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ARAŞTIRMA / RESEARCH

Ceranib-2 inhibits HIF1-α gene expression and induces apoptosis in HepG2 cells

Ceranib-2 HIF1-α gen ekspresyonunu inhibe eder ve HepG2 hücrelerinde apoptozu indükler

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Abstract

Purpose: The aim of this study is to investigate the apoptotic effect of a novel anti-cancer drug, ceranib-2 and impact on HIF-1α levels on HepG2.
Materials and Methods: The cell line was treated in vitro with 0,1, 1, 5, 10, 25 and 50 μM ceranib-2 for 24 and 48 hours and cell viabilitiy was determined. mRNA levels of acid ceramidase, caspase-3, caspase-8, caspase-9, Cyc1, HIF-1α and TNF-α were measured by qPCR.
Results: Ceranib-2 at 10 μM concentration reduced the viability by about 58 % after 24 and 48 hours. The same dose increased mRNA level of caspase-3 and no change was detected on caspase-8 when compared to the control group after 24 hours. No difference was detected on caspase-3, but caspase-8 mRNA level increased after 48 hours with ceranib-2 at 10 μM concentration. Caspase-9 mRNA levels did not differ after 24 and 48 hours. Ceranib-2 at 10 μM concentration lowered mRNA level of Cyc1 against the control group after the 24- hour treatment. ASAH mRNA level was reduced after the 48-hour treatment with 10 μM ceranib-2. Reduction of ASAH indicated that 10 μM ceranib-2 could inhibit ceramidase after 48 hours and this may elavate ceramide concentration. TNF-α mRNA increased after 24 and 48 hours, but HIF-1α expression was low after 24 hours when compared to the control group.
Conclusion: We have found that ceranib-2 induces apoptosis in HepG2, thus ceranib-2 may play an anti-cancer role at 10 μM concentration.
Keywords: HepG2, apoptosis, HIF-1α, TNF-α, ASAH

Öz

Amaç: Bu çalışmanın amacı, yeni bir anti-kanser ilaç olan seranib-2' nin apoptotik etkisini ve HIF-lα düzeylerinin HepG2 üzerindeki etkisini araştırmaktır.
Gereç ve Yöntem: Hücreler 0, 1, 1, 5, 10, 25 ve 50 μM seranib-2 ile 24 ve 48 saat süreyle muamele edildi ve hücre canlılığı belirlendi. Asit seramidaz, kaspaz-3, kaspaz-8, kaspaz-9, Sitokrom C1, HIF-1α,ve TNF-α mRNA seviyeleri qPCR ile ölçüldü.
Bulgular: 10 μM konsantrasyonda Ceranib-2; 24 ve 48 saat tedaviden sonra hücre canlılığını yaklaşık % 58 azalttı. Aynı doz, kaspaz-3'ün mRNA seviyesini arttırdı ve 24 saat sonra kontrol grubuna kıyasla kaspaz-8 üzerinde herhangi bir değişiklik tespit edilmedi. Kaspaz-3'te herhangi bir değişiklik saptanmazken kaspaz-8 mRNA seviyesi, 48 saat sonra 10 μM seranib-2 konsantrasyonunda arttı. Kaspaz-9 mRNA seviyeleri 24 ve 48 saat sonra değişmedi. 10 μM konsantrasyonda Ceranib-2, 24 saatlik tedaviden sonra kontrol grubuna karşı Sitokrom C1'in mRNA seviyesini düşürdü. ASAH mRNA seviyesi, 10 μM seranib-2 ile 48 saatlik tedaviden sonra azaltılmıştır. ASAH' ın azaltılması, 10 μM seranib-2'nin 48 saat sonra seramidazı inhibe edebileceğini ve bunun seramid konsantrasyonunu artırabildiğini gösterdi. TNF-α mRNA seviyesi hem 24 hem de 48 saat sonra arttı, ancak HIF-lα mRNA seviyesi, 24 saat sonra kontrol grubuna kıyasla düşüktü.
Sonuç: Seranib-2' nin HepG2'de apoptozu indüklediğini bulduk, bu nedenle seranib-2' nin 10 μM konsantrasyonda anti-kanser bir rolü olabilir.
Anahtar kelimeler: HepG2, apoptoz, HIF-lα, TNF-α, ASAH

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common cancer among other cancers and the third most common cause of cancer-related death worldwide. Several pathogenic mechanisms and factors associated with HCC have been reported, but mechanisms for the formation and treatment are still under investigation[26]. Many molecules and mechanisms involved in apoptosis are of interest in cancer treatment studies. One of them, sphingolipids, is a structural element of cell membrane and bioactive molecules that regulate the basic cellular processes[3,4].

As membrane and intracellular lipids sphingolipids organize various pathways on cell survival. Acting on a complex system these play an active role in many cellular functions such as growth, adhesion, migration, autophagy, necrosis and apoptosis. Over the past two decades studies with bioactive sphingolipids played an important role in enhancing our understanding about pathogenesis and treatment of different cancers[13-6585]. Ceramide can be produced by hydrolysis of the sphingomyelin with the enzyme sphingomyelinase, the breakdown of complex sphingolipids or the transformation of certain molecules such as sphingosine[12]. Ceramide may then interact with many enzymes to turn into glycosphingolipids and sphingosine1 phosphate. Ceramide is an important bioactive lipid molecule in various aspects of cancer biology including cell proliferation, migration, senescence, inflammation and apoptosis[3,4]. Especially in cancer cells, cellular ceramide content can be regulated and pro-apoptotic pathways can be activated by using ceramide analogs or ceramidase inhibitors. On the other hand, a reduction in ceramide concentration impairs ceramide signalling and results in resistance to apoptosis in cancer cells. Anti-cancer drugs are reported to cause apoptosis and inhibit cell cycle by increasing ceramide level in the cell[11]. Therefore, small, drug-like anticancer agents have been developed for the inhibition of acid ceramide. One of these substances is ceranib-2, a new anti-cancer drug that targets acid ceramidase enzyme[11].

In the complex metabolic pathway of sphingolipids, ceramidases (CDases) have a key role in many human cancers[9]. Therefore, ceramidases are important targets for the development of anticancer drugs because inhibition of the activity of ceramidases causes ceramide accumulation[26-3]. Furthermore, the destruction of ceramides by ceramidases showed that tumor cells are freed from apoptosis[11]. It was observed that the activity of ceramidase enzyme decreased in the serovanate-treated SCOV3 human ovarian adenocarcinoma cells[11]. Although exposure to ceramide-producing sphingomyelinase was found to induce apoptosis in cancer cells, overexpression of ceramidases was associated with increased resistance to apoptosis resulting in poor prognosis in a variety of cancers, including melanoma, prostate cancer and colon cancer [1]. Nonetheless, despite the known actions of sphingolipid-mediated signaling in cancer cells, the effects of ceramidase inhibition are poorly understood. Therefore, inhibition of ceramidase activity could offer a potential alternative target for liver cancer treatment[11].

Since there are no other studies encountered on the potential effects of ceranib-2 on HCC cell line HepG2, this study was designed to test the possible effect of ceranib-2 on viability as well as on caspase-3, 8, 9, cytochrome c1 (Cyc1), TNF-α, HIF1-α and acid ceramidase (ASAH) expressions. Although several studies have reported that ceramide metabolism can be associated with HCC, the underlying mechanisms still remain unknown.

MATERIALS AND METHODS

Cell survival

Human liver carcinoma cells HepG2 were used in this study. Since the cells are purchased commercially, ethics committee approval is not required and the study was organized in accordance with Helsinki protocols. Human liver carcinoma cells HepG2 (the American Type Culture Collection - ATTC; Rockville, MD) was cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma; St. Louis, MO) containing 10% fetal bovine serum, 100 U/mL penicillin and 100 µg/mL streptomycin. Following detachment from the flasks with 0.25 % trypsin+EDTA (Sigma), collected cells were centrifuged at 1200 rpm and 4 °C for 5 minutes, counted with a CEDEX (Roche; Mannheim, Germany) cell counter and then seeded overnight in 96 well plates (approximately 10^4 cells per 0.25 mL). Ceranib-2 (Cayman Chemical, MI) was dissolved in dimethyl sulfoxide (DMSO, Sigma) and DMEM was applied for optimal dilution. HepG2 cells were treated with 0,1, 1, 5, 10, 25 and 50 µM ceranib-2 for 24 and 48 hours and cell viability was determined by 3-3-(4,5-D-methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, thiazolyl blue (MTT)

test at 550 nm with a microplate reader (BioTek; Winooski, VT) method, in vitro. A minimum of three independent assays were run to statistical analysis.

Measurement of mRNA levels by using quantitative reverse transcription PCR

The mRNA levels of caspase-3, 8, 9, Cyc1, ASAH, TNF-α and HIF1-α in relation to the housekeeping gene (β-actin) were determined by qRT-PCR with Syber green prop. qRT-PCR data were collected by using Thermo StepOnePlus. Gene expression levels were reported by using the median as a point estimator and the range of values. qRT-PCR results were calculated on GraphPad software program. Transcript data were expressed relative to the control (set to 1) ± standard deviation. Following determination of ASAH, caspase-3, 8, 9, HIF-1α, and TNF-α mRNA expressions β-actin as a reference gene, the data obtained from qRT-PCR were calculated from $2^{-\Delta\Delta ct}$ formula. ASAH primer sequence: forward AGTTGCGTCGCCTTAGTCCT; reverse TGCACCTCTGTACGTTGGTC, caspase-3 primer sequence: forward TGGTTCATCCAGTCGCTTTG; reverse CATTCTGTTGCCACCTTTCG, caspase-8 primer sequence: forward CTGCTGGGGATGGCCACTGTG; reverse TCGCCTCGAGGACACGCTCTC, caspase-9 primer sequence: forward CGAACTAACAGGCAAGCAGC; reverse ACCTCACCAAATCCTCCAGAAC, Cyc1 primer sequence: forward AAGGGAGGCAAGCACAAGACTG; reverse CTCCATCAGTGTATCCTCTCCC, HIF-1α primer sequence: forward TATGAGCCAGAAGAACTTTTAGGC; reverse CACCTCTTTTGGCAAGCATCCTG

TNF-α primer sequence: forward CTCTTCTGCCTGCTGCACTTTG; reverse ATGGGCTACAGGCTTGTCACTC, Beta Actin primer sequence: forward CACCATTGGCAATGAGCGGTTC; reverse AGGTCTTTGCGGATGTCCACGT.

Statistical analysis

Statistical analysis was performed using GraphPad6 software. Data are presented as mean ±SEM or median, %25, %75. Kolmogorov Smirnov test were used to evaluate the normality of the data. Parametric and non-parametric data were analysed using one – way ANOVA test and Tukey or Kruskal-Wallis and Dunn's test respectively. Differences with P-values < 0.05 were considered significant.

RESULTS

In our study 10 μM ceranib-2 concentration decreased the viability of HepG2 cells in 24 and 48 hours. This impact shows 58 % difference when compared to the control group (Fig.1.).

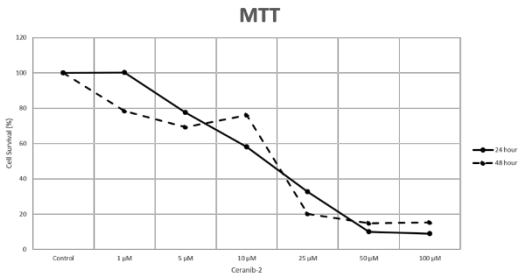

Figure 1. Survival effects of six different ceranib-2 concentrations on HepG2 cells for 24 and 48 hours.

According to RT-PCR results, while mRNA levels of caspase-3 increased statistically ($p<0.05$ Fig.2.A), caspase-8 levels did not change after the 24-hour application of 10 μM ceranib-2 when compared to the control group (Fig.3.A). No difference for caspase-3 amount in 48 hours was detected (Fig.2.B), but there was a statistically significant reduction in caspase-8 mRNA level ($p>0.05$) after the 48-hour treatment with ceranib-2 at 10 μM concentration (Fig.3.B). Although there were little rises, no statistical differences were calculated in caspase-9 mRNA level after 24 and 48-hour applications of 10 μM ceranib-2 (Fig.4.A-B). Ceranib-2 at 10 μM concentration decreased mRNA abundance of Cyc1 vs control group ($p<0.05$) after the 24-hour treatment (Fig.5.A) but no change was detected after 48 hours (Fig.5.B). Abundance of ASAH mRNA did not change in 24 hours (Fig.6.A), but reduced after the 48-hour treatment with 10 μM ceranib-2 ($p<0.05$ Fig.6.B). TNF-α mRNA increased after 24 and 48 hours ($p<0.05$ Fig.7.A-B), but HIF-1α expression was low after 24 hours when compared to the control group ($p<0.0001$ Fig.8.A).

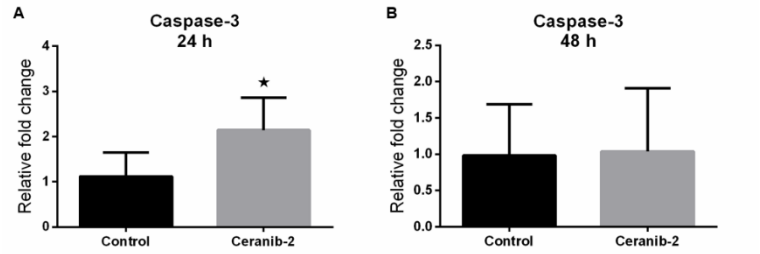

Figure 2: Expressions of caspase-3 mRNA at 24 (A) and 48 (B) hours (p<0.05, p>0.05).

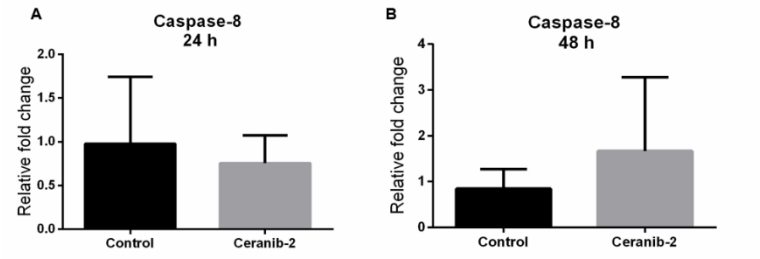

Figure 3. Expressions of caspase-8 mRNA after 24 (A) and 48 (B) hours (p>0.05, p>0.05).

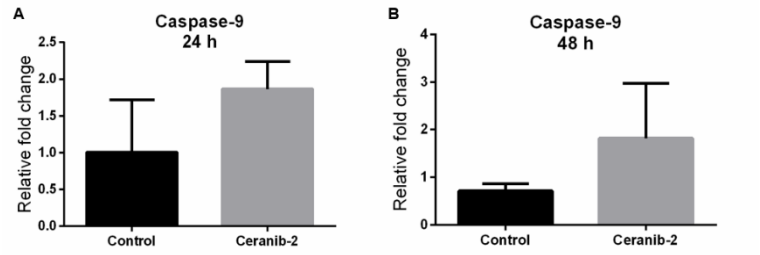

Figure 4. Expressions of caspase-9 mRNA after 24 (A) and 48 (B) hours (p>0.05, p>0.05).

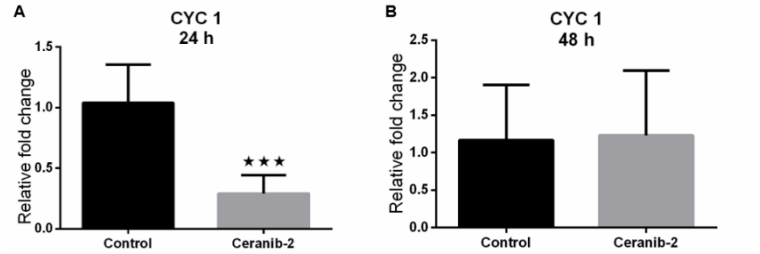

Figure 5. Expressions of Cyc1mRNA after 24 (A) and 48 (B) hours (p<0.05, p>0.05).

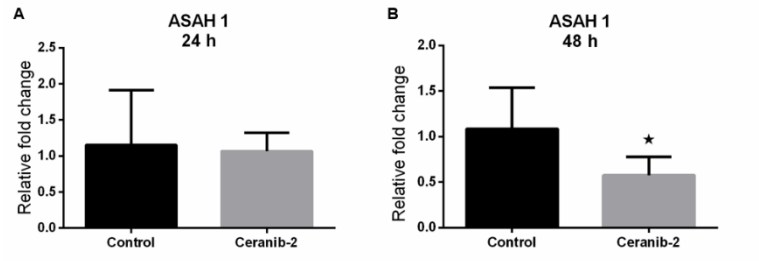

Figure 6. Expressions of ASAH mRNA after 24 (A) and 48 (B) hours (p>0.05, p<0.05).

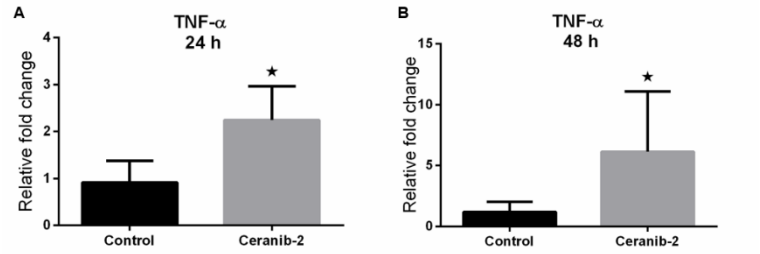

Figure 7. Expressions of TNF-α mRNA after 24 (A) and 48 (B) hours (p<0.05, p<0.05).

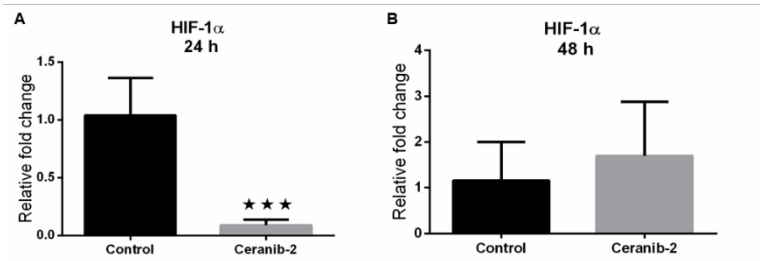

Figure 8. Expressions of HIF-1α mRNA after 24 (A) and 48 (B) hours (p<0.05, p>0.05).

DISCUSSION

The results of our study on human liver carcinoma cells indicate that ceranib-2 may have anti-cancer effect depending on time and dose. Supporting our cell survival results, Kus et al. found in human prostate cancer cell lines LNCaP and DU145 that ceranib-2 has cytotoxic effects [10]. In addition, Draper et al. identified ceranib-2 as a potent ceramidase inhibitor of human ovarian adenocarcinoma cells (SKOV3), and found an inhibition of cell proliferation with an IC_{50} of 28 μM [11]. In our study, ceranib-2 indicated cytotoxic effect with an IC_{50} of 10 μM. It is possible that ceranib-2 inhibited cell proliferation and led to cell death at the dose of 10 μM.

According to our RT-PCR results, mRNA level of caspase-3 increased at 24 hour, while levels of caspase-8 and 9 did not show clear differences at 24 hour with the presence of 10 μM ceranib-2 concentration. At 48 hour, the mRNA levels of caspase-3 and 9 did not significantly change. Compared to control groups, although the mRNA level of Cyc1 decreased only at 24 hour, the mRNA

level of ASAH showed a decrease with concentration of 10 μM after 48 hours. While the amount of TNF-α mRNA increased after both 24 and 48 hours, HIF-1α mRNA level increased after only 24 hours.

Raisova et al. in their study on human keratinocytes and melanocytes observed that D-e-MAPP and D-NMAPPD enzymes suppressed the acid ceramidase activity. They concluded that higher ceramide inhibitors could be a new treatment group for antiproliferative and cytotoxic drugs [1-3]. In aggrement with their conclusion, ceramidase inhibitor ceranib-2 at 10 μM concentration did not change the expression of ASAH mRNA at 24 hour, but at 48 hour it decreased when compared to the control group. Our results showed that caspase 3 level in HepG2 cells increased by ceranib-2 treatment at 24 hour. Although statistically not significant, caspase 9 expression levels also elevated by ceranib-2 treatment of HepG2 cells.

It was indicated that caspase activation involved in apoptosis is preceded by the elevation of ceramide which is a ubiquitous regulator of cellular stress and found to be an inducer of apoptosis caused by specific stress stimuli [9]. Many reports demonstrated that ceramide can activate caspase cascades[9] and the ability of ceramide to induce apoptosis has been demonstrated in many cell types[1,2-15]. In parallel with previous studies, this study confirmed ceramide elevation by apoptotic cell death in HepG2 cells under ceranib-2 treatment. Likewise, it has been reported that ceramide treatment increases the expressions of caspase 3 and 9 in human epithelial lung carcinoma (H1299) cells[7]. Reduction of ceramidase indicates that the production of ceramide and thus apoptosis is increased. In this context, in our study, ceranib-2 treatment in liver carcinom cells line increased the levels of caspase 3 in accordance with the literature. However, in our study, mRNA levels of Cyc1 decreased at 24 hour, but did not change compared to control group at 48 hour. There are two ways in which mammalian cells mediate apoptosis: the extrinsic route, the pathway mediated by death receptors such as the TNF-α family and the intrinsic pathway also known as Bcl-2, is referred to mitochondrial route mediated by Cyc1 [2] It is possible that apoptosis might be mediated by TNF-α via the extrinsic pathway, but not by Cyc1. Via the enzyme sphingomyelinase TNF-α is known to induce the production of ceramide which may be transformed in turn into different sphingolipid intermediates including sphingosine, sphingosine-1-phosphate (S1P) and ceramide-1-phospahate (C1P) [6]. For this purpose, when we studied the levels of TNF-α mRNA involved in the extrinsic pathway, we observed an increase of it in the ceranib-2 treates groups at both 24 and 48 hours. At the same time, the decrease in caspase 8 mRNA levels in our study also supports that the extrinsic pathway is more active.

Cancer cells in hypoxic regions are a clinical problem because they are more resistant to radiotherapy and many chemotherapeutic drugs than cancer cells in normoxic regions, thereby increasing the likelihood of tumor recurrence[6]. Zhang et al. showed that HIF1 alpha protein was expressed in HCC and positive rate of HIF1 alpha protein in HCC tissues was higher than that in normal hepatic tissues[4]. Although several genes such as HIF-1α have been proposed for hypoxia-induced chemoresistance in some types of cancer[28], the molecular mechanism underlying drug resistance caused by hypoxia continues to be described. In accordance with this information, ceramidase inhibitor ceranib-2 at 10 μM concentration reduced the expression of HIF-1α mRNA at 24 hour, but at 48 hour it decreased when compared to the control group. Hypoxia represents a potent therapeutic goal given that it plays an important role in tumor development and treatment resistance, and that hypoxia levels are more severe in most cancers than in normal cells. One approach to targeting hypoxia is small molecule inhibitors against molecular targets that play a role in the survival of hypoxic cells. The present interest focuses on inhibition of HIF1. In view of our results, ceranib-2 may reduce hypoxia in HCC because it inhibits HIF-1α gene expression and can be used as a therapeutic biological agent.

In the present study, ceranib-2 decreased the viability of HepG2 cells at 10 μM concentration after 24 and 48 hours. This effect shows a difference of 58% when compared to the control group. When the effects of ceranib-2 on caspase 3, 8, and 9 mRNA expressions, which are ASAH and apoptotic markers, were investigated, it was observed that anti-cancer and apoptotic activity may be time-dependent at a concentration of 10 μM.

The limitation of this study may be that a higher ceranib-2 dose should be used and that ceramide levels should also be analyzed. In addition, protein datas is required to reveal the exact mechanisms involved ceranib-2 and HCC in future work. Accordingly, the effect mechanism of ceranib-2

should be clearly identified and its role on different pathways with varied methods should be the subject of research for future studies.

Yazar Katkıları: Çalışma konsepti/Tasarımı: RÖ; Veri toplama: RÖ, MÖ; Veri analizi ve yorumlama: RÖ; Yazı taslağı: RÖ; İçeriğin eleştirel incelenmesi: RÖ; Son onay ve sorumluluk: RÖ, MÖ, AK, CCME, NE; Teknik ve malzeme desteği: NE; Süpervizyon: -; Fon sağlama (mevcut ise): yok.
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Author Contributions: Concept/Design : RÖ; Data acquisition: RÖ, MÖ; Data analysis and interpretation: RÖ; Drafting manuscript: RÖ; Critical revision of manuscript: RÖ; Final approval and accountability: RÖ, MÖ, AK, CCME, NE; Technical or material support: NE; Supervision: -; Securing funding (if available): n/a.
Ethical Approval: Human liver carcinoma cells HepG2 were used in this study. Since the cells are purchased commercially, ethics committee approval is not required and the study was organized in accordance with Helsinki protocols.
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