the cytoplasm to the nucleus, inducing the gene expression modulation of a wide range of genes [6]. Stress stimuli appear to override the sequestration of FOXO by growth

factors in mammalian cells [6,27]. Hence in cells with high insulin signaling levels, overexpression of JNK upstream kinase is enough to promote FOXO translocation to the nucleus [28]. The binding of FOXO with 14-3-3 protein contributes to the export of FOXO from the nucleus, in response to AKT phosphorylation. There is also evidence showing that JNK phosphorylates 14-3-3 proteins releasing 14-3-3 substrates, including FOXO factors [29]. This suggests that JNK regulates FOXO subcellular localization, even in the presence of growth factors, by a mechanism independent of AKT but that also involves14-3- 3 proteins [2,6].

The balance between FOXO nuclear export or import, creates an environment that defines the amount of FOXO in the nucleus [12]. FOXO proteins may contribute to the regulation of multiple metabolic pathways important for the adaptation to fasting and feeding in the liver [30] (Fig. 2 and table 1).

1.2.1.2 FOXO transcriptional activity regulation

Acetylation. FOXO transcription factors regulate gene expression positively and negatively [30]. They bind to DNA target sites, stimulate transcription of target genes, interact with and modulate the function of other transcription factors and co-activator proteins important for the regulation of gene expression in the liver [9,30]. Deacetylation by SIRT1, a nicotinamide adenine dinucleotide (NAD)-dependent histone deacetylase [27,31], and glycosylation [32] are necessary for FOXO1 transcriptional activity [16]. Calorie restriction, starvation, oxidative stress and resveratrol have been described as SIRT1 inducers [33,34],

In growing cells, FOXO proteins are predominantly cytoplasmic. Nuclear import results in association of FOXO with proteins that have histone acetylase activity, such as CREB binding protein (CBP/p300) [31]. Since acetylation of FOXO reduces transcriptional activity, acetylated and JNK-phosphorylated nuclear FOXO recruits Silent Information Regulator 1 (SIRT1), a constitutive nuclear deacetylase [11,35]. SIRT1, which is present in the nucleus, binds directly to FOXO1 through a conserved LXXLL motif and catalyzes its deacetylation, associated with an increase in FOXO transcriptional activity [35]. The presence of SIRT1 into the nucleus is not sufficient to initiate FOXO1-SIRT1 interaction, owing to the recruitment of SIRT1 is oxidative

stress dependent [31,36]. Also, it was shown that SIRT1 levels increase in fasting conditions with a contribution to gluconeogenesis [37].

Enzyme	Phosphorylation	Effect	Reference
AKT/SGK	T24, S256, S319	-	[16]
CK1	S322, S325	-	[16]
DYRK1	S329	-	[16]
CDK2	*S249*	-	[16]
ERK	S246, S284, S295, S326 S413, S415, S429, S467 S475	-	[17]
p38α	S284, S295, S326, S467 S475	-	[17]
CDK1	S249	+	[25]
JNK	ND	+	[22,24]
MST-1	S212	+	[38]

Table 1. FOXO1 phosphorylation sites**.** FOXO1 phosphorylations sites. The site numbers are for human FOXO family member. (-), Inhibition of FoxO; (+), activation of FOXO; ND, not described yet. This table is inspired by the paper from Calnan DR et al, with updates and modifications.

Enzyme	Metylation	Acetylation	Ubiquitinaltion	Effect	Refer.
SKP2			S256	-	[15]
PRMT1	Arg248,Arg250				[39]
CBP/300		K242 K245 K262		-	[16]
PCAF		*K294* *K274*		-	[16]
Oxidative stress		*K559*		+	[16]
SIRT1		*K274* *K294*		+	[16]

Table 2. FOXO1 post-translational modifications (PTM). The site numbers are for the human FOXO family member. (-), Inhibition of FoxO; (+), activation of FOXO; black, PTM verified in cells; blue, expected site based on sequence alignment with other FOXO family members; red, removal of PTM. Italics—PTM identified *in vitro*, not verified in cells. This table is inspired by the paper from Calnan DR et al, with updates and modifications.

1.2.2 Physiological roles of FOXO proteins

The functions of FOXO proteins are diverse. They are distributed in several tissues such as liver, muscle, adipose tissue and pancreas, contributing to the regulation of metabolism [40]. At the cellular level, FOXO proteins can inhibit cellular proliferation, promote apoptosis and enhance resistance to oxidative stress. At the level of complex organisms, alterated FOXO activity might result in the development of neoplastic diseases and disorders of metabolism, including diabetes [9]. We focus our investigation on liver cells, chosen for the evaluation of the effect of Benzyl isothiocyanate (BITC) produced by myrosinase treatment of benzyl glucosinolate in this study.

1.2.2.1 Gluconeogenesis

The adaptation to feeding and fasting state in liver is accomplished by the modulatory actions of insulin, where FOXO1 plays a critical role. During feeding, insulin increases glucose uptake into hepatocytes, suppresses gluconeogenesis and glycogenolysis, and up regulates glycogen synthesis [12].

In fasting, the catabolic hormone glucagon and stress hormone cortisol stimulate the gluconeogenic gene transcription by triggering the activation of transcriptions factors, including cAMP response element binding protein (CREB), cAMP-responsive element binding protein hepatocyte specific (CREBH), nuclear receptors such as hepatocyte nuclear factor 4 alpha (HNF4α) [41], estrogen receptor related gamma (ERRγ), and FOXO proteins. The activation of transcriptional co-activators for these factors is necessary, and their activities or expression levels are also regulated by catabolic hormones. At the present CBP/p300, CREB regulated transcription co-activator 2 (CRTC2) [42], peroxisome proliferator activated receptor gamma co-activator 1 alpha (PGC-1α) [43], and SIRT1 [42] are known to be critical co-activators for gluconeogenesis (refer to figure 3 for a more detailed explanation).

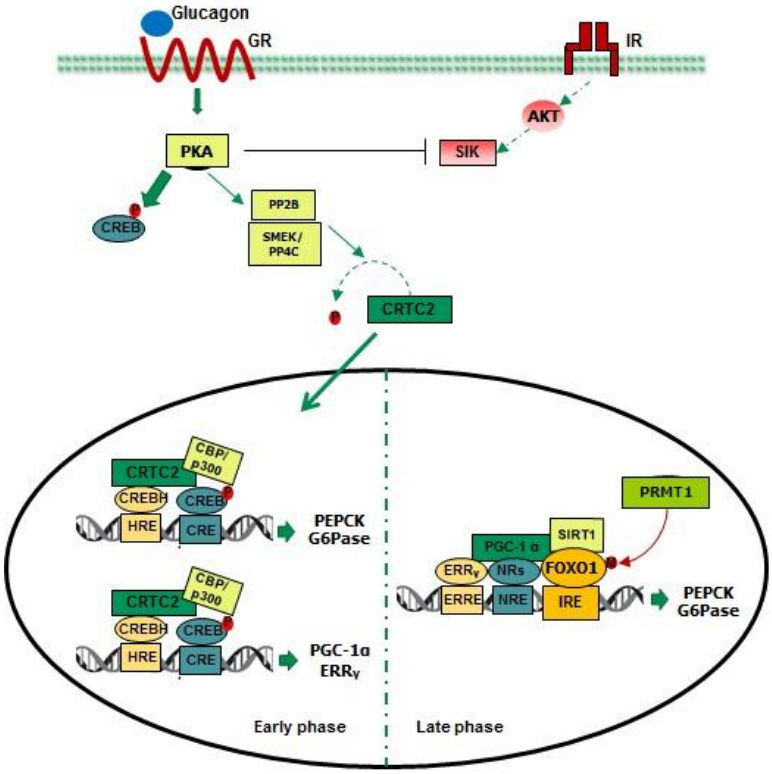


Figure 3. Transcriptional activation of hepatic gluconeogenesis. Under fasting conditions, the presence of glucagon activates Protein Kinase A (PKA), which phosphorylates CREB, inducing association with its co-activator CBP/p300 onto the chromatin. PKA inactivates salt-inducible kinase (SIK) promoting dephosphorylation of CRTC2 by serine/threonine phosphatases suppressor of MEK null 1/ protein phosphatase 4 (SMEK/PP4C) and protein phosphatase 2 B (PP2B), which induce CRTC2 nuclear translocation and interaction with CREB. The interaction of CREB-CRTC2 induces gluconeogenesis through expression of PEPCK and G6Pase and their transcriptional regulators, during the later phase of fasting PGC-1α and ERR_{γ}. In the later phase of gluconeogenesis protein arginine N-methyltransferase 1 (PRMT1) methylates arginine residues of FOXO1, preventing AKT-dependent phosphorylation. SIRT-1 deacetylates and activates FOXO1, promoting PEPCK and G6Pase gene expression [44]. This figure was adopted and modified from Oh, K.J. *et al.* 2013 [45].

Several studies in mice have shown that in partial or total absence of FOXO1, a reduced hepatic glucose production is displayed via impaired fasting-dependent gluconeogenesis in the liver [46-48]. Samuel *et al.* showed an improved hepatic and peripheral insulin action in high-fat diet-induced obese mice upon antisense oligonucleotide-mediated inhibition of FoxO1 [49]. He suggests an association between increased FoxO1 activity and impaired ability of insulin to regulate hepatic glucose metabolism, contributing to abnormal metabolism in diabetes. In addition, induction of Foxo1 in mouse liver elevates hepatic glucose production, by stimulating glucose 6 phosphatase (G6pase) and phosphoenol pyruvate carboxy kinase (Pepck) expression [50].

PEPCK catalyzes the formation of phosphoenolpyruvate, whereas G6Pase removes the phosphate from glucose-6-phosphate, before glucose is released into the blood [50]. FOXO1 binds to the insulin response elements (IREs) in the promoter-regions of genes encoding PEPCK and G6Pase, activates transcription through these elements, and this activation is blocked by insulin [41,51]. In the case of PEPCK, it has been reported that upregulation of gene expression seems to be depend on FOXO1 in excess of the normal cellular concentration. When FOXO1 is at wild-type levels, insulin repression of transcription occurs in a FOXO1-independent manner [51].

FOXO1 has been linked with the late phase of gluconeogenesis [42,50]. Glucagon effects are attenuated during late fasting, SIRT1 deacetylates CRTC2, promotes its ubiquitinylation, deacetylates and activates FOXO1, leading to the expression of gluconeogenic enzymes. The disruption of SIRT1 activity, by liver-specific knockout of SIRT1 gene or by administration of SIRT1 antagonists, increased CRTC2 activity and glucose output, while SIRT1 agonists reduced them [42]. The cooperative regulation of FOXO1 shows how the compensatory activities of these gluconeogenic regulators maintain energy balance under different phases of fasting [47] (Fig. 3).

Transcriptional inhibitors are also involved in the regulation of hepatic gluconeogenesis. Orphan nuclear receptors DAX-1/ SHP (dosage-sensitive sex reversal, adrenal hypoplasia critical region, on chromosome X, gene 1/ Small heterodimer partner) and transcription factor 7-like 2 (TCF7L2) have been identified as antagonists of PEPCK and G6Pase gene expression [45]. Studies in mice show that acute depletion of DAX-1 [52] and TCF7L2 promote hyperglycemia and glucose intolerance, concomitant with increased gluconeogenic gene expression [45]. There is evidence showing that DAX-1 blocks the association of HNF4-α and PGC-1-α on the gluconeogenic promoters, such G6Pase and PEPCK, reducing their gene expression [52]. Regarding TCF7L2, its expression is increased under feeding conditions, reducing the binding of CREB and Foxo1 over the gluconeogenic promoters in mice liver, leading to inhibition of gluconeogenesis. Overexpression of nuclear TCF7L2 in the liver ameliorates hyperglycemia, with reduced hepatic gluconeogenesis in the diet-induced-obesity mice, suggesting that TCF7L2 is critical in the regulation of glycaemia in mammals by modulating hepatic gluconeogenesis *in vivo* [45].

The reciprocal regulation of FOXO1 in the modulation of glucose production plays a critical role in maintaining blood glucose levels within a narrow physiological range in different metabolic states [53].

1.2.2.2 Antioxidant and detoxification response

Reactive oxygen species (ROS) are normal by-products produced by cellular functions or generated as signaling molecules in various intracellular processes, including cell proliferation, migration, and apoptosis [2]. They also include hydrogen peroxide (H_2O_2), hydroxy radicals (HO.) and superoxide (O_2^-) [54] (Fig. 4). Oxidative stress occurs within cells when the balance between ROS and antioxidant defense is abolished, either by a reduction in the cellular antioxidant detoxification activity or through an increase in oxygen radicals [55]. There are multiple sources of ROS in the cells, such as mitochondrial dysfunction, increased metabolic activity, starvation, altered glucose levels, oncogenic activity, decreased antioxidant capacity, or through crosstalk with immune cells [56].

Oxidative stress leads to relocalization and activation of FOXO1 in the nucleus, even in presence of growth factors [2,27]. In response to oxidative stress, the protein kinases JNK and MST1 (mammalian Ste20-like kinase) phosphorylate FOXO1, and disrupt the FOXO1-14-3-3 protein complex, thereby promoting FOXO1 nuclear accumulation [22,24,38] (Fig. 1B). Thus, the oxidative stress-activated MST1 and JNK pathways, directly antagonize the insulin/growth factor-activated PI3K–AKT/SGK pathway, and through FOXO1 transcriptional activity modulate apoptosis or stress resistance by the gene expression of FOXO1 target genes [16,57].

It is well established that in response to oxidative stress, FOXO proteins induce manganese superoxide dismutase (MnSOD) and catalase [58,59], which breakdown H_2O_2 into water and oxygen [54]. Decomposition of H_2O_2 is also mediated by other enzymes. In addition to catalase, several peroxiredoxins and GSH peroxidases, localized either in cytosol or mitochondria, catalyze the breakdown of H_2O_2 and organic hydroperoxides (Fig.4) [56,60].

Although there is no recognized sequence in MnSOD promoters for FOXO1 binding up to the date, it is known that FOXO3 directly binds to the MnSOD promoter at

FOXO binding elements, increasing its gene expression [58]. The functional significance of FOXO1 in regulating oxidative stress was demonstrated in mice hematopoietic stem cells, and mice osteoblasts cells. There, the lack of FoxO increased ROS levels, decreased expression of antioxidant proteins such as GSH, and increased apoptosis, which suggested a critical role of FoxO1 in stress resistance [61,62].

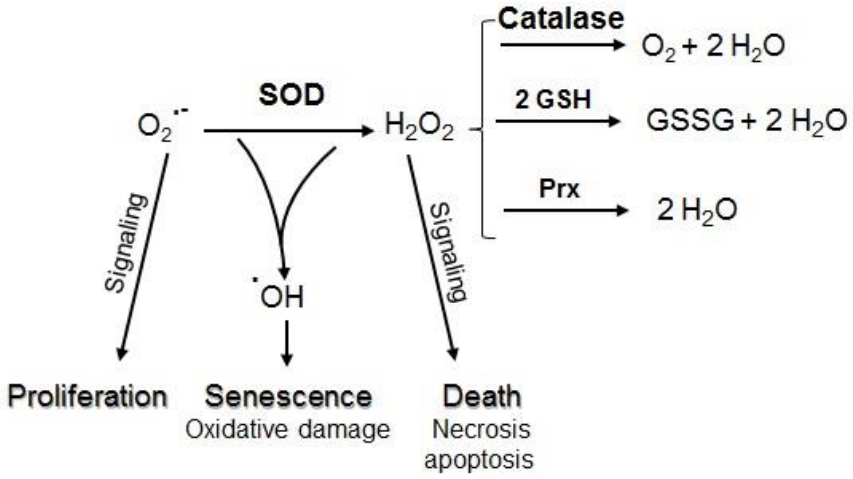


Figure 4. Major ROS in cells: superoxide (O2.-), hydrogen peroxide (H2O2), and hydroxyl ions (.OH). O_2 are mainly generated by mitochondria or NADPH oxidase enzymes and induce cell proliferation. $O_2^{\cdot-}$ is dismutated to H_2O_2 whose major role is in inducing apoptotic or necrotic cell death. Both $O_2^{\cdot-}$ and H_2O_2 can form macromolecule damaging hydroxyl ions that can contribute to senescence and cell death. Antioxidant enzymes such as superoxide dismutase (SOD), catalase and glutathione/glutathione peroxidase (GSH/Prx) systems are responsible for the hydrolysis of H_2O_2 to water and oxidized GSH, which protect the cells against the ROS and allow the cell to keep the balance between ROS and antioxidant capacity. Adapted from Storz P *et al.*

1.2.2.3 Lipid metabolism

The role of FOXO proteins in the lipid metabolism is unclear. Studies in diabetic mice show that FOXO1 induces an up-regulation in the lipid metabolism pathways such as lipogenesis and hepatic triglyceride accumulation [48,53], although subsequent studies have observed down-regulation of the same pathways as well [30,63]. Zhang *et al.* [63] showed that loss of hepatic FoxO1 and FoxO3 in mice *in vivo,* synergistically enhanced the expression of a number of proteins required for lipid synthesis, including sterol regulatory element binding protein (SREBP-1c), acetyl-CoA carboxylase-a (ACC), and fatty acid synthase (FAS). Conversely, overexpression of FoxO1 and FoxO3 reduced their gene expression *in vitro.* The physiologic significance of enhanced lipogenesis in these studies is unclear, since

the anabolic cellular activity in fasting conditions is low, where FOXO1 activity is expected [64].

1.2.2.4 Autophagy and adaptation to starvation

Autophagy, is a process characterized by the lysosomal degradation of cytoplasmic constituents of the cell necessary for the maintenance of cellular homeostasis during energy starvation or stress [65]. A dysregulated autophagy has been implicated in the pathogenesis of T2D, neurodegenerative diseases, cancer, aging, and infection [66,67]. In autophagy, part of the cytoplasm is surrounded by a double membrane, presumably from the endoplasmic reticulum (ER), to form an autophagosome that fuses with lysosomes after which the sequestered material is degraded. Autophagy-related (ATG) proteins contribute with the formation of double membrane around cytoplasm to form the autophagosome. Autophagy is inhibited by Mammalian target of rapamycin (mTOR) signaling pathway via both short-term and long-term regulatory mechanisms [68]. Short-term inhibition can be produced by mammalian target of rapamycin complex 1 (mTORC1) which, by phosphorylation, produces inhibition of ULK1. Long-term regulation occurs via the transcription factors FOXO1 and FOXO3 [68].

FOXO proteins promote adaptation to starvation and fasting, by the induction of the autophagy pathway through transcription dependent and independent mechanisms: 1) induce the transcription of BECLIN1, autophagy-related protein 8 (ATG8) [12], microtubule-associated protein 1A/1B-light chain 3 (LC3), GABA(A) Receptor-Associated Protein Like 1 (GABARAPL-1), phosphatidylinositol 3-kinase catalytic subunit type 3 (VPS34), ULK2 and autophagy-related protein 12 (ATG12), which are factors of the autophagy pathway [69]; 2) increasing the levels of the amino acid glutamine that inhibits mTORC1 signaling activity [70], decreasing the negative regulation on autophagy; 3) the cytoplasmic acetylated form of FOXO1 directly interacts with ATG7, a key regulator of the formation of the autophagosome [71] (refer to figure 5 for a more detailed explanation).

The transcription activity of FoxO3 has been shown as FoxO1 dependent, since the absence of FoxO1 reduces the gene expression of vps34, atg12, and gabarapl1 genes in mice livers [72]. In response to starvation or oxidative stress, FOXO3

transcriptionally activated class I PI3K catalytic subunit PIK3CA phosphorylates and up-regulates AKT activity, which catalyzes the phosphorylation of FOXO1 inducing its nuclear exclusion. In the cytoplasm, FOXO1 is dissociated from SIRT2 and interacts with ATG7 inducing autophagy [71,73]. The figure 4 illustrates the autophagy pathway and the steps in which FOXO proteins, especially FOXO1, play an important role.

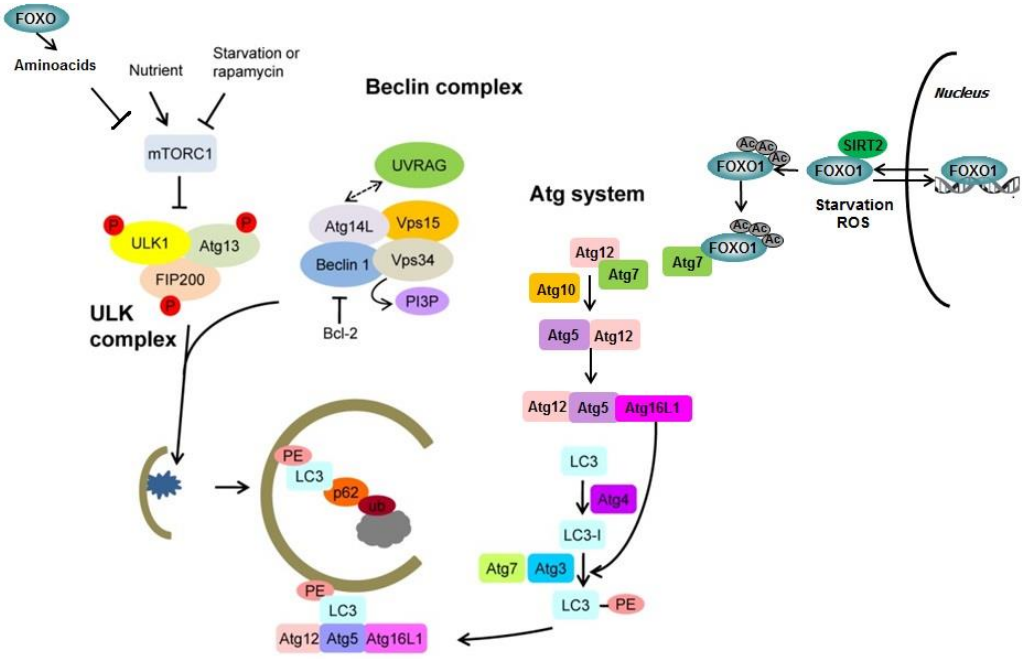


Figure 5. Autophagy modulation by FOXO proteins.The amino acid synthesis modulation by FOXO proteins, starvation or rapamyicin treatment, inhibits mammalian target of rapamycin complex 1 (mTORC1) whereas nutrients availability induce it. mTOR induces dephosphorylation of UNC51-like kinase 1 (ULK1), which phosphorylates autophagy-related gene 13 (Atg13) and focal adhesion kinase family interacting protein of 200 kD (FIP200), activating autophagy. The autophagosome formation starts with Vps34, Vps15, and Atg14Lor alternatively, with Vps34, Vps15, and ultraviolet radiation resistance-associated gene (UVRAG). All of them are induced by Beclin 1, which is liberated from B-cell lymphoma 2 (Bcl-2) [65]. Phosphatidylinositol- 3-phosphate (PI3P) produced by Vps34 contributes with the recruitment of Atg proteins to initiate autophagosome formation. FOXO nuclear proteins, such as FOXO3, contribute with the transcription of some mediators of the autophagic pathway. However, under stress conditions or starvation, the presence of FOXO1 in the cytoplasm and its acetylation by SIRT2 is necessary for the binding to Atg7 [65], which activates Atg 12 and which is transferred by Atg10 to Atg5. The Atg12-Atg5 dimer will be joined by Atg16 to form an Atg12-Atg5-Atg16 multimer complex, which is localized to membranes of early autophagosomes. LC3-I is cleaved by Atg4 to produce LC3-which will be conjugated to phosphatidylethanolamine by Atg7 and Atg3, binding to the autophagosomal membrane [72]. LC3-II, is a receptor for p62 which binds to ubiquitinated proteins for proteolytic degradation. This figure was adopted and modified from Quan W *et al.* [65].

1.2.2.5 mTOR regulation

Mammalian target of rapamycin (mTOR) is the catalytic subunit of two distinct complexes called mTOR complex 1 (mTORC1) and mTORC2 [74]. In mammals, mTOR specifically associates with regulatory-associated protein of mTOR (RAPTOR) to form mTORC1, whereas mTORC2 associates with rapamycin-insensitive companion of mTOR (RICTOR) [75]. Although RAPTOR determines the subcellular localization of mTORC1 and contributes to the amino acids sensing, the binding with these factors function as scaffolds for assembling the complexes and for binding substrates and regulators [76-78]. Because mTORC1 is activated by nutrients, growth factors, cellular energy status, and FOXO proteins have a role in its modulation, this theses will be focused on this complex (and not on mTORC2).

The mTORC1 pathway has an important role in energy balance and coordinates cell growth based on the nutritional and energy status of the cell. mTOR senses the cell's energy status by the ATP/AMP ratio for protein synthesis, and integrates growth stimulus with cell cycle progression, increasing phosphorylation and activation of molecules crucial to protein translation in the presence of abundant growth factors and nutrients [79]. Conversely, during periods of starvation or growth factor withdrawal, as well as in response to oxidative stress and infection, the phosphorylation of mTOR factors can be decreased and the autophagy is upregulated [80,81].

mTOR is part of the PI3K/AKT/mTOR pathway. mTORC1 is upstream activated in the presence of growth factors (insulin, insulin-like growth factor, platelet-derived growth factor), mitogens, hormones, or nutrients [82]. The phosphorylation and activation of PI3K and AKT/PKB leads to the inhibition of tuberous sclerosis complex (TSC) composed of TSC1 and TSC2, by directly phosphorylating the GTPase-activating protein TSC2. When released from TSC inhibition, GTP-binding protein (Rheb) is free to activate mTORC1 [79]. Activation of mTORC1, results in phosphorylation of proline-rich AKT substrate of 40 kDa (PRAS40) by mTORC1. PRAS40 is a component and substrate of mTORC1, which exert an inhibitory action on mTORC1 activity [83]. The phosphorylation by mTORC1 leads to dissociation of PRAS40 from the complex and relieves the inhibitory constraint on mTORC1 activity [83,84] (Fig. 6).

Subsequently activated mTORC1 phosphorylates 70 kDa ribosomal S6 kinase (p70S6K) and eIF4E binding protein 1 (4E-BP1). Phosphorylation of 4E-BP1 on four sites by mTORC1 blocks its inhibitory ability, promoting its dissociation from eukaryotic translation initiation factor 4E (eIF4E), allowing eIF4E to recruit the translation initiation factor eIF4G to the 5′ end of most mRNAs [85]. The dephosphorylation of 4E-BP interferes with translation initiation by binding and sequestering the eIF-4E mRNA cap-binding protein and preventing its assembly into the eIF-4F cap-binding complex, thus blocking cap-dependent mRNA translation [81]. S6K1 phosphorylation by mTORC1 promotes mRNA translation by phosphorylating or binding multiple proteins, including eukaryotic elongation factor 2 kinase (eEF2K), S6K1 Aly/REF-like target (SKAR), 80 kDa nuclear cap-binding protein (CBP80; also known as nCBP1) and eIF4B, which collectively affect translation initiation and elongation [75] (Fig. 6).

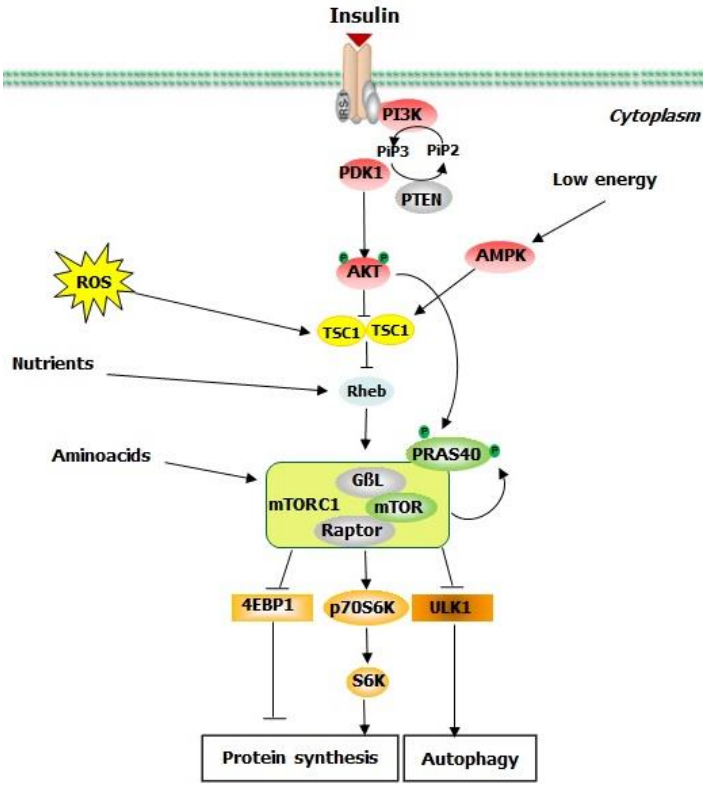


Figure 6. mTORC1 pathway regulation. The insulin stimulus activates the PI3K/AKT pathway. Activated AKT promotes the phosphorylation of Thr246 on PRAS40. In addition, AKT inhibits the activity of the tuberous sclerosis complex (TSC1)/TSC2 complex, which induces mTORC1 activation. Activated mTORC1 phosphorylates PRAS40, 4E-BP1, and ribosomal protein S6 kinase 1 (S6K1) [83]. Phosphorylation of PRAS40 results in dissociation of the mTORC1 complex. Phosphorylation of 4E-BP1 and S6K1 regulates, among others, mRNA translation. The presence of amino acids induces activity of mTORC1 complex as well [83]. In contrast to growth factors, cellular stress like hypoxia and energy deprivation promote the activity of the TSC1/TSC2 complex via AMP-activated protein kinase, (AMPK)-dependent and independent phosphorylations of TSC2, thus resulting in inhibition of the mTORC1-pathway and promoting, among other functions [74].

A major regulator of autophagy is mTORC1 [86,87]. When nutrients are available, mTORC1 inhibits autophagy by phosphorylating ULK1/2 and blocking the formation of the active ULK1/2 complex. Starvation conditions inhibit the mTORC1–ULK1/2 interaction, which de-represses ULK1/2 and induces activation of the autophagy cascade [88]. The regulation of mTORC1 activity is modulated by its subcellular localization, perhaps most notably its association with the lysosome [70].

Recently van der Vos *et al.* showed a link between FOXO proteins transcription activity and mTORC1 modulation. FOXO proteins, FOXO3 and FOXO4, induced the transcription of glutamine synthetase gene and enzyme activity and, therefore, glutamine production. Increased glutamine levels inhibited mTOR activity, as observed by a reduction in the phosphorylation of S6K1 kinase, and the re-localization of mTOR from the lysosomes to the cytoplasm [89].

Alterations in the mTOR pathway have been involved in a wide variety of diseases such as cancer and diabetes, and inhibitors of mTOR have shown promising results in animal studies [90]. Since FOXO proteins under some conditions behave as inhibitors of mTORC1, the understanding of the mechanism behind this regulation is necessary.

1.2.2.6 Proliferation and survival

Depending on the conditions and cell type, FOXO transcription factors FOXO 1/3/4 mediate cell proliferation and survival, through the gene expression of different factors of cell cycle arrest, DNA repair, and apoptosis [6]. In response to oxidative stress, FOXO induces cell cycle arrest in G1 phase, by repressing the expression of G1 cyclins D1 and D2, cell cycle positive regulators [91,92], and by inducing CDK inhibitor Cyclin-Dependent Kinase Inhibitor 1B (P27, Kip1) [93] and DNA damage-inducible gene 45α (GADD45α), which is responsible for growth arrest and DNA repair [94]. FOXO factors also bind to the promoter of cyclin-dependent kinase inhibitor 1A (p21, Cip1), a cell cycle inhibitor, and induce cell cycle arrest at the G1/S transition and subsequent quiescence [92,93]. In addition, FOXO proteins are also implicated in regulating the expression of genes, such as cyclin B1, cyclin G2 and M-phase inducer phosphatase 2 (Cdc25B), important for G2/M cell-cycle phase transition [95].

Altogether, FOXO proteins modulate cell cycle arrest at the G1/S and G2/M transitions, which are critical checkpoints in the cellular response to stress [96]. FOXO-induced cell cycle arrest may allow time for repair of damaged DNA, as well as for detoxification of cells [93]. The ability of FOXO proteins to control cell cycle progression and to promote apoptosis has illustrated the potential of FOXO in becoming an important component for new strategies directed against tumorigenesis, and can be directed also to the prevention or treatment of diabetes whose complications have been related with ROS production.

1.3 Secondary plant metabolites and effects on health

Epidemiological and animal studies have shown that consumption of food rich in fruits and vegetables decreases the risk of chronic diseases [97]. Especially in T2D, the primary prevention is considered as a public health priority. In fact, intervention trials show that the dietary modification can delay or prevent the development of diabetes type 2 [98]. Some studies show an inverse association between vegetables intake and T2D prevention [99], although the underlying mechanisms and the effect of individual antioxidant compounds are still unclear [100].

Besides cofactors and antioxidant vitamins, vegetables and fruits contain additional bioactive compounds such as phytochemicals (e.g. polyphenols or glucosinolates as source for isothiocyanates), which can induce adaptive stress response pathways able to modulate the expression of heat-shock proteins, antioxidant enzymes and anti-apoptotic proteins [101]. In plants, these substances behave as toxins, protecting against insects and other damaging organisms. These bioactive compounds can be extremely toxic when they are consumed at high concentrations by humans (carcinogenic, neurotoxic and/or cardio toxic). However, in relatively low concentrations obtained by normal plant consumption, these substances are not toxic and can induce adaptive stress responses with beneficial effects [101]. This hormetic effect has been attributed to several phytochemicals such as polyphenols and glucosinolates, from which only the effect of the first has been evaluated in the prevention or treatment of T2D [102,103].

1.3.1 Glucosinolates

Glucosinolates (GLS) are secondary plant metabolites, most of them being isolated from cruciferous or Brassicaceae plant family (e.g., broccoli, cabbage, brussels sprouts, watercress*, Nasturtium*, garden cress, kale, cauliflower) [104]. Around 150 different naturally-occurring GLSs have been recognized so far, although their content varies among different cruciferous vegetables [105,106]. Broccoli is a rich source of the glucosinolate glucoraphanin (22mg/100g) and glucobrassicin (17mg/100g). Cabbage is rich in sinigrin (17mg/100g) [106], and garden cress possesses a high content of glucotropaeolin (1000mg/100g) [107,108].

GLS share a chemical structure consisting of a glucose residue linked via a sulfur atom to an aglycone, plus a variable R group derived from amino acids [109]. GLS are chemically unreactive, strongly acidic and water-soluble. Owing to their negative log P (octanol–water partition coefficient) values, they require active transport to cross cell membranes [110].

In plants, glucosinolates are accompanied by β-thioglucosidases which hydrolyze the glucose moiety to an unstable aglycone that can rearrange to form isothiocyanates, nitriles, and other products [111]. Myrosinases and GLS are located in different compartments, but they come in contact upon tissue damage, e.g. through chewing or cutting, resulting in rapid hydrolysis [109] (Fig. 7).

Mammalian tissues do not contain myrosinases, but conversion of GLS to isothiocyanates (ITC) is possible in humans through the bacterial microflora of the gastrointestinal tract. This effect has been observed upon reduction of the bowel microflora by mechanical cleansing and antibiotic treatment, which almost eliminates ITC production [111,112]. According to their hydrolysis products, GLS are classified into aliphatic and aromatic, terminally-unsaturated, β-hydroxy-, and indolyl GLS [110] (Fig. 7).

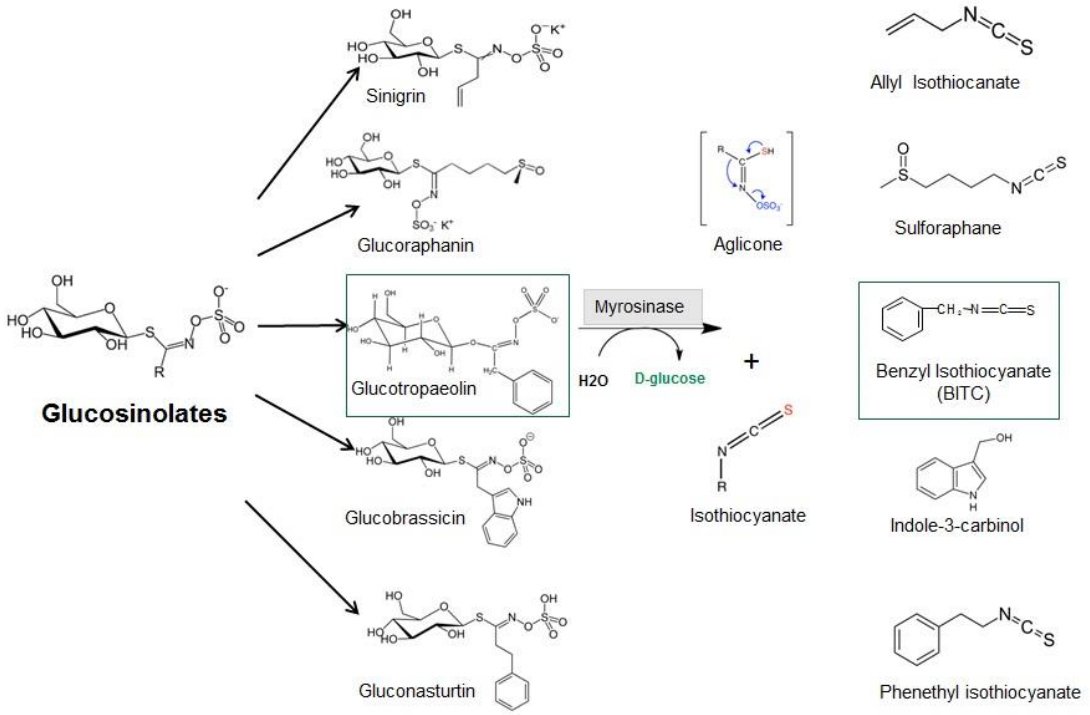


Figure 7. Glucosinolates (GLSs) and myrosinase. The figure presents the chemistry of the most representative GLSs and their hydrolysis to the respective isothiocianates (ITC). Shown in frame: benzyl isothiocyanate (BITC) extracted from *Nasturtium* (Indian cress; *Tropaeolum majus L.*).

1.3.2 Isothiocyanates

ITCs products are responsible for most of the biological activities of GLSs and the biological effect depends on the side-chain structure [106]. ITCs possess a reactive central carbon atom in the –N=C= S group, which reacts with oxygen-, sulfur-, or nitrogen-centered nucleophiles, giving rise to carbamates, thiocarbamates, or thiourea derivatives [110]. Owing to the positive log P of ITCs, they can cross biological membranes. Therefore, they are passively absorbed into epithelial cells of the gastrointestinal tract [107]. In the cytoplasm, they are immediately conjugated with GSH to form the metabolite ITC–GSH [110].

1.3.2.1 Biological activities of Isothiocyanates

Studies with animal models have demonstrated the inhibition of carcinogen-induced tumor formation by ITCs, suggesting a cancer protective effect of cruciferous vegetable consumption [106]. ITCs from Brassica species are involved in different

and interconnected signaling pathways which have a role in detoxification, inflammation, apoptosis, cell cycle and epigenetic regulation, among others [106].

The biological effect of ITCs vary according with the side-chain structure. *In vitro* studies show that cell accumulation and enzymes induction differ according with the type of ITCs [113]. Since benzyl-isothiocyanate (BITC) was chosen in the current study to evaluate the effect of glucosinolates on the pathways related with the T2D prevention, this review will focus in the biological effects of this substance.

In vitro studies in animal and human cells have demonstrated the inhibitory effects of BITC on initiation, growth, and metastasis of pancreatic, breast, and bladder cancers cells [114]. In cancer cells, BITC induces selective cell cycle arrest and apoptosis through induction of p53, JNK and p38 MAPKs [115]. In addition BITC has shown to inhibit G2/M cell cycle progression by up-regulation of the G2/M cell cycle arrest-regulating genes p21 and GADD45 [115,116]. Thus, it inhibits cyclin-dependent kinase activity and promotes apoptosis [115,117]. The cytotoxic effect of BITC has been observed in proliferating cells more than in the quiescent cells [118].

BITC treatment induces cellular stress itself, as observed by the reduction in the pool of cellular thiol compounds, such as GSH, and the increase of ROS upon exposure to BITC [115,119]. Depletion of GSH weakens the cell antioxidative capacity and induces oxidative stress. Although a moderated oxidative stress induction has positive effects on the cells, due to the increase in phase II enzymes and MnSOD expression observed in some studies, it is noteworthy that the effect in antioxidant resistance induction decreases at high concentrations of BITC [120]. The source of ROS under BITC stimulus results from increase in nitric oxide (NO) [121] and in oxygen production on the mitochondria, by a disruption in the mitochondrial respiration [122].

Despite of the modulatory effect of ICTs on cytoprotective enzymes and inflammatory processes [3,111], only few studies have linked them with T2D diabetes prevention. There is one clinical trial with broccoli (*Glucoraphanin* and *Glucobrassicin*), but information about the effect of garden cress or *Nasturtium* (*Glucotropaeolin*) is not yet available. Bahadoran Z. *et al.* evaluated the administration of broccoli sprouts powder (BSP) containing high concentration of sulphoraphane (10 g/d and 5 g/d BSP), on insulin resistance (IR) and some oxidative stress parameters in T2D

patients. After 4 weeks treatment they found a reduction in serum insulin, improvement of IR, decrease in serum malondialdehyde and oxidized low density lipoprotein (OX-LDL) concentrations and increased antioxidant capacity [3],[123], suggesting that ITCs can be considered as good candidates for the treatment or prevention of T2D.

1.3.2.2 ICTs and cytotoxicity

Even though several studies show the protective effect of Brassica vegetables against different type of cancer, some reports have documented genotoxic effects in bacteria and mammalian cells [124]. Few reports show that BITC is cytotoxic and clastogenic only *in vitro* [125,126] and to observe cytotoxic effects *in vivo* animals have to be exposed to high concentrations of the substance. Kassie F. *et al* showed *in vivo* that DNA damage by BITC is observed only when rats are exposed to high dose of BITC (220mg/kg body) [127]. The discrepancy between the genotoxic effects of BITC seen under *in vitro* and *in vivo* conditions lie in the ability of the living animal to detoxify the compound, which counteracts the toxicity by induction of its antioxidant capacity (e.g α-tocopherol, β-carotene and antioxidant enzymes) [127].

Brassica vegetables besides glucosinolates contain also polyphenols, flavonoids and other antigenotoxic substances. The presence of these protector factors accompanied by a poor *in vivo* genotoxic effect by BITC indicates that the consumption of BITC by humans is probably not associated with an increased risk of genetic damage [127].

1.4 Motivation of the project and Aims

The search for bioactive compounds from plants for the prevention or treatment of T2D is necessary, not only because the possibility of finding dietary agents less expensive, safer, and more readily available than synthetic agents, but because the understanding of the mechanisms underlying the positive effects of vegetables and fruits consumption in the prevention of chronic diseases is needed.

Therefore the current study evaluated the ability of benzyl isothiocyanate (BITC) extracted from *Nasturtium* (Indian cress; Tropaeolummajus L.) to modulate i) the insulin-signaling pathway, ii) the intracellular localization of FOXO1 and, ii) the expression of proteins involved in glucose and lipid metabolism, ROS detoxification and cell cycle arrest and DNA repair, looking for the possibility of T2D prevention.

For this purpose an optical screening platform for compounds that translocate FOXO1 protein labeled with GFP (green fluorescent protein) to the nucleus in engineered cell cultures of human osteosarcoma cells (U-2OS) was established. Using this system aglycons of glucosinolates from Tropaeolum majus were identified as potent activators of FOXO1 translocation. Following identification of BITC as promoter of FOXO1 translocation, *In vitro* assays with HepG2 and U-2OS cells were performed to evaluate the effect of BITC on:

i) FOXO1 mobility from cytoplasm to the nucleus and its relation with oxidative stress induction.
ii) Protein and gene expression of insulin signaling pathway factors and FOXO1 target proteins.
i) Gene expression of factors involved in glucose and lipid metabolism, ROS detoxification, cell cycle arrest and DNA repair upon FOXO1, AKT, NRF2 and SIRT1 knock-down conditions.

Chapter 2

2. Materials and Methods

2.1 Materials

2.1.1 Table 3. Cell lines

Cell type	Supplier
Human hepatoma cells (HepG2)	ECACC (European Collection of Cell Cultures)
Human osteosarcoma cells (U-2OS)	ECACC (European Collection of Cell Cultures)

2.1.2 Table 4. Enzymes, PCR supplies and reaction kits

Compound/reaction kit	Supplier
BCA protein assay kit	BIO RAD
CellTiter96 Aqueous Cell Proliferation assay	Promega (USA)
Dharmafect4	Dharmacon Thermo Scientific
High Capacity cDNA Reverse Transcription Kit	Applied Biosystems (UK)
NON-Target siRNA	Thermo Scientific
Nucleospin RNA II total RNA isolation kit	Machery-Nagel (Germany)
ON-TARGETplus siRNA	Thermo Scientific
Power SYBR green PCR Master Mix	Applied Biosystems (UK)

siRNA, Small interfering RNA.

2.1.3 Table 5. Target specific silencing RNA sequences

Target	Oligo sequence (5' to 3')
FOXO1	5′-GCGCUUAGACUGUGACAUG-3′
	5′-GAGGUAUGAGUCAGUAUAA-3′
	5′-UGACUUGGAUGGCAUGUUC-3′
	5′-GGACAACAACAGUAAAUUU-3′
FOXO3a	5′-UAACUUUGAUUCCCUCAUC-3′
	5′-CGAAUCAGCUGACGACAGU-3′
	5′-GUACUCAACUAGUGCAAAC-3′
	5′-GCACAGAGUUGGAUGAAGU-3′
AKT	5′-CAUCACACCACCUGACCAA-3′
	5′-ACAAGGACGGGCACAUUAA-3′

	5′-CAAGGGCACUUUCGGCAAG-3′
	5′-UCACAGCCCUGAAGUACUC-3′
SIRT1	5′-GCAAAGGAGCAGAUUAGUA-3′
	5′-GCGAUUGGGUACCGA-3′
	5′-GGAUAGGUCCAUAUACUUU-3′
	5′-CCACCUGAGUUGGAUGAUA-3′
NRF2	5′-CACCUUAUAUGUCGAAGUU-3′
	5′-UGGAGUAAGUCGAGAAGUA-3′
	5′-GAGUUACAGUGUCUUAAUA-3′
	5′-UAAAGUGGCUGCUCAGAAU-3′
Non-Targeting Sequences (NT)	5′-UAAGGCUAUGAAGAGAUAC-3′
	5′-AUGUAUUGGCCUGUAUUAG-3′
	5′-AUGAACGUGAAUUGCUCAA-3′
	5′-UGGUUUACAUGUCGACUAA-3′

FOXO1/3a, Forkhead box protein O1 or 3a; AKT,Protein kinase B; SIRT1, NAD-dependent deacetylase sirtuin-1; NRF2, Nuclear factor (erythroid-derived 2)-like 2.

2.1.4 Table 6. Primary Antibodies

Antibody	Dilution	Supplier
ACC rabbit Antibody	1:1000	Cell Signaling Technology (USA)
AKT (Protein Kinase B)(pan) mAB	1:1000	Cell Signaling Technology (USA)
alpha-Tubulin rabbit Antibody	1:1000	Cell Signaling Technology (USA)
FASN rabbit Antibody	1:1000	Cell Signaling Technology (USA)
FOXO1 FKHR (H128) rabbit Antibody	1:200	Santa Cruz Biotechnology (USA)
G6Pase rabbit Antibody	1:200	Santa Cruz Biotechnology (USA)
GADD45α rabbit Antibody	1:200	Santa Cruz Biotechnology (USA)
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) rabbit mAB	1:1000	Cell Signaling Technology (USA)
p21 rabbit Antibody	1:200	Santa Cruz Biotechnology
PCK-1 rabbit Antibody	1:1000	Abcam (UK)
PCK-2 rabbit Antibody	1:1000	Abcam (UK)
Phospho-AKT(Ser473) rabbit mAB	1:1000	Cell Signaling Technology (USA)
Phosphoenolpyruvate carboxykinase PCK-2 rabbit Antibody	1:1000	Cell signaling Technology (USA)

Phospho- FOXO1(Thr24)/FOXO3a (Thr32) rabbit Antibody	1:1000	Cell Signaling Technology (USA)
SRXN1 Goat antibody	1:300	Santa Cruz Biotechnology (USA)

ACC, Acetyl-CoA-Carboxylase; FASN, Fatty acid-synthetase; G6Pase, Glucose-6-phosphatase; GADD45, Growth Arrest / DNA Damage Repair; p21, cyclin-dependent kinase inhibitor 1A; SRXN1, Sulfiredoxin-1; PCK-1, Phophoenolpyruvate carboxkinase.

2.1.5 Table 7. Secondary Antibodies

Antibody	Dilution	Supplier
IRDye 800 CW conjugated Goat Anti rabbit IgG	1:20000	LI-COR Biosciences for ODISEY (USA)
IRDye 680 CW conjugated Goat Anti Mouse	1:20000	LI-COR Biosciences for ODISEY (USA)

2.1.6 Table 8. Primers for qRT-PCR

Target	Oligo name	Oligo sequence (5' to 3') Fwd	Oligo sequence (5' to 3') Rev
60S ribosomal protein L32	hRLP-32	CAACGTCAAGGAGCTGGAAGT	TTGTGAGCGATCTCGGCAC
Acetyl-CoA-carboxylase	hACC	TCGCTTTGGGGGAAATAAAGTG	ACCACCTACGGATAGACCGC
B-Cell Lymphoma 6 Protein	hBCL6	ACCTGCAGATGGAGCATGTTG	TGAGGAACTCTTCACGAGGAGG
Catalase	hCAT	GCGGTCAAGAACTTCACTGAGG	GGTGTGAATCGCATTCTTAGGC
Cyclin G2	hCCNG2	GGCTACCCCGGAGAATGATAA	CCAGGACAAAAGTTTCAGTGCA
DNA damage-binding protein 1	hDDB1	GTCCCCTCAATTCAGATGGCT	TGTGCGAATGTGCAGCTTCT
Fatty acid-synthetase	hFASN	AGACACTCGTGGGCTACAGCAT	ATGGCCTGGTAGGCGTTCT
Forkhead box O1	hFOXO1	GGCTGGAAGAATTCAATTCGTC	ACCCTCTGGATTGAGCATCCAC
Forkhead box O3	hFOXO3	CATGGCAAGCACAGAGTTGGA	CGGCTTGCTTACTGAAGGTGAC
Glucose-6-phosphatase	hG6Pc	CCCCTGATAAAGCAGTTCCCT	ATACACCTGCTGTGCCCATG
Glutathione peroxidase 2	hGPX-2	GTGCTGATTGAGAATGTGGC	AGGATGCTCGTTCTGCCCA
Growth Arrest / DNA Damage Repair	hGADD45A	GTGCTGGTGACGAATCCACATT	TCAGATGCCATCACCGTTCAG
NAD(P)H dehydrogenase (quinone 1)	hNQO1	CATCACAGGTAAACTGAAGGACCC	CTCTGGAATATCACAAGGTCTGCG
nuclear factor (erythroid derived)-like2	hNRF2	AACTACTCCCAGGTTGCCCA	CAAGTGACTGAAACGTAGCCGA
cyclin-dependent kinase inhibitor p21/CIP1	hCDKN1A	CAAAGGCCCGCTCTACATCTT	AGGAACCTCTCATTCAACCGC
Cyclin-dependent kinase inhibitor 1B p27/KIP1	hCDKN1B	GGCTAACTCTGAGGACACGCAT	AAGAATCGTCGGTTGCAGGT
Phosphoenolpyruvat-carboxykinase	hPEPCK	AAGTATGACAACTGCTGGTTGGC	ATAACCGTCTTGCTTTCGATCCT
Proteinkinase B	hAKT1	GCTTCTATGGCGCTGAGATTGT	TGATCTTAATGTGCCCGTCCTT
NAD-dependent deacetylase sirtuin-1 (Sirtuin1)	hSIRT1	ATGCTGGCCTAATAGAGTGGCA	CCTCAGCGCCATGGAAAAT
Sulfiredoxin-1	hSRXN1	CTCAGTGCTCGTTACTTCATGGTC	GTTTGGCCCTTCCTCTTCCTCC
Superoxide dismutase	hSOD2	GCCTGCACTGAAGTTCAATGG	CGTTTGATGGCTTCCAGCA
Autophagy related protein 5	hATG5	GGCCATCAATCGGAAACTCA	AGCCACAGGACGAAACAGCTT
Autophagy related protein 12	hATG12	ATCAGTCCTTTGCTCCTTCCC	CGCCTGAGACTTGCAGTAATGT
BECLIN1	hBECLIN1	ACCATCCAGGAACTCACAGCTC	CCTGGCGAGGAGTTTCAATAAA
GABA receptor-associated protein 1	hGABARAPL1	TGCCCTCTGACCTTACTGTTGG	TACAGTTGGCCCATGGTAGCA
PI3-kinase, catalytic subunit type 3	hPIK3C3	GCTGCAGTGGTGGAACAGATT	GGTTGCTCCTAACGTGGTTTTC
UNC51-like kinase 1 ULK1	hULK1	AAGTTCGAGTTCTCCCGCAA	TGAGACTTGGCGAGGTTCTTC

Sequestosome-1		hSQSTM1		AATCAGCTTCTGGTCCATCGG		CCGTGCTCCACATCGATATCA

All primers were diluted in DEPC-treated water to a stock solution of 0.5 µmol/mL

2.1.7 Table 9. Chemicals

Chemical	Supplier
Acetic acid	Merck (Germany)
Acrylamid 30%	Carl Roth (Germany)
Ammonium persulfate (APS)	Carl Roth (Germany)
Benzyl glucosinolate 10mM	Leibniz-Institute of Vegetable and Ornamental Crops Großbeeren/Erfurt e.V
Blot stripping buffer	Thermo scientific (USA)
Bovine Serum Albumin BSA	Sigma- Aldrich (USA)
Complete EDTA-free Protease Inhibitor Cocktail	Roche (Germany)
DAPI 4',6- Diamidino-2-phenylindole	Sigma- Aldrich (USA)
DL-Dithiothreitol solution	Sigma- Aldrich (USA)
DMEM (Dulbecos Modified Eagle Medium) with 4.5g/L D-glucose, 3.7g/L NaHCO3, stable glutamine, and Na-pyruvate	Biochrom (Germany)
Ethanol absolute	Biochrom (Germany)
Fetal calf serum (FCS)	Applichem (Germany)
Formaldehyd	Thermo Fisher Scientific (Waltham, USA)
Geneticin G418 sulfate (0.4mg/ml)	Sigma- Aldrich (USA)
Insulin solution human 10mg/ml in 25 mM Hepes pH:8.2	Calbiochem (Germany)
Lysis buffer	Sigma- Aldrich (USA)
EMEM (EBSS Eagle´s Minimum Essential Medium with Earle´s Balanced Salt Solution), stable glutamine, 2.2g $NaHCO_3$, 1g/L D-glucose, supplemented with 1% NEAA supplement	Cell signaling (USA)
Methanol	Carl Roth (Germany)
Myrosinase 1mg	Sigma-Aldrich (USA)
Non-esencial aminoacids (NEAA)	Bichrom (Germany)
PBS	GIBCO (UK)
Phosphatase inhibitor cocktail	Roche (Germany)
Prestained protein Ladder page ruler	Thermoscientific (USA)
PVDF-membranes (Immobilon-FL) 0,45µM	Millipore

TEMED- tetramethylethylenediamine	Carl Roth (Germany)
TrypLE express	GIBCO (UK)
Tween	Molecular bioproducts (USA)
SDS (Dodecyl Sulfate Sodium Salt)	Merck (Germany)

Benzyl glucosinolate was kindly provided by Monika Schreiner and Inga Mewis from the Leibniz-Institute of Vegetable and Ornamental Crops Großbeeren/Erfurt e.V. It was extracted from *Tropeaolum majus L.* (*Nasturtium*) plants grown in a greenhouse for 3 months.

2.1.8 Table 10. Buffers and solutions

All buffers and solutions were prepared with Millipore® filtered water. The pH was adjusted with HCl 0.1M or NaOH 0.1M.

Buffers and solutions	Ingredients
Coomasie	Methanol 90ml, Acetic acid 20ml, Coomassi Brillant 0.25g, H_2O 90ml
Destaining solution	7% Acetic acid; 30% Ethanol
Lower Tris ph 8,8	1495mM Tris Base, pH: 8.8
Running buffer	247mM Tris, 1918mM Glycin, 1% SDS, pH: 8.6
SDS-PAGE sample buffer	40% Glycerol; 240 mM Tris/HCl pH 6.8; 8% SDS; 0.04% bromophenol blue; 5% beta-mercaptoethanol
Tris buffered saline (TBST)	100mM Tris-HCl pH 8, 150mM NaCl with 0.1% Tween
Towbin-buffer	25mM Tris, 192mM glycine, 0.1% SDS, 14% methanol
Upper Tris	500mM Tris, pH: 6.8

2.1.9 Table 11. Software

Software	Supplier
BD AttoVision version 1.6/435	Becton Dickinson Biosciences, (USA)
BD Image Data Explorer	Becton Dickinson Biosciences (USA)
2100 Expert Software Version 2.5	Agilent Technologies (USA)
ODYSSEY software	LI-COR Biosciences (USA)
GraphPad Prism version 6.02	GraphPad Prism (USA)
SPSS version 16.0	SPSS (USA)
ViiA™ Software version 1.2	Applied Biosystems (USA)

2.2. Methods

2.2.1 Cell lines and culture

Cell culturing was carried out under humid atmosphere, at 37°C with a 5% CO_2 content. For the experiments, U-2OS cells (human osteosarcoma cell line) and HepG2 (human hepatocellular carcinoma cell line) were used. U-2OS was cultivated using DMEM with 4.5g/L D-glucose, 3.7g/L $NaHCO_3$, stable glutamine, and Na-pyruvate supplemented with 10% FCS (Fetal calf serum) and HepG2 was cultivated in EMEM, stable glutamine, 2.2g $NaHCO_3$, 1g/L D-glucose, supplemented with 1% NEAA (Non-Essential Amino acids) and 10% FCS. For passaging of cells trypsin in TrypLE express was used.

2.2.2 Cell transfection and colony growth

2.2.2.1 U-2OS (human osteosarcoma cell line)

The cells were transfected with DNA plasmids using Lipofectamine 2000 in Opti-MEM® I Reduced-Serum Medium (Invitrogen) according to the manufacturer´s description. A stable transfection of U-2OS for constitutive expression or green fluorescence protein (GFP) labelled FOXO1 is obtained using pEGFP-N1 as vector, with an insertion of FOXO1 cDNA (complete FOXO1 coding sequence (CDS)) in front of the CDS of EGFP. The vector used for the transfection was a gift as courtesy from Terry Unterman (University of Illinois at Chicago, USA).

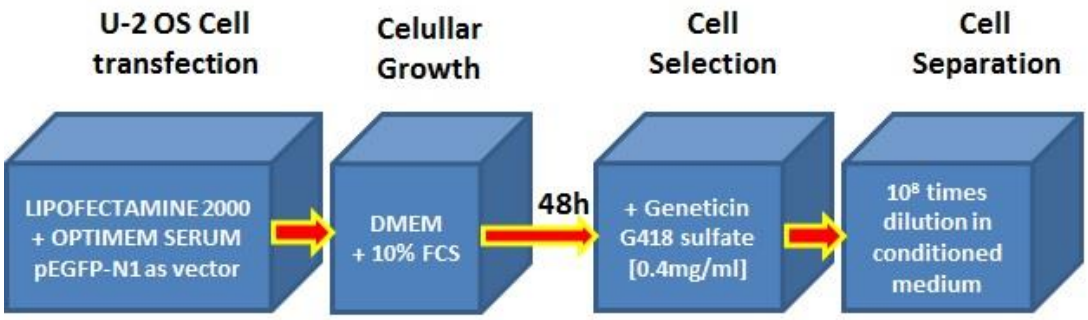


Figure 8. Cells growth procedure prior fluorescent microscopy measurement

The whole procedure of colony cell growth is illustrated in Figure 8. Following transfection, cells were grown in DMEM + 10% FCS. Geneticin G418 sulfate (Calbiochem) at 0.4mg/ml was added after 48h, in order to select those genetically engineered cells carrying the plasmid with encoded resistance. Cell separation was obtained through diluting up to $1:10^8$ in 96-well plates coated with Poly-D-lysine and growth in conditioned medium. Separating cells over 3 passages resulted in homogeneous cell colonies expressing FOXO1-GFP, controlled by fluorescence microscopic live cell imaging. Figure 9 shows fixed and stained cells with DAPI for nuclear staining (Fig. 9).

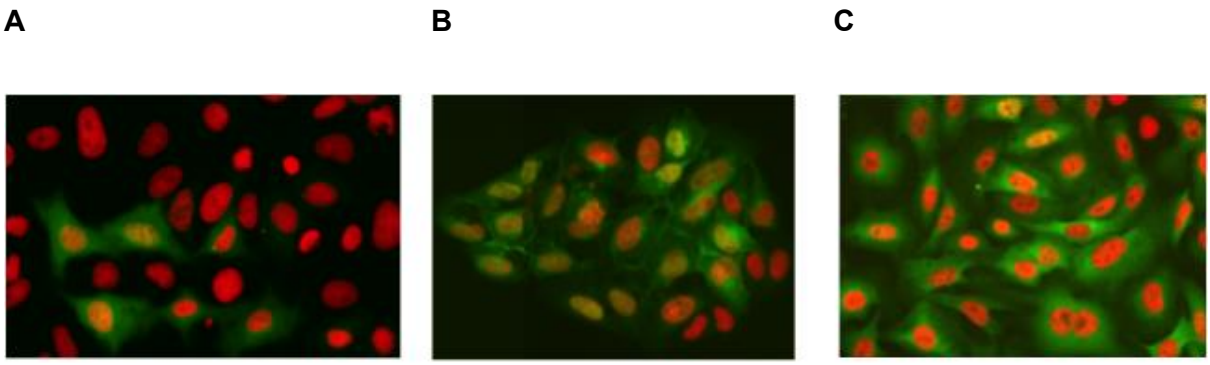


Figure 9. Transfection of human osteosarcoma cells U-2OS with pEGFP-FOXO1wt (wild type). A.Transient transfection of human osteosarcoma cells U-2OS with pEGFP-FOXO1wt. B. Colony of stable transfected U-2OS following selection with G418. C. Cloned U-2OS with constitutive expression of FOXO1-GFP. Colour coding: nuclear staining (DAPI) in red; FOXO1-GFP in green and overlay in orange.

2.2.2.2 HepG2 cells (Human hepatoma cell line)

For the transfection of silencing RNA (siRNA), combined DharmaFECT4 (D4) transfection reagent and siRNAs ON-TARGETplus SMARTpool siRNA were applied. SMARTpool siRNA contained four SMARTselection-designed siRNAs, pooled for efficient knock-down of each analyzed target: FOXO1, AKT, SIRT1, NRF2, and NON-Target siRNAs NT were used for control transfections. The target specific silencing RNA sequences and NT are described in Table 5. SiRNAs were dissolved and diluted according to the description of the manufacturer. Their transfections were performed 24h after seeding in 12 well plates, 100000 cells per well each, in EMEM with FCS 10%. Transfection reagent D4 was initially diluted in EMEM without FCS and subsequently incubated for 5 minutes at RT. Afterwards, it was mixed with siRNAs and followed by additional 20 minutes incubation at room temperature. The general procedure for cell transfection is depicted in Figure 10.

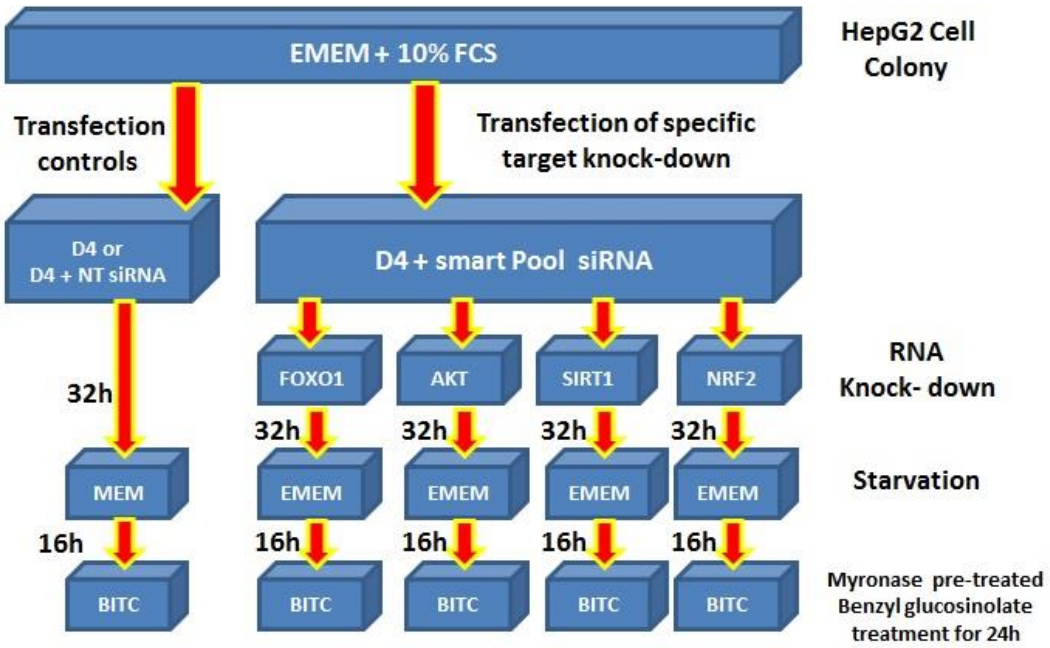


Figure 10. General procedure for the siRNA transfection. Prior to transfection HepG2 cells were incubated with EMEM medium with 10% FCS. For transfection, control samples were treated with D4 or alternatively, a mixture of D4 and NT-siRNA. Smart pool siRNAs combined with D4 were selected for knock-down the specific targets FOXO1, AKT, SIRT1 and NRF2. In addition, siRNA treatment for the mixtures of NRF2/FOXO1, NRF2/AKT, SIRT1/FOXO1 and SIRT1/AKT mixtures was also tested. Cell transfection was carried out during 32h. The cells were starved for 16 in EMEM without FCS reaching 48h after transfection described for an effective RNA-knock-down and protein reduction as well. Afterwards, transfected cells were stimulated for 24h with 30µM benzyl-glucosinolate pretreated with myrosinase to obtain benzyl- isothiocianate (BITC).

2.2.3 Cells treatment with BITC and insulin

To evaluate the effect of BITC on FOXO1 translocation stably transfected U-2OS cells were used for the microscopic analyses due to these cells grow in mono layer and their well defined structure can be identified by fluorescence microscopy. HepG2 cells were selected to evaluate the effect of BITC on gene and protein expression to observe liver specific gluconeogenic gene expression.

2.2.3.1 U-2OS cells

Prior cell incubation with test compounds, growth of sub-confluent U-2OS cells was controlled for 16h by an initial reduction from 10% FCS to 2% FCS, and further to 0% FBS in DMEM for 1h starvation.

2.2.3.2 HepG2 cells

HepG2 cells were starved for 16h in EMEM with 1g/L glucose without FCS. The cells stimulation was performed with benzyl glucosinolate dissolved in FCS-free medium and treated with myrosinase at 37°C during 15min, which allows benzylisothiocianate formation (BITC). Insulin stimulations were performed with 100nM human insulin in FCS-free medium.

2.2.4 RNA isolation, cDNA synthesis and quantitative real time PCR (qRT-PCR)

2.2.4.1 RNA isolation

Total RNA was extracted upon 24h incubation with BITC and insulin, using for this task the Nucleospin RNA II total isolation kit from Machery-Nagel. RNA purity and quantity were assessed using the NanoDrop 2000 UV/VIS spectrophotometer fabricated by Thermo Scientific. The optical density (OD) was measured at different wave lengths: 230 nm (absorption of contaminants & background absorption), 260 nm (absorption maxima of nucleic acids), 280 nm (absorption maxima of proteins). An optical density ratios OD260/280 and OD260/230 higher than 1.8 was assumed to be suitable for gene expression measurements, total RNA concentration was quantified by measuring the extinction at 260 nm [128].

2.2.4.2 cDNA synthesis

RNA was reverse transcribed with the High Capacity cDNA Reverse Transcription Kit from Applied Biosystems. For each 1 µg of RNA a total volume of 10 µl master mix was used. The master mix contained: 2 µl 10x reverse transcription (RT) buffer, 0.8 µl 25x dNTPMix, 2µl 10xRT random primers, 1µl multiscribe reverse transcriptase, 1 µl RNAse inhibitor and 3.2 µl nuclease-free H_2O. The reverse transcription or cDNA synthesis was performed during incubation at 37°C for 2h and stopped at 85°C for 5 minutes.

2.2.4.3 Quantitative real time PCR (qRT-PCR)

For quantitative real-time PCR, samples were diluted 40-fold in DNase/RNase free water. The Power SYBR green PCR Master Mix from Applied Biosystems was used for quantitative real time (qRT) analysis for measurement with the Vii7 Real-Time PCR System from Life Technologies in 384 well-plates. Reactions were carried out in triplicates. A standard curve with cDNA from control cells was prepared for each primer pair. The final volume of each reaction was 5 µl, consisting of: 1µl cDNA, 2µl SYBR® Green PCR Master Mix, 0.35 µl forward and reverse primer (diluted 1:40 pM) and 1,3µl RNase/DNase free Water.

The reactions were carried out 10 min at 95°C, followed by 40 alternated cycles of 95°C for 15 sec and 60°C for 35 sec. For melting curve analysis, the reactions were warmed up to 95°C for 15 sec, then cooled down to 60°C for 15 sec and heated up again to 95°C for 15 sec. For relative quantification of cDNA transcripts, five standards at different cDNA concentrations of 0.25, 0.0625, 0.015625, 0.00390625 and 0.000976563 were used. The Primers for qRT-PCR summarized in table 9 were designed using Primer Express and purchased from Invitrogen™. The RPL32 housekeeping gene was used for normalization as an endogenous control. The mean value was used for further calculations. Reported levels of mRNA are expressed as fold changes and compared to untreated control cells.

2.2.5 Western-blot

Upon incubation with the compounds lysis buffer was used (Signaling Technology) protecting the cells with protease and phosphatase-inhibitors from Roche. Protein was quantified using a BCA protein assay kit from BIO RAD in a 96-well plate. 5 µL of Bovine serum albumin (BSA) diluted in phosphate buffered saline (PBS) and lysis buffer was used as a standard with a range of concentration from 5-0.25 mg/mL it was pipetted into triplicate wells for a standad curve. Triplicates of each 5 µL of diluted samples were used as well. Afterwards, reagents A and B were added according to the kit conditions. In order to allow colour development, samples were incubated under dark conditions for 15 minutes at room temperature. Absorbances were read at 750 nm wavelength using the Wallac VICTOR plate reader. Protein quantity was determined through interpolation of the standard curve.

Each 20 µg of protein were denaturated in SDS-PAGE sample buffer at 95°C for 5 minutes and subsequently separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), with 10% acrylamide for resolving gel, 5% acrylamide for stacking gel, or 4%-20% BIO RAD for a gradient gel. Samples were electrophoresed at 120V in running buffer 1X using the Biorad Mini -PROTEAN gel electrophoresis system.

Blotting of proteins was carried out by electrotransfer in Towbin-buffer into PVDF-membranes (Immobilon-FL) from Millipore, using a wet transfer system from BIO RAD during 90 minutes at 80 Voltage. After transfering, 5% non-fat milk in tris buffered saline, supplemented with 0.1% Tween (TBST) was used for the membranes blocking for 1h at room temperature (RT). Membranes were washed 3 times for 5 minutes in TBST and incubated afterwards, with specific primary antibodies. According to the instructions given by the provider antibodies were diluted in TBST with 5% bovine serum albumin (BSA), followed by 16h incubation at 4°C.

Dilution of primary antibodies is described in table 6. As constitutively expressed housekeeping protein glyceraldehyde-3-phosphate dehydrogenase (GAPDH) rabbit mAB, and alpha-Tubulin rabbit antibody were used. Membranes were rinsed three times with TBST for 5 minutes each and incubated for 1h under light protection with the secondary antibody IRDye 800CW conjugated goat anti rabbit IgG from LI-COR for ODYSSEY infrared imaging system, which was diluted 1:20000 in TBST with 5%

non-fat milk. Membranes were scanned with the LI-COR Bioluminescence imager and analysed using ODYSSEY software.

2.2.6 Path scan intracellular signaling array

A PathScan Signaling Array Kit from Cell Signaling Technology® with a fluorescent readout [129] for the simultaneous detection of the phosphorylated signaling molecules protein kinase B AKT (Thr308), AKT (Ser473), ribosomal Protein S6 (Ser235/236), adenosine monophosphate-activated protein kinase AMPKα (Thr172), proline-rich AKT substrate of 40 kDa PRAS40 (Thr246), mammalian target of rapamycin mTOR (Ser2481), glycogen synthase kinase 3 GSK-3α (Ser21), GSK-3β (Ser9), 70 kDa ribosomal S6 kinase p70S6K (Thr389), p70S6K (Thr421/ser424), BCL2-Associated agonist of cell death BAD (Ser112), MAPK activated protein kinase-1a RSK1 (Ser380), phosphatase and tensin homolog PTEN (Ser380), 3-phosphoinositide dependent protein kinase-1 PDK1 (Ser421), extracellular-signal-regulated kinase ERK1/2 (Thr202/Tyr204), eIF4E binding protein 14E-BP1 (Thr37/46) and stress-activated kinases/ c-Jun N-terminal kinase SAPK/JNK (Thr183/Tyr185).

Cell lysates were obtained from HepG2 treated with BITC 30 µM, insulin 100 nM and insulin+BITC for 30 minutes. The 1X cell lysis buffer provided by the kit supplemented with phenylmethylsulfonyl fluoride (PMSF) to a final concentration of 1mM and phosphatase-inhibitor was used for the cells lysis. Upon sonication and centrifugation, the clear supernatant was used for protein content determination. Samples were diluted in array diluent buffer to 1 mg/ml concentration. For blocking single nitrocellulose-pads with specific antibodies spotted in duplicates on membranes mounted on glass slides, these were connected with a multi-well gasket. After treatment with 100 µl array blocking buffer per well for 15 minutes, 16h incubation at 4°C with each 75 µl diluted lysate was performed.

The pads were washed four times with 100 µl array wash buffer and incubated with 75 µl of the provided detection antibody cocktail for 1h at RT. The samples were again washed four times and incubated with 75 µl DyLight 680-linked streptavidin for 30 minutes, protected from light. After four washing cycles of 5 minutes duration each, the gasket was removed. The slide was rinsed in deionized water for 10

seconds and dried. LI-COR Bioluminescence imager and ODYSSEY software were used to scan and analyse the slides.

2.2.7 Cell viability assay

The viability of HepG2 cells after 24 hours incubation with selected BITC concentrations of 1 µM, 2 µM, 5 µM, 10 µM, 20 µM, 50 µM, and 100 µM was tested with the CellTiter96 Aqueous Cell Proliferation assay from Promega. Each 20000 HepG2 cells per well were seeded in 96-well plates from Greiner. and grown in EMEM + 10% FBS. After 5 hours cells were starved for 16h in a EMEM without FBS. After starvation 1 µM, 2 µM, 5 µM, 10 µM, 20 µM, 50 µM and 100 µM of benzyl glucosinolate and myrosinase pretreated benzyl glucosinolate (BITC), were added each in triplicates.

Following cell incubation of 24h, 20µM of PMS-electron coupling reagent (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt (MTS)/ phenazine methosulfate, was supplied to each well. Afterwards, cells were incubated at 37°C under an atmosphere with 0.5% CO_2 content, for 1 to 4 hours. Formazan, a MTS bioreduction product, was measured at 490 nm wavelength after 1h and 4h with the Wallac VICTOR plate reader.

2.2.8 FOXO-translocation assay

Stably transfected U-2OS cells with pEGFP-FOXO1 expressing FOXO1 with C-terminalyl tagged GFP were used for FOXO1-GFP visualization by fluorescence microscopy. Zeiss “Axio Observer.Z1” inverted fluorescent microscope with a cell incubation chamber was used for visualization of FOXO1-GFP. Seeding was performed in black, clear bottom 96-well plates with poly-D-lysine coating from BD. Each well contained between 100000 and 150000 cells in 100µl DMEM + 10% FBS solution. The FBS content of the DMEM medium was reduced progressively to 2% after 6h, up to 0% after 16h. Starvation lasted 1h prior cell treatment with test substances.

Life cell images were recorded every minute up to 1 h with a filter for GFP, for tracing intracellular translocation of FOXO1-GFP after treatment of cells with: a) insulin

100nM; b) BITC 30 to 100 µM; c) insulin+BITC; d) NAC (N acetyl cysteine); e) BITC+NAC; f) insulin+NAC and g) BITC+insulin+NAC.

For defining nuclear areas, we used the following procedure: a) Fixing cells with 4% paraformaldehyde in PBS; b) Staining of nuclei for 30 minutes with a solution of 200nM DAPI (4´,6-diamidino-2-paraphenylindole, from Invitrogen) and 0.3% TritonX-100; c) Two step rinsing in PBS and d) Fluorescence microscopic UV-Exposition in the BD Pathway 435 Bioimager from Becton Dickinson with the filter for DAPI followed by two expositions for GFP. Exposition for GFP-Nuc (nuclear) and GFP-Cyto (cytoplasmic) were acquired each for 0.5 seconds followed by DAPI exposition 0.05 seconds for each well per probe cycle. Quantification of GFP signals in nuclear areas and cytoplasm was analysed using BD AttoVision version 1.6/435.

For cell segmentation we applied the provided method Cyto-Nuc Ring Band, with the shape Ring (2outputs) Band analysing the DAPI channel for defining nuclear areas. Further analyses were performed with the BD Image Data Explorer from BD Biosciences on the measured regions of interest (Roi). Ratios of GFP_Nuc_intensity divided by GFP_Cyt_intensity as Ratio Nuc/Cyt were calculated with the average parameter values per well, standard errors over distribution in cells of selected wells were calculated also.

Ratios lower than 1 reflected a predominant cytoplasmic localization of FOXO1-GFP. Ratios higher than 1 showed a nuclear accumulation of FOXO1. An induced translocation factor could be calculated after substance treatment by normalization versus untreated cells.

2.2.9 Data handling and statistical analyses

Fluorescent microscopic data were analysed with the BD image data explorer. For Western blots and path scans quantification the integrated intensities were corrected by background substraction and normalized with housekeeping proteins and untreated control cell phosphorylation respectively.

Relative mRNA expression was measured by cDNA amplification quantified with ViiA7 RUO (software for real time PCR system version 1.2) by comparison to standard cDNA dilution for amplification. One-way ANOVA with appropriate post hoc

tests (Bonferroni and Dunnett's Multiple Comparison post-hoc test) or 2-tailed unpaired *t* Test were used for statistical analysis using SPSS. A p value lower than 0.05 was assumed to be significant. All results are presented as means + standard error of the mean (SEM). The median inhibitory concentration EC50 values were calculated using GraphPad Prism 5.0 software (San Diego, CA, USA).

Chapter 3

3. Results

FOXO proteins have an important role in mediating the effects of insulin on gene expression and metabolism, and alterations in FOXO1 function might contribute to metabolic alterations in diabetes [9]. The understanding of FOXO1 modulation by BITC could contribute with the development of effective nutritional strategies to treat or prevent diabetes and other disorders of metabolism. In this study the effect of BITC on FOXO1 translocation, gene and protein expression of FOXO1 target proteins and the factors behind of BITC modulation were evaluated in U-2OS and HepG2 cells.

3.1. BITC induces FOXO1 translocation in U-2OS stably transfected with pEGFP-FOXO1

All data shown in this section were recorded for cells treated according to the procedure described in numeral 2.2.8. Following 1h of starvation, stimulation of stably transfected cells was performed with benzyl glucosinolate (1 μM-100 μM), pre-incubated for 15 minutes with myrosinase, for 1 hour. Cells were fixed and stained with DAPI for identification of nuclear areas by fluorescence microscopic detection, for segmentation of cells and calculation of GFP-intensity ratios in nuclei and cytoplasm (Nuc/Cyt). The whole data analysis for all FOXO1-GFP expressing cells was performed by BD Image Data Explorer, including: fluorescence microscopic detection of nuclei, segmentation of cells, quantification of measured GFP intensities in nuclear and cytoplasmic areas, and calculation of the GFP-ratio nucleus/cytoplasm.

Fluorescent microscopy images are shown on Figure 11. After treatment with BITC FOXO1 translocation was induced in stable transfected U-2OS-FOXO1-GFP cell cultures. Translocation from cytoplasm into nucleus, was observed dependent on BITC concentration. Unstimulated cells show FOXO1-GFP predominantly localized on the cytoplasm (Fig. 11A). By contrast, stimulation with BITC at concentrations of 1, 10 and 100μM (Figs. 11B-D, respectively) resulted in a dose-dependent translocation to the nucleus.

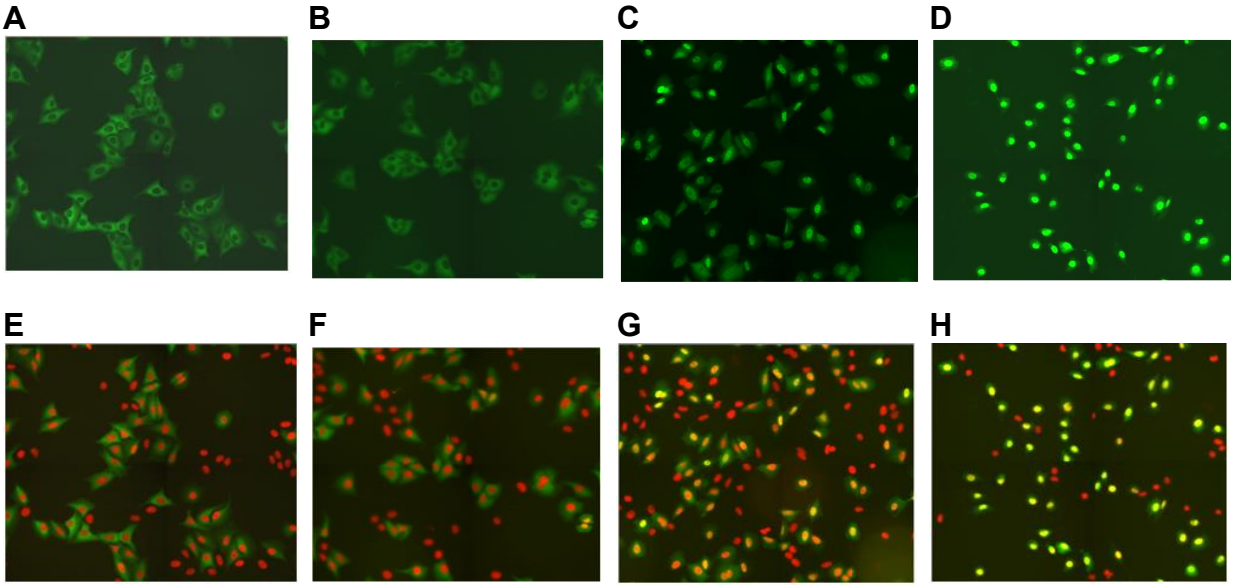


Figure 11. FOXO1 translocation in U-2OS-FOXO1-GFP cells. Figures 11 A-D show FOXO1-GFP expressing cells after 1h incubation with BITC at concentrations of 0, 1, 10 and 100μM, respectively. Figures 11 E-H show nuclear DAPI-staining (red) changing to orange-yellow in overlays with GFP.

The DAPI-staining shown in the figures 11 E-H indicates the nuclei in red when the cells are unstimulated and FOXO1 is located in the cytoplasm indicated by the green colour, but when BITC was added at the concentrations 10 and 100 μM the nuclei become orange or green, respectively, as a result of the FOXO1 translocations and the overlay with GFP.

A significant FOXO1 nuclear accumulation with increasing BITC concentration was observed (Fig. 12). Nuclear accumulation was estimated by measuring the ratio of FOXO1 nuclear/FOXO1 cytosolic (FOXO1 Nuc/Cyt) after 1h treatment. A slight increase up to 1.14-fold was observed for BITC concentrations below 10 μM, becoming progressively more significant (up to 3.59-fold) for concentrations between 10 μM and 50 μM. Further increase in BITC concentration did not modify nuclear accumulation of FOXO1 significantly.

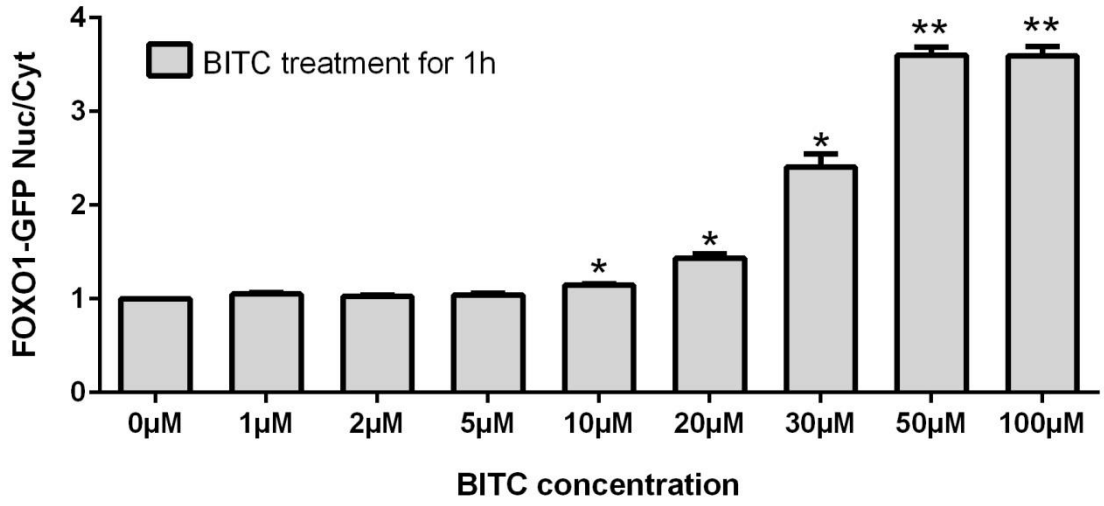
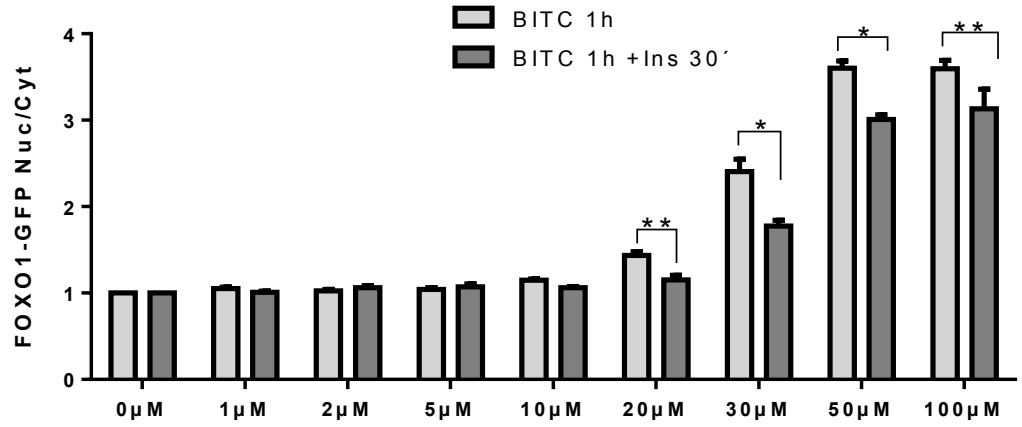


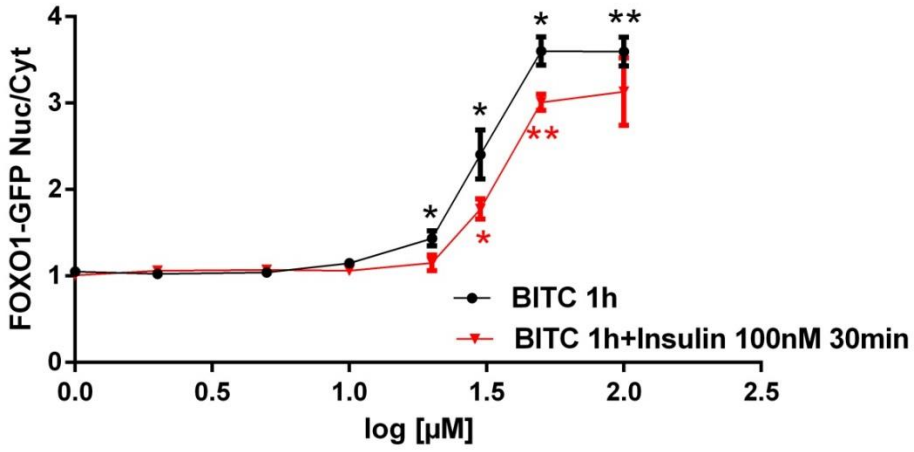
Figure 12. U-2OS-FOXO1-GFP cells treated 1 hour long with BITC at selected concentrations. Results are presented as mean values + standard error (SEM) of at least 4 independent treatments. Significant differences vs control are labelled for *(p<0.05) and **(p<0.01) respectively. One way ANOVA was used for the nonlinear regression analysis with posthoc Bonferroni or Dunnett T3 for multiple comparisons.

It is known that in response to insulin, FOXO1 is phosphorylated by AKT and excluded from the nucleus, inducing its degradation in the cytoplasm [2]. Therefore, the effect of insulin on the BITC-FOXO1 translocation induction was evaluated. The BITC-dose dependency in the range 1µM to 100µM during 1h and the reversibility of FOXO-1 translocation into nuclei by adding 100nM insulin for 30 minutes into parallel cell cultures were evaluated. The results are summarized in Figure 13.

A



B



	BITC 1h	BITC+Insulin
EC50	29.39	33.14

Figure 13. U-2OS-FOXO1-GFP cells treated with BITC 1-100µM and addition of 100nM insulin. A) Representation using bar diagram with BITC (grey) and BITC+insulin (dark grey). B) Insulin competition. The curves show the relative Nuclei/Cytoplasm intensity of FOXO1-GFP before (black curve) and after insulin addition (red curve). Results are presented as mean values + standard error (SEM) of at least 4 independent treatments. Significant differences vs control are labelled for *(p<0.05) and **(p<0.01) respectively. One way ANOVA with posthoc Dunett T3 for multiple comparisons was used for the nonlinear regression analysis.

Without pre-incubation with BITC, insulin reduced the ratio of FOXO1-GPF-intensity (FOXO1 Nuc/Cyt) significantly from 0.75 to 0.69 (Data not shown), which reflects an insulin-induced nuclear export of FOXO1 under these conditions. Insulin 100nM applied after 30 minutes after BITC partially reversed FOXO1-translocation induced by BITC 10 µM to 100 µM which was significant for the doses 20-100 µM. The half maximal effective concentrations (EC50) values were 29.4 µM and 33 µM for BITC and BITC+insulin, respectively. A competition between nuclear import by BITC and export by insulin (Fig. 13 A-B) can be deduced from these data.

3.2. Cell viability

Cell viability assays up to 100µM BITC were performed for insulin sensitive HepG2 cells expressing endogenous FOXO1 (See Figure 14). Using the cell proliferation assay from Promega we observed that cell survival rates up to 50 µM were not

affected. A significant reduction of about 34% on cell viability was found after 24h for 100 µM BITC but not for 100 µM benzyl glucosinolate.

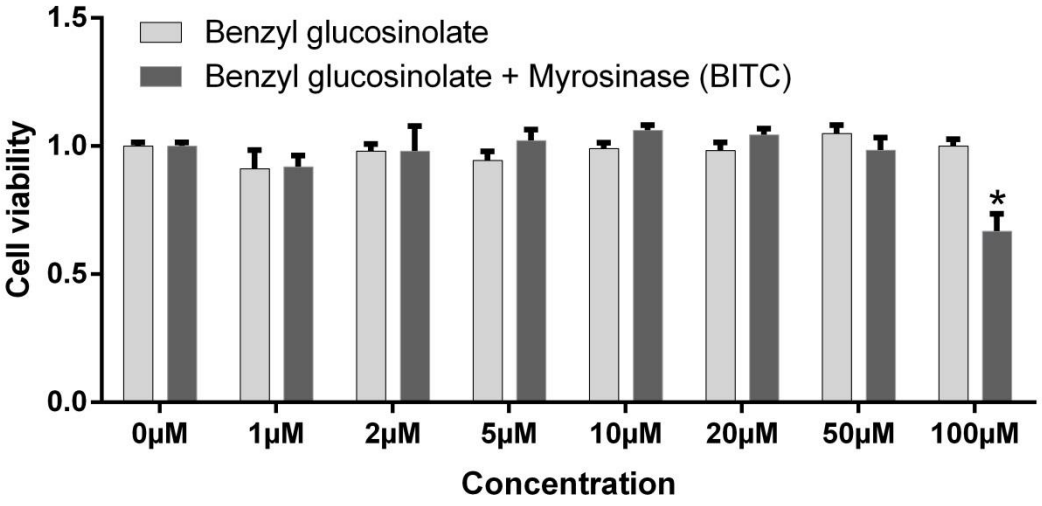


Figure 14. HepG2 cells survival. It was measured using the CellTiter96 AQuous ONE Solution from Promega applied to benzyl glucosinolate and BITC pre-treated cells. Following 4h incubation at 37°C with 20µM of PMS-electron coupling reagent (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy phenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt (MTS)/ phenazine methosulfate, the optical density (OD) of the MTS bioreduction product formazan was measured at 490 nm wavelength. OD mean values were normalized to mock treated cells (100% survival). Results are presented as mean values + SEM of 3 independent treatments with * (p<0.05) significant differences vs control. One way ANOVA (posthoc Bonferroni for multiple comparisons) was used for the nonlinear regression analysis.

3.3. BITC modulates gene expression of FOXO1 target genes

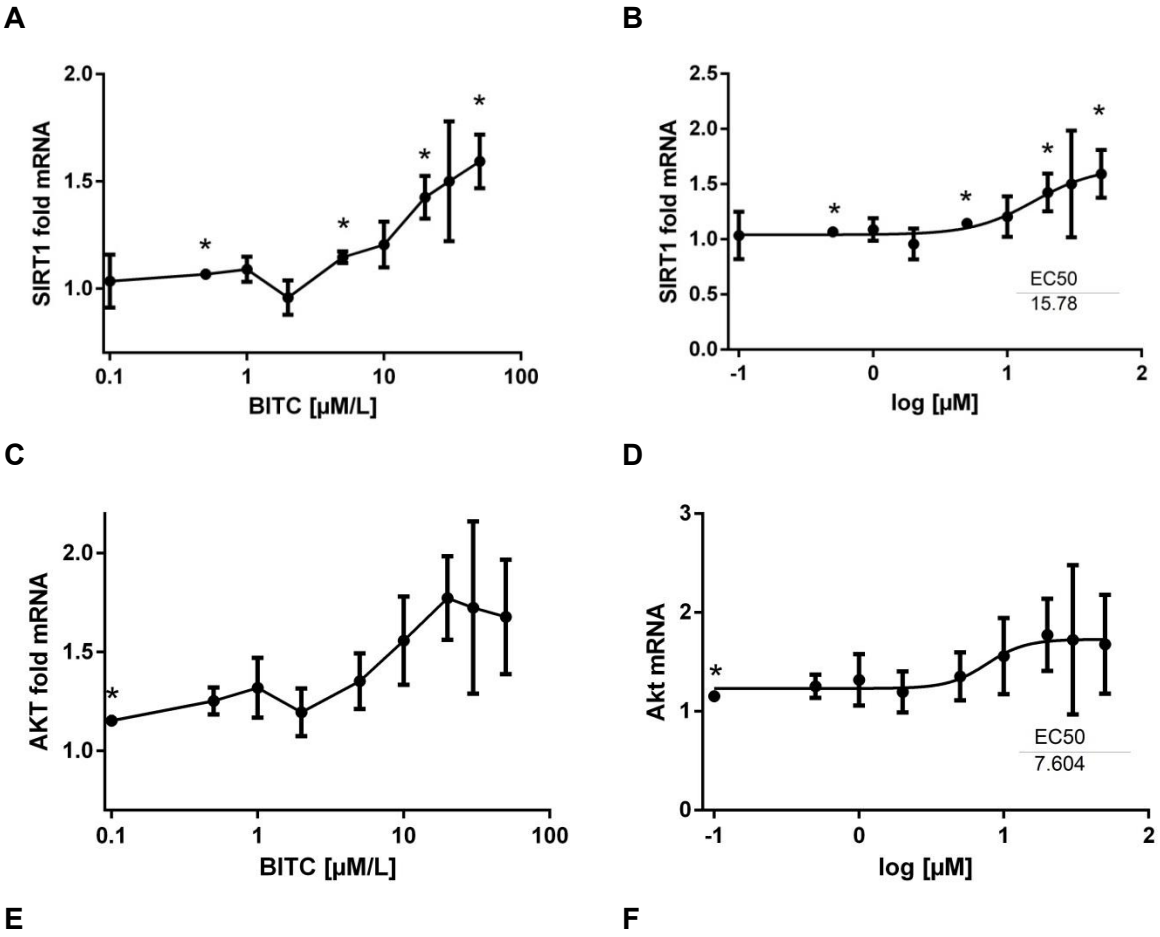
The FOXO1 transcription factor is linked to the regulation of gluconeogenesis, antioxidant and detoxification response, lipid metabolism, autophagy and cell proliferation. Since BITC was able to induce FOXO1 nuclear accumulation, the gene expression of different FOXO1 target genes was evaluated.

For all the time- and dose-dependent analyses described in this section, HepG2 cells were cultivated in 12-well plates in EMEM + 10% FCS and starved without FCS during 16h before stimulation. BITC was applied in the range of 0.1µM-100µM for 24h in the dose-dependent analyses and 30µM was used for time-dependent evaluations with an incubation period of 2h to 24h. Total RNA was extracted and reverse transcribed. qRT-PCR was performed using SYBR green analysed in ViiA7. Three independent experiments were performed with different passages of HepG2 for the dose-dependent evaluations and four for the time-dependency analyses. qRT-

PCR was run in triplicates using cDNA from control cells for standard dilutions. Results are presented as fold mRNA expression, normalized to the housekeeping gene RPL32 and unstimulated control cells.

3.3.1 Expression of FOXO1 transcription factor and interacting SIRT1 and AKT

The factors SIRT1, AKT and FOXO1 were evaluated in HepG2 cells after BITC treatment, at concentrations ranging from 0.1 µM to 50 µM during 16h. The results are depicted in Figure 15.



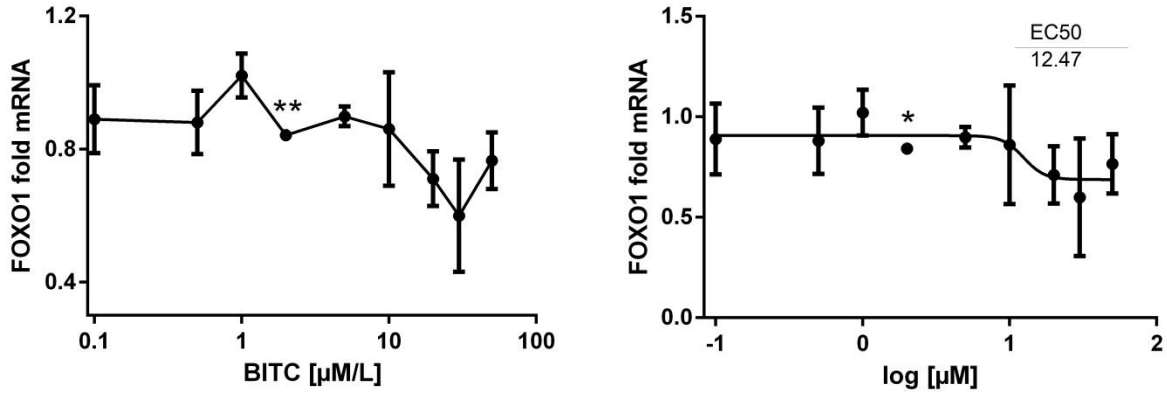


Figure 15. SIRT1, AKT and FOXO1 gene expression in HepG2 cells modulated by BITC. The gene expression at selected BITC concentrations (0.1 µM to 50µM) is shown for SIRT (Figures A, B); AKT (Figures C, D) and FOXO1 (Figures E, F). Results are presented as fold mRNA expression, normalized to the housekeeping gene RPL32 and the control. One way ANOVA (posthoc Bonferroni or Dunnett T3 for multiple comparisons) was used for the nonlinear regression analysis. The statistical significance level was set at $p<0.05$. An asterisk indicates a significant difference in the mean value + SEM of gene expression versus control *($p<0.05$) and **($p < 0.01$).

In figures 15 A and 15 B, a dose dependent up-regulation of SIRT1 mRNA was observed after BITC stimulation (0.5 to 50 µM). The effect is significant for concentrations of 0.5, 5, 20 and 50 µM (i.e. 1.6-fold for 50 µM and EC50=15.78 µM). AKT mRNA was significantly induced at 0.1µM. This effect is more pronounced with increasing concentrations up to 20 µM (1.7-fold EC50=7.6 µM) but not significant (Figures 15C and 15D). A significant down-regulation of FOXO1 mRNA at 2 µM BITC was observed. This trend includes a reduction by 0.59-fold at 30µM BITC (not significant) and EC50=12,5 µM. Absolutely no effect of BITC on FOXO3 gene expression was registered (Data not shown).

3.3.2 Gluconeogenic enzymes

BITC reduced markedly the gene expression of PEPCK and G6Pase in a dose dependent manner as deduced from figures 16A and 16B, respectively. For instance, with concentrations from 0.5 µM to 20 µM and 24 hours treatment, PEPCK was down-regulated significantly (i.e. 0.28-fold for 20 µM as observed in Fig. 16A). However, this effect became less pronounced and lost significances for concentrations between 30 µM and 100 µM (Fig. 16A). The effect of BITC 30µM on the gene expression of both enzymes was continuously monitored from 2h until 24h. For PEPCK, after 2h and 6h significant differences were obtained (p=0.02 and p=0.01, respectively) (Fig. 16C).

A clear dose dependent reduction in the gene expression of G6Pase was achieved with 0.1 µM to 100 µM BITC (0.24-fold with 20 µM) with significances in 20 µM and 50 µM (Fig. 16B). The reduction was observed from 6h to 24h stimulation and reached significance at 24h (Fig. 16D). In order to evaluate the effective dose of BITC, EC50 was determined for both, PEPCK (EC50=3.7 µM) and G6Pase (EC50=3.4 µM) illustrated in figures 16E and 16F, respectively.

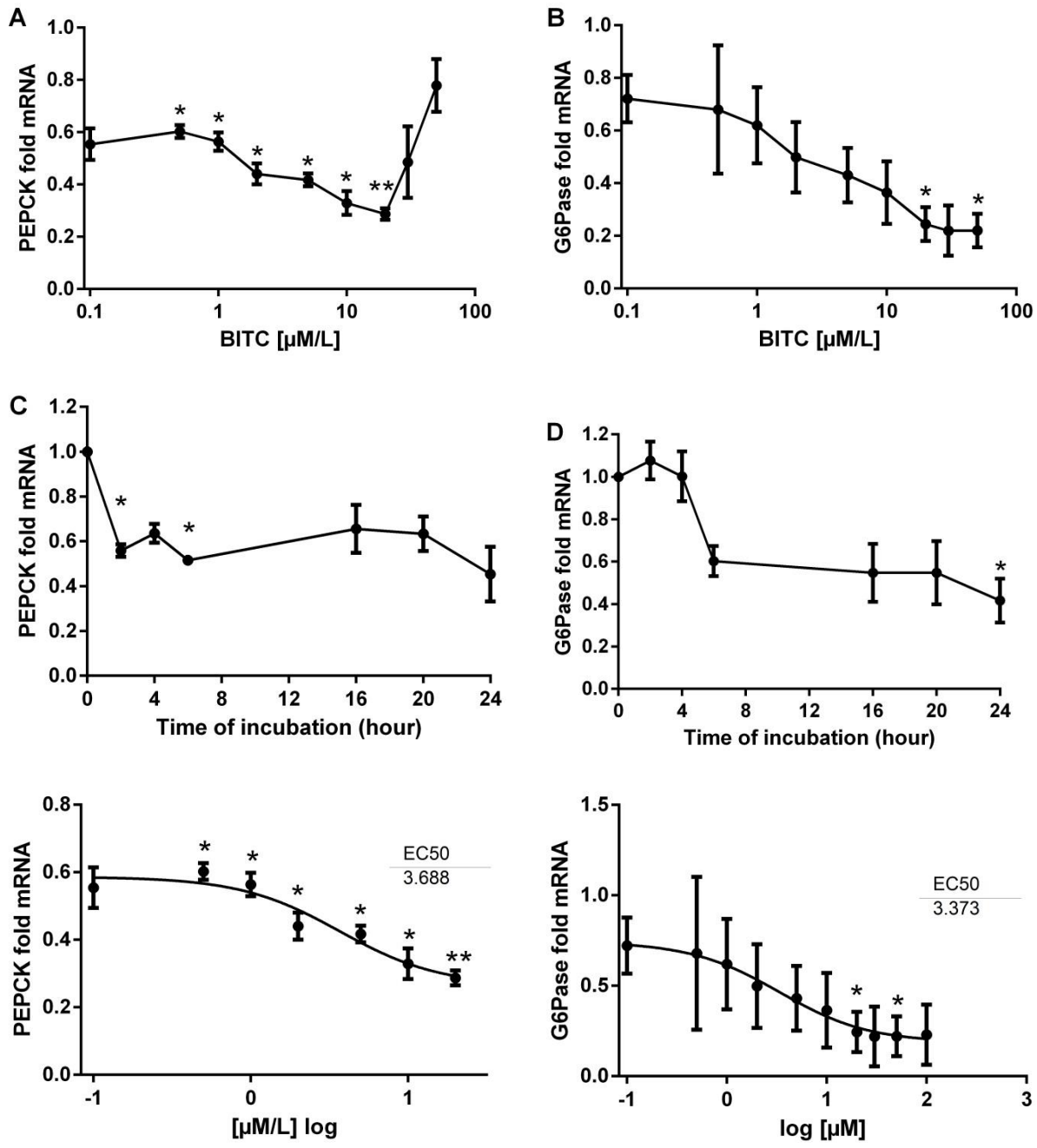


Figure 16. Gluconeogenic gene expression in HepG2 cells modulated by BITC. Dose-dependency is shown for gluconeogenic enzymes A) PEPCK and B) G6Pase. Time dependent analyses for C) PEPCK and D) G6Pase, respectively. E) PEPCK and F) G6Pase EC50, data for EC50 are presented in logarithmic scale. Results are

presented as fold mRNA expression, normalized to the housekeeping gene RPL32 and the control. One way ANOVA (posthoc Bonferroni or Dunnett T3 for multiple comparisons) was used for the nonlinear regression in dose and time-dependency analyses. The statistical significance level was set at $p<0.05$. Asterisks indicates a significant difference in the mean value + SEM of gene expression versus control *($p<0.05$) and **($p< 0.01$).

3.3.3 Antioxidant and detoxification enzymes

Figure 17 illustrates the gene expression analyses of antioxidant enzymes CAT and SOD. Although CAT and SOD have similar function regarding antioxidant response, an opposite effect after BITC stimulation was observed at relatively high concentrations. BITC induced a dose-dependent down-regulation of CAT with a significant reduction (around 50%) for concentrations higher than 10µM (Figure 17A). Conversely, SOD expression was significantly reduced with low concentrations but increased from 10µM BITC on, reaching a 2.5-fold maximum at 50µM, with a further decrease for the concentration of 100µM (Figure 17B). The decrease observed with the highest concentration is probably related to the toxicity produced by BITC with high concentrations, observed in the viability analyses (Fig. 14). The figures 17C and 17D show the EC50s for CAT (EC50=41µM) and SOD (EC50=8µM), respectively. More interestingly, time-dependent analyses showed that this effect just started after 16h for both enzymes, as deduced from figures 17E (CAT) and 17F (SOD), and persists after 24 hours of incubation.

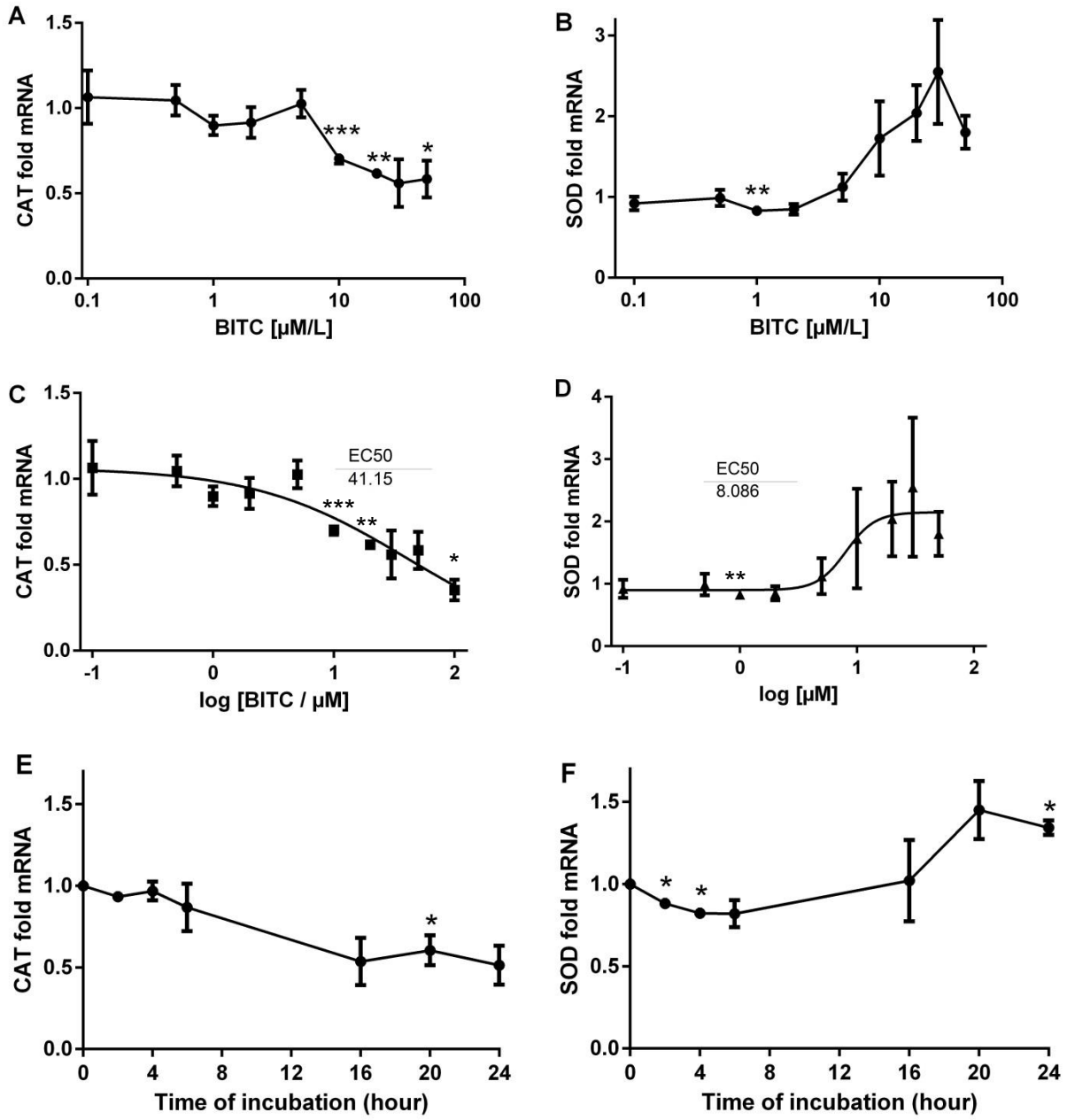


Figure 17. Antioxidant gene expression in HepG2 cells modulated by BITC. Dose-dependency is shown for antioxidant enzymes A) CAT and B) SOD. C) CAT and D) SOD EC50 presented in logarithmic scale. Time dependent analyses for E) CAT and F) SOD, respectively. Results are presented as fold mRNA expression, normalized to the housekeeping gene RPL32 and the control. One way ANOVA (posthoc Bonferroni or Dunnett T3 for multiple comparisons) was used for the nonlinear regression in dose and time-dependency analyses. The statistical significance level was set at $p<0.05$. Asterisks indicate a significant difference in the mean value ± SEM of gene expression versus control *($p<0.05$), **($p<0.01$) and ***($p<0.001$).

Detoxification pathways were analyzed through gene expression analyses of NRF2, SRXN1, NQO1 and GPX-2. HepG2 cells were treated with 30µM BITC during 24h and mRNA was extracted for qRT-PCR analyses. Figure 18 shows the effect of BITC on detoxification enzymes. NRF2 is not influenced by the presence of BITC. All

remaining detoxification enzymes SRXN1, NQO1, GPX-2 exhibit non-significant 2.9, 1.19, 1.21-fold increases, respectively.

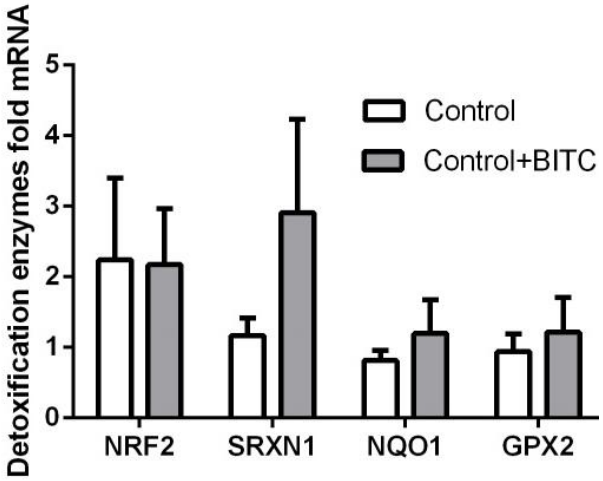


Figure 18. Detoxification enzymes gene expression in HepG2 cells modulated by BITC. Results are presented as fold mRNA expression, normalized to the housekeeping gene RPL32 and the control. No significances were found by Student's *t*-test which was used for the analyses.

3.3.4 Lipid metabolism enzymes

Figure 19 illustrates the gene expression analyses of ACC and FASN. The gene expression of both was slightly elevated after stimulation with increasing concentrations of BITC (Figures 19A and 19B). However, the statistical analysis did not show any significance for this trend. This phenomenon was observed in both cases after long term exposition: 20h for ACC (Figure 19E) and 16h for FASN (Figure 19F). The EC50 values for ACC and FASN were quite similar: 20.7 µM and 20.2 µM, respectively (Figures 19C and 19D).

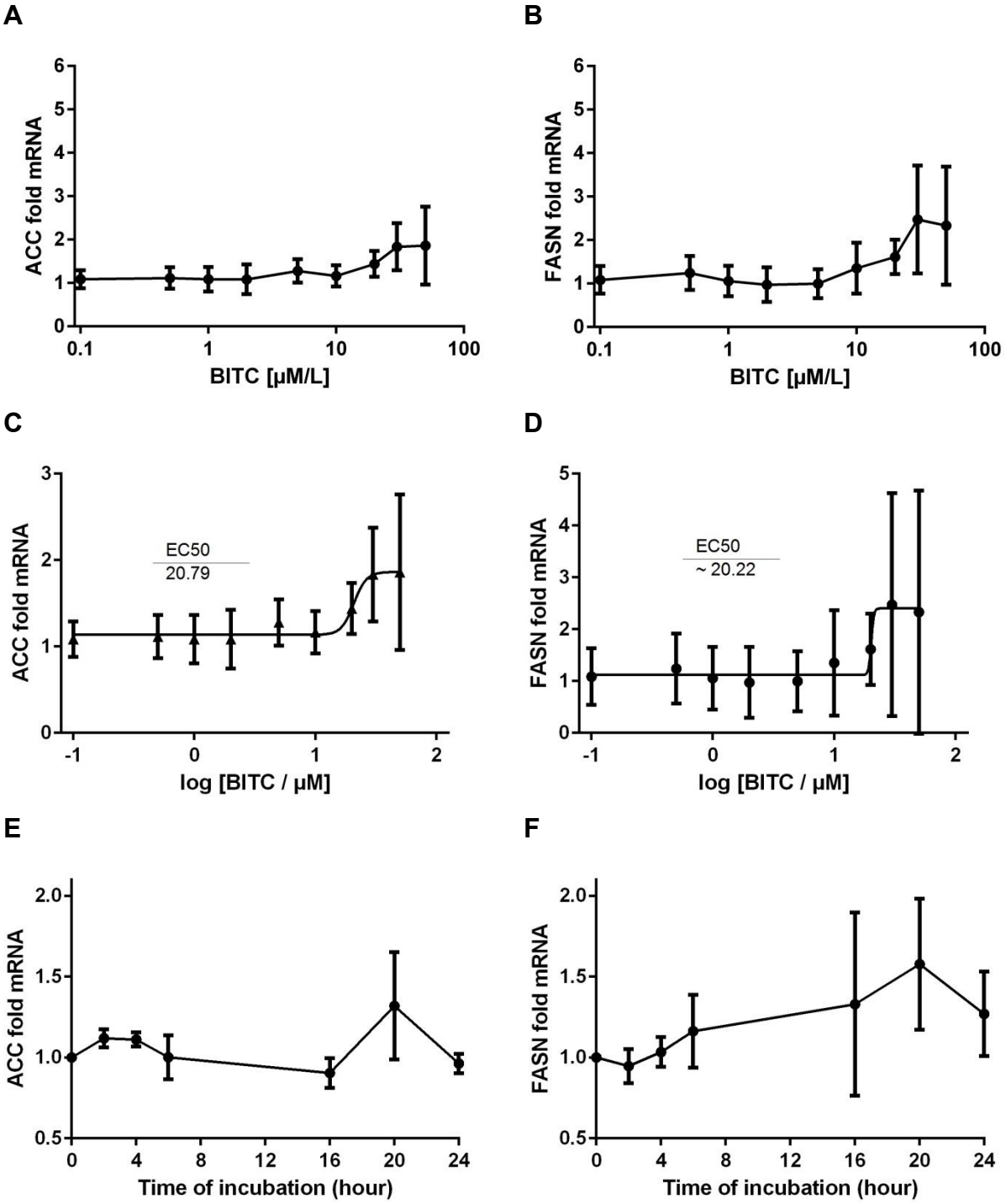


Figure 19. Lipogenic gene expression in HepG2 cells modulated by BITC. Figures 8A-B and E-F show dose- and time dependency for ACC and FASN. The EC50 for ACC (Figure E) and FASN (Figure F) are also presented. Results are presented as fold mRNA expression, normalized to the housekeeping gene RPL32 and the control.

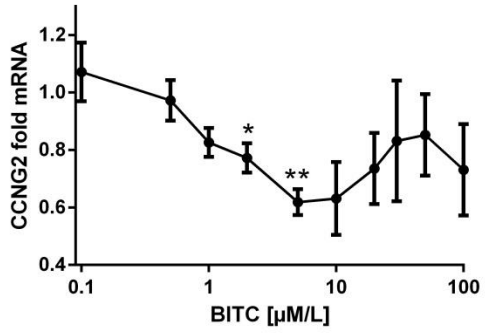
3.3.5 Cell cycle arrest and DNA damage repair enzymes

Figure 20 depicts the gene expression for cell cycle arrest and DNA damage repair enzymes CCNG2, P21/CIP, P27/KIP and GADD45. Upon BITC stimulation, CCNG2 mRNA expression was down-regulated for concentrations higher than 1 µM (Fig 20A). A significant reduction from 0.67 to 0.61-fold was estimated for 2 µM and 5 µM BITC (EC50 0.7 µM) (Figure 20C). The effect was significant after 6h stimulation and continued up to 24h, when the cells were stimulated with 30 µM BITC (Fig. 20E).

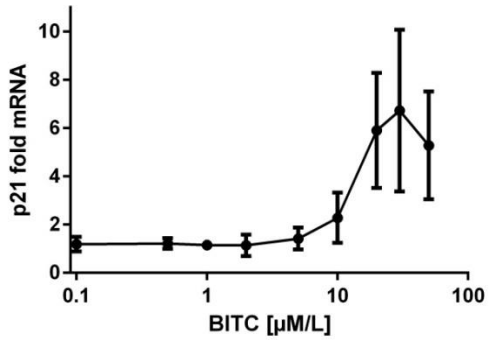
A dose-dependent up-regulation in p21 mRNA expression was observed for BITC 20 µM to 50 µM (Figure 20B), with an EC50 of 11.7 µM (Fig. 20D). Time-dependent analyses of stimulated cells with 30µM BITC (Fig. 20F), evidenced a significant up-regulation at 2h (1.09-fold) and 20h (2.23-fold). However, a slight down-regulating effect in the dose- and time- dependency analyses (Figures 20G, 20I and 20K) for p27 remains unclear and without significances.

Regarding GADD45, BITC induced an increase in the mRNA expression in a dose dependent manner with significances for concentrations of 5 µM and 30 µM (1.2-fold and 2.9-fold respectively) (Fig. 20H), showing an EC50 of 12.3 µM (Fig. 20J). This up- regulation was significant after 2h and 16h stimulation (Fig. 20L).

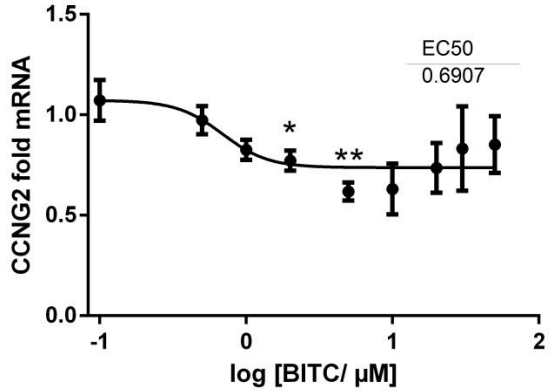
A



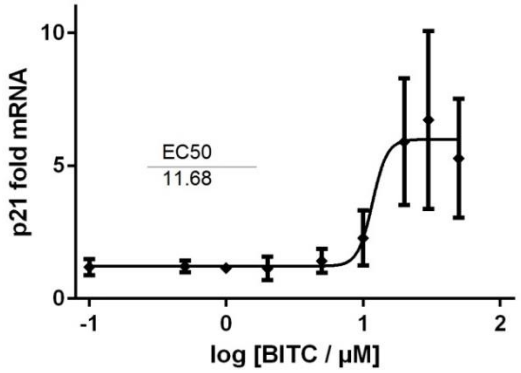
B



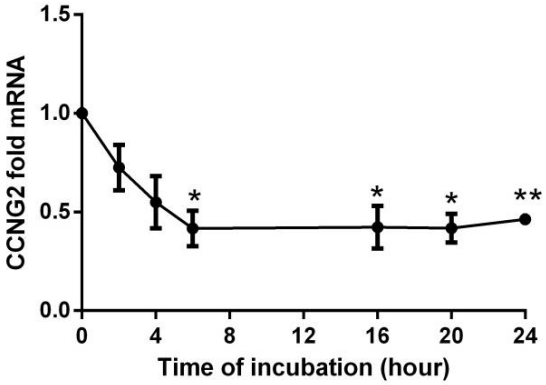
C


CCNG2 fold mRNA
EC50
0.6907
log [BITC/ µM]

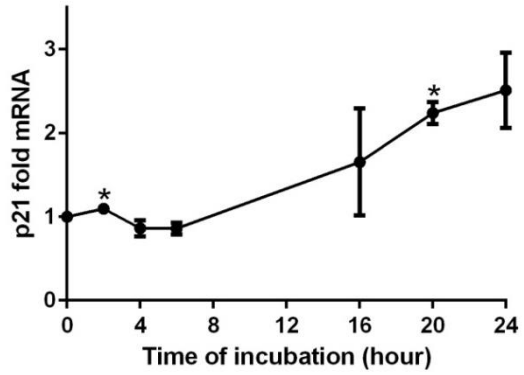
D


p21 fold mRNA
EC50
11.68
log [BITC / µM]

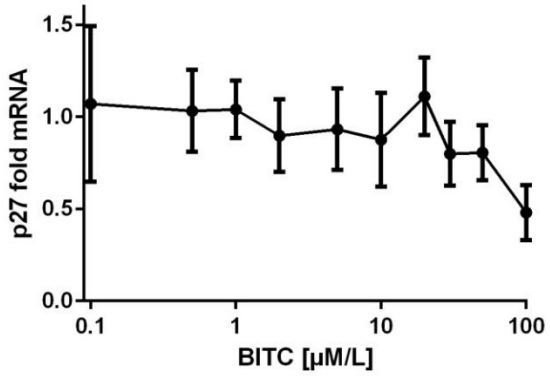
E


CCNG2 fold mRNA
Time of incubation (hour)

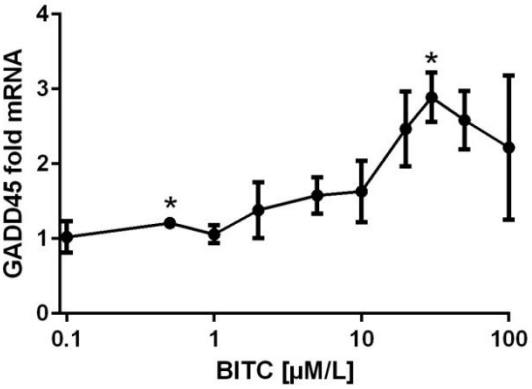
F


p21 fold mRNA
Time of incubation (hour)

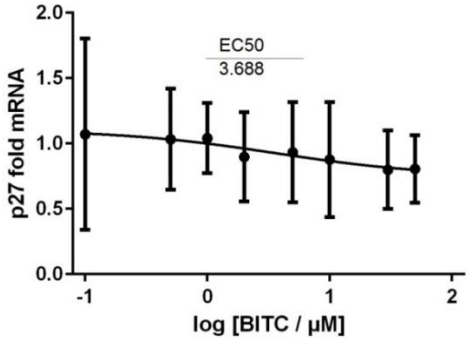
G


p27 fold mRNA
BITC [µM/L]

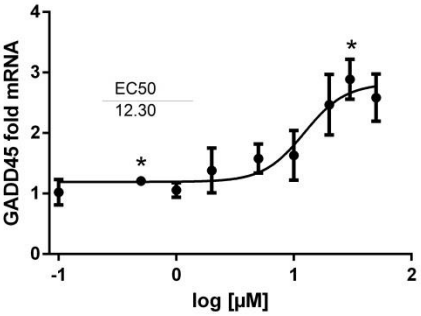
H


GADD45 fold mRNA
BITC [µM/L]

I


p27 fold mRNA
EC50
3.688
log [BITC / µM]

J


GADD45 fold mRNA
EC50
12.30
log [µM]

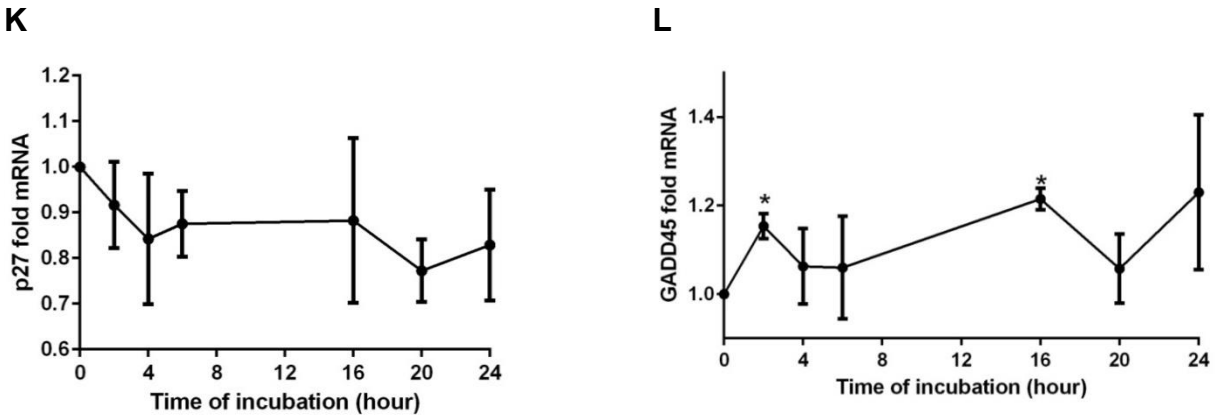


Figure 20. Cell cycle arrest and DNA damage repair gene expression in HepG2 cells modulated by BITC. Dose dependencies are shown in figures A, B, G, H; EC50 in figures C, D, I, J and time dependencies in the figures E,F, K, L respectively. Results are presented as fold mRNA expression, normalized to the housekeeping gene RPL32 and the control. One way ANOVA (posthoc Bonferroni or Dunnett T3 for multiple comparisons) was used for the nonlinear regression in dose and time dependecy analyses. The statistical significance level was set at P<0.05. Asterisks indicate significant differences in the mean value ± SEM of gene expression versus control *(p<0.05), **(p < 0.01) and ***(p <0.001).

Taken together the time dependency analyses showed conclusively that BITC modulated in a short term (after 2 hours) the gene expression of PEPCK and CCNG2 (down-regulation), ACC and GADD45 (up-regulation). Long term modulations (after 16 hours) were observed for G6Pase and CAT (down-regulation) and FASN, SOD and p21 (up-regulation).

3.3.6 Autophagic pathway

Evaluations of the effect of BITC on autophagic pathways were carried out for SQSTM1, PI3K3, ATG5, ATG12, BECLIN1, GABARAPL-1 and ULK1 mRNA expression. HepG2 cells were treated with 30µM BITC during 24h and mRNA was extracted for qRT-PCR analysis (Figure 21).

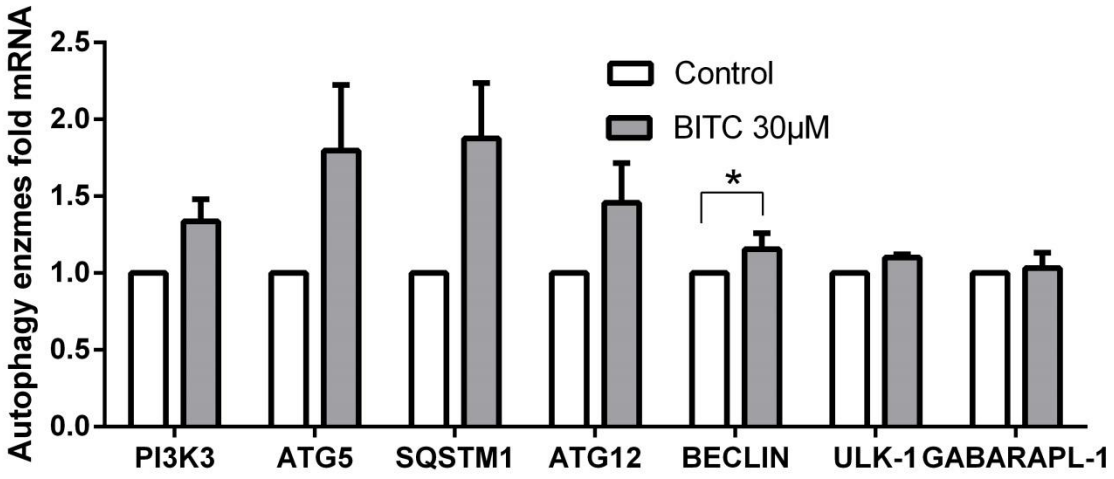


Figure 21. Autophagy gene expression in HepG2 cells modulated by BITC. Results are presented as fold mRNA expression, normalized to the housekeeping gene RPL32 and the control (Non target) without BITC treatment. The statistical significance level was set at $p<0.05$. Assterisks indicate a significant difference in the mean value + SEM of gene expression versus control *($p<0.05$) of ≥ 4 independent experiments. Unpaired Student's *t*-test was used for the analyses.

A significant 1.2-fold induction for BECLIN1 mRNA was found ($p=0.02$). Although all the remaining factors PI3K3, SQSTM1, ATG5, ATG12 ULK1 and GABARAPL-1 were apparently induced, no statistical significances were deduced from these data (Fig. 21).

3.4. Effect of BITC upon siRNA knock-down

In order to find an explanation of the underlying mechanisms for BITC gene expression modulation, the transcription factors FOXO1 and NRF2, and modulators such as AKT and deacetylating SIRT1 were knocked-down. It is well established that AKT is a FOXO1-inhibitor whereas SIRT1 acts as an activator [16]. In this way, expression of the responsible genes for gluconeogenesis (PEPCK and G6Pase), antioxidant and detoxification response (CAT, SOD GPX-2, SRXN and NQO1), cell cycle arrest and DNA damage repair (CCNG2, GADD45, p21 and p27) was evaluated under knock-downs of these factors.

For small interfering RNA analysis (siRNA), HepG2 cells grown in EMEM with 10% FCS, were treated with DharmaFECT4 (D4) transfection reagent or transfection mixtures of D4 and NT-siRNA for transfection controls. Cells were transfected with

D4 and smart pool siRNAs for FOXO1, AKT, SIRT1, NRF2, and their combinations NRF/FOXO1, NRF2/AKT, SIRT/FOXO1 and SIRT1/AKT. Following 48h transfection (32h in EMEM + FCS 10% followed by 16h EMEM without FCS for starvation of cells), cells were treated with BITC 30 µM for 24h, followed by RNA extraction and gene expression analyses.

Table 12 Gene expression changes (%) after FOXO1, AKT, SIRT1 and NRF2 knock-down and their stimulation with BITC

si RNAs	FOXO1		AKT		SIRT1		NRF2	
	Basal	BITC	Basal	BITC	Basal	BITC	Basal	BITC
Control (NT)	o	-5,36	0	*19,18	0	*52,30	0	45,55
FOXO1	**-45,30	**-52,48	27,33	30,92	*131,81	37,03	155,80	39,29
AKT	-4,72	-5,14	***-71,90	***-70,69	*32,58	*§50,36	67,45	99,15
SIRT1	33,19	48,45	27,12	-4,58	***-42,71	**-50,93	96,21	21,16
NRF2	37,45	-1,13	25,58	9,57	64,24	4,89	*-39,77	*-64,56
NRF2/FOXO1	32,41	6,26	16,62	59,90	120,25	36,54	46,88	16,58
NRF2/AKT	77,94	-5,20	**-65,20	***-67,83	158,44	50,76	58,88	*-59,64
SIRT1/FOXO1	50,99	-39,25	2,67	-7,62	52,83	-31,24	105,15	25,81
SIRT1/AKT	136,04	42,72	**-64,61	**-64,06	0,72	-26,82	123,03	39,30

Table 12. Gene expression changes (%) after FOXO1, AKT, SIRT1 and NRF2 knock-down and stimulation with BITC. Gene expression changes (%) after FOXO1, AKT, SIRT1 and NRF2 knock-down and stimulation with BITC. Mean mRNA levels of at least 4 independent experiments (n ≥ 4) are shown as percentage change of basal expression in untreated cells of transfection controls. The knock-down efficiencies for each factor are shown in blue in single transfections and grey in double transfections. The statistical significance level was set at $p < 0.05$ (Student's *t* test). *p <0.05, **p < 0.01 and ***p <0.001 vs control cells transfected with Non Target siRNA (NT) normalized with RPL32 and control. Significance regarding to the cells treated with FOXO1, AKT, SIRT1 and NRF2 is showed as (§).

Table 12 summarizes the BITC-modulated gene expression under FOXO1, AKT, SIRT1, NRF2 knocked-down conditions, the gene expression of FOXO1 target genes under NRF2/FOXO1, NRF2/AKT, SIRT1/FOXO1 and SIRT1 AKT are shown in the supplements. As deduced from the data, FOXO1 was efficiently knocked-down 45% (p=0.003) and its gene expression was not significantly affected by other factors knocked-down. The stimulation with BITC in the presence of the FOXO siRNA reduced FOXO1 about 52% (p<0.01) and this reduction is similar to that observed in the control treated with the non-target siRNA (-45.3%).

AKT was significantly reduced (72%, p<0.001) upon AKT siRNA treatment and after siRNA combinations for NRF/AKT and SIRT1/AKT (65% and 64%, respectively).

According to these analyses AKT basal gene expression was FOXO1, SIRT1 and NRF2 independent. BITC increased AKT mRNA about 20% (p<0.05) in control cells and this effect was abolished after AKT, NRF/AKT and SIRT1/AKT knock-down (p<0.00001, p<0.005, p<0.02, respectively).

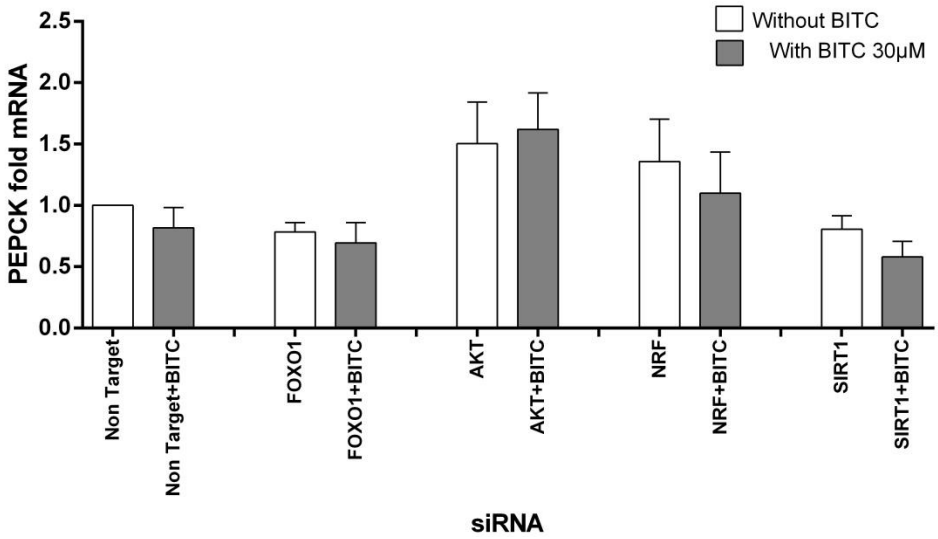
Upon the siRNA treatment SIRT1 was successfully reduced by 43% (p=0.001), and significantly increased by FOXO1 and AKT knock-down 131% and 32%, respectively (p=0.019 and p=0.015, respectively). After BITC stimulation SIRT1 was up-regulated significantly in control cells (p<0.05), but this effect was abolished after its knock-down (50% reduction, p<0.01). BITC increased SIRT1 by 37% and 50% upon FOXO1 and AKT siRNA treatment, respectively, which was significant for AKT siRNA regarding control cells and control in the presence of AKT siRNA, suggesting that the effect of BITC on SIRT1 is FOXO1 and AKT independent (Table 12).

NRF2 was knocked down by 40% (p=0.05) in control cells and not affected by FOXO1, AKT and SIRT1 knock down. In control cells BITC up-regulated NRF2 gene expression by 45% (not significant), but upon NRF2 siRNA this effect was significantly abolished by 65% (p<0.05) regarding control cells without siRNA transfection (Table 12). The abolishment in the induction of NRF2 after NRF2 knock-down and BITC stimulus could suggest that BITC is NRF2 dependent.

3.4.1 Gluconeogenic enzymes

Figure 22 shows the results obtained for the gluconeogenic genes PEPCK and G6Pase upon the siRNA treatment. None of the factors knocked-down had significant effects on PEPCK gene expression, although under AKT knock-down an increasing trend in PEPCK mRNA was observed indicating a higher expression rate under reduced AKT leading to lower phosphorylating capacity for inactivation of FOXO1. BITC reduced PEPCK gene expression 18% and 21% in control and after FOXO1 knock-down, but without significance, suggesting that there are additional factors implied in the modulation of its gene expression.

A



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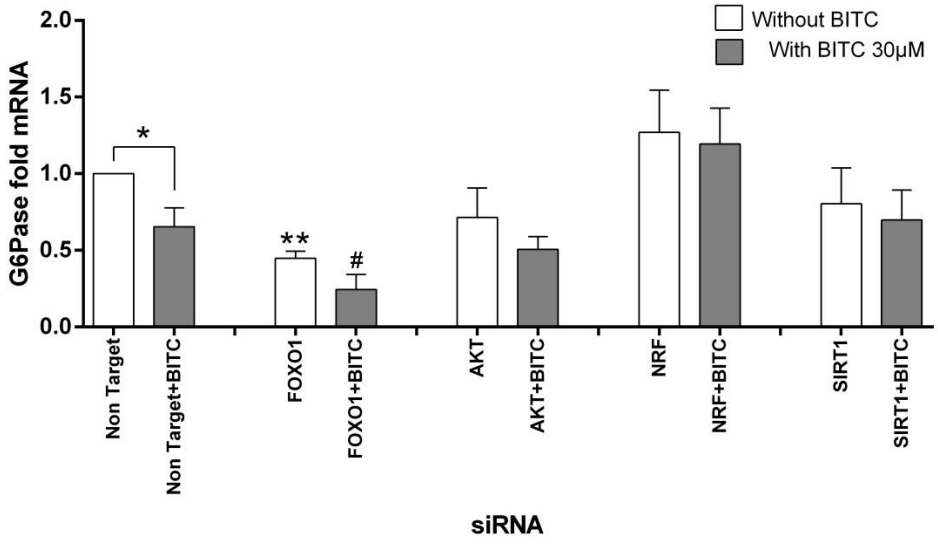


Figure 22. HepG2 cells siRNA analyses and gluconeogenic enzymes gene expression. HepG2 cells siRNA analyses and gluconeogenic enzymes gene expression. A) PEPCK and B) G6Pase for evaluating BITC influence on gluconeogenesis under FOXO1, AKT, NERF2 and SIRT knock-down conditions. The statistical significance level was set at $p<0.05$ (Unpaired Student's *t* test). Significances are shown as *$p<0.05$ and **$p<0.01$ vs control cells treated with NT "*"or NT+BITC "#", normalized with GAPDH and versus control. Data shown as mean of fold mRNA + SEM from $\geq$ 4 independent experiments.

G6Pase was reduced by 55% after FOXO1 knock-down (p=0.007, Fig. 22B), which confirms the role of FOXO1 in its gene expression modulation. A non significant up-

regulation upon NRF2 and the combination of NRF2/FOXO1 was observed and none effect under AKT and SIRT1 knock-down was found.

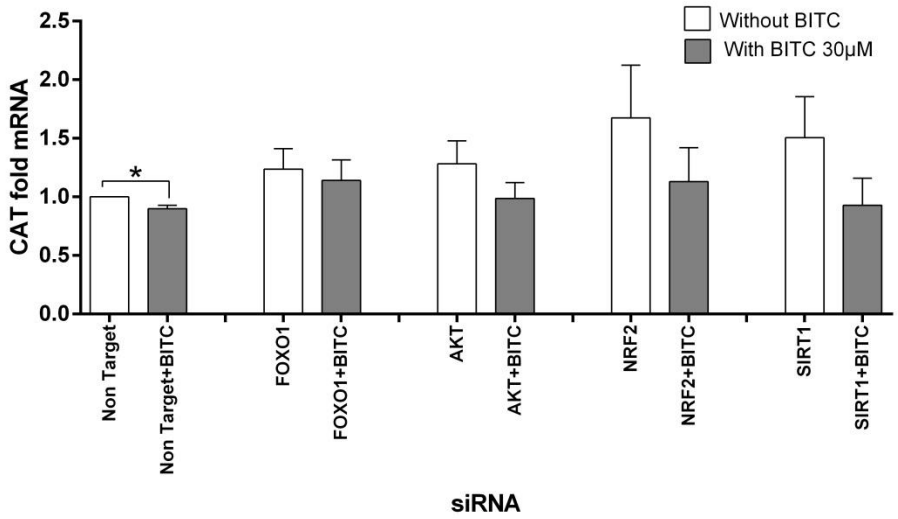
Upon BITC treatment G6Pase was significantly down-regulated 35% in control cells (p<0.05) and 62% upon FOXO1 knock-down (p<0.05) regarding to the cells without siRNA treatment and stimulated with BITC, in the last case the significance reached by BITC regarding to the unstimulated cells was reduced to less than half of expression rate reached under FOXO1 knock-down.

Under AKT knock-down G6Pase was slightly reduced by BITC, but without significance (Fig. 22B). The treatment with NRF2 siRNA and combination of siRNAs for NRF2/FOXO1 and NRF2/AKT abolished the significant reduction reached by BITC in control cells, increasing by 2 fold the G6Pase gene expression rate (See in supplements). These results suggest that NRF2 and AKT could be involved in the BITC modulatory effect on G6Pase gene expression.

3.4.2 Antioxidant and detoxification enzymes

Figure 23 illustrates the antioxidant enzymes CAT and SOD gene expression modulation after FOXO1, AKT, NRF2 and SIRT1 knock-down. As observed in the time- and dose- dependent analyses (Fig. 23A and B), BITC had opposite effects on CAT and SOD gene expression in control cells at the dose of 30μM, reducing CAT and increasing SOD mRNA. CAT was not influenced by BITC after inhibition of FOXO1, AKT, NRF2 and SIRT1 (Fig. 23A), while the 72% reduction of AKT by the siRNA treatment (shown in Table 12) increased significantly the SOD gene expression in BITC treated cells (Fig. 23B). The combinations of siRNA for FOXO1, AKT, NRF2 and SIRT1 did not show any clear effect (Data shown in supplements).

A



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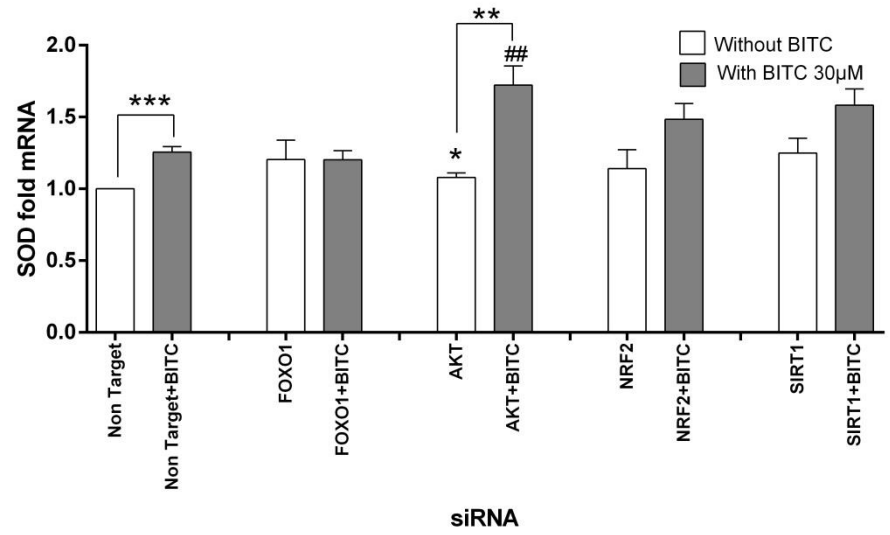
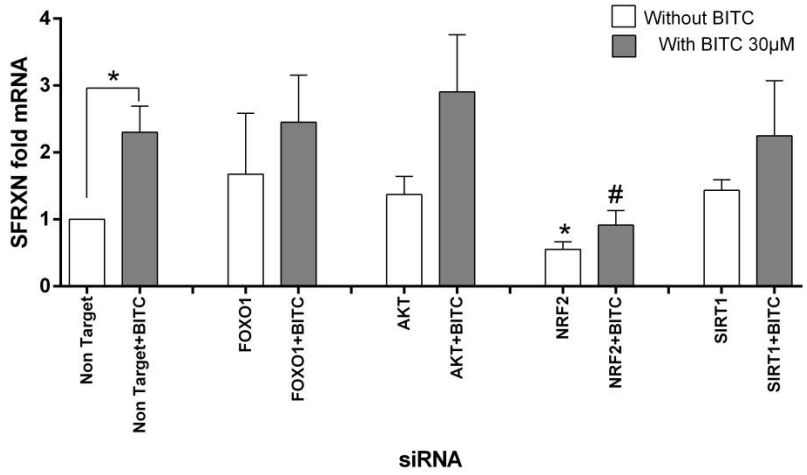


Figure 23. HepG2 cells siRNA analysis and antioxidant enzymes gene expression. A) CAT and B) SOD genes expression under FOXO1, AKT, NERF2 and SIRT knock-down conditions. The statistical significance level was set at $p<0.05$ (Unpaired Student's *t* test). Significances are shown as *$p<0.05$, ** $p<0.01$ and *** $p<0.001$ vs control cells treated with NT "*"or NT+BITC "#". Data shown as mean of fold mRNA + SEM from $\geq$ 4 independent experiments.

The influence of BITC on detoxification enzymes GPX-2, SFRXN and NQO1 analyses are shown in figure 24.

A



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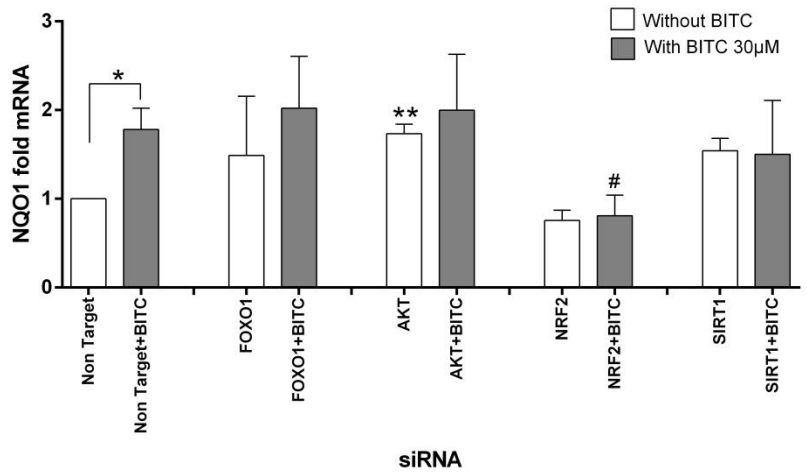


C

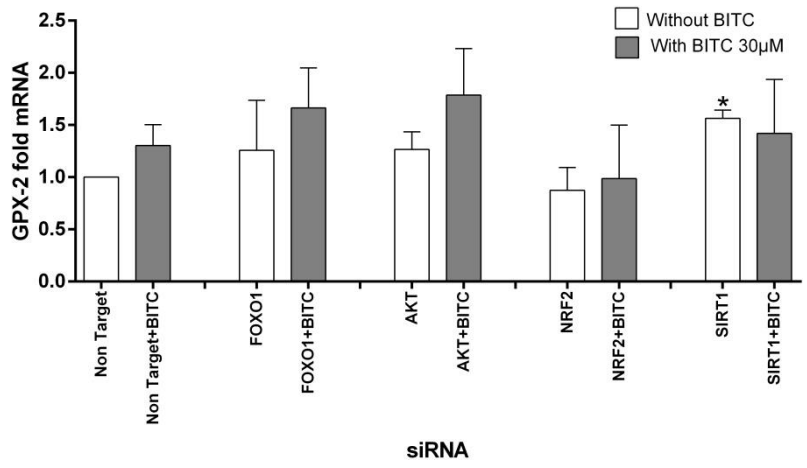


Figure 24. HepG2 cells siRNA analysis and detoxification enzymes gene expression. A) GPX-2, B) SFRXN and C) NQO1 gene expression under FOXO1, AKT, NRF2 and SIRT knock-down conditions. The statistical significance level was set at $P<0.05$ (unpaired Student's *t* test). Significances are shown as $*P<0.05$, $** P<0.01$ and $*** P<0.001$ vs control cells treated with NT "*"or NT+BITC "#". Data shown as mean of fold mRNA + SEM from $\geq$ 4 independent experiments.

As expected, detoxification enzyme SRXN was significantly down-regulated (45%) after NRF2 knock-down, since SRXN is known for being a NRF2 target factor [5] (Fig. 24A). After BITC stimulation, SRXN mRNA was significantly up-regulated in control cells. However, it was significantly reduced 60% under NRF2 knock-down conditions. This strong reduction upon BITC stimulus suggests a NRF2 dependency.

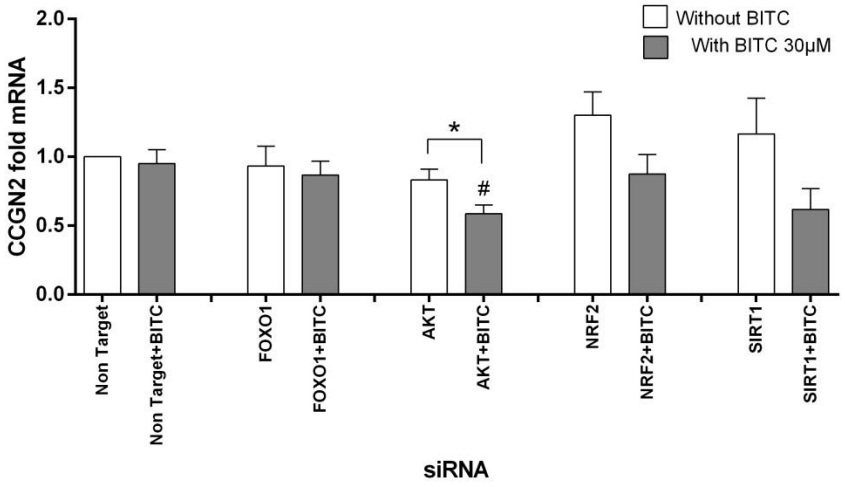
Regarding NQO1, NRF2 knock-down reduced its gene expression about 24% without significance, while AKT siRNA increased NQO1 expression significantly (P<0.005). After BITC stimulation NQO1 was significantly up-regulated by 78% in control cells, and this effect was abolished by NRF2 knock-down (Fig. 24B) suggesting a NRF2 dependency.

Figure 24C shows that GPX-2 gene expression was not altered after FOXO1, AKT and NRF2 knock-down, except for the case of SIRT1 knock-down, which induced an up-regulation on untreated cells. Conversely, the treatment with BITC induced an up-regulation in all the cases, except for SIRT1 siRNA treated cells, but no statistical significance was found.

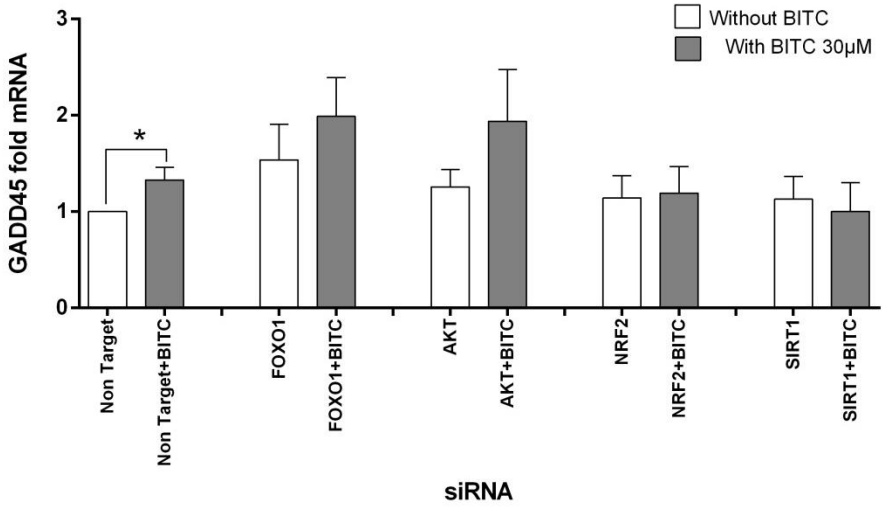
3.4.3 Cell cycle arrest and DNA damage repair enzymes

Information about the influence of BITC on the enzymes: CCNG2, GADD45, p21 and p27 is shown in figure 25. None of the knock-down factors FOXO1, AKT, NRF2 and SIRT1 had any significant effect on the basal gene expression of CCNG2, GADD45 and p21. The treatment with BITC reduced significantly the CCNG2 gene expression upon AKT knock-down conditions (Fig. 25A). BITC induced a significant increase in GADD45 mRNA in control cells (Fig. 25B) in agreement with previous analyses (Fig. 20H), but this induction was abolished upon NRF2- and SIRT- knock-down indicating NRF2 and SIRT1 mediating the BITC effect on GADD45 induction. No clear effect on p21 mRNA was observed (Fig. 25C). Only under NRF2 and SIRT1 knock-down conditions a significant up-regulation in p27 was observed in untreated cells (Fig. 25D) which was not found after BITC stimulation showing basal levels of p27 expression .

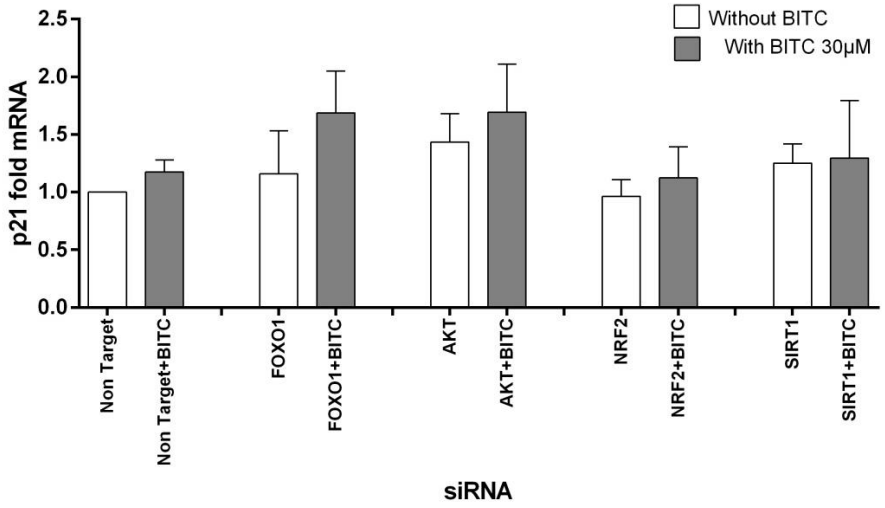
A


Without BITC
With BITC 30μM
CCGN2 fold mRNA
2.0
1.5
1.0
0.5
0.0
*
#
Non Target
Non Target+BITC
FOXO1
FOXO1+BITC
AKT
AKT+BITC
NRF2
NRF2+BITC
SIRT1
SIRT1+BITC
siRNA

B


Without BITC
With BITC 30μM
GADD45 fold mRNA
3
2
1
0
*
Non Target
Non Target+BITC
FOXO1
FOXO1+BITC
AKT
AKT+BITC
NRF2
NRF2+BITC
SIRT1
SIRT1+BITC
siRNA

C


Without BITC
With BITC 30μM
p21 fold mRNA
2.5
2.0
1.5
1.0
0.5
0.0
Non Target
Non Target+BITC
FOXO1
FOXO1+BITC
AKT
AKT+BITC
NRF2
NRF2+BITC
SIRT1
SIRT1+BITC
siRNA

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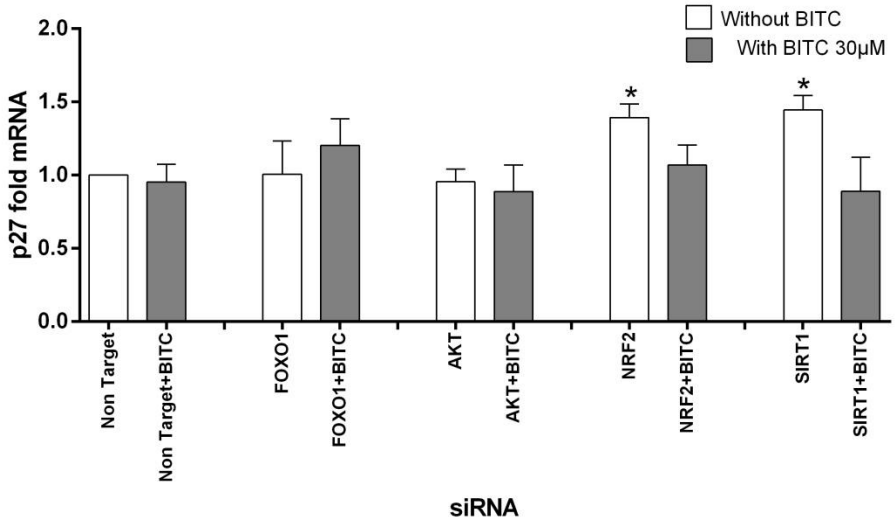
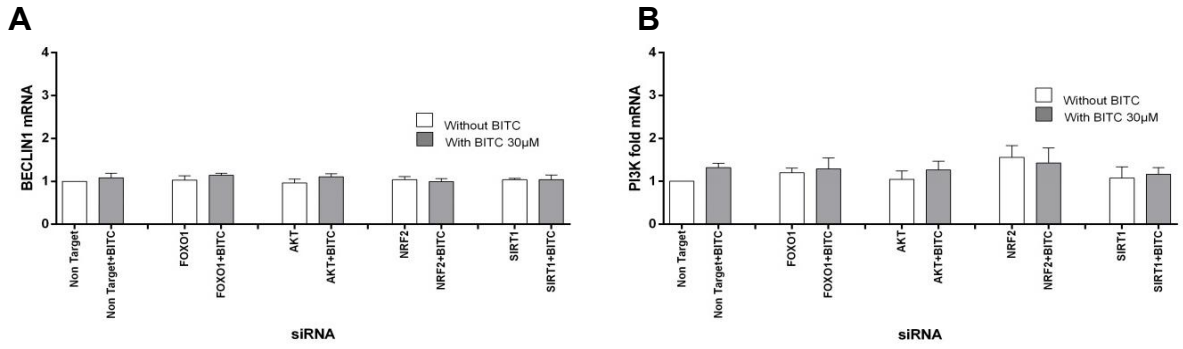


Figure 25. HepG2 cells siRNA analysis of cell cycle and DNA damage repair enzymes. A) CCNG2, B) GADD45, C) p21, D) p27 gene expression under FOXO1, AKT, NERF2 and SIRT knock-down conditions. The statistical significance level was set at $P<0.05$ (Unpaired Student's *t* test). Significances are shown as *$P<0.05$ vs control cells treated with NT "*"or NT+BITC "#".Data shown as mean of fold mRNA + SEM from $\geq$ 4 independent experiments.

3.4.4 Autophagy inducing gene expression

The factors involved in the autophagy pathway BECLIN1, PI3K3, SQSTM1, ATG5, ATG12, ULK1 and GABARAPL-1 were evaluated under FOXO1, AKT, SIRT1, NRF2 knock-down conditions. None of the analysed knocked-down conditions was involved in the gene expression modulation of factors involved in autophagy, but all factors were apparently induced by BITC, although without significance (Figure 26).



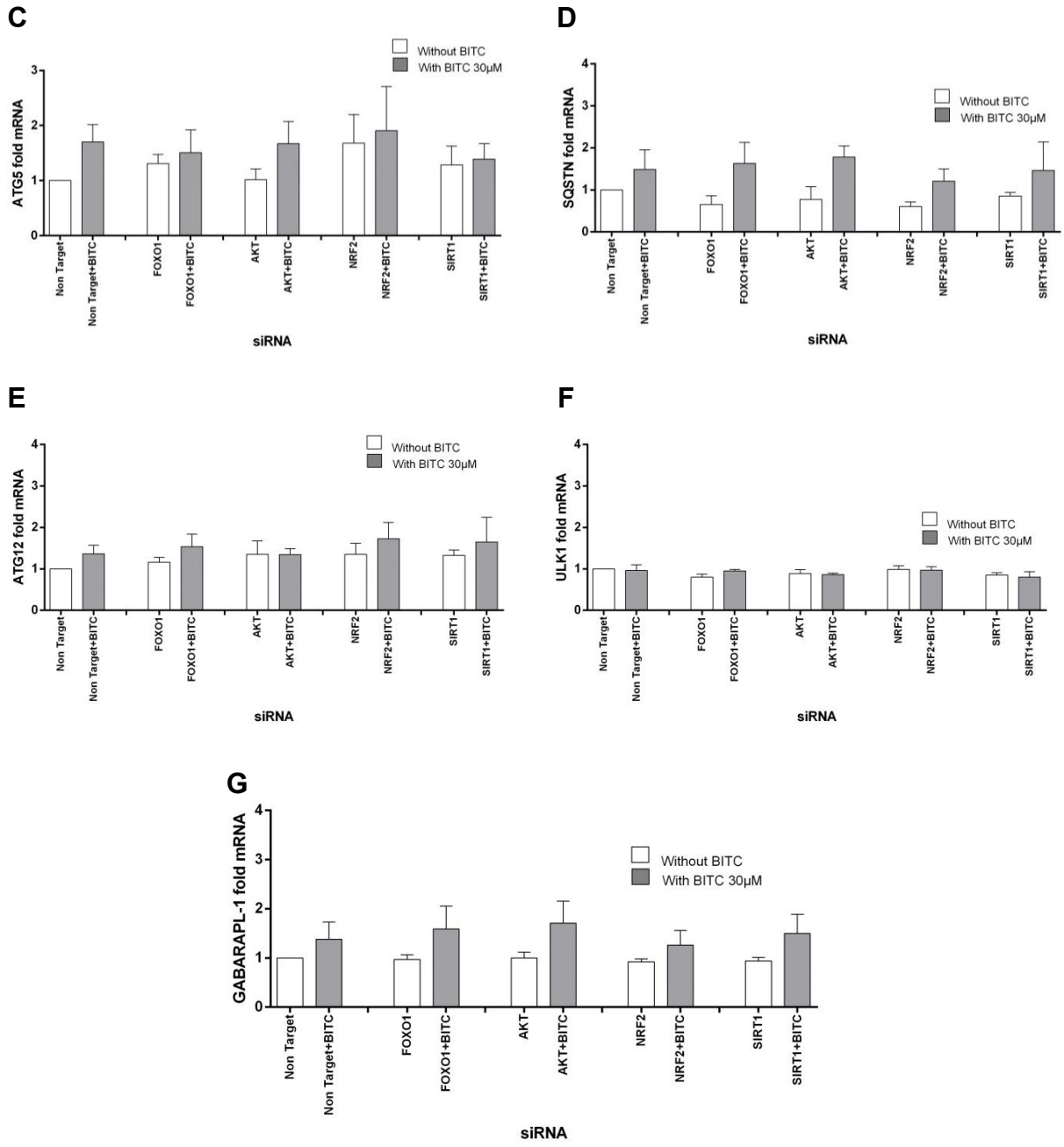


Figure 26. HepG2 cells siRNA analysis and autophagy pathway. A) BECLIN1; B) PI3K; C) ATG5; D) SQSTM1; E); ATG12; F) ULK1; G) GABARAPL-1 gene expression under FOXO1, AKT, NERF2 and SIRT knock-down conditions. Specific mRNA expression was normalized to housekeeping RPL32. The statistical significance level was set at $P<0.05$ (Unpaired Student's *t* test). Data are shown as mean of 4 independent experiments + SEM (standard error of mean).

3.5. Effect of BITC on protein expression

3.5.1 AKT and FOXO1

To elucidate the mechanism by which BITC modulates FOXO1 translocation and activation, the phosphorylation and protein expression of AKT and FOXO1 and some FOXO1 target proteins were determined by Western immuno-blot. The results are presented on Figure 27.

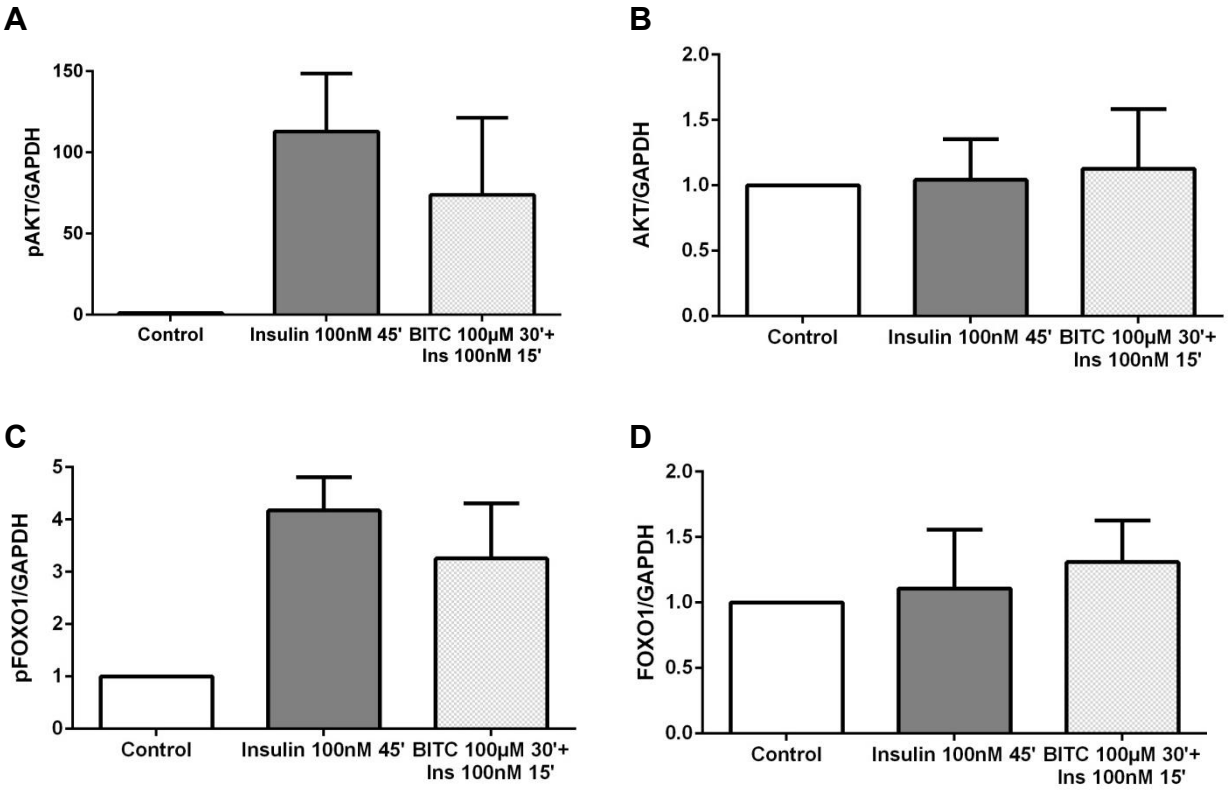


Figure 27. AKT and FOXO1 phosphorylation upon BITC and insulin treatment. HepG2 cells were incubated with 100nM insulin for 15 minutes, following pre-incubation with BITC 100µM for 30 minutes. Cells were lysed in the presence of phosphatase- and protease-inhibitors, ultrasonicated and in the supernatant after centrifugation the protein concentration was measured. Western immuno-blots were performed with antibodies against pAKT (Phospho AKT), pFOXO1 (Phospho FOXO1) and GAPDH (housekeeping protein). Data are shown as mean of 3 independent experiments + SEM (standard error of mean). One way ANOVA (posthoc Bonferroni or Dunnett T3 for multiple comparisons) was used for the analysis.

Figure 27A shows a non-significant induction of AKT-phosphorylation in HepG2 cells treated with insulin alone in comparison with untreated cells used as control. Following a BITC pre-incubation, a slight but non-significant inhibition of AKT-phosphorylation in the HepG2 cells was observed. The total AKT levels remained constant upon the different treatments as was expected (Fig. 27B).

Figure 27C shows an induction of FOXO1-phosphorylation in HepG2 cells treated with insulin alone in comparison with untreated (control) cells, which is expected as AKT phosphorylates FOXO1 in response to insulin. Cells pre-incubated with BITC, showed a reduction of FOXO1-phosphorylation compared to insulin treatment (not significant). Total FOXO1 remained constant after the different treatments (Fig. 27D).

Owing to the fact that the exposition of 100nM BITC for 24h was shown to be slightly toxic for HepG2 cells an additional experiment for evaluating FOXO1 phosphorylation following a pre-incubation with 30µM BITC for 24 hours was performed. The figure 28 shows a significant reduction in pFOXO1 as compared to untreated cells.

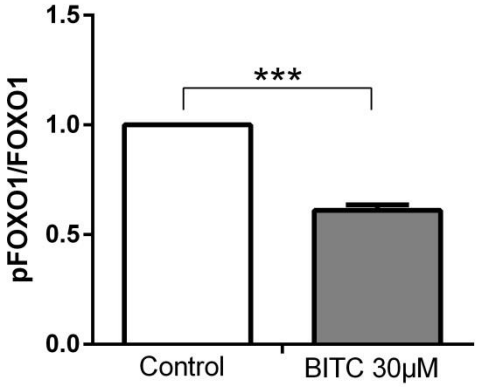


Figure 28. FOXO1 phosphorylation upon BITC treatment for 24h. Immunodetections with were made on HepG2 cells incubated with 30µM BITC for 24h. Cells were lysed in the presence of phosphatase- and protease-inhibitors, ultrasonicated and in the supernatant after centrifugation the protein concentration was measured. Western immuno-blot was performed with antibodies against pFOXO1 (Phospho FOXO1) and Total FOXO1 used for normalization of pFOXO1. Data are shown as mean of 3 independent experiments + SEM (standard error of mean).The statistical significance level was set at $P<0.05$ (Student's *t* test). Significances are shown as *** P <0.001 vs control cells.

3.5.2 FOXO1 target proteins

The expression of FOXO1 target proteins was evaluated after incubation of HepG2 cells with BITC 30 µM for 24h (Figure 29). Figure 28A depicts the gluconeogenic enzymes. A significant reduction in PEPCK and G6Pase expression after treatment with BITC is shown, suggesting an effect of BITC on the levels of these proteins. The results are in agreement to those observed on the gene expression analyses detecting mRNA levels (Figure 16A and B), in which a down-regulation was also found. After 24h stimulation, BITC induced a significant increase in GADD45 and a

non-significant reduction in the expression of both, p21 and FASN (Fig. 29B and 29C). The effect of BITC on GADD45 protein agrees with previous results obtained in the gene expression analysis based on mRNA detection (Figure 20 F and H).

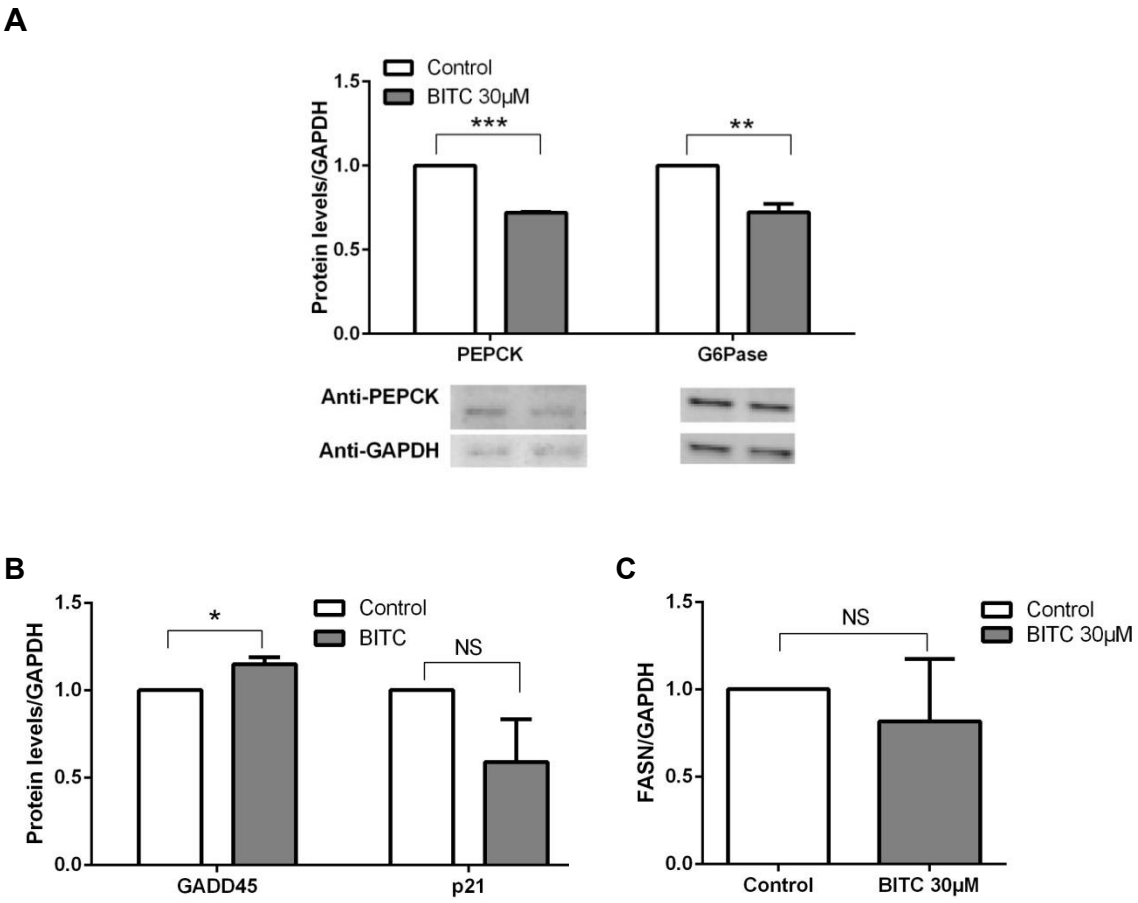


Figure 29. HepG2 cells Western-blot analyses for evaluating protein levels of FOXO1 targets. For immunodetections HepG2 cells incubated with 30 μM BITC for 24h. Cells were lysed in the presence of phosphatase- and protease-inhibitors, ultrasonicated and in the supernatant after centrifugation the protein concentration was measured. Western immuno-blots were performed with antibodies anti-PEPCK, anti-G6Pase, anti-GADD45, anti-p21 or anti-FASN. Results are normalized with respect to total GAPDH. Data are shown as mean of 3 independent experiments + SEM (standard error of mean). The statistical significance level was set at $p<0.05$ (Student's *t* test). Significances are shown as $*p<0.05$, $**p<0.01$, $***p<0.001$ vs control cells.

3.6. Effect of BITC on AKT intracellular signaling

In order to clarify the effects of BITC on the AKT pathway, we evaluated the phosphorylation of AKT (Thr308), AKT (Ser473), PTEN (Ser380), PDK1 (Ser421), AMPKα (Thr172), PRAS40 (Thr246), mTOR (Ser2481), GSK-3α (Ser21), GSK-3β (Ser9), p70S6K (Thr389), p70S6K (Thr421/ser424), BAD (Ser112), RSK1 (Ser380), ERK1/2 (Thr202/Tyr204), 4E-BP1 (Thr37/46) and SAPK/JNK (Thr183/Tyr185). HepG2 cells were treated with BITC 30μM, insulin 100nM and insulin+BITC for 30 minutes. The results are shown in Figures 30-32.

3.6.1 AKT pathway

AKT is activated by phosphorylation at two distinct sites: Thr308 by the plasma membrane residing kinase PDK1, and Ser473 by the mTORC2 complex [18]. BITC induced a non-significant reduction in the phosphorylation of Thr308 and a significant decrease in Ser473 (Fig. 30A and B). As expected, incubation with insulin increased AKT phosphorylation, which was significantly reversed by BITC for Ser473, but not for Thr308 when incubated in combination with insulin. In addition, no clear effect of BITC on PTEN and PDK phosphorylation was observed (Data not shown). All together, these results show that 30μM BITC affects significantly only AKT phosphorylation at Ser473 and is able to reduce the phosphorylation in the presence of insulin as well.

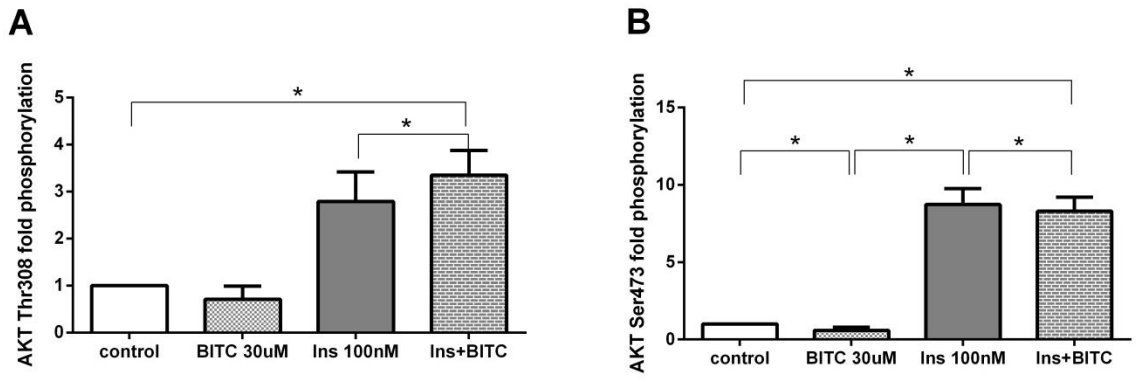
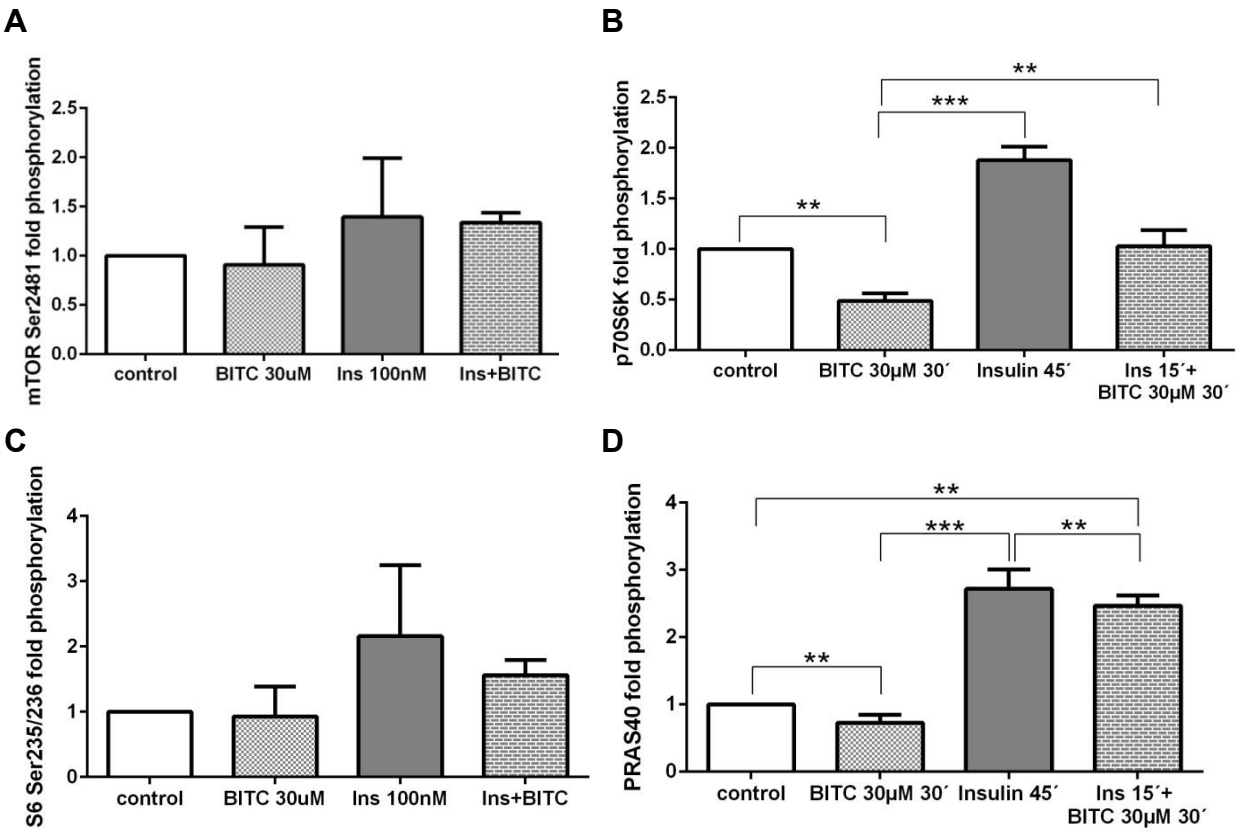


Figure 30. AKT phosphorylation upon BITC and insulin treatment (PathScan Signaling array analysis). HepG2 cells were incubated with BITC 30μM, insulin 100nM and insulin+BITC for 30 minutes. PathScan Signaling Array Kit with a fluorescent readout was used for the simultaneous detection of the phosphorylated AKT (Thr308) and AKT (Ser473). Data are shown as mean of fold phosphoralation normalized to untreated control + SEM from 3 independent experiments. One way ANOVA (posthoc Games-Howell for multiple comparisons) was used for the analysis. Significances are shown as *P <0.05 vs control cells.

3.6.2 mTOR pathway

The mammalian target of rapamycin (mTOR) signaling pathway senses and integrates a variety of environmental cues for regulating organismal growth and homeostasis [130]. The auto-phosphorylation of mTOR at Ser2481 correlates with the levels of its activation, which activates the downstream factor p70 S6 kinase, which in turn activates protein synthesis [6]. Based on this previous evidence, a faint reduction in mTOR (Ser2481) phosphorylation and a significant reduction of the signaling molecules p70 S6K (Thr389), S6 Ribosomal Protein (Ser235/236) and PRAS40 (Thr246) phosphorylation upon BITC treatment was observed, the results are presented in figure 31.



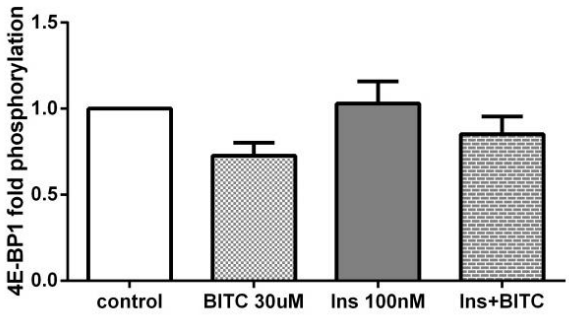


Figure 31. mTORC1 signaling pathway phosphorylation (PathScan Signaling array analysis). HepG2 cells were incubated with BITC 30μM, insulin 100nM and insulin+BITC for 30 minutes. PathScan Signaling Array Kit with a fluorescent readout was used for the simultaneous detection of the phosphorylated molecules mTOR (Ser2481), p70S6K (Thr389), S6 Ribosomal Protein (Ser235/236), PRAS40 (Thr246) and 4E-BP1 (Thr37/46). Data are shown as fold phosphorylation versus unstimulated controls of 3 independent experiments + SEM. One way ANOVA (posthoc Bonferroni or Dunnett T3 for multiple comparisons) was used for the analysis. Significances are shown as *P <0.05, **P <0.01, *** P <0.001 vs control cells.

mTOR phosphorylation was not significantly increased after the treatment with insulin (Fig. 31A) as expected, and although it was not significantly modified by the BITC treatment its downstream factor p70 S6 kinase was significantly reduced 0.5 fold (Fig. 31B) and was able to antagonize the effect of insulin (2-fold induction by insulin reversed to basal phosphorylation) when the cells were incubated with the combination of BITC and insulin (Fig. 31B). A non-significant decreasing trend in the other mTORC1 downstream factor, 4E-BP1, upon BITC treatment was also observed. The p70S6 kinase-downstream factor S6 was slightly reduced after BITC treatment. Incubation together with insulin reversed the phosphorylation reached with insulin alone, although without significance (Fig. 31C).

PRAS40 is a component and substrate of mTORC1 whose phosphorylation by AKT and by mTORC1 itself results in dissociation of PRAS40 from mTORC1, relieving an inhibitory constraint on mTORC1 activity [7]. Figure 31D reveals that PRAS40 was significantly affected by BITC treatment. After 30 min stimulation its phosphorylation was reduced by 30% in cells treated with BITC alone, and by 25% in cells treated with BITC and insulin together.

Upon BITC stimulation a tendency towards an inhibition of AMPKα (Thr172) and to the induction of BAD (Ser112) was found (Data shown in supplements). No effect was found on PTEN (Ser380), PDK1 (Ser421), GSK-3a (Ser21), GSK-3b (Ser9) and RSK1 (Ser380).

Kinases ERK1 and ERK2 regulate multiple cellular functions. They are involved in a broad range of cellular processes such as proliferation, differentiation, and motility [131]. Although they are not components of the AKT signaling network, ERK1 and ERK2 kinases are central in the Ras/MAP kinase signaling module [131] and there is evidence that shows that ERK1/2 phosphorylates FOXO1 promoting its exclusion from the nucleus [17]. On the other hand, JNKs, are members of a larger group of serine/threonine (Ser/Thr) protein kinases, known as the MAPK family, which are activated following the sensing of internal stress events [132]. Under these circumstances, JNK activation may have important cellular consequences, such as FOXO1 phosphorylation and alterations in gene expression, cell death or altered cellular proliferation [23,24]. Figure 32 summarizes the experimental results of evaluating BITC- induced phosphorylation of these kinases.

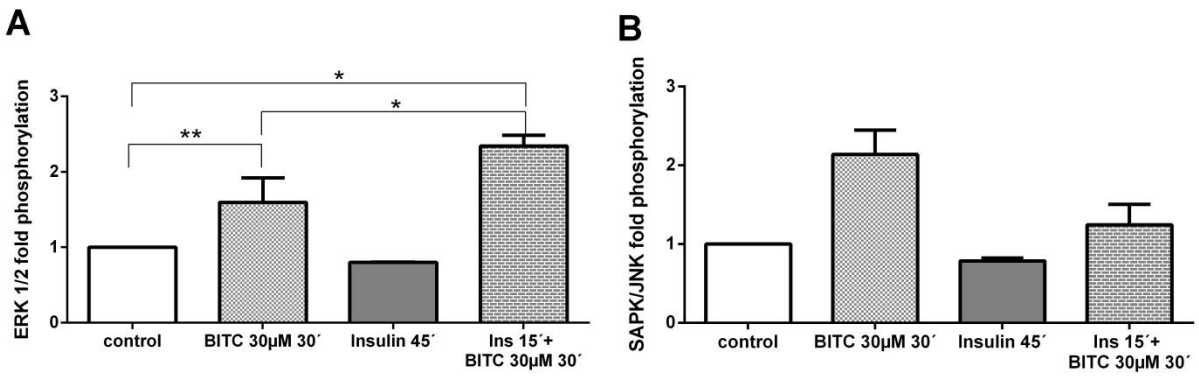


Figure 32. MAPKs ERK and JNK phosphorylation upon BITC and insulin treatment (PathScan Signaling array analysis). HepG2 cells were incubated with BITC 30μM, insulin 100nM and insulin+BITC for 30 minutes. PathScan Signaling Array Kit with a fluorescent readout was used for the simultaneous detection of the phosphorylated molecules ERK1/2(Thr202/Tyr204), SAPK/JNK (Thr183/Tyr185). Data are shown as fold phosphorylation versus unstimulated controls of 3 independent experiments + SEM. One way ANOVA (posthoc Bonferroni or Dunnett T3 for multiple comparisons) was used for the analysis. Significances are shown as *P <0.05 and ** P <0.01.

Figure 32A indicates that stimulation with BITC increased significantly ERK 1/2 phosphorylation 1.5 fold (P<0.05), as compared with control cells. BITC also induced by >2 fold the phosphorylation when cells where stimulated with combined insulin and BITC. The case of JNK is illustrated in Figure 32B. Although not significant, JNK phosphorylation was 2.13 fold increased after the stimulation with BITC. An induction was also found in cells treated with insulin and BITC together, in comparison with

cells treated only with insulin. The increase in MAPKs expression upon the treatment with BITC suggests an apparent stress.

3.7. Oxidative stress induction by BITC in U2-OS-FOXO1-GFP cells

In order to clarify the apparent oxidative stress observed upon BITC treatment, it was investigated whether the generation of intracellular oxidative stress is part of the mechanism by which BITC induces FOXO1 translocation and activation. Therefore stably transfected U2-OS-FOXO1-GFP cells were treated with BITC 30 µM for 2h and 24h following antioxidant N-acetyl-cysteine (NAC) 5 mM preincubation for 30´ to avoid cellular ROS accumulation. BITC induced FOXO1 translocation was reduced under antioxidant protection. The results are shown in Figure 33.

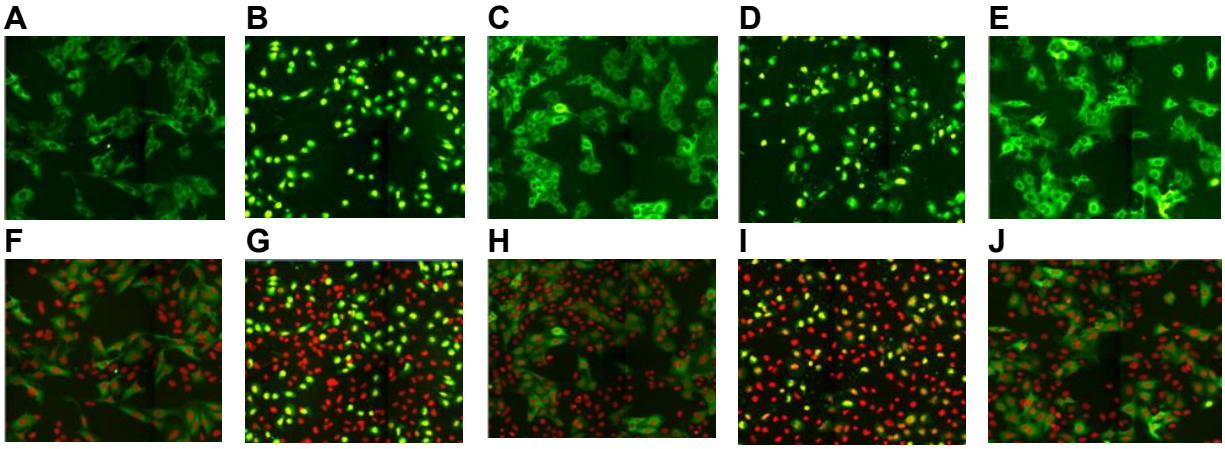


Figure 33. FOXO1 translocation in U2-OS-FOXO1-GFP cells after antioxidant protection with N-acetyl cysteine (NAC). NAC (5mM) was added to the medium 30 minutes prior to the BITC treatment in order to inhibit the reactive oxygen species production, and remained in the medium throughout the experiments. Images A and F were recorded on untreated control cells. Stimulation of stably transfected cells after 16h of starvation was done with BITC 30µM (B and G), BITC+NAC (C and H), insulin+BITC (D and I), and BITC+ insulin +NAC (E and J) for 2h. Cells were fixed and stained with DAPI for identification of nuclear areas by fluorescence microscopic detection, for cell segmentation and calculation of GFP-intensity ratios in nuclei and cytoplasm (Nuc/Cyt). Figures F-G show nuclear DAPI-staining (red) changing to orange-yellow in overlays with GFP.

Figures 33A and 33F (with DAPI overlay) clearly show that FOXO1 is located in the cytoplasm. Upon BITC treatment (Figs. 33B, 33G) or BITC+insulin (Figs. 33C, 33H) FOXO1 translocated to the nucleus, as previously shown in the first translocation analysis (Figure 11). By contrast, when BITC was incubated in combination with NAC (Figs. 33D, 33I) or insulin plus NAC (Fig. 33E, 33J), FOXO1 was located in the cytoplasm.

A time dependent FOXO1 translocation can be followed in the figures 34A and B. There, the nuclear and cytoplasmic GFP-fluorescence intensity ratios of FOXO1 after 2h (A) (B) stimulation with different agents are shown.

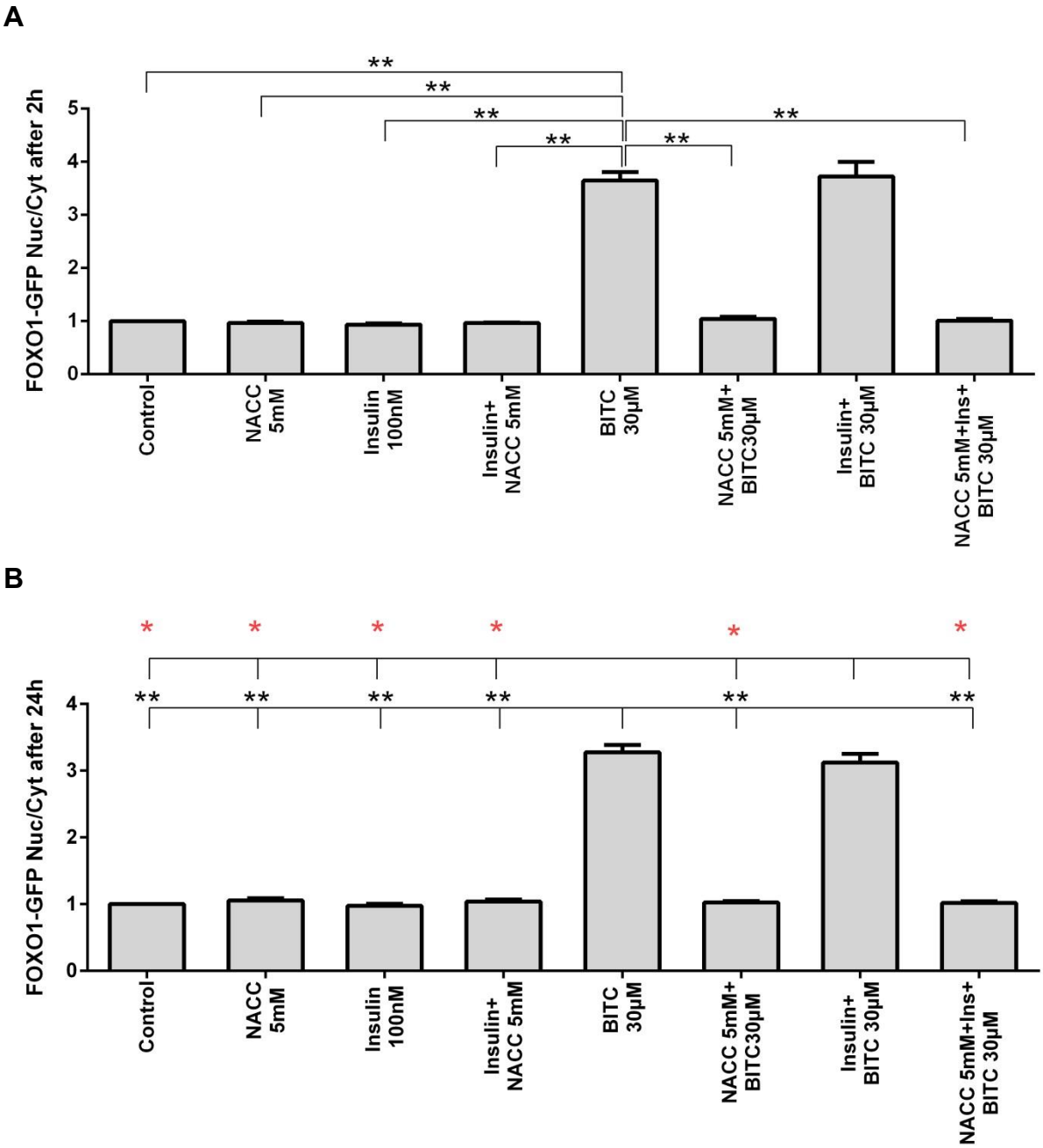


Figure 34. Time dependency analyses in U2-OS-FOXO1-GFP cells upon BITC and antioxidant protection. Stimulation with insulin 100nM, BITC 30µM, insulin+BITC, NAC 5 mM (N acetyl cysteine), BITC+NAC, insulin+NAC and BITC+insulin+NAC after A) 2h and B) 24h. Data are shown as mean of 4 independent experiments + SEM normalized to untreated control cells. One way ANOVA (posthoc Dunnett T3 for multiple comparisons) was used for the analysis. Significances are shown as *P <0.05, **P <0.01, *** P <0.001 vs BITC (black) or vs insulin (red).

The treatment with BITC or BITC + insulin induced a significant increase in FOXO1 translocation ($p<0.05$) after 2h and 24h stimulation (Figs. 34A and B), in agreement with previous analyses (Figure 10). However, when the cells were incubated with antioxidant protection by NAC, NAC+insulin, NAC+BITC and NAC+Iinsulin+BITC no effect on FOXO1 translocation was observed. These results show that BITC acts in HepG2 cells via oxidative stress, which can be inhibited by incubation with antioxidative NAC.

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Chapter 4

4. Discussion

4.1. BITC induces FoxO1 translocation in U2OS stably transfected with pEGFP-FoxO1

FOXO1 is a key factor in the insulin signaling pathway which is affected in type 2 diabetes [4]. In presence of insulin FOXO1 is negatively regulated by phosphorylation. This causes the sequestration of FOXO1 in the cytoplasm, thereby preventing FOXO1 from transactivating its target genes in the nucleus [16]. The mobility of this factor is mandatory for its activation or deactivation, which affects FOXO1 dependent transcription [15]. Starvation and substances such as resveratrol or H_2O_2 have been shown to induce FOXO1 nuclear accumulation and activation [133,134].

This work has shown that in U2-OS cells stably transfected with pGFP-FOXO1, BITC converted by myrosinase from benzyl glucosinolate extracted from *Nasturtium* seeds, is able to promote FOXO1 nuclear accumulation in a dose dependent manner preventing FOXO1 degradation in the cytoplasm (Fig. 11A-D). These results are supported by previous studies from Boreddy *et al*. They showed by immunofluorescence analysis in pancreatic cells, an increased FOXO1 nuclear accumulation and FOXO1 dependent transcription upon the stimulation with BITC 10μM [135].

The FOXO1 nuclear accumulation observed in this study could be the result of a reduction in its phosphorylation by AKT (the main pathway of FOXO nuclear exclusion and degradation) or by ERK [12]. FOXO1 phosphorylation by AKT at Thr-24, Ser-256, and Ser-319 [16], ERK [17], and CDK2 induces FOXO1 export from the nucleus to the cytoplasm and the activation of these kinases normally correlates with a reduction of nuclear FOXO1 [12]. Also the increase in FOXO1 translocation from cytoplasm into the nucleus could be produced by additional post-translational modifications that control the FOXO1 nuclear import increasing its transcriptional activity. For instance phosphorylation by JNK [22], p38 [17] and Cyclin dependent kinase 1 (CDK1) [25] have been described as inducers of FOXO1 nuclear accumulation in response to oxidative stress and other stimuli.

In the frame of this work it was also found that BITC was able to antagonize the effect of insulin, since incubation with BITC 30 µM or 50µM in combination with insulin 100 nM induced FOXO1 accumulation by 1.7- and 3-fold respectively (Fig. 13). These results also show a competition between nuclear import by BITC and export by insulin. Incubation with insulin increased the EC50 for FOXO1 translocation from 29 µM to 33 µM (Fig. 13B), compared to the stimulation with BITC 10, 20, 30 or 50 µM alone and in combination with insulin 100nM. After 50 µM BITC a plateau in FOXO1 import was observed, probably because higher concentrations of BITC were shown to be toxic in cell cultures reducing survival rates (see Fig. 14).

4.2. Cell viability

In the viability analysis (Fig. 14), HepG2 cells exposed to 1-50 µM BITC or benzyl glucosinolate (without the pre-incubation with myrosinase) for 24h, showed similar cell survival rates to the control. Just stimulation with highly concentrated 100 µM BITC (but not with 100 µM benzyl glucosinolate) was toxic for the cells. This is probably due to conversion of benzyl glucosinolate to the isothiocyanate form by myrosinase, which is associated to the production of reactive oxygen species ROS and cell death [106].

These findings apparently differ from previous studies that have reported high levels of cytoxicity after incubation with other kinds of isothiocyanates such as sulphoraphane, PEITC and BITC [136,137]. Jakubikova *et al.* showed in primary myeloma and tumor cells, a significant reduction in the cell vitality after the stimulation with sulphoraphane and PEITC for 24h at concentrations from 1.6, 12.5, 25 to 50 µM [137]. Borges A. *et al.* have found a drastic reduction in cell viability in mouse lung fibroblasts after its exposition for 72h to 50, 100, 500 and 1000µg/ml BITC [136]. The reason for the discrepancy with those reports could lie in the variations in the expression of enzymes involved in the detoxification of ITCs, which is different between the cells and higher in liver cells [127] and also the different type of isothiocyanate used, the range of concentrations and time of exposition to the substance.

4.3. BITC modulates gene expression of FOXO1 target genes

4.3.1 FOXO1 interacting transcription factors: SIRT1

FOXO transcription factors regulate positively and negatively the gene expression [30]. They bind to DNA target sites, stimulate transcription of target genes, interact with and modulate the function of other transcription factors and co-activator proteins important for the regulation of gene expression in the liver [9,30]. The interaction of FOXO1 with other proteins such as AKT and SIRT1 are necessary for FOXO1 nuclear exclusion or accumulation, respectively [138]. The FOXO1 deacetylation by SIRT1, is necessary for the transcriptional activity [16].

In the present study it is shown that BITC induces expression of SIRT1 mRNA in HepG2 cells. The significant up-regulation is dose dependent. Up to the date, no study had evaluated the effect of isothiocyanates on SIRT1 gene expression. The induction of SIRT1 gene expression in this study could be responsible for the FOXO1 nuclear accumulation together with nuclear SIRT1, an important factor for FOXO1 deacetylation, which in most of the studies induces the transcription of FOXO1 target genes [133]. In this regard, it should be emphasized that the net outcome of its modification is likely to be rather complex as FOXO1 can be both an activator [19] and a suppressor of gene expression [133].

FOXO1 was down regulated upon BITC treatment. Regarding to these result one might speculate that i) because FOXO1-dependent transcriptional activity depends on its protein phosphorylation and acetylation, the down-regulation of its mRNA level induced by BITC might not be relevant to modulate the gene expression modulation of FOXO1 target genes or ii) this down-regulation shows that perhaps there is an additional factor modulated by BITC responsible for the transcriptional activity observed in this study.

FOXO proteins have been involved in the regulation of gene expression of genes related to glucose metabolism [30,46], cell cycle arrest [31], DNA damage repair, lipid metabolism [30] and antioxidant response [56]. Previous studies provide evidence that in the absence of insulin stimulation or in the fasting state, FOXO1 induces glucose production through the gene expression of G6ase and PEPCK [30], reduces lipogenesis via inhibition of ACC and FASN [63], increases oxidative stress resistance mediated by the induction of catalase [59] and SOD [139], modulates the

cell cycle by up-regulation of CCNG2 [95] and promotes cell cycle arrest and DNA damage repair by induction of p27/KIP1 [135], p21/CIP [135,140] and GADD45 respectively [27].

4.3.2 Gluconeogenic enzymes

Although FOXO1 activators have been described as inducers of gluconeogenesis [46,133,141] it is demonstrated in this work that the activation of FOXO1 by BITC promotes a dose dependent down-regulation of gluconeogenesis, as confirmed by the reduction in PEPCK and G6Pase gene and protein expression (Fig. 16A and B). BITC reduced PEPCK mRNA within the first 6 hours of stimulation (early phase of gluconeogenesis), while G6Pase was down-regulated from 6 hours to 24 hours (late phase of gluconeogenesis). These findings reveal for the first time BITC as an inhibitor of gluconeogenesis. This could be considered as a positive factor in T2D prevention, since increased hepatic glucose production is a major feature of the disease [142]. This statement is supported by Bahadoran Z. *et al.*, who found a significant reduction in fasting glucose and insulin resistance after the treatment of type 2 diabetic patients with 5 and 10 g/d broccoli sprouts powder for 4 weeks [3]. The observed reduction in PEPCK and G6Pase in the present analyses could be attributed either to FOXO1 acting as inhibitor of gluconeogenesis upon BITC stimulus or BITC modulating gluconeogenic enzymes gene expression in a FOXO1 independent manner.

Regarding the first possibility there is evidence, which shows that FOXO1 acts as transcriptional activator in some situations and as repressor in other ones [41,143]. Moreover, direct DNA binding is not a prerequisite for modulating the transcription of gene targets [2]. For instance, in the fasting state FOXO1 localizes in the nucleus and its interaction with HNF-4 factor induces G6Pase promoter and inhibits glucokinase promoter (GK), whereas in the fed state the phosphorylated form of FOXO1 induces dissociation from HNF-4 inhibiting G6Pase and promoting GK transcription [41]. FOXO1 is responsible for sensing the insulin signal translation in response to fasting/feeding, and when present in the nucleus it modulates positively or negatively additional factors which can result in opposite transcriptional outcomes [41,45].

With respect to the last possibility recent studies might support this hypothesis. Experiments made with liver-specific FoxO1 triple knockout (TLKO) mice, displayed an appropriate response to feeding and to insulin itself. In addition, both the expression of gluconeogenic genes and the hepatic production of glucose were significantly suppressed by feeding or by insulin treatment [144].

These results suggested that the AKT-FOXO1 cascade might not be the only driver of the transition from fasting to feeding [145]. An induction of gluconeogenesis inhibitors mediated by BITC could be also considered. Transcriptional inhibitors are also involved in the regulation of hepatic gluconeogenesis. Orphan nuclear receptors DAX-1/ SHP and TCF7L2 are well documented [45,52]. In the normal physiological conditions DAX-1 blocks the association of HNF4 and PGC-1α on the gluconeogenic promoters of G6Pase and PEPCK, and TCF7L2 leads to a reduced binding of CREB and FOXO1 over the gluconeogenic promoters [45].

FOXO1 transcriptional activity has been related to the late phase of gluconeogenesis [35,42], were it interacts with other transcription factors as PGC-1α, ERRγ and HNF4 inducing G6Pase and PEPCK gene expression [39,64]. In the current study the BITC dependent down-regulation in PEPCK and G6Pase was observed within the first 6h and up to 24h, which suggests that additional factors different from FOXO1 could be involved in the gluconeogenic response to BITC.

4.3.3 Antioxidant and detoxification enzymes

The progression and complications of diabetes are linked to oxidative stress [2] through the glucose auto-oxidation, overproduction of ROS by mitochondria, non-enzymatic glycation, and the polyol pathway [146]. FOXO transcription factors are critical regulators of cell fate and play a major role in the oxidative stress responses modulating antioxidant and detoxification enzymes in order to protect the cells from ROS [2,56,139]. Owing antioxidant enzymes CAT and SOD and detoxification proteins NQO1, GST and GPX-2 have an established role in the oxidative stress resistance and have been linked with FOXO1 activity as well [59]. Therefore, the potential modulatory effect of BITC on their gene expression was evaluated in the frame of this work.

CAT has been described as a FOXO1 target gene, which is induced after FOXO1 activation. In this study BITC reduced significantly CAT gene expression in a dose dependent manner after 16h incubation, suggesting that another unknown pathway affected by BITC, is eventually regulating CAT gene expression. This point needs further investigation.

According to the analyses done in the present study, BITC had a two-phase effect in SOD gene expression. It starts with a significant reduction (p=0.01) at low doses of BITC (1 μM and 5 μM), followed by a dose dependent up-regulation at higher concentrations (10-50 μM). The down-regulation in SOD could be comparable to that reported by Zhu Y. *et al.* who found a reduction of SOD activity upon the stimulation with BITC (2 μM and 5 μM) after 24h [147]. Up to the current date there are no reports evaluating the gene expression of SOD upon high concentrations of BITC. The results suggest that SOD is up regulated as a compensatory response to the oxidative stress conditions produced by BITC, as observed in preliminary studies [147,148].

The detoxification enzymes SRXN1, NQO1 and GPX-2 mRNA were up-regulated upon BITC stimulation. Similar results have been reported for the isothiocyanate sulforaphane, known for its NRF2 dependency in the induction of detoxification enzymes [149]. Sulforaphane promotes NRF2 nuclear import by the interaction with its cytoplasmic repressor kelch-like ECH-associated protein 1 (KEAP1). In the nucleus NRF2 heterodimerizes with small Maf (V-maf musculoaponeurotic fibrosarcoma oncogene homolog) proteins and binds to antioxidant response elements (AREs), inducing the expression of several cytoprotective genes [149]. These facts suggest that the effect of ITCs, probably BITC as well, on NRF2 modulation are at post-translational levels, which could explain the lack of effects on NRF2 gene expression upon BITC stimulus.

4.3.4 Lipid metabolism enzymes

Hyperlipidemia is one the features of T2D as well. There is evidence showing that in fasting conditions or in absence of insulin/IGF, FOXO1 activation leads to the inhibition of lipogenesis, reducing the gene expression of lipogenic genes [63]. In this study, despite the FOXO1 nuclear accumulation, non-significant increases in ACC

and FASN mRNAs were observed at high BITC concentrations. There is no information about the direct effect of BITC on ACC and FASN gene expression, although other ITCs have shown a reductive effect. Rodríguez-Cantú L. *et al.* found a down-regulation in FASN after feeding syrian hamsters with dietary-induced hypercholesterolemia with ITC glucoraphanine-rich and sulforaphane-rich broccoli sprouts extract [150].

4.3.5 Cell cycle arrest and DNA damage repair enzymes

FOXO1 up-regulates the gene expression of some of the responsible factors for cell cycle arrest such as p21CIP, GADD45 and CCNG2 in cancer cells [95] having a critical role in diabetes- enhanced apoptosis [2]. In the current study the same proteins were evaluated upon the stimulation at different BITC concentration. A dose dependent induction in p21 and GADD45 mRNAs was found, both with similar response profiles. The up-regulation in p21 was significant at 30 µM and for GADD45 with 20 µM. Both effects were observed at 2h and 20h, respectively. The EC50s for the BITC induction of both proteins were identical (EC50=12µM). The gene expression analysis for GADD45 was coherent with its up-regulation observed in the protein expression analyses (Fig. 28B). In the case of p21, Western blot analyses failed to give any clear effect.

The results are in agreement with those reported by various authors showing an increase in these proteins for BITC-treated tumor cells [135],[116] at similar concentrations [116], although none of these studies have been performed in the context of T2D. Interestingly, the experiments performed in this study showed a significant down-regulation of CCNG2 mRNA in contrast to the report from Martínez-Gac *et al.* who reported an induction of CCNG2 upon FOXO1 activation in NIH 3T3 cells [95]. Possibly other unknown pathways affected by BITC interfered with the modulation of CCNG2, an issue that needs further investigation.

4.3.6 Autophagy pathway

Autophagy is essential for maintaining cellular health. There is evidence showing that it is reduced in conditions of insulin resistance and hyperinsulinemia, conditions associated to the retention of misfolded large molecules and dysfunctional cellular organelles [72]. FOXO proteins modulate the gene expression of some factors involved in the autophagic pathway [151]. FoxO3 up-regulates the gene expression of autophagy factors such as LC3, Gabarapl-1, Vps34, Ulk2 and Atg12 in mouse cells [69], and this transcriptional activity is FoxO1 dependent [73]. In addition, Zhao *et al.* showed that the interaction of the cytoplasmic form of FOXO1 with ATG7 is a critical step in the induction of autophagy [71]. This evidence suggests that FOXO1 has both transcription-dependent and -independent roles in autophagy [152].

In this study the effect of BITC on the gene expression of SQSTM1, PI3K3, ATG5, ATG12, BECLIN1, GABARAPL-1 and ULK1 was evaluated [66,72,73,153]. Upon BITC 30 μM for 24h we found a significant induction in BECLIN1, and an increasing trend for all other investigated genes except for ULK1, which was reduced. A previous study showed that under stress conditions, BITC induces autophagy in cancer cells [73]. Up to the present there is few information about the effect of ITCs on the different factors of the autophagic pathway, although some evidence shows that ULK1 is down-regulated upon the stimulation with BITC in human prostate cancer cell lines [154] and ATG5 is a critical factor in phenethyl isothiocyanate-mediated autophagic and apoptotic cell death [153]. All together these results suggest that BITC could be an inducer of autophagy probably mediated by the up-regulation in BECLIN1.

4.4. siRNA Analyses

For evaluating the mechanism underlying the gene expression modulation upon the stimulation with BITC in HepG2 cells, the transcription factors FOXO1 and NRF2 and modulators such as SIRT1 and AKT were knocked-down. The cells were stimulated with BITC and gene expression for all the previously mentioned enzymes was performed.

4.4.1 Gluconeogenic enzymes

PEPCK and G6Pase are considered as FOXO1 target genes. Many studies emphasize that FOXO1 is an important factor in their transcription [45]. Nevertheless, in the present study the effect of FOXO1 knock-down (45% after FOXO1 siRNA transfection) on PEPCK (18% reduction) upon FOXO1 siRNA transfection (Fig. 22 A and B) was not as strong as expected. The data indicate that under reduced levels of FOXO1 other factors may play a role in its modulation. The factors FOXO3, FOXO6, CREB and CREBH have been described as important PEPCK modulators [45,51,145,155]. Possibly under FOXO1 knock-down conditions other adaptive mechanisms modulate the PEPCK gene expression [47].

In this work it we recognized an increasing trend in PEPCK mRNA under AKT and NRF2 siRNA conditions. This observation supports the idea that FOXO1 stays in the nucleus modulating PEPCK gene expression when AKT is inhibited, also suggesting that NRF2 might play a role in the modulation of gluconeogenesis. Recently Uruno *et al.* showed a reduction in Pepck mRNA when Nrf2 was induced in diabetic mice [156]. In our analyses SIRT1 failed to give an effect on PEPCK gene expression, indicating that FOXO1 deacetylation by SIRT1 is not critical for PEPCK modulation.

In this study the effect of BITC on PEPCK gene expression is independent from FOXO1, NRF2 and SIRT1. AKT knock-down abolished the inhibitory effect of BITC on PEPCK expression (not statistically significantly in the siRNA analysis, but evident in the gene expression analysis), implying that BITC might be acting via AKT modulation.

Our siRNA analyses confirmed the role of FOXO1 in G6Pase gene expression reported previously [45]. Upon FOXO1 siRNA transfection G6Pase mRNA was reduced by 55%. AKT and SIRT1 failed to give any effect and NRF2 knock-down induced an increase in its gene expression, which may be explained by the same mechanism reported by Uruno *et al.* in the case of PEPCK [156].

According to these results the effect of BITC on G6Pase is partially FOXO1 dependent, and AKT and SIRT1 independent. Upon FOXO1 siRNA treatment the effect of BITC on G6Pase gene expression was reduced by 62% compared to control cells treated with BITC and 45% compared with the FOXO1 siRNA treated cells, showing that FOXO1 plays a role in the BITC-induced G6Pase gene expression

modulation. Upon NRF2-siRNA treatment the inhibitory effect of BITC on G6Pase was abolished, suggesting that NRF2 might be involved in G6Pase-BITC modulation. Aleksunes L. *et al.* showed an increase in G6Pase gene expression in Nrf2-null mice, indicating that Nrf2 influences the transcriptional regulation of hepatic metabolic genes [157].

Summarizing the BITC effect on gluconeogenic genes we conclude that it is independent from all the factors knocked-down except AKT for PEPCK, FOXO1 for G6Pase and NRF2 for both PEPCK and G6Pase. It is noteworthy that PEPCK and G6Pase are regulated both directly and indirectly via transcriptional and posttranscriptional mechanisms [45], which can compensate their functions in absence or reduction of a single factor, making it difficult to determine the relative contribution of individual transcription factors to these processes [47].

4.4.2 Antioxidant and detoxification enzymes

The siRNA analyses showed that CAT and SOD basal gene expression is independent from all the knocked-down factors (Fig 23 A and B). CAT is reduced and SOD induced upon BITC stimulation as was demonstrated in the gene expression analyses, although this effect is FOXO1, AKT, NRF2 and SIRT1 independent. These results put forward that additional factors are responsible for their modulation in the presence of BITC. For instance FOXO3 has been described as SOD and CAT inducer under oxidative stress conditions [135]. There is evidence that it can be modulated by BITC, inducing oxidative stress response resistance, therefore although not evaluated in our study, FOXO3 could be involved in the antioxidant response to BITC observed in this work [158].

The down-regulation in SFRXN, NQO1 and GPX-2 upon NRF2-siRNA in control cells was expected since these factors are known as NRF2 down stream genes (Fig 24 A, B and C). Experiments regarding the effects of BITC on SFRXN, NQO1 and GPX-2 showed that it is NRF2 dependent, as observed by the abolishment in the up-regulation of these genes after the NRF2 siRNA treatment. These findings confirm the role of ITCs on NRF2 transcriptional activity modulation. One can speculate that BITC could act on KEAP1, relieving its repression of NRF2 in cytoplasm and promoting its nuclear import and transcriptional activity.

An up-regulation in NQO1 after AKT knock-down was also found, suggesting that AKT might be involved in its gene expression modulation. Recently, Li *et al.* showed in HepG2 cells that the ATK pathway is inlvolved in NRF2 transcription modulation [159].

4.4.3 Cell cycle repair and DNA damage repair enzymes

The experimental results show that CCNG2, GADD45 and p21 mRNAs expression are independent from the investigated knocked-down factors (Fig 25 A-D). With respect to CCNG2 and p21, recent studies have shown that other transcription factors like FOXO3 or FOXO4 play a role in their transcriptional regulation [95,140]. Although FOXO1 is required for the full activation of the p21 promoter upon fasting, loss of FOXO1 does not completely suppress the fasting-induced activation of p21 expression in mice [140]. Although GADD45 has been described as a FOXO1 target protein, a previous reports has shown that it is also activated by FOXO4 [160], which could explain the observed BITC effects on GADD45 during the present study.

It is known that p27 is induced under stressful conditions [158]. Therefore the increase of p27 upon NRF2 siRNA treatment could be related to the increase in the oxidative stress produced by the down-regulation of antioxidant and detoxification NRF2 target genes [159]. The up-regulation of p27 upon SIRT1-siRNA treatment is unexpected since the deacetylation of FOXO transcription factors such as FOXO1 or FOXO3 by SIRT1 has been described as necessary for the induction of p27 [158].

4.4.4 Autophagy pathway

In this study BITC induced a significant up-regulation in BECLIN1 and a trend towards an increase in other factors of the autophagic pathway (Fig. 21), but this effect was FOXO1 and AKT independent (Fig. 26). Recent evidence shows that the cytoplasmic form of FOXO1 is necessary for the transcription and activation of some autophagy factors [73]. Zhou *et al.* showed that in the FOXO3-induced autophagy FOXO3 up-regulated PI3K-AKT1 pathway activity promoting phosphorylation and nuclear export of FOXO1 to the cytoplasm where it promoted autophagy. Since in this study a BITC-induced FOXO1 translocation and nuclear accumulation was

observed, it is possible that the increase in some autophagic factors by BITC is related to additional factors different from FOXO1 or FOXO3.

The induction of autophagy by BITC could antagonize the consequences of insulin resistance in T2D, since in the presence of insulin resistance and hyperinsulinemia autophagy is suppressed, which can contribute to the retention of misfolded large molecules and aged/dysfunctional cellular organelles associated with the development of many health problems [72].

4.5. Effect of BITC on protein expression

4.5.1 AKT and FOXO1

The state of phosphorylation of FOXO1 determines its subcellular localization. FOXO1 is negatively regulated by the PI3K–AKT signaling pathway in response to insulin by the phosphorylation in Thr 24 [16]. FOXO1 phosphorylation induces its export from the nucleus preventing FOXO1 target gene transcription and sequestration in the cytoplasm where it is degraded. In this study, levels of phosphorylated FOXO1 (pFOXO1) were induced upon incubation with insulin, and reduced after the BITC stimulus (Fig 26 A), while FOXO1 protein levels remained constant. The up-regulation after insulin stimulation was expected, nevertheless its reduction upon BITC incubation was less than anticipated. Since fluorescence microscopic detection analysis showed that FOXO1 protein was located in the cytoplasm and migrated into the nucleus upon BITC stimulation, a reduced level of pFOXO1 was expected after BITC stimulus, because phosphorylation is considered the main, although not the only [22,161], mechanism for FOXO1 export and sequestration in the cytoplasm [16].

The faint reduction in pFOXO1 by BITC (100 μM) found in this study might be related to the cell toxicity produced by high concentrations of BITC, observed in the viability assays (Fig. 14), or with additional mechanisms for the FOXO1 nuclear import, such as phosphorylation by other proteins at different amino acids not evaluated in this study.

To complement the previous findings, the effect of BITC on pFOXO1 levels was evaluated stimulating the cells with BITC 30μM for 24h (Fig. 27), the same

concentration used for the gene expression analyses, and a significant reduction in pFOXO1 was observed. In this regard it is important to underline that the incubation period of 24h is remarkably longer than the 30min used in the first experiments which could make these analyses not comparable to each other.

The levels of pAKT Ser473 (important for AKT inactivation) [18], but not total AKT, showed a decreasing trend upon BITC stimulus in combination with insulin. In the same way Path Scan analyses showed that BITC 30 μM reduced pAKT in a significant manner when the data were analysed by one way ANOVA with the post hoc test Games-Howell, but not with Dunnett, which is more strict. Also BITC in combination with insulin was able to reduce the phosphorylation induced by insulin alone.

Most of the studies [79], but not all [154] evaluating the effect of BITC on AKT phosphorylation show a reduction in pAKT. In this study even when BITC induced FOXO1 translocation only a faint reduction in AKT phosphorylation was observed, which might suggest that there is an additional mechanism regulating FOXO1 mobility. Most of the evidence regarding the effect of BITC on the AKT pathway come from β-cells and breast cancer cells which might explain the differences found in the AKT modulation as well.

4.5.2 mTOR pathway

Path Scan analyses were used to address additional mechanisms, which could be involved in the FOXO1 activation and the gene expression modulation of FOXO1 target genes. In this study, the phosphorylation levels of mTORC1 were evaluated.

Although phosphorylation levels of mTORC1 upon BITC stimulus were slightly reduced compared to the control and the insulin stimulation, its downstream factor p70S6K [162] was significantly reduced and BITC was able to reverse the level of phosphorylation reached by insulin. A reducing trend in the phosphorylation levels of the S6 and 4EBP, mTORC1 downstream factors [74], was observed as well.

PRAS40 a component and substrate of mTORC1 [83], exerts an inhibitory effect on mTORC1 activity in an unphosphorylated form [75]. In response to insulin and growth factors it is phosphorylated by AKT in Thr246, which weakens the interaction with

mTORC1 and leads to mTOR driven anabolism, as well as energy storage and consumption [75]. The results of this study showed that BITC reduced PRAS40 phosphorylation and reversed the effect reached by insulin in a significant manner (p=0.001). These findings suggest that BITC exerts an inhibitory effect on the mTOR pathway possibly mediated by a reduction of PRAS40 phosphorylation which promotes autophagy and cell death. AKT is a PRAS40 inhibitor. Since in the current study AKT was slightly reduced it is not straightforward to conclude that inhibition of PRAS40 takes place through the inhibition of AKT. Possibly, additional factors different from AKT, may play a role in Thr246 PRAS40 phosphorylation and regulation. Thus, another study reported that PRAS40 phosphorylation in Thr246 might be modulated by AKT independent mechanisms [83]. These findings agree with previous studies revealing that BITC inhibits mTORC1 by a reduction of its phosphorylation [154], or alternatively, through a reduction in the phosphorylation of its down stream targets p70s6K and 4E-BP1 [148] in human prostate cancer cells [154] and breast cancer cells [148]. The inhibition of mTORC1 might be related to the autophagy induction by BITC and could have positive consequences in diabetic patients, in which the autophagy pathway is reduced as a consequence of the hyperinsulinemia [72,74].

4.5.3 ERK1/2 and JNK phosphorylation

The MAPKs, ERK and JNK, participate in the transduction of signals from the surface to the interior of the cell inducing the appropriate gene expression events as homeostatic response [132]. ERK and JNK are activated by stress stimuli [163],[132]. In this study, the phosphorylation of ERK1/2 and JNK (not significant) was increased upon BITC incubation. No change was observed in response to insulin, however the combined incubation with BITC and insulin resulted in an increased effect. There is evidence showing that MAPKs such as JNK and ERK are responsible for FOXO1 modulation [17],[22],[24]. JNK induces FOXO1 nuclear import enhancing the glucose production in hepatitis C virus-infected cells [22] or enhancing angiogenesis in fibroblasts [17]. ERK in contrast to JNK, promotes FOXO1 nuclear export [17], by phosphorylation in nine serine residues, different from those three residues that can be phosphorylated by AKT, which were not evaluated in this study [164]. However, the FOXO1 translocation and nuclear accumulation observed in the current work

suggests that the possible stimulus of ERK on FOXO1 phosphorylation was not sufficient to inhibit FOXO1 mobility and transcriptional activity. We can speculate that the increase in ERK phosphorylation could be linked to the inhibition in gluconeogenesis found in this study. Previous studies have reported that activation of the MAPK/ERK pathway can also inhibit expression of PEPCK and G6Pase genes, most likely through a mechanism independent of insulin [164].

The induction of these MAPKs has been related as well to the NRF2 phosphorylation by different stimuli, which induces gene expression of phase II metabolizing enzymes [132]. The inducer effect of isothiocyanates such as PEITC or sulforaphane on MAPKs has been demonstrated previously in the cancer prevention in other studies [132].

4.6. Oxidative stress induction by BITC in U2-OS-FOXO1-GFP cells

In this study we observed that the oxidative stress produced by BITC induces FOXO1 translocation and activation. The translocation from cytoplasm into the nucleus was completely inhibited in those cells stimulated with the antioxidant N-acetyl cysteine. This finding confirms the role of ITC in the induction of an oxidative stress response which displays several protective pathways in the cell [119,121]. This hormetic behavior of ITCs and BITC has been largely explored in cancer cells [113,120,165].

4.7. Conclusions and Perspectives

Altogether, the present study provides insights about the effect of BITC on the PI3K/AKT/FOXO1 pathway. BITC induces oxidative stress and it is responsible for FOXO1 nuclear import and accumulation, which is inhibited by the presence of the antioxidant NAC. The oxidative stress conditions probably promote inhibition of AKT and FOXO1 phosphorylation. The FOXO1 nuclear import and accumulation allows FOXO1 to partially inhibit gluconeogenesis via reduction of G6Pase gene expression.

Most of the effects of BITC on FOXO1 target genes were FOXO1, AKT, NRF2 and SIRT1 independent. Although it could be concluded from these findings that the gene expression of factors described as FOXO1 target genes (PEPCK, CAT, SOD,

CCNG2, p21CIP, p27KIP, and GADD45) is modulated by additional factors in absence of FOXO1, BITC can modulate the gene expression of FOXO1 target genes by targetting, probably, other FOXO proteins, which can compensate their function under knock-down conditions.

The conclusion from these results is that BITC has an antidiabetic effect observed by the down-regulation of gluconeogenic enzymes; promotes antioxidant resistance expressed by the up-regulation in SOD and detoxification enzymes; modulates autophagy by induction of BECLIN1 and down-regulation of the mTORC1 pathway; and promotes cell cycle arrest and DNA damage repair by the up-regulation in p21CIP and GADD45. Except NRF2, none of the factors knocked-down were involved in BITC modulatory effects gene expression (Fig.36).

These results show that BITC mimics the fasting state, in which insulin stimuli are absent and FOXO proteins remain in the nucleus modulating gene expression of their target genes, with the advantage of down-regulating gluconeogenesis instead of increasing it. These effects suggest that BITC might be considered as a promising substance in the prevention or treatment of T2D. Therefore the factors behind of its modulatory effects merit further investigation.

As was shown in this document most of the studies evaluating the effects of BITC have been performed in the context of cancer. Since the final purpose of the search of bioactive compounds from plants for chronic disease prevention is their consumption by humans, the effects of BITC on T2D prevention should be considered in *in vivo* studies.

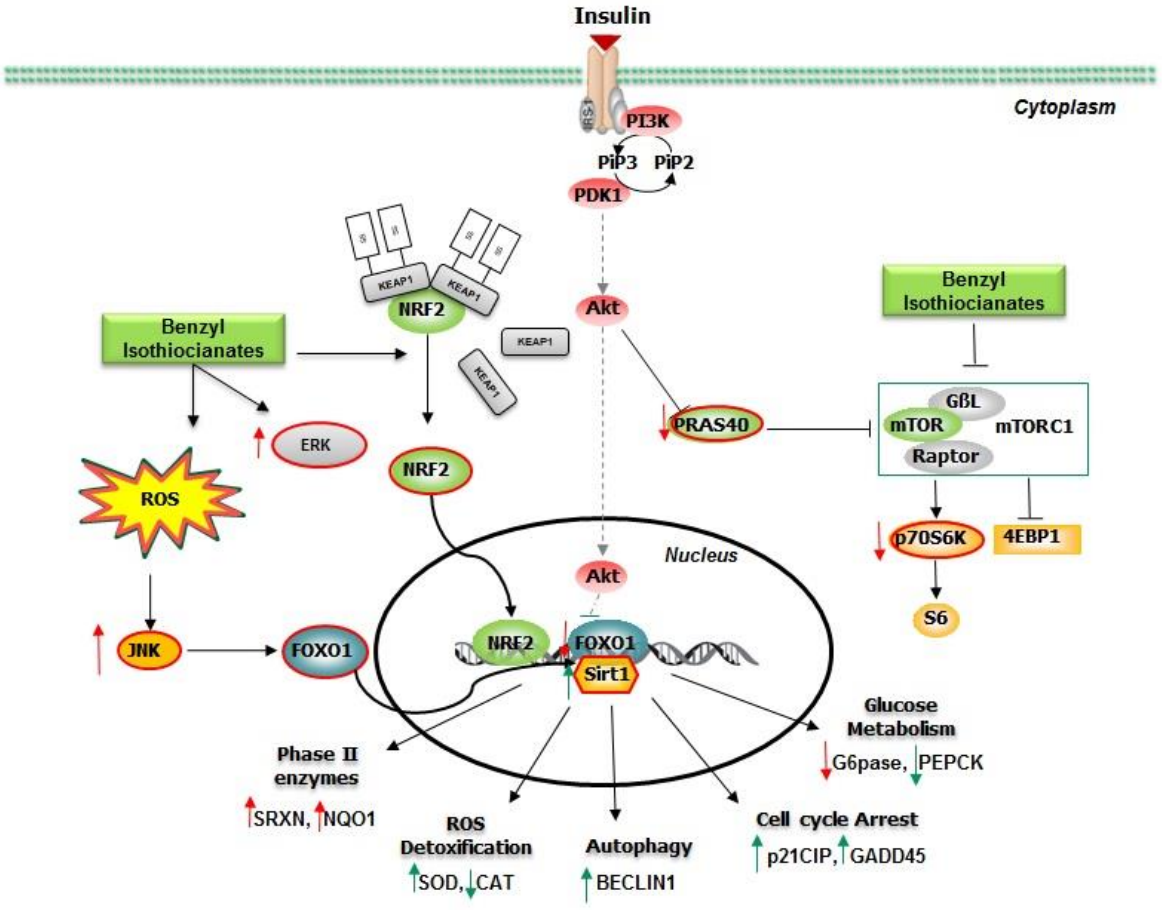

Figure 35. Effect of BITC on HepG2 cells. BITC acts in Hep G2 cells through oxidative stress, which probably induces ERK and JNK phosphorylation. FOXO1 translocates into the nucleus were it is activated modulating the gene expression of several pathways: It reduces gluconeFigure 35. Effect of BITC on HepG2 cellsogenesis by down-regulation of G6Pase and PEPCK; promotes cell cycle arrest by induction of p21CIP and GADD45; induces autophagy by up-regulation of BECLIN1; reduces some mTOR factors and stimulates oxidative stress resistance and detoxification inducing SOD, SRXN and NQO1. The presence of FOXO1 is necessary for BITC induced G6Pase down- reguation and NRF2 for the up-regulation in the detoxification enzymes SFRXN and NQO1.

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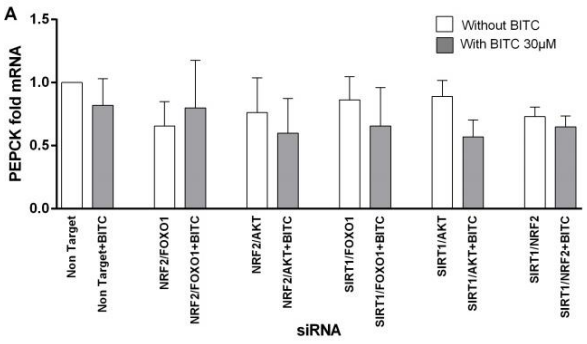
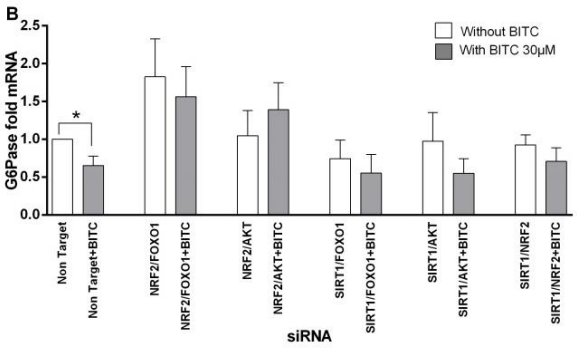
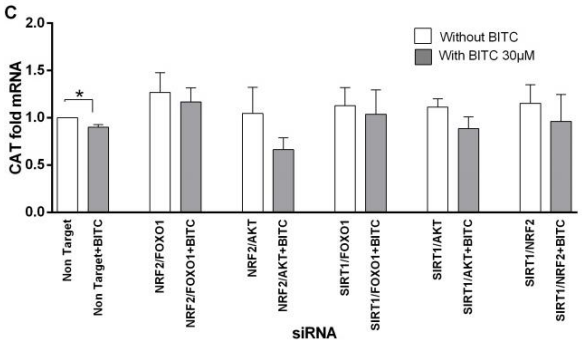
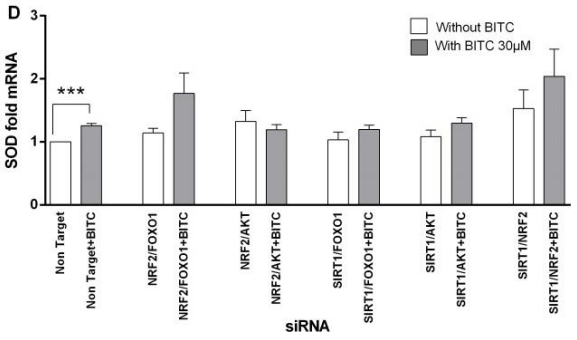
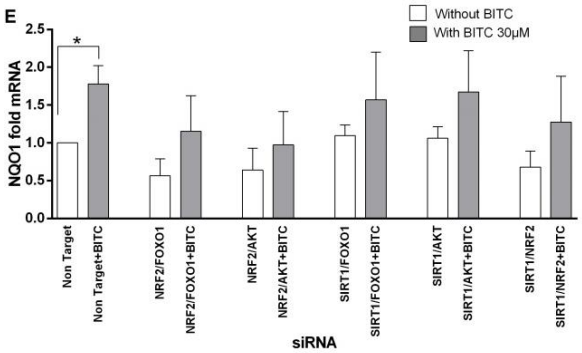
Supplements

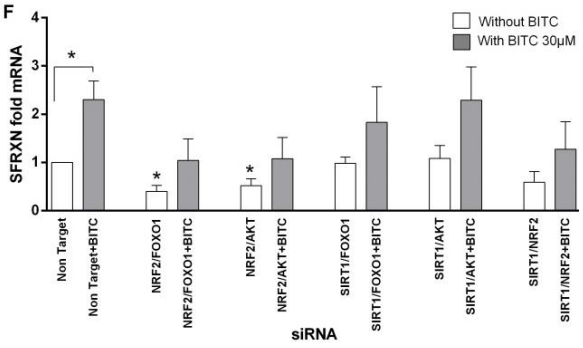


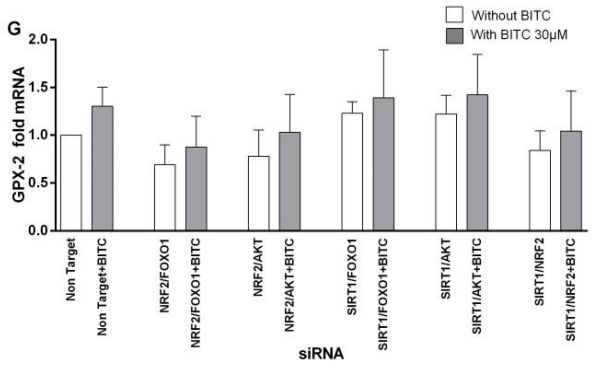








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NRF2/FOXO1+BITC
NRF2/AKT
NRF2/AKT+BITC
SIRT1/FOXO1
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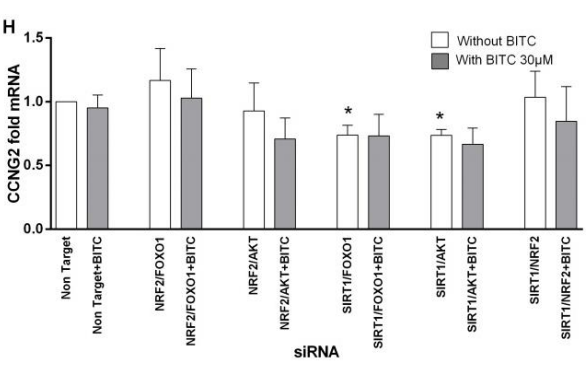
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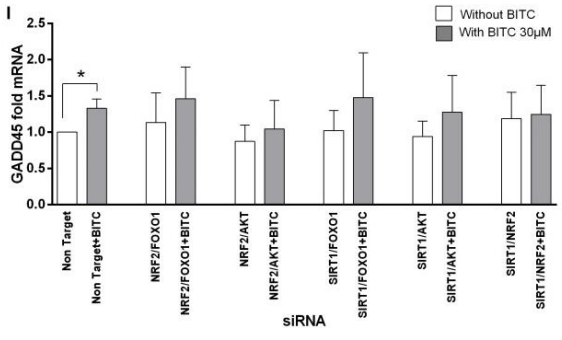
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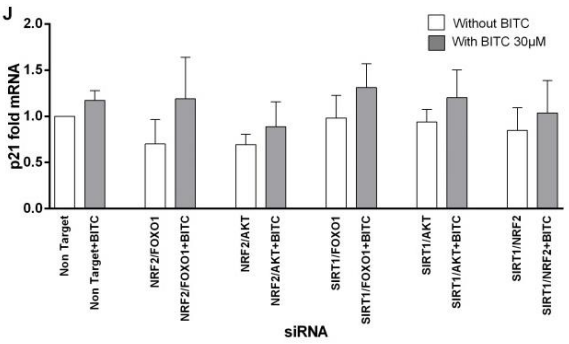
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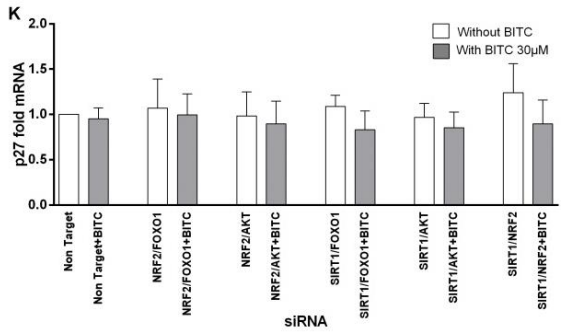
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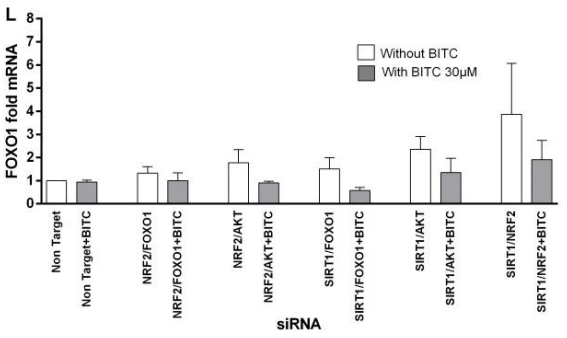
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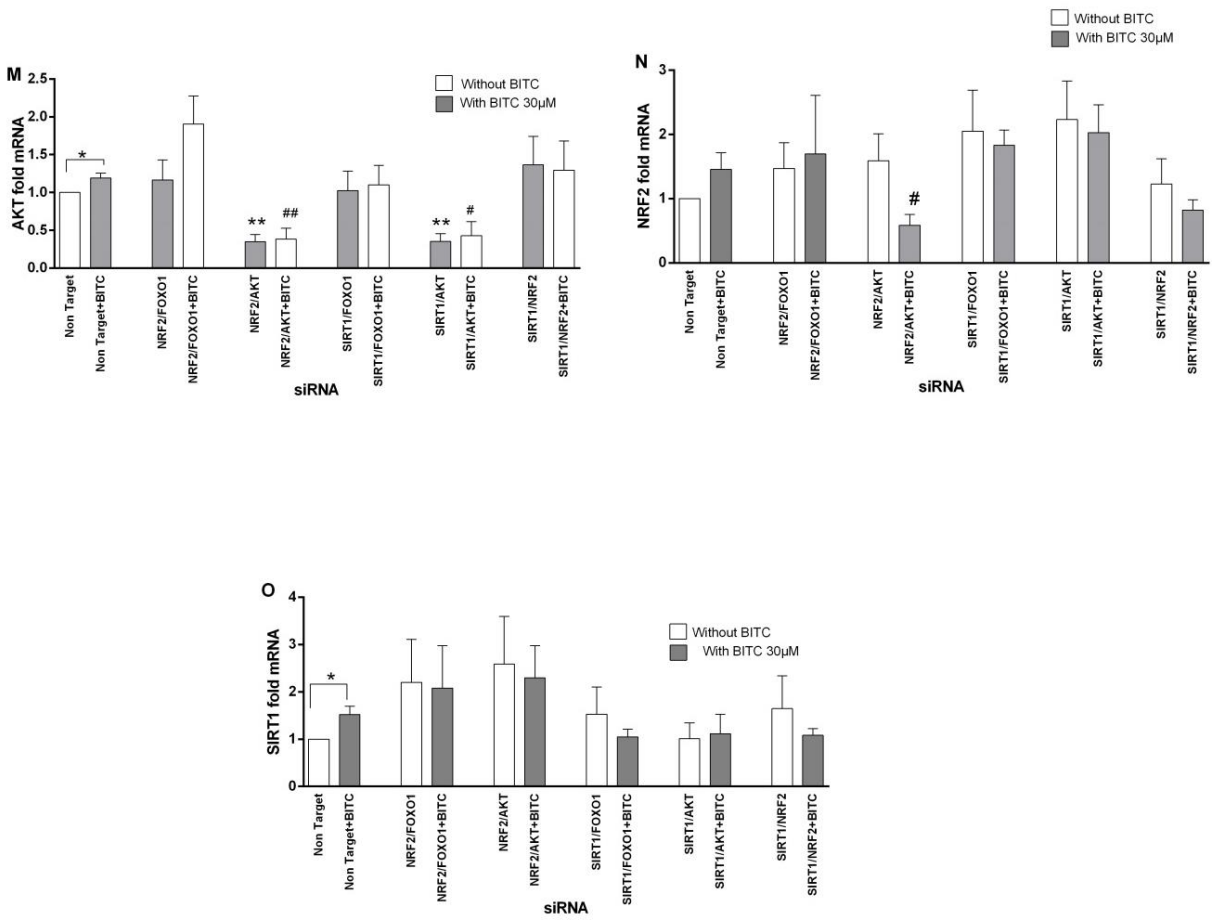

Figure 36. HepG2 cells siRNA analyses and gluconeogenic enzymes gene expression. A) PEPCK; B) G6Pase; C) CAT; D) SOD; E); NQO1; F) SFRXN; G) GPX-2; H) CCNG2; I) GADD45; J) p21; K) p27; L) FOXO1; M) AKT; N) NRF2; O) SIRT1 gene expression under FOXO1, AKT, NRF2 and SIRT1 siRNA combinations treatment. The statistical significance level was set at $p<0.05$ (Unpaired Student's *t* test). Significances are shown as $*p<0.05$ and $**p<0.01$ vs control cells treated with NT "*"or NT+BITC "#", normalized with GAPDH. Data shown as mean of fold mRNA + SEM from $\geq$ 4 independent experiments.

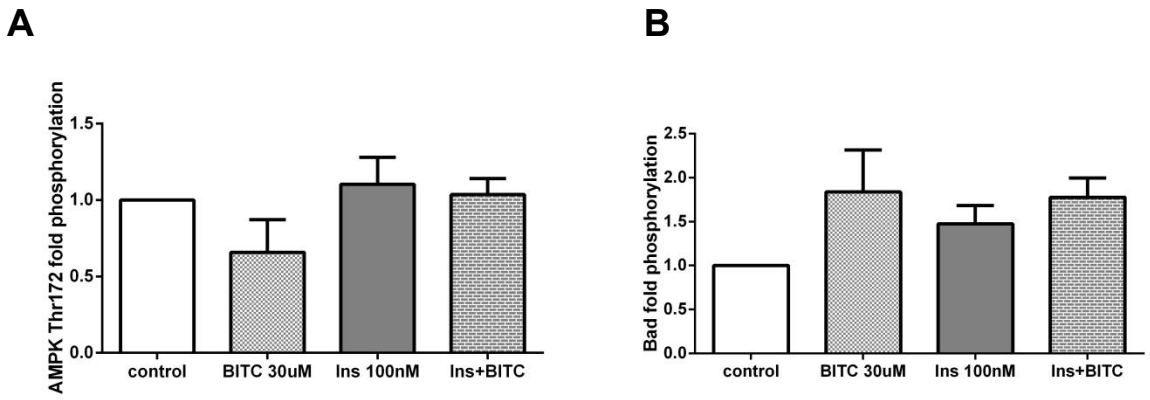

Figure 37. AMPK and BAD phosphorylation upon BITC and insulin treatment (PathScan Signaling array analysis). HepG2 cells were incubated with BITC 30μM, insulin 100nM and insulin+BITC for 30 minutes. PathScan Signaling Array Kit with a fluorescent readout was used for the simultaneous detection of the phosphorylated AMPK (Thr172) and BAD (Ser112). Data are shown as fold phosphorylation versus unstimulated controls of 3 independent experiments + SEM. One way ANOVA (posthoc Bonferroni or Dunnett T3 for multiple comparisons) was used for the analysis.

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Declaration to the Thesis

This work has done in the Department of Clinical Nutrition at the German Institute of Human Nutrition, Potsdam-Rehbrücke under the supervision of Prof. Dr. Andreas Pfeiffer. I assure that I completed this work myself with the indicated means. This work has not been submitted to any other university.

Potsdam 5.03.2014

Valentina Guzmán Pérez