Inhibition of Grb2-mediated activation of MAPK signal transduction suppresses NOR_1/CB1954-induced cytotoxicity in the HepG2 cell line

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Received March 12, 2012; Accepted June 22, 2012

DOI: 10.3892/ol.2012.774

Abstract. The nitroreductase oxidored-nitro domain containing protein 1 (NOR_1) gene may be involved in the chemical carcinogenesis of hepatic cancer and nasopharyngeal carcinoma (NPC). We have previously demonstrated that NOR_1 overexpression is capable of converting the monofunctional alkylating agent 5-(aziridin-1-yl)-2,4-dinitrobenzamide (CB1954) into a toxic form by reducing the 4-nitro group of CB1954. Toxic CB1954 is able to enhance cell killing in the NPC cell line CNE_1; however, the underlying mechanisms remain unknown. Using cDNA microarrays and quantitative real-time PCR, we previously discovered that NOR_1 increases the expression of growth factor receptor-bound protein 2 (Grb2) mRNA by 4.8-fold in the human hepatocellular carcinoma cell line HepG2. In the present study, we revealed that NOR_1 increased Grb2 protein expression by 3-fold in HepG2 cells. Additionally, we demonstrated that NOR_1 enhanced CB1954-induced cell killing in HepG2 cells, and cell cytotoxicity was inhibited with the tyrosine kinase inhibitor genistein, or by stable transfection of Grb2 small hairpin RNA (shRNA) $pU6^{+27}$-shGrb2 to silence the expression of Grb2. Western blot analysis revealed that Grb2 downregulation may reduce the activity of the mitogen-activated protein kinase (MAPK). Inhibiting the activation of MAPK using the methyl ethyl ketone (MEK) inhibtor PD98059 suppressed CB1954-induced cell killing. These results suggested that the NOR_1 gene enhances CB1954-mediated cell cytotoxicity through the upregulation of Grb2 expression and the activation of MAPK signal transduction in the HepG2 cell line.

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Key words: NOR_1, Grb2, MAPK, siRNA

Introduction

The oxidored-nitro domain containing protein 1 (NOR_1) gene was previously cloned in our laboratory at the Third Xiangya Hospital (Hunan, China) using suppression subtractive hybridization and cDNA microarrays (1,2). By examining the human genome working sequence, it has been identified that that the NOR_1 gene is located on 1p34.3 and contains 10 exons and 9 introns. Additionally, the PROSITE database identified two possible cAMP and cGMP-dependent protein kinase phosphorylation sites, two tyrosine phosphorylation sites and four N-myristoylation sites in NOR_1. This gene, has an important oxidored-nitro domain and shares 38% homology with bacterial nitroreductase (NTR); therefore, the gene was named NOR_1, as approved by The HUGO Gene Nomenclature Committee (2). Cell cytotoxicity assays have demonstrated that the NOR_1 gene has similar functions to NTR, which is able to enhance 5-(aziridin-1-yl)-2,4-dinitrobenzamide (CB1954)-induced cell killing (3); however, the signaling mechanisms by which NOR_1 enhances CB1954-induced cell killing remain unknown.

N-nitroso compounds (NOCs), including nitrosamines, either present in food or formed endogenously, have been suggested as etiological factors in hepatic carcinoma, stomach cancer, nasopharyngeal carcinoma (NPC) pathogenesis and various other types of cancer (4-6). Humans are exposed through ingestion or inhalation to preformed NOCs in the environment and through the endogenous nitrosation of amino precursors in the body. *In vivo* mechanisms suggest that the formation of NOCs may involve chemical and enzymatic nitrosation, which is particularly dependent on the presence of nitrate reductase and nitroreductase. As a consequence, endogenous nitrosation may occur at various sites within the body, including the oral cavity, liver, stomach, urinary bladder and at other sites of infection or inflammation (7).

Growth factor receptor-bound protein 2 (Grb2) is an adaptor protein that links the associated downstream molecules on receptor tyrosine kinases (8-10). Earlier studies have demonstrated that in addition to the overexpression of receptor tyrosine kinases, overexpression of downstream proteins, including Grb2, may also induce the upregulation of signaling pathways associated with cell transformation. In human breast

cancer cell lines (MCF-7, MDA-MB-361 and MDA-MB-453), overexpression of Grb2 correlated with increased complex formation between Grb2 and SOS (11-13). Overexpression of Grb2 protein in the fibroblast cell line NIH 3T3, potentiated the activation of mitogen-activated protein kinase (MAPK) (14). Additionally, overexpression of Grb2 has been demonstrated in chemical carcinogen-induced liver tumorigenesis. In N-ni trosodimethylamine (NDMA)-induced A/J mice at 1 year of age, overexpression of Grb2 was identified in liver lesions, preneoplastic foci, adenomas and carcinomas. These results suggested that the upregulation of Grb2 is an early event within liver carcinogenesis (15).

In a previous study, using cDNA microarray and quantitative real-time PCR analysis of HepG2 cells, we revealed that NOR_1 produced a 4.8-fold increase in Grb2 mRNA levels (16). Grb2 is known to transduce activated tyrosine kinase signaling to Ras, which subsequently facilitates the activation of downstream signaling pathways, including Ras and MAPK. In our study, we identified that NOR_1-enhanced CB1954-induced cell killing and overexpression of NOR_1 upregulated Grb2 expression in HepG2 cells (3,16). Thus, Grb2 may play a role in NOR_1-enhanced CB1954-induced cell killing. In this study, we examined the involvement of Grb2 and the downstream MAPK in NOR_1-enhanced CB1954-induced cell cytotoxicity in the hepatic carcinoma cell line HepG2.

Materials and methods

Cell lines, reagents, plasmids and antibodies. A NOR_1 stably-transfected cell line was previously built at our laboratory (16). Genistein and PD98059 were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The small hairpin RNA (shRNA) Grb2-encoding construct used for Grb2 knockdown ($pU6^{+27}$-shGrb2) and its control (empty vector, $pU6^{+27}$-shControl) were purchased from Panomics, Inc. (Fremont, CA, USA). Antibodies specific for Erk1/2 and phosphorylated Erk1/2 were purchased from Cell Signaling Technology Inc. (Beverly, MA, USA). Antibodies specific for Grb2 and β-actin were purchased from BD Transduction Laboratories (San Diego, CA, USA) and Sigma Chemical Co., respectively. Anti-mouse and anti-rabbit secondary antibodies conjugated with horseradish peroxidase were purchased from Amersham Pharmacia Biotech (Piscataway, NJ, USA).

Cell culture and cell cytotoxicity. The human hepatocellular carcinoma cells, HepG2, were maintained in RPMI-1640 supplemented with 10% fetal calf serum (FCS) in a humidified culture incubator at 37˚C with 5% CO_2 and 95% air. Cell cytotoxicity assays were conducted as previously described (3). HepG2 cells grown to ~80% confluence were washed with PBS and treated with a signal transduction inhibitor and/or CB1954. Measurements were collected from 10-12 individual microscopic fields in each experiment and data were summarized from 3-5 experiments.

Stable transfection of $pU6^{+27}$ plasmids into HepG2 cells. HepG2 cells were initially seeded in 6-well tissue culture plates at $1x10^5$ cells/well in 1.5 ml RPMI-1640 containing 10% FCS. Cells were grown at 37˚C in a CO_2 incubator until ~75% confluence was reached. Stable transfection of HepG2 cells with $pU6^{+27}$-shGrb2 or $pU6^{+27}$-shControl plasmid was conducted with 3 μg of linearized vectors using Lipofectamine™ 2000. After two days, cells were incubated in the presence of neomycin (0.5-1 mg/ml). Once all untransfected HepG2 cells were killed by neomycin, HepG2 cells resistant to neomycin were isolated, grown and examined using western blot analysis. Clones expressing minimal endogenous Grb2 (HepG2-shGrb2) were selected and propagated for further experiments.

Western blot analysis. Cells were lysed in ice-cold lysis buffer containing 150 mM NaCl, 20 mM Tris (pH 7.5), 1 mM $MgCl_2$, 1 mM PMSF, 1% Triton X-100, 0.5% sodium deoxycholate, 2 mM sodium orthovanadate, 25 mM sodium fluoride, 1% aprotinin and 10 μg/ml leupeptin. The protein concentration of the supernatant was determined using the Bio-Rad DC Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA). Equal concentrations of total protein (5-10 μg) were aliquoted and prepared for electrophoresis by adding 2X SDS-PAGE loading buffer and boiling. Samples were electrophoresed on 10% polyacrylamide gels (Bio-Rad Laboratories) and transferred to nitrocellulose membranes (Schleicher & Schuell Bioscience, Inc., Keene, NH, USA) for western blot analysis. Membranes were blocked in Tris-buffered saline containing 20 mM Tris (pH 7.6) and 150 mM NaCl, with 0.1% Tween 20 (TBST) containing 5% non-fat dry milk (Bio-Rad Laboratories). Primary antibodies (anti-Grb2, 1:5000; anti-phosphorylated MAPK, 1:5000; anti-MAPK, 1:1000; anti-β-actin, 1:7500) diluted in TBST were then added. Membranes were washed and incubated with secondary antibodies conjugated with horseradish peroxidase. Protein bands were detected via enhanced chemiluminescence (Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA) and images were scanned using an AlphaImager densitometer (Alpha Innotech Corp., San Leandro, CA, USA).

Statistical analysis. Multiple group comparisons were conducted using ANOVA and the Student's t-test for pairwise comparisons. Group differences resulting in $P<0.05$ were considered to be statistically significant.

Results

Western blot analysis demonstrates that NOR_1 overexpression enhances the expression of Grb2. In our previous study, we revealed that overexpression of NOR_1 increased the expression of Grb2 mRNA by 4.8-fold in HepG2 cells. To examine whether NOR_1 alters the protein level of Grb2, we used NOR_1-overexpressed HepG2 cells as the experimental group, empty plasmid vector pcDNA3.1(+)-transfected HepG2 cells and wild-type (WT) HepG2 cells as the control groups. Western blot analysis demonstrated that NOR_1 increases the expression of Grb2 protein by approximately 3-fold (Fig. 1).

NOR_1 overexpression enhances CB1954-induced cell killing in HepG2 cells. To examine whether NOR_1 overexpression increases CB1954-induced cell killing of HepG2 cells, the

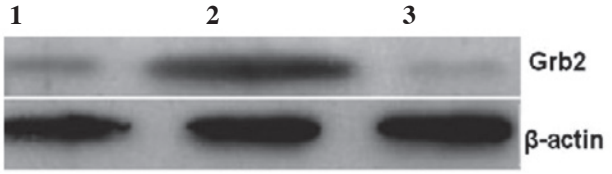


Figure 1. NOR_1 overexpression enhances the expression of Grb2 protein. Lane 1, wild-type HepG2 cells; lane 2, pcDNA3.1(+)-NOR_1-HepG2 cells; lane 3, pcDNA3.1(+)-HepG2 cells. β-actin was used as a protein loading control. Grb2, growth factor receptor-bound protein 2; NOR_1, oxidored-nitro domain containing protein 1.

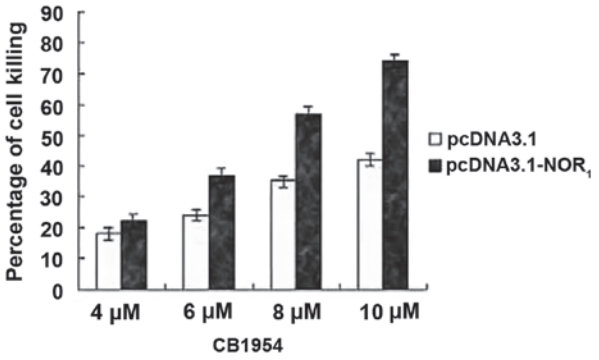


Figure 2. NOR_1 overexpression enhances CB1954-induced cell killing. Cell killing assays were conducted in pcDNA3.1(+)-HepG2 and pcDNA3.1(+)-NOR_1-HepG2 cells. Cell killing was determined using trypan blue exclusion 2 days after the cells were incubated with various concentrations of CB1954 (4-10 μmol/l). NOR_1, oxidored-nitro domain containing protein 1.

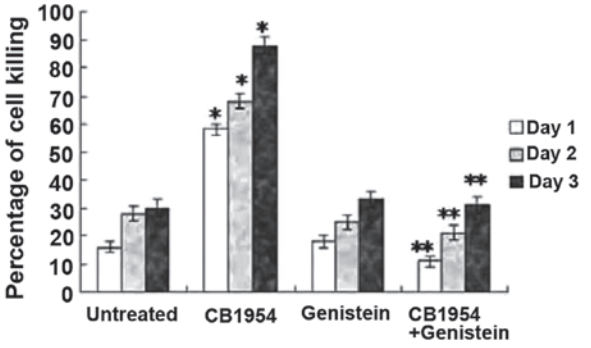


Figure 3. CB1954-induced cell killing is tyrosine kinase dependent. pcDNA3.1(+)-NOR_1-HepG2 cells were treated with 6 μmol/l CB1954 and/or 5 μmol/l genistein at various experimental time points. Each bar represents the mean accounts of cell killing from 10-12 microscopic fields ± SEM on the day indicated. *Significantly different compared with the untreated group ($P<0.05$). **Significantly different compared with the CB1954-treated group. NOR_1, oxidored-nitro domain containing protein 1; SEM, standard error of mean.

HepG2 cell line was transfected with the recombinant plasmid pcDNA3.1(+)-NOR_1 or plasmid pcDNA3.1(+). When the HepG2 cells reached ~80% confluence, they were incubated with various concentrations of prodrug CB1954 (4-10 μmol/l). Cell viability was determined after 2 days using Trypan Blue exclusion. As shown in Fig. 2, expression of the NOR_1 gene in the HepG2 cell line markedly enhanced CB1954-induced cell killing ($P<0.05$).

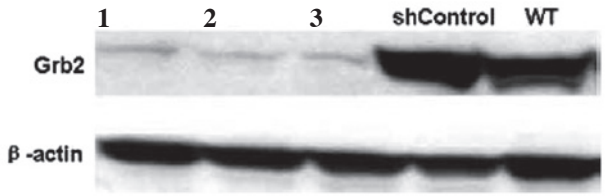


Figure 4. Generation of shRNA Grb2 transfected stable HepG2 cell line. Three clones resistant to neomycin were screened for Grb2 expression using western blot analysis. Lane 3 was selected for the subsequent stages of the experiment. β-actin was used as a protein loading control. Grb2, growth factor receptor-bound protein 2; shControl, small hairpin empty vector $pU6^{+27}$ control; WT, wild-type.

Genistein inhibits NOR_1-enhanced CB1954-induced cell killing. Since the NOR_1 gene has two tyrosine phosphorylation sites and NOR_1 gene overexpression enhanced the expression of Grb2 at mRNA as well as protein level, and as Grb2 plays an important role in tyrosine kinase receptor involved signal transduction, we anticipated that NOR_1 enhanced CB1954-induced cell killing may depend on tyrosine kinase activity. Therefore, we assayed levels of cell killing in pcDNA3.1(+)-NOR_1-HepG2 cells treated with the tyrosine kinase inhibtor genistein in the absence or presence of CB1954. As we anticipated, treatment with genistein alone did not alter cell killing; however, in the presence of CB1954, genistein treatment reduced cell killing comparable to that of untreated control cells (Fig. 3). These results indicate that NOR_1 enhanced CB1954-induced cell killing is dependent on tyrosine kinase activity.

Silencing Grb2 protein expression using shRNA Grb2 inhibits CB1954-induced cell killing. pcDNA3.1(+)-NOR_1-HepG2 cells were WT or stably transfected by $pU6^{+27}$-shGrb2 or $pU6^{+27}$-shControl. Three clones resistant to neomycin and expressing $pU6^{+27}$-shGrb2 were screened for Grb2 expression using western blot analysis (Fig. 4). The effects of Grb2 downregulation on CB1954-induced cell killing of pcDNA3.1(+)-NOR_1-HepG2 cells were examined. In the absence of CB1954, shGrb2 did not alter HepG2 cell killing; however, in the presence of CB1954, shGrb2 decreased the level of cell killing to that of the untreated control group (Fig. 5). The shControl did not alter cell motility in either the presence or absence of CB1954 (Fig. 5). These results indicate that Grb2 mediates cell killing induced by CB1954 in pcDNA3.1(+)-NOR_1-HepG2 cells.

Grb2 mediates the activation of MAPK by CB1954. Western blot anlysis demonstrated that shGrb2 treatment reduced Grb2 protein expression (Fig. 6A) and CB1954 increased phosphorylated-MAPK levels (Fig. 6B) in pcDNA3.1(+)-NOR_1-HepG2 cells. However, whether or not their activation is dependent on Grb2 was unknown. Therefore, we determined whether CB1954-mediated activation of MAPK is altered by shGrb2. In the presence of CB1954, shGrb2 decreased Grb2 protein level by 4-fold (Fig. 6A). Compared with CB1954 treatment alone, shGrb2 also decreased CB1954 mediated activation of MAPK by approximately 3-fold (Fig. 6B and C). These results indicate that Grb2 mediates CB1954-induced activation of MAPK.

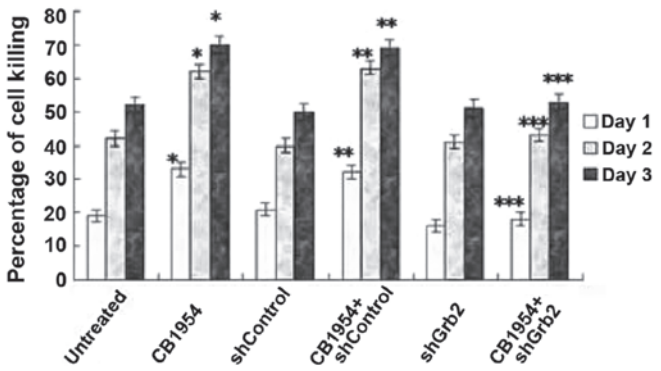
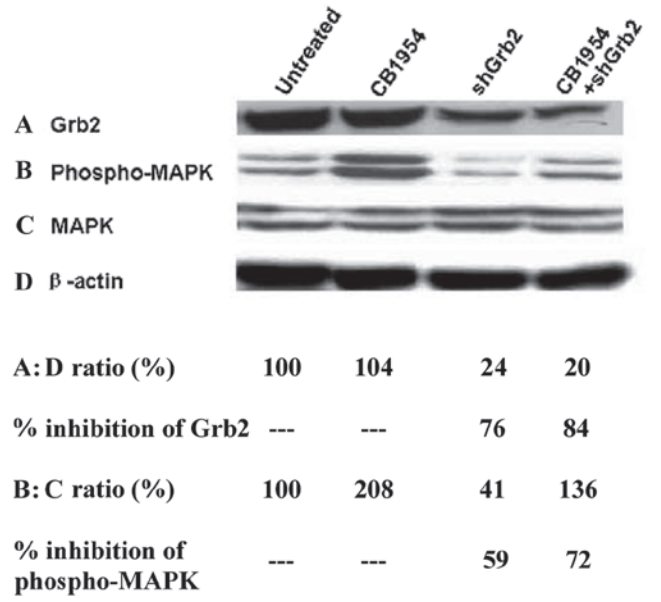


Figure 5. Grb2 downregulation inhibits CB1954-induced cell killing in HepG2 cells. HepG2 cells were treated with 6 μmol/l CB1954 and/or 3 μg shGrb2 or shControl at various experimental time points. *Significantly different compared with the untreated group (P<0.05). **Significantly different compared with the CB1954+shGrb2-treated group (P<0.05). ***Significantly different compared with the CB1954-treated group (P<0.05). shControl, small hairpin empty vector $pU6^{+27}$ control; Grb2, growth factor receptor-bound protein 2.



A: D ratio (%)	100	104	24	20
% inhibition of Grb2	---	---	76	84
B: C ratio (%)	100	208	41	136
% inhibition of phospho-MAPK	---	---	59	72

Figure 6. Grb2 downregulation inhibits MAPK activity in CB1954-stimulated pcDNA3.1(+)-NOR_1-HepG2 cells. HepG2 cells were treated with 6 μmol/l CB1954 and/or 3 μg shGrb2 for 2 days. Equal amounts of protein (10 μg) were loaded for SDS-PAGE. (A) Anti-Grb2 was used to determine levels of endogenous Grb2 in specified treatment. (B) Anti-phosphorylated-MAPK was used to determine MAPK activity. (C) Anti-MAPK was used to determine endogenous levels of MAPK in specified treatment. (D) Anti-β-actin was used as a protein loading control. Grb2, growth factor receptor-bound protein 2; sh, small hairpin; MAPK, mitogen-activated protein kinase. NOR_1, oxidored-nitro domain containing protein 1.

PD98059 inhibits CB1954-induced cell killing of HepG2 cells. To determine whether MAPK mediates CB1954-induced cell killing, pcDNA3.1(+)-NOR_1-HepG2 cells were treated with PD98059. PD98059 inhibits methyl ethyl ketone (MEK) activity and consequently inhibits the activation of its downstream kinase, MAPK. In the presence of CB1954, PD98059 inhibited the cell killing of HepG2 (Fig. 7). These data indicate that CB1954-induced HepG2 cell killing is mediated by the MEK/MAPK pathway.

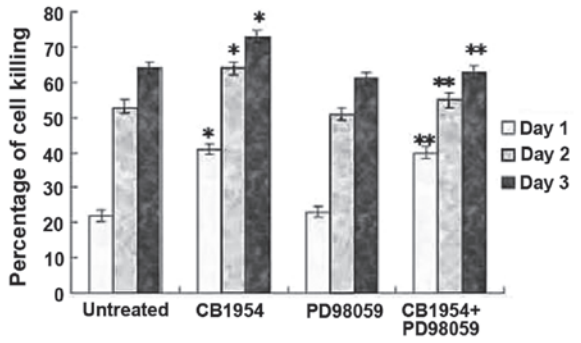


Figure 7. CB1954-induced HepG2 cell killing is dependent on the MEK/MAPK pathway. pcDNA3.1(+)-NOR_1-HepG2 cells were treated with 8 μmol/l CB1954 and/or 1 μM PD98059 at the experimental time period. Each bar represents the mean cell killing from 10-12 microscopic fields ± SEM on the day indicated. *Significantly different compared with the untreated group (P<0.05). **Significantly different compared with the CB1954-treated group (P<0.05). MEK, methyl ethyl ketone; MAPK, mitogen-activated protein kinase; NOR_1, oxidored-nitro domain containing protein 1; SEM, standard error of mean.

Discussion

CB1954 is an antitumor prodrug which recently entered clinical trials in combination with *Escherichia coli* (*E. coli*) NTR as a potential gene-directed enzyme prodrug therapy (GDEPT) (17-19). Nitroreduction of CB1954 by *E. coli* NTR results in the formation of cytotoxic 4-hydroxylamine and the reduction of a 2-nitro group to either 2-hydroxylamine or 2-amine, which are potent cytotoxins (20).

Certain studies have demonstrated that the human liver is capable of aerobic reductive bioactivation of CB1954 to cytotoxic metabolites *in vitro*, possibly involving multiple enzymes, including nitrate reductase and NTR (20). The NOR_1 gene is the first member of the nitroreductase family which has been cloned from human tissue and has a similar function to the reduction of the nitro of NTR (2). Our previous study demonstrated that NOR_1 overexpression is able to convert CB1954 into a toxic form by reducing the 4-nitro group of CB1954, which subsequently enhances cell killing in CNE_1 cells. In this study, we revealed that NOR_1 also enhanced CB1954-induced cell killing in HepG2 cells, and demonstrated that this process may be due to the upregulated expression of Grb2 and the activiation of MAPK signal transduction.

The Grb2 gene is highly conserved among numerous species and the Grb2 protein is an ubiquitously expressed adapter protein. Overexpression of the Grb2 protein has been identified in breast cancer cells, hepatic carcinoma cells and other cancer tissue specimens. The Grb2 protein contains one Src Homology 2 (SH2) domain situated between two Src Homology 3 (SH3) domains, and is known to use its SH2 domain to bind to phosphorylated tyrosine residues of the YXNX motif located in target growth factor receptors (8,21). However, the NOR_1 gene does not contain this motif and our experiments revealed that NOR_1 does not contact Grb2 directly by coimmunoprecipitation (data not shown). However, the NOR_1 gene may increase Grb2 expression at the mRNA and protein level; thus, it is possible that NOR_1 does not form a complex with Grb2, but regulates it to mediate Grb2 signaling.

In conclusion, we used a tyrosine kinase inhibtor (genistein), a MEK inhibtor (PD98059) and shRNA Grb2 to examine the mechanisms of NOR_1 function. We demonstrated that the NOR_1 gene enhanced CB1954-induced cell cytotoxicity through upregulating the expression of Grb2 and activiating MAPK signal transduction. Accordingly, inhibitors targeting the NOR_1/Grb2/MAPK pathway have the potential to be selective therapeutic or chemopreventive modalities against hepatic cancer development.

Acknowledgements

This study was supported by grants from the National Natural Sciences Foundation of China (Nos. 3030083, 81072270 and 81101828), the Fundamental Research Funds for the Central Universities (No. 2011JQ030) and the Hunan Provincial Natural Science Foundation of China (No. 07JJ3036).

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