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Effect of a Proprietary *Commiphora mukul* Gum Resin Extract and Medium-Chain Triglyceride Preparation (GU-MCT810) on hypoxia-inducible factor-1 pathway in HepG2 cell line

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Abstract

The heterodimeric transcription factor HIF-1 is responsible for the regulation of genes that facilitate adaptation and survival of cells under hypoxic conditions. HIF-1 gene expression is also associated with angiogenesis, glucose transport, nitric oxide synthase activity and cell proliferation through the regulation of hundreds of genes associated with HIF-1 pathway. GU-MCT810 is a nutraceutical ingredient complex that includes a *Commiphora mukul* (guggul) extract prepared by a supercritical CO_2-co-solvent extraction with ethanol and medium chain triglyceride (MCT) oil composed of C8 and C10 fatty acids. Since cancer cells use glycolytic pathway, 2-deoxyglucose (2-DG) has been reported to inhibit the glycolysis. We have investigated the anticancer potential of GU-MCT810 with and without 2-DG in HepG2 human hepatoma cell line. Even though GU-MCT810 and 2-DG are individually weakly cytotoxic, the combination is synergistic with combination index (CI) values of 0.21, 0.22 and 0.88 at IC_{50}, IC_{75} and IC_{90} levels, respectively. The combination also showed a synergistic inhibitory effect on ATP-synthesis in HepG2 cells. GU-MCT810 inhibits $CoCl_2$-induced HIF-1 expression significantly in a dose-dependent manner with complete inhibition at 50 µg/ml concentration. GU-MCT810 upregulates Bax and p21 genes and down regulates Bcl-2, BNIP3 and mutant p53 genes associated with apoptosis. It also down regulates proteins associated angiogenesis (VEGF, VEGF-R), cell proliferation (IGF-2), glucose transport (GLUT1) and adaptogenesis (HSP70 and HSP90). These results indicate that GU-MCT810 can be combined with 2-DG for inhibition of HIF-1 pathway genes which would be useful for elimination of refractory cancer cells present in the hypoxic region of human tumors.

Keywords: HIF-1, GU-MCT810, 2-deoxyglucose, synergism, combination index, gene expression, western blot hybridization

Introduction

Hypoxia-inducible factor-1 (HIF-1), a key heterodimeric transcription factor, is responsible for the regulation of genes that facilitate adaptation and survival of cells as well as the whole organism under conditions of change from normoxia (~21% O_2) to hypoxia (~1% O_2) [1,2]. HIF-1 can also interact with enzymes and other transcription factors to control vascularization and tissue growth. Since microenviroments surrounding tumors are extremely hypoxic, cell proliferation often is made possible by HIF-1 activation which leads to increased angiogenesis and, thus, an increased oxygen supply to the area [3-5]. Because of this role in hypoxia, HIF-1 plays a critical part in tumor growth and proliferation [6]. As the tumor develops and grows, a hypoxic environment is created because of the extreme energy demands of the numerous, rapidly dividing cells. Angiogenesis is often induced by cellular masses to meet the needs for increased oxygen, energy and blood supplies [3,7,8]. Concurrently, HIF-1 contributes to the shift to anaerobic glycolysis. The importance of this transcription factor in tumor cell survival is also reflected by the finding that the levels of HIF-1 in glioma cells increase proportionally with the grade of the tumor [9]. It is also reported that certain chemicals such as cobalt chloride, nickel chloride and desferrioxamine can act as hypoxia mimicking agents and these agents can be used for HIF-1 investigations [10]. In fact, cobalt chloride increases the stability of HIF-1 , and its effect on the survivability of cell under normoxic conditions [11].

The inhibition of HIF-1 pathway is a desirable objective for treatment of diseases like cancer. The biology of cancer growth and metastases provide multiple points where HIF-1 inhibition offers an opportunity for therapeutic intervention. HIF-1 has been found to regulate the shift within the tumor cells to anaerobic glycolysis for primary energy requirements, to activate vascular endothelial growth factor (VEGF) and angiogenesis. Consequently, down regulation of the HIF-1 complex may suppress cancer progression. Several small molecular inhibitors of HIF-1 pathway have been identified, and topotecan, a topoisomerase inhibitor, decreases cellular accumulation of HIF-1 as it alters the ribosome entry site on the HIF-1 mRNA molecule, preventing translation [12,13].

GU-MCT810 is a proprietary nutraceutical ingredient complex that includes a *Commiphora mukul* (guggul) extract prepared with and a supercritical CO_2-co-solvent extraction with ethanol and medium chain triglyceride (MCT) oil composed of C8 and C10 fatty acids. GU-MCT810 was shown to promote hypolidemic effects *in vitro* as it reduces low-density lipoprotein cholesterol and increases the high-density lipoprotein/low-density lipoprotein ratio. Additionally, GU-MCT810 inhibits adipocyte differentiation, increases AMPK phosphorylation and AMPK kinase activity and inhibits phosphorylated form of mTOR expression [14]. It also up regulates the expression of LXR, PPAR , BABP and SHP genes associated with the lipid metabolism. In this investigation we have studied the effect of GU-MCT810 on the expression of HIF-1 pathway-associated genes in human HepG2 cell line for its use in cancer therapy.

Materials and Methods

Reagents: GU-MCT810 is manufactured by Flavex Naturextrakte GmbH, Rehlingen, Germany. Its extraction, preparation and quality details have been described in our earlier publications [15,16]. Other reagents like 2-Deoxyglucose (2-DG) and $CoCl_2$ were purchased from Sigma Aldrich Chemical Co., St. Louis, MO.

Cell line: Human hepatoma cell line (HepG2) was grown in Eagle's minimum essential medium supplemented with 10% fetal bovine serum, 100 U/ml of streptomycin and100 µg/ml of penicillin in a 5% CO_2 humidified incubator maintained at 37 C.

Cytotoxicity assay: HepG2 cells were treated with increasing concentrations of GU-MCT810, 2-DG and their combination at 37 C for 72 h in a CO_2 incubator. The cytotoxicity of HepG2 cells against single agent as well as the combination with 2-DG was analyzed by MTT assay using the Cell proliferation kit I from Roche Biochemicals, IN [17].

Synergy analysis with 2-deoxyglucose

To determine the synergistic/additive/antagonistic effect between and GU-MCT810 and 2-DG, cytotoxicity data were analyzed further using CompuSyn software (ComboSyn, Inc. Paramus, NJ). The program is based on Chou and Talalay's [18] multiple drug effect equations and it defines synergism as more-than-expected additive effect and antagonism as a less-than-expected additive effect [18,19]. The combination index was calculated by the Chou-Talalay equations for multiple drug effects, which take into account both potency (inhibitory concentration values) and shape (slope, m) of dose-effect curve.

ATP analysis

HepG2 cells (5 x10^3 /100 µl/well) were incubated in a 96-well plate overnight in low glucose medium at 37 C in a CO_2 incubator. On the next day, cells were treated with increasing concentrations of GU-MCT810, 2-DG or GU-MCT810 +2-DG combination for 5 h. The plate was kept for 10 min at room temperature for equilibration and 100 µl of Cell Titer-Glo reagent (Promega Corporation, Madison, WI) was added into each wells. The plate was incubated again for 10 min at room temperature for stabilization of luminescent signal and 100 µl of sample was transferred to opaque-walled 96-well plates, the luminescence of which was read in a Veritas luminometer (Turner Biosystems, Inc., Sunnyvale, CA). The percentage of ATP inhibition in each sample was calculated based on untreated sample and plotted against drug concentrations.

HIF-1 assay

Log phase HepG2 cells (4x10^6/5 ml) were plated in 5 ml of EMEM and treated with 150 µM $CoCl_2$ with increasing concentrations of GU-MCT810 (0-100 µg/ml) at 37 C for 72 h in a CO_2 incubator along with an untreated control sample. Total cellular protein was extracted with 0.5 ml of Invitrogen protein extraction buffer (Invitrogen, Federick, CA) according to the manufacturer's instructions. The protein concentration was determined and cellular extract equivalent to 25 µg protein was analyzed for the expression of HIF-1 using the human HIF-1 ELISA kit (R&D systems, Minneapolis, MN) according to manufacturer's instructions. The cellular HIF-1 expression (pg/ml) was plotted against various treatment groups.

RNA extraction and RT-PCR

HepG2 cells (4x10^6) were plated in 5 ml of EMEM and treated with 150 mM $CoCl_2$ and increasing concentrations of GU-MCT810 (0-100 µg/ml) at 37 C for 72 h in a CO_2 incubator along with an untreated control sample. Total RNA was extracted from the cells and 5 µg RNA was reverse transcribed to synthesize cDNAs. The mRNA expression of genes associated with HIF-1 pathway was analyzed by reverse transcriptase-polymerase chain reaction using 0.4 µg of cDNA using gene-specific primers [14,15]. The expression of house-keeping genes, glyceraldehydes 3-phosphate dehydrogenase (GAPDH) and β-actin were also amplified as controls. The gene expression was quantified using gel pictures from three separate experiments by the UNSCAN-IT Gel software (Silk Scientific, Inc., Orem, UT).

Western blot hybridization

HepG2 cells (4 x10^6/5ml) were treated with 150 µM $CoCl_2$ and increasing concentrations of GU-MCT810 (0-50 µg/ml) for 72 h, and total cellular protein was extracted with 0.5 ml of Invitrogen protein extraction buffer (Invitrogen, Federick, CA) according to the manufacturer's instructions. The protein concentration was determined and 100 µg protein was separated on 10% sodium-dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The separated protein was blotted on a nitrocellulose filter, which was hybridized with antihuman monoclonal/polyclonal antibodies specific to each protein (Cell Signaling Technology, Beverly, MA; Life Technology corporation, Grant Island, NY; EMD Millipore, Billerica, MA) in a western blot procedure and detected using the alkaline phosphatase color detection kit (Bio-Rad laboratories,

Hercules, CA). The relative expression of proteins compared to untreated control sample was quantified using UNSCAN-IT Gel software (Silk Scientific Inc. Orem, UT) and the fold-level changes in protein expression were plotted against CA concentrations.

Cytotoxicity of GU-MCT810

GU-MCT810 appears to have very little cytotoxic effect against HepG2 cells *per se* (Figure. 1).

Results

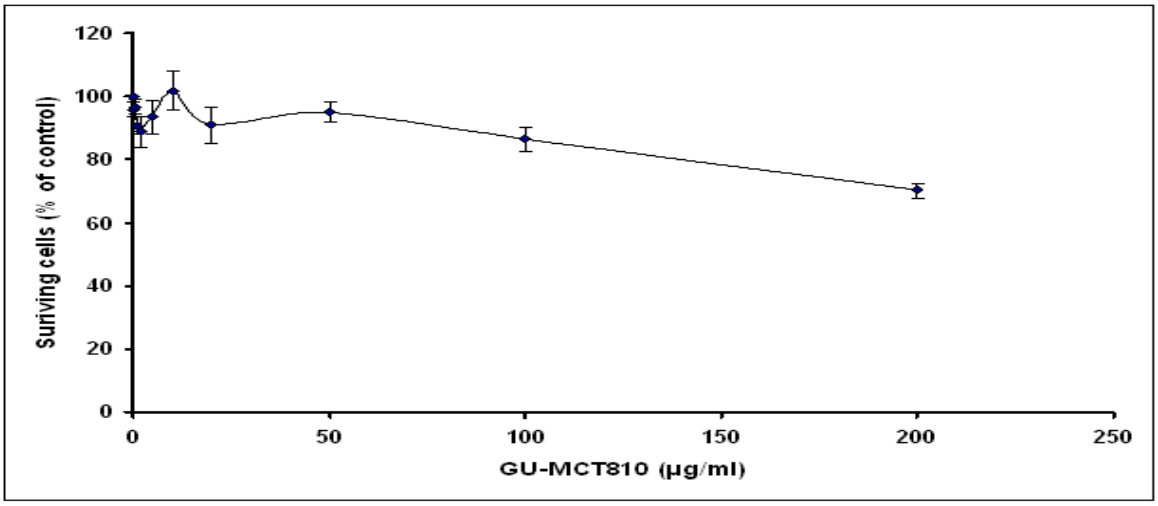


Figure. 1: Cytotoxicity of GU-MCT810 in HepG2 hepatoma cell line. The tumor cells were treated with increasing concentrations of GU-MCT810 for 72 h and cytotoxicity analyzed by MTT assay. The mean percentage of surviving cells (n=4) and standard deviation estimates were plotted against GU concentrations.

Synergy between GU-MCT810 and 2-deoxy glucose

The dose-effect curve, medium-effect curve, DRI (dose-reduction index) and isobologram of interaction between GU-MCT810 and 2-DG analyzed by CompuSyn software are presented in Figure. 2 and Table 1. Even though GU-MCT810 and 2-DG as individual agents were weakly cytotoxic, their combination showed significant cytotoxicity to U-87MG cells as indicated by dose-effect and medium-effect curves. The combination index (CI) values were 0.21, 0.22 and 0.88 at IC_{50}, IC_{75} and IC_{90} levels (all <1), respectively, indicating a strong synergistic effect between GU-MCT810 and 2-DG for cytotoxicity in HepG2 cells.

Table 1. Dose-effect relationships of GU-MCT810 (G), 2-deoxyglucose (2-DG) and G + 2-DG in cancer cell lines

Drug	Dm (µg/ml)	m	r	CI IC_{50}	CI IC_{75}	CI IC_{90}	DRI-2-DG IC_{50}	DRI-2-DG IC_{75}	DRI-2-DG IC_{90}
G	193.85	0.23	0.75						
2-DG	102.49	0.67	0.97						
G + 2-DG	28.38	0.66	0.88	0.21	0.22	0.88	4.91	5.07	5.22

Dm, Median-effect dose that produces 50% cell death; m, The shape parameter for dose-effect curve. The m value is the slope of the median-effect plot; r, The conformity parameter for goodness of fit. It is the linear correlation coefficient by the median-effect plot; CI, A quantitative measure of the degree of drug interaction in terms of synergism and antagonism for a given endpoint of the effect measurement. (Chou and Talalay, 1981);DRI, Dose-Reduction Index , A measure of how many folds the dose of each drug in a synergistic combination may be reduced at a given effect level when compared with the doses of each drug alone.

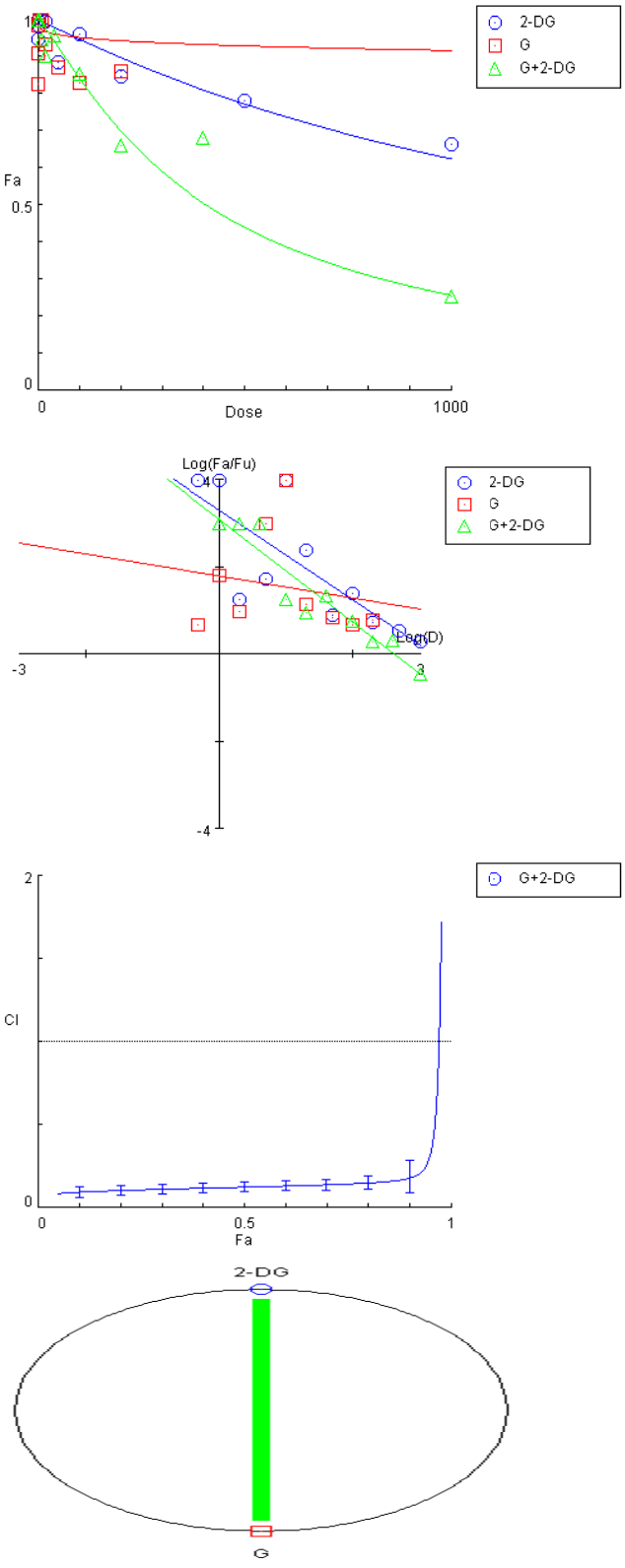


Figure. 2: CompuSyn analysis of cytotoxicity data for determining synergism/additiveness/antagonism between GU-MCT810 (G) and 2-deoxyglucose (2-DG) in HepG2 cell line. A, Dose-effect plot for 2-DG, G and G-2-DG; B, Medium-effect plot for 2-DG, G and G+2-DG; C, Isobologram showing the combination Index values (CI) below 1 indicating synergism between G+2-DG; D, Polygonogram indicating the synergistic effect between G and 2-DG (green color indicates synergism).

Inhibition of ATP synthesis

The effect of GU-MCT810, 2-DG and GU-MCT810 +2-DG combination on ATP synthesis is shown in Figure. 3. 2-DG (a glycolytic inhibitor) and GU-MCT810 have weak inhibitory effect on ATP synthesis up to the 200 µg/ml dose. However, when the two agents were combined in equal proportions, a significant but synergistic effect on ATP synthesis was noticed.

Inhibition of $CoCl_2$-induced HIF-1 -expression by GU-MCT810

HepG2 cells express very little HIF-1 expression and upon treatment with 150 µM $CoCl_2$, significant induction of HiF-1 is noticed with about 880 pg/ml of HIF-1 (Figure. 4). Cells treated with rapamycin (positive control) and $CoCl_2$ showed about 25% inhibitionof HIF-1 . In the HepG2 cells treated with $CoCl_2$ and increasing concentrations of GU-MCT810 for 24 h, a dose-dependent inhibition of HIF-1 was noticed. Treatment of cells with10 µg/ml GU-MCT810 has reduced the HIF-1 by 40% and complete inhibition at 50 µg/ml concentration.

mRNA expression

We have analyzed the mRNA expression of HIF-1 pathway genes that are associated with the cellular functions such as nitric oxide synthesis (iNOS and eNOS), glucose transport (GLUT1 and GLUT3), angiogenesis (VEGF, VEGF-R, MMP-2, TIMP1 and TIMP2), cell proliferation (IGF-2), adaptogenesis (HSP70, HSP90), and apoptosis (*Bax, Bcl-2, BNIP3, p21, p53, p16* and *c-myc*) along with two house keeping genes (GAPDH and β-actin). These results are presented in Figure. 5a & b and the quantified plots are given in Figure. 5c-e. GU-MCT810 treatment affects the expression of GAPDH which is grouped as one of HIF-1 pathway genes. The expression of β-actin, another housekeeping gene, is not affected by GU-MCT810 treatment. HIF-1 mRNA expression increased with $CoCl_2$ and GU-MCT810 treatment showed a significant inhibition of HIF-1 mRNA. HIF-1β also increased with $CoCl_2$ treatment and inhibited by 50 µg/ml level of GU-MCT810 as well as 0.02 µg/ml of rapamycin (Figure. 5a &c). Of the two glucose transporters analyzed, only GLUT3 mRNA was altered by GU-MCT810 and not GLUT1. It also altered the expression of two matrix metalloproteinase associated genes (TIMP2 and MMP-2) significantly and not TIMP1. Among two important angiogenesis-associated genes, VEGF-R mRNA expression was inhibited significantly by $CoCl_2$+ GU-MCT810 treatment. Among the two

nitric oxide synthase genes (NOS) studied, inducible form (iNOS) showed an increase with $CoCl_2$ treatment which got inhibited significantly with 50 µg/ml of GU-MCT810. On the other hand, endogenous NOS (eNOS) showed a non- significant decrease with $CoCl_2$ treatment which got reestablished with increasing doses of GU-MCT810.

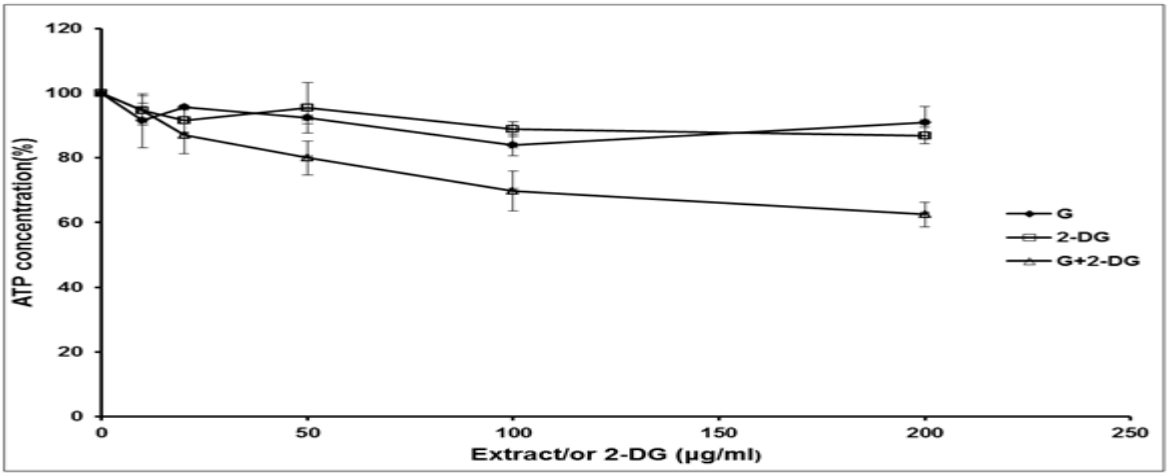


Figure. 3: ATP assay in HepG2 cells treated with GU-MCT810 (G), 2-deoxyglucose (2-DG) and G+2-DG. The inhibition of ATP with the combination of G+2-DG was significantly better than single agents.

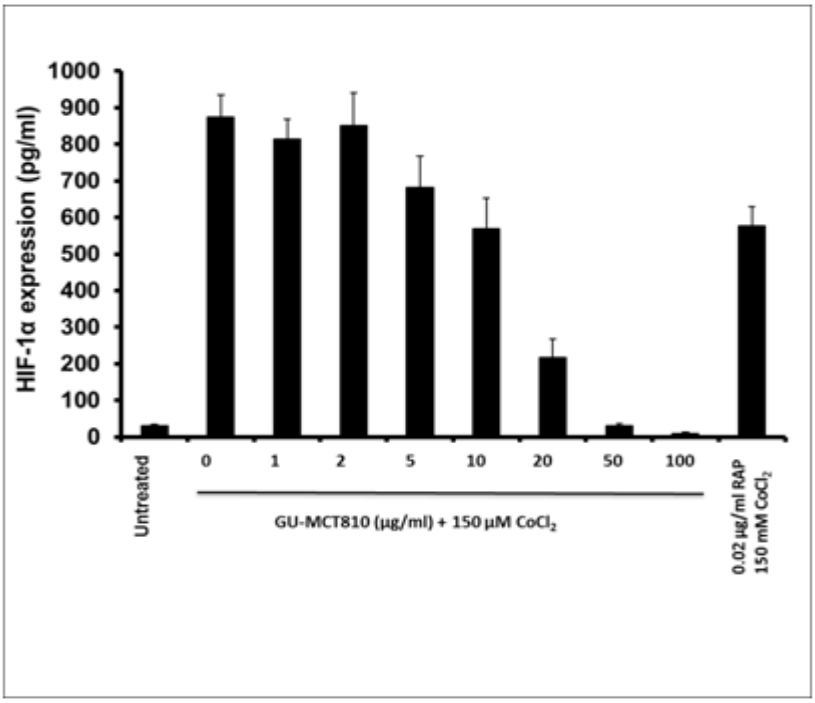
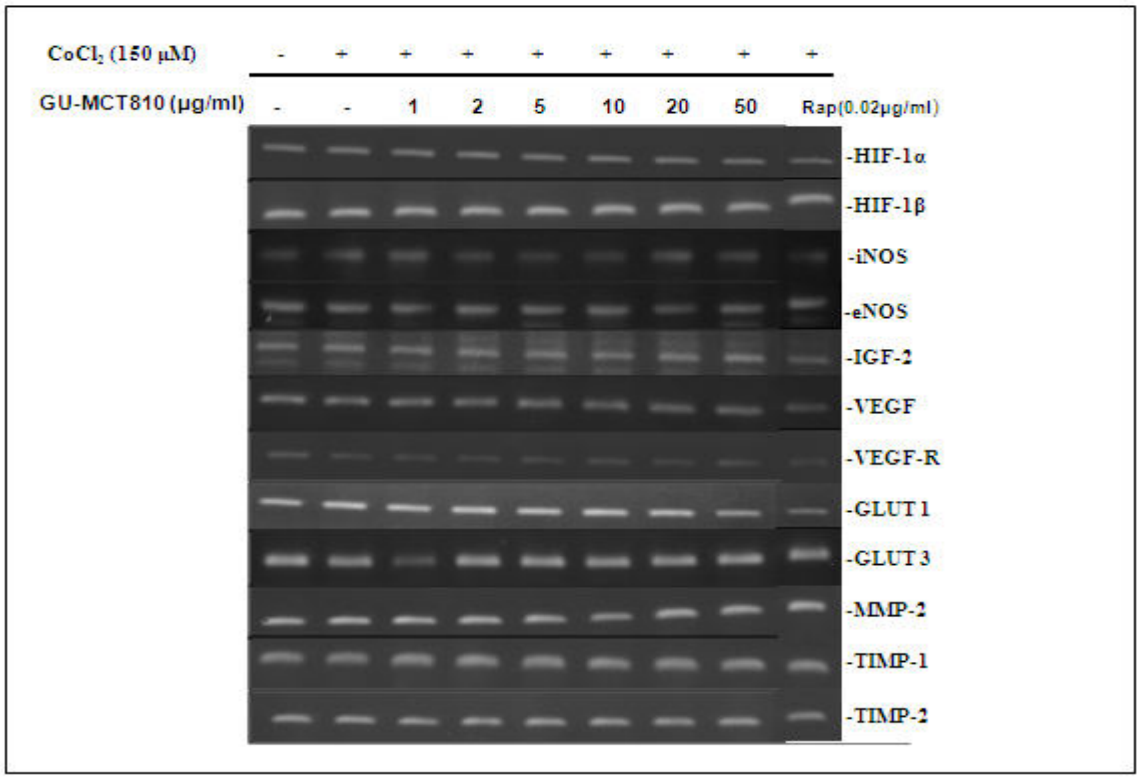
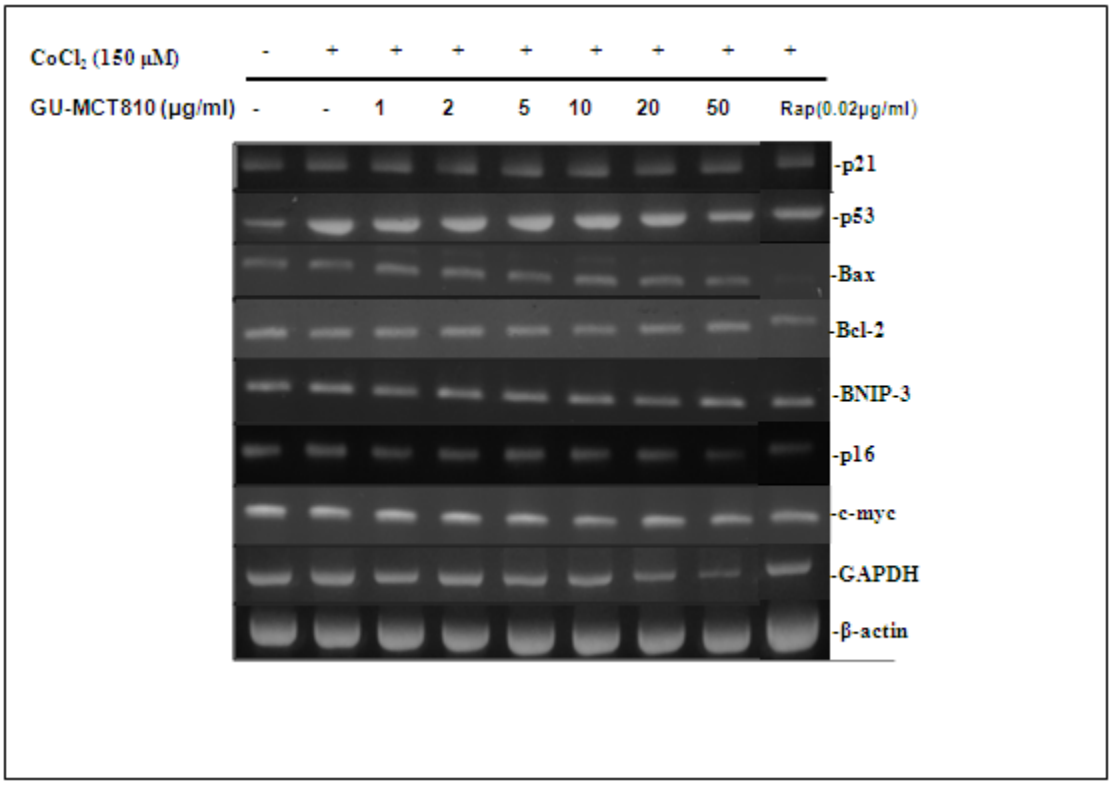


Figure. 4: Inhibition of $CoCl_2$-induced HIF-1 expression by GU-MCT810 in HepG2 cells. A dose-dependent inhibition was noticed with increase in GU-MCT810 concentration. Cells treated with $CoCl_2$ and 0.02 µg/ml rapamycin (RAP) was used as a positive control which showed about 25% HIF-1 inhibition.


CoCl2 (150 μM) - + + + + + + + +
GU-MCT810 (μg/ml) - - 1 2 5 10 20 50 Rap(0.02μg/ml)
-HIF-1α
-HIF-1β
-iNOS
-eNOS
-IGF-2
-VEGF
-VEGF-R
-GLUT 1
-GLUT 3
-MMP-2
-TIMP-1
-TIMP-2


CoCl2 (150 μM) - + + + + + + + +
GU-MCT810 (μg/ml) - - 1 2 5 10 20 50 Rap(0.02μg/ml)
-p21
-p53
-Bax
-Bcl-2
-BNIP-3
-p16
-c-myc
-GAPDH
-β-actin

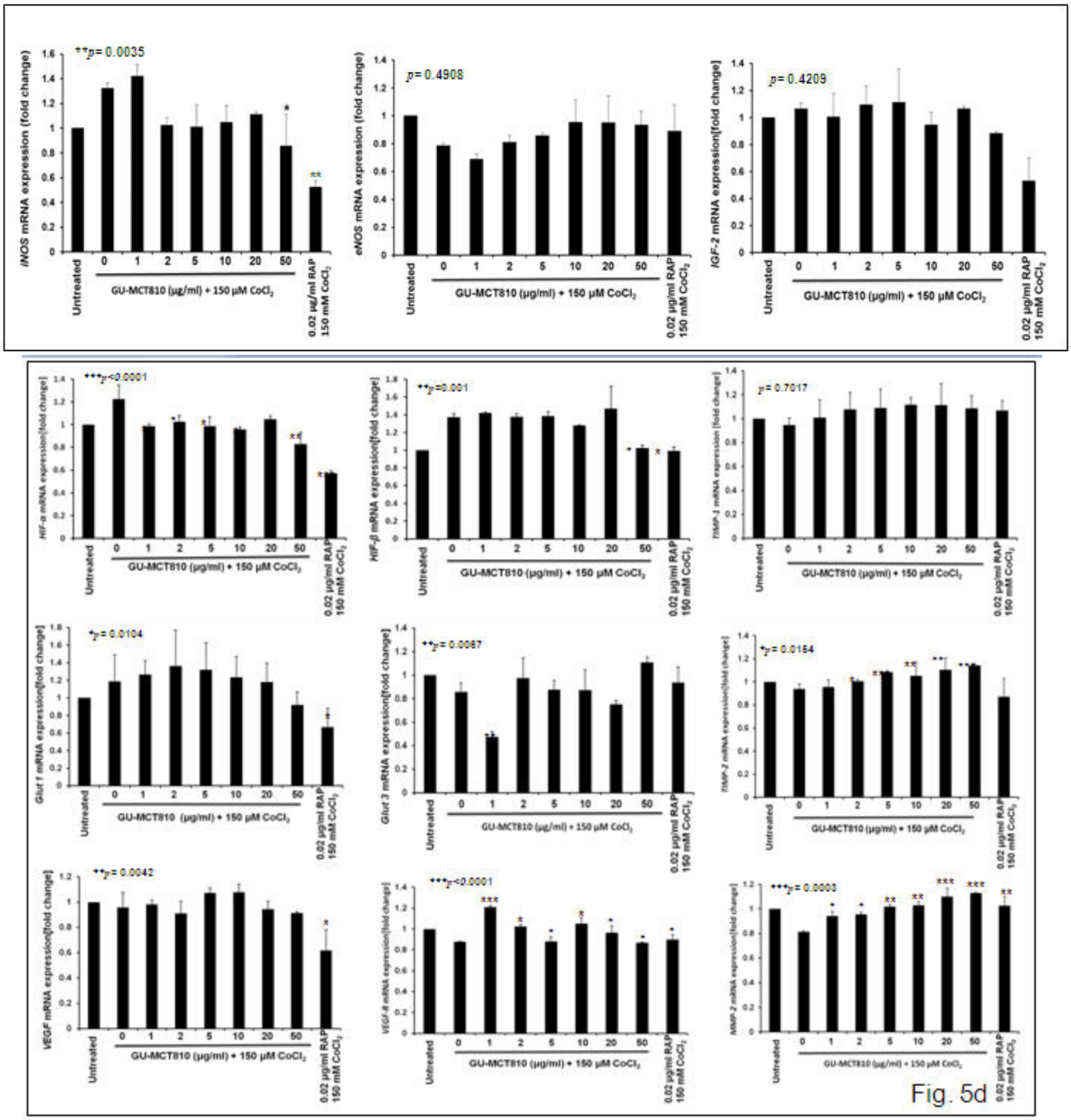

iNOS mRNA expression (fold change)
**p= 0.0035
eNOS mRNA expression (fold change)
p= 0.4908
IGF-2 mRNA expression[fold change]
p= 0.4209
HIF-α mRNA expression[fold change]
***p<0.0001
HIF-β mRNA expression[fold change]
**p=0.001
TIMP-1 mRNA expression [fold change]
p= 0.7017
Glut 1 mRNA expression[fold change]
*p= 0.0104
Glut 3 mRNA expression[fold change]
**p= 0.0087
TIMP-2 mRNA expression[fold change]
*p= 0.0164
VEGF mRNA expression[fold change]
**p= 0.0042
VEGF-R mRNA expression[fold change]
***p<0.0001
MMP-2 mRNA expression[fold change]
***p= 0.0003
Untreated
0 1 2 5 10 20 50
GU-MCT810 (µg/ml) + 150 µM CoCl2
0.02 µg/ml RAP 150 mM CoCl2

Fig. 5d

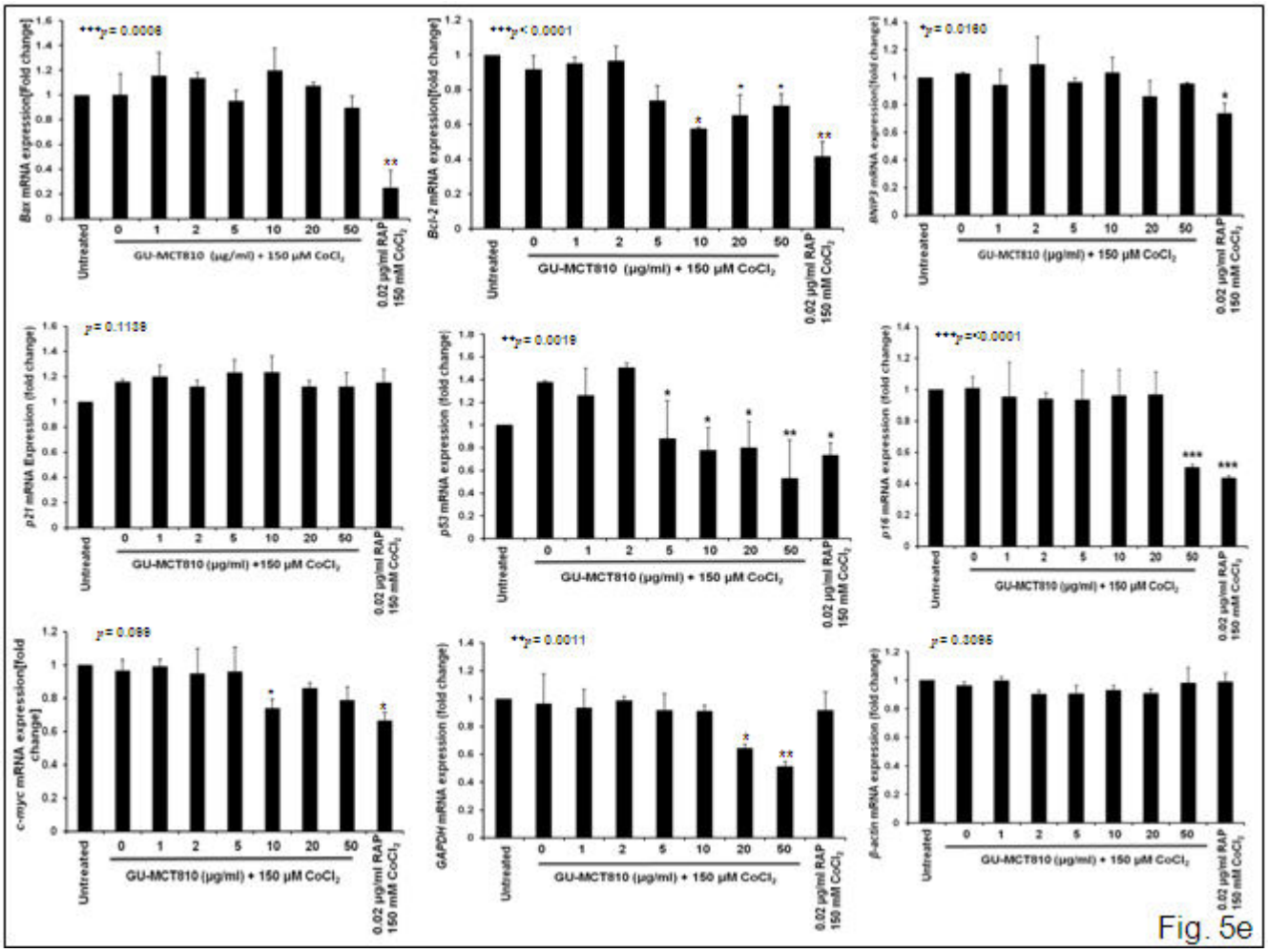


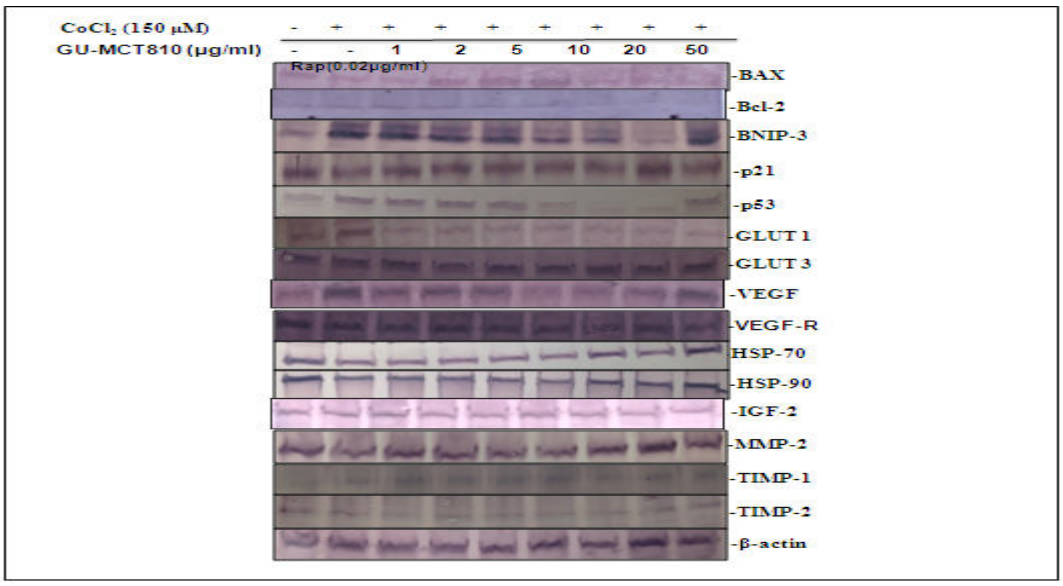
Figure. 5: (a) Effect of GU-MCT810 on the expression of HIF-1-associted genes analyzed by RT-PCR assay in HepG2 cells. Cells were co-incubated with $CoCl_2$ + increasing doses of GU-MC810 and gene expression patterns analyzed by RT-PCR (b) Effect of CoCl2 and GU-MCT810 on apoptosis-associated genes. (c) Quantification of expression of HIF-1-associated genes by UNSCAN-IT gel software. (d) Quantification of expression of apoptosis-associated genes by UNSCAN-IT gel software. (e) Quantification of expression of nitric oxide synthase genes and cell proliferation association genes. The relative expression of genes (fold-increase or -decrease) is plotted against GU-MCT810 concentrations. The significant difference between treatments was compared by 1-way analysis of variance with Tukey's multiple comparison test (Graphpad Prism software, La Jolla, CA). (*p<0.05; **p<0.01; ***p<0.001).

Of the apoptotic genes studied, $CoCl_2$ + GU-MCT810 treatment affected the mRNA expression of genes such as Bax, Bcl-2, BNIP3, p53 and p16. Mutant p53 mRNA increased with $CoCl_2$ treatment which was subsequently inhibited with increasing concentrations of GU-MCT810. While p21 mRNA level did not change with $CoCl_2$ and/or GU-MCT810, p16 mRNA was down regulated at 50 µg/ml concentration of GU-MCT810.

Protein expression

The results of western blot analysis associated with HIF-1 pathway are presented in Figure. 6a and the quantifications are given in Fig. 6b&c. GU-MCT810 treatment of HepG2 cells have affected the translation of several genes associated with apoptosis such as Bcl-2, Bax, BNIP3, p21 and p53. Bax and p21 expression decreased with $CoCl_2$ treatment which then showed a dose-dependent increase with GU-MCT810 treatment. On the other hand, Bcl-2, BNIP3 and p53 showed an increase with $CoCl_2$ treatment, which was inhibited in a dose-dependent manner with GU-MCT810 treatment (Figure. 6b). The protein expression of angiogenic markers, VEGF and VEFG-R, was inhibited by GU-MCT810 treatment, although VEGF showed an initial increase with $CoCl_2$ treatment. IGF-2 protein also got elevated with $CoCl_2$ treatment which then got inhibited with GU-MCT810 treatment. Of the glucose transporters, only GLUT1 protein expression was inhibited

by GU-MCT810 treatment. Similarly, both HSP70 and HSP90 protein expression was down regulated by the GU-MCT810.



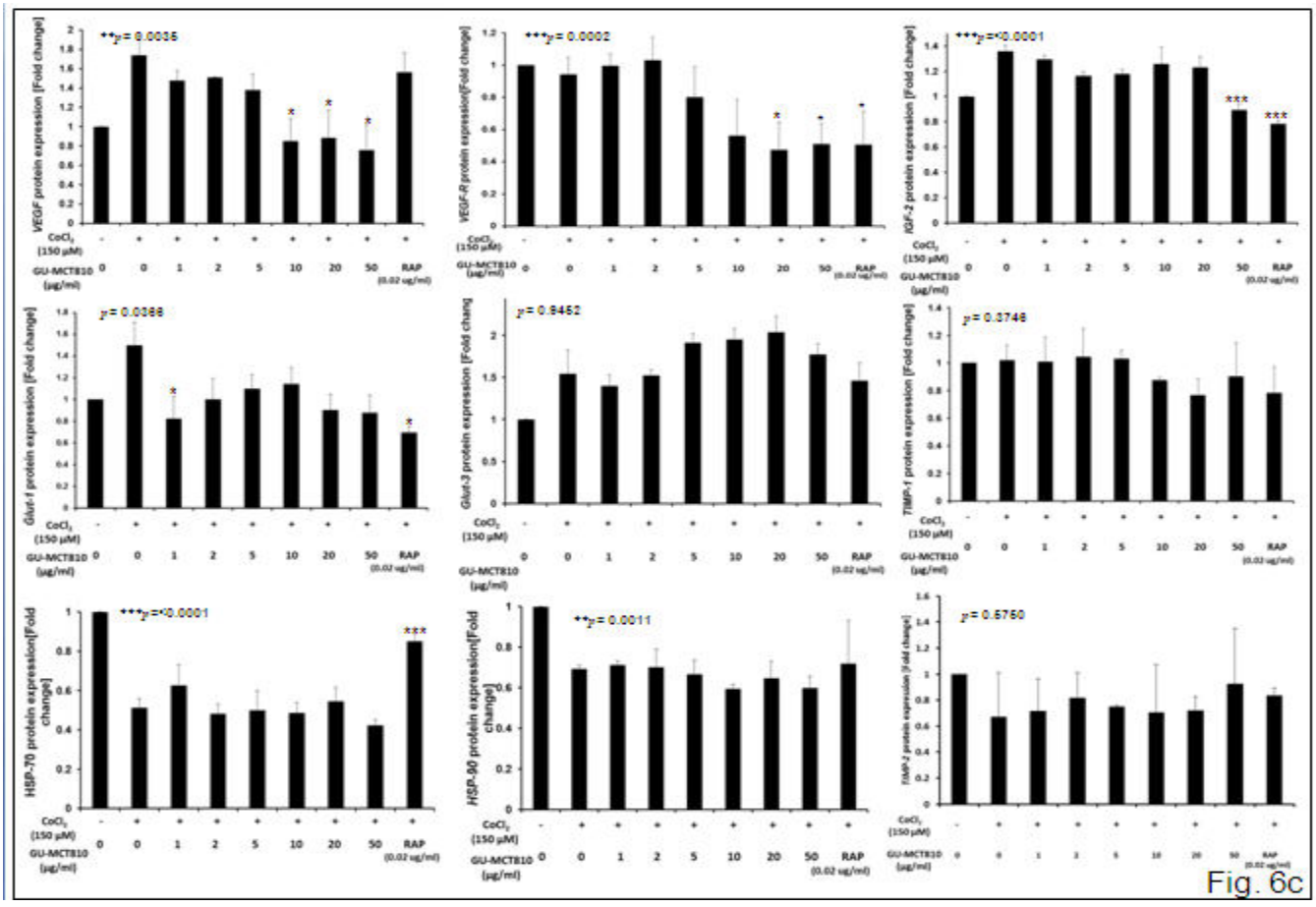


Fig. 6c

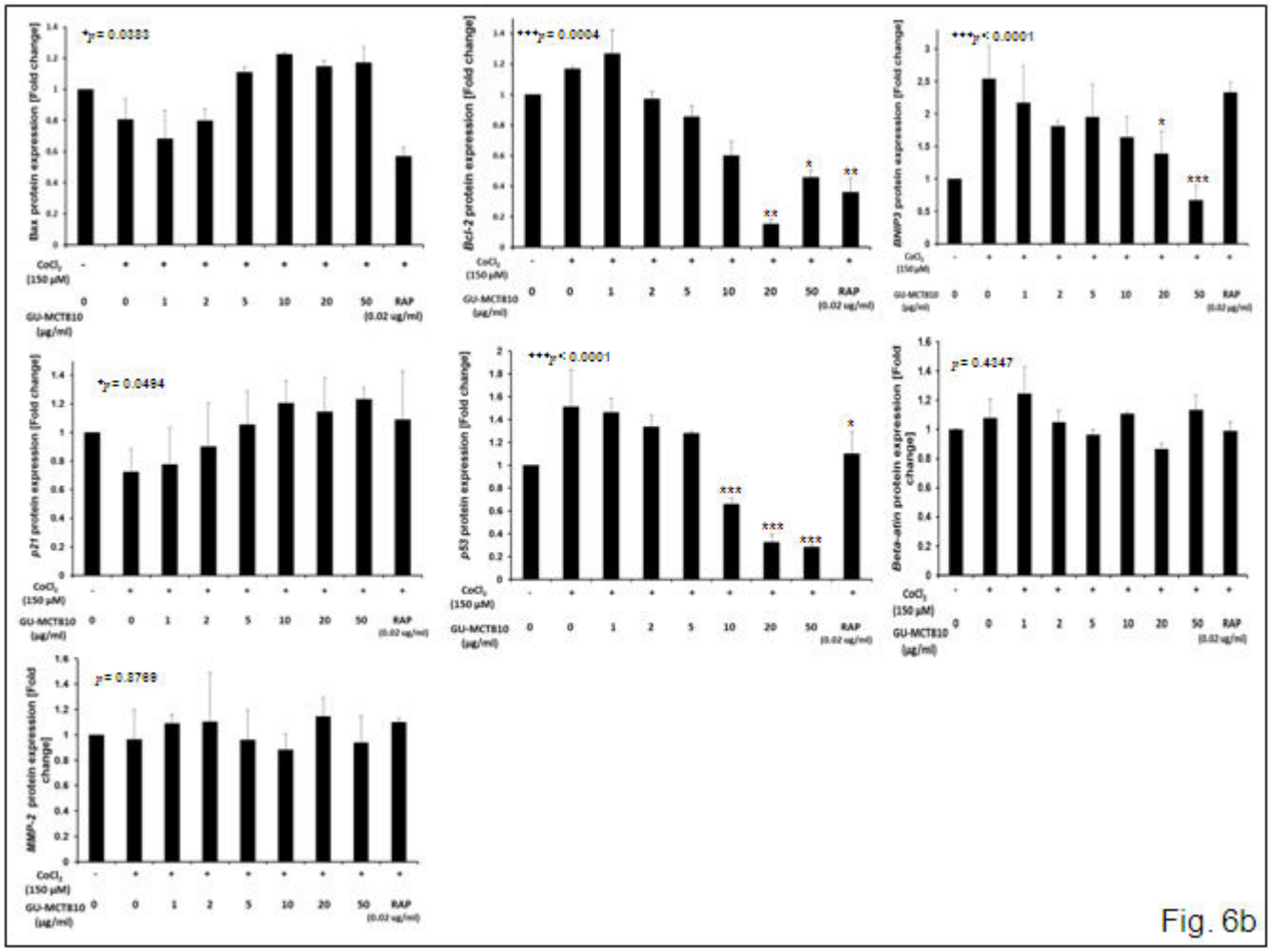


Figure. 6: (**a**) Effect of GU-MCT810 on protein expression of HIF-1-associated genes analyzed by western blot hybridization. (b&c) Quantification of western blots using UNSCAN-IT gel software. The relative levels of protein expression (fold-increase or -decrease) are plotted against GU-MCT810 concentrations. The significant difference between treatments was compared by 1-way analysis of variance with Tukey's multiple comparison test (Graphpad Prism software, La Jolla, CA). (*p<0.05; **p<0.01; ***p<0.001).

Discussion

Overexpression of HIF-1 is usually noticed in various human cancers, probably as a consequence of intra-tumoral hypoxia or genetic alteration [20,21]. The interior of the tumor mass is progressively hypoxic with increasing size until adequate blood vessels are formed within the tumors. Hypoxic conditions within tumors can result in increased HIF-1stability and activity, which is a positive factor for tumorigenesis [22]. It has been suggested that disruption of the HIF-1 pathway might be effective in the treatment of pancreatic cancer, which works through the suspension of glucose metabolism [23] and rendering the cells susceptible to apoptosis. Moreover, several novel therapeutic agents such as Herceptin, Iressa, Calphostin C, Wortamannin and Rapamyicn that target signal-transduction pathways have been shown to block HIF-1 function and have also been shown to possess antiangiogenic effects. Many small molecular inhibitors of HIF-1 transcriptional activation pathway have also been identified and shown to decrease HIF-1 levels, inhibit the expression of VEGF and other HIF-1 target genes, impair xenograft growth and vascularization, and inhibit angiogenesis [24].

In the present investigation, GU-MCT810 and 2-DG as single agents have failed to show any appreciable cytotoxicity in HepG2 cells. However, when these two agents were combined in treatment, this combination was significantly cytotoxic. GU-MCT810 + 2-DG combination showed a strong synergism (CI values ranged from 0.21 to 0.88) for cytototxic effect in HepG2 cells. Cancer cells in general exhibit increased substrate level glycolysis and depend less on oxidative phosphorylation for ATP production [25,26]. Consequently they require a high uptake of glucose and accelerated rates of glycolysis to survive. Hypoxia inside tumor leads to the production of hypoxia inducible factors

that stimulate enhanced anaerobic glycolysis. Moreover, the cells within the hypoxic environment are often slowly proliferating and resistant to cytotoxic therapies. Since these cells are more dependent upon on glycolysis for survival they may be hypersensitive to glycolytic inhibitors like 2-DG [27]. 2-DG is a synthetic glucose analogue that is phosphorylated by hexokinase upon transport into cells, but cannot be fully metabolized [25,28). 2-DG can also cause inhibition of protein glycosylation that induces endoplasmic reticulum stress and gives rise to activation of the unfolded protein [29,30]. As a single agent, 2-DG has been shown to inhibit cell growth in a number of cancers, and to enhance the therapeutic efficacy of chemotherapeutic drugs in human xenografts [31-34]. However, 2-DG has been reported to protect cancer cells from death by activation of the Akt and mitogen-activated protein kinase (MAPK) pathways [35]. In a recent study we reported that GU-MCT810 has hypolipidemic effect in HepG2 cells and increased AMPK kinase activity. Furthermore, GU-MCT81 inhibited adipocyte differentiation and increased cellular glucose uptake in 3T3LI preadipocytes [14]. It is quite possible that GU-MCT810 also increases 2-DG uptake further supporting the inhibition of glycolysis. The synergistic effect between GU-MCT810 and 2-DG on the cytotoxicity supports the potential for reduction of slow growing resistant tumor cells present in the hypoxic environment inside the inner regions of human tumors. Upon transport into the cells, 2-DG is phosphorylated by hexokinase to 2-DG-phosphate. However, unlike glucose-6-phosphate, 2-DG-phosphate cannot be further metabolized by phosphohexose isomerase, which converts glucose-6-phosphate to fructose-6-phosphate [28]. 2-DG-phosphate is trapped and accumulated in the cells, leading to inhibition of glycolysis mainly at the step of phosphorylation of glucose by hexokinase. Inhibition of this rate-limiting step by 2-DG causes a depletion of cellular ATP, leading to blockage of cell cycle progression and cell death *in vitro* [36]. In the present investigation, GU-MCT810 +2-DG combination has produced a synergistic inhibitory effect on cellular ATP as compared to single agents. It is quite possible that the synergistic cytotoxic effect of this combination may be due to the increased cellular ATP depletion of cancer cells [25].

GU-MCT810 treatment of $CoCl_2$-challenged HepG2 cells showed a dose-dependent inhibition of HIF-1 expression in the present investigation. It is known that $CoCl_2$ can act as hypoxia mimicking agents [10]. It is also well established that HIF-1 promotes the expression of genes encoding proteins that increase the cellular supply of oxygen and promote survival in periods of cellular hypoxic stress [37-40]. This transcription factor has been shown to regulate more than 100 downstream genes with varying functions such as angiogenesis, glucose metabolism, apoptosis, erythropoesis, cell proliferation and survival [41]. Gene expression analysis performed in HepG2 cells treated with $CoCl_2$+ GU-MCT810 treatment showed that $CoCl_2$ activated HIF-1 and HIF-1β genes were down regulated by GU-MCT810 at mRNA level. Also ELISA results showed that at the protein expression level, HIF-1 was down regulated. Several authors have described the changes in mRNA expression of both HIF-1 and HIF-1β subunits [42-44]. Under normoxic conditions, when HIF-1 and HIF-1β are constitutively transcribed and translated, the abrogation of HIF-1 activity results from constitutive HIF-1 degradation [45]. Our results also show that genes such as Bcl-2, P53 and VEGF were down regulated by GU-MCT810 at mRNA level and protein level. Then there are genes such as VEGF-R, GLUT1 and IGF-2 that were down regulated at the protein level and not at mRNA level. This discrepancy is plausible because of post-translational modification of these proteins [46,47]. Further, there are genes such as Bax, GLUT3 and P53 whose protein expression increased with no clear cut difference in mRNA levels which could also be attributed to post translational modification and stabilization [48,49]. In conclusion, the results show that GU-MCT810 affects the HIF-1 pathway affecting several genes involved in apoptosis, angiogenesis, and glucose transport and cell proliferation. The combination of GU-MCT810 and 2-DG is synergistic for cytotoxic effect and it may inhibit HIF-1 pathway, the gene expression patterns of which has to be investigated further. Also the inhibition of HIF-1 by GU-MCT810 will be useful for elimination of drug resistant cells present in the hypoxic environment of the tumor.

Author Contributions

Dr. Cheppail Ramachandran, Ms. Gilda M. Portalatin, Enrique Escalon and Dr. Steven J. Melnick were responsible for study design, experimentation, and manuscript preparation. Dr. Karl-Werner Quirin formulated and prepared the GU-MCT810 for the investigation. All authors have read and approved the article.

Declaration of Conflicting Interests

The authors declare the following potential conflicts of interest with respect to the research, authorship, and/or publication of this article: Dr. Steven J. Melnick is the founder of Dharma Biomedical LLC, which is an evidence-based ethnobotanical and evochemical nutraceutical and drug discovery company operating on a for-profit basis. Dr. Karl-Werner Quirin is the Chief Executive Officer of Flavex Naturextrakte GmbH, a company producing specialty botanical extracts for cosmetics and food supplements on the basis of supercritical CO_2 extraction. Dr. Enrique Escalon has no conflict of interest. Dr. Cheppail Ramachandran and Ms. Gilda M. Portalatin are employees of Dharma Biomedical LLC.

Ethical Approval

Since this investigation did not involve human subjects or animal species, approvals from the Institutional Committees were not required.

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