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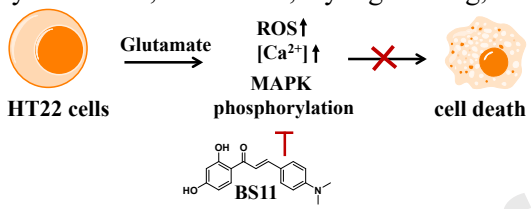

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Synthesis and Biological Evaluation of Isoliquiritigenin Derivatives as a Neuroprotective Agent against Glutamate mediated Neurotoxicity in HT22 Cells

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ABSTRACT

Glutamate-induced neurotoxicity is characterized by cellular Ca^{2+} uptake, which is upstream of reactive oxygen species (ROS)-induced apoptosis signaling and MAPKs activation. In the present study, we synthesized isoliquiritigenin analogs with electron-donating and electron-withdrawing functional groups. These analogs were evaluated for neuroprotective effect against glutamate-induced neurotoxicity in HT22 cells. Among these analogs, compound **BS11** was selected as a potent neuroprotective agent. Cellular Ca^{2+} concentration, ROS level, MAPKs activation and AIF translocation to the nucleus were increased upon treatment with 5 mM glutamate. In contrast, we identified that compound **BS11** reduced the cellular Ca^{2+} concentration and ROS level upon glutamate exposure. Western blot analysis showed that MAPK activation was decreased by treatment with compound **BS11**. We further identified that cotreatment of compound **BS11** and glutamate inhibited translocation of AIF to the nucleus.

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Oxidative stress has been known as the primary cause of neuronal cell death, which is implicated in neurodegenerative condition[1,2] such as ischemic stroke[3], amotrophic lateral sclerosis (ALS)[4,5,6], Parkinson's disease[7,8] and Alzheimer's disease[9,10]. Glutamate is an endogenous excitatory neurotransmitter in the mammalian central nervous system. High concentration of glutamate in the central nervous system induce excitotoxicity and oxidative stress, resulting in neuronal loss.[11] In glutamate-mediated neurotoxicity, glutamate induces the accumulation of intracellular reactive oxygen species (ROS) by inhibition of cysteine uptake into cells via the cysteine/glutamate antiporter.[12,13] Therefore, elimination of intracellular ROS by antioxidants, such as N-acetyl cysteine, can reduce glutamate-induced neuronal cell death.[14,15]

Glutamate-induced oxidative stress activates various signaling pathways including the c-Jun *N*-terminal kinase (JNK) and p38 mitogen-activated protein kinase (MAPK) pathways, which affect inflammation, differentiation, proliferation and cell death.[16,17] The prolonged activation of MAPKs including p38, ERK, and JNK can induce neuronal cell death.[18] Although the increase in intracellular ROS is implicated in neuronal cell death via MAPK activation, this is not the only mechanism of glutamate-induced neuronal cell toxicity; caspase-independent apoptotic pathways mediated by translocation of apoptosis inducing factor (AIF) from the mitochondria to the nucleus are also invovled.[19,20,21,22] Therefore, preventing elevated ROS levels by chemical agents can provide a potential therapeutic opportunity for treating neuronal diseases.

Isoliquiritigenin is a natural chalcone and is known as a major biologically active component of *Glycyrrhizae radix* (licorice roots).[23,24] Isoliquiritigenin has various biological activities including anticancer[25,26], antiinflammatory[27,28], neuroprotective effects[29,30]and NRF2 activation effects.[31,32,33] Recent studies reported that isoliquiritigenin attenuates 6-hydroxydopamine-induced neurotoxicity and shows neuroprotective effects against glutamate-mediated oxidative stress in a mouse hippocampal neuronal cell line (HT22).[34] A recent study reported that metabolites of isoliquiritigenin also potently protect against glutamate-induced neuronal cell death.[35] Therefore, we believed that analogs of isoliquiritigenin may have potent protective effects against glutamate-induced neurotoxicity in HT22 cells. Despite the promising pharmacology of isoliquiritigenin, the structure activity relationships of isoliquiritigenin analogs have not been thoroughly studied yet. Therefore, we decided to synthesize isoliquiritigenin analogues and evaluate their protective effects against glutamate-induced neurotoxicity in a mouse hippocampal neuronal cell line.

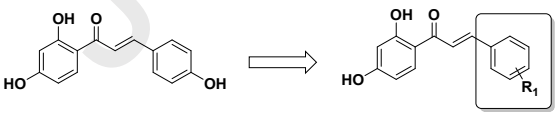
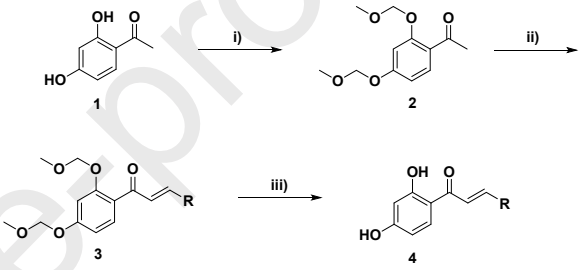


Figure 1. Structural modification of isoliquiritigenin.

Isoliquiritigenin has a chalcone structure with 2'-,4'-,4-hydroxyl group.[23] We synthesized eighteen isoliquiritigenin analogs including isoliquiritigenin **BS1** as shown in Figure 2. At first, intermediate **2** was synthesized from 2,4-dihydroxyacetophenone using chloromethyl methyl ether and diisopropylethylamine (DIEA) with high yield. Intermediate **3** benzaldehydes under basic condition. Deprotection of the methyl ether group of intermediate **3** using 3N HCl afforded final compounds **4** with high yields (Scheme 1). All final compounds were analyzed by ^{1}H-NMR, ^{13}C-NMR, and LC-MS. We initially introduced various aromatic aldehydes that possess electron-withdrawing or electron-donating group. We assumed that the electronic properties of aldehydes would influence the protective properties of these derivatives.

These synthetic isoliquiritigenin derivatives were evaluated for protective effects against HT22 cell death by glutamate induced oxidative stress. HT22 cells were treated with various concentration of glutamate to identify the optimal concentration that induces about 50-60% of cell viability. In the cell based assay, 5mM glutamate was selected as the best condition to induce cell death by glutamate toxicity in HT22 cells. Previous study reported that 5mM glutamate treatment seems optimal condition in HT22 cells.[36] To compare protective effects of compounds against glutamate-induced cell death, we carried out cell viability assay using calcein AM and propidium iodide (PI) double staining.



Scheme 1. The synthetic scheme of isoliquiritigenin derivatives. Reagent and condition; i) DIEA 3.0eq, MOMCl 3.0eq, CH_2Cl_2, RT, overnight ii) 8N NaOH, CH_3CH_2OH, RT, overnight iii) 3N HCl, CH_3OH, 60 ^{o}C, 3h

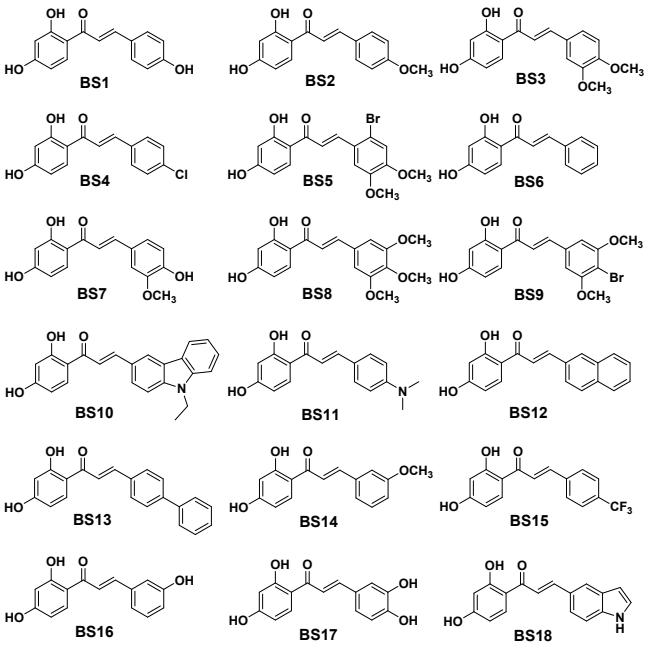


Figure 2. The structure of synthetic isoliquiritigenin derivatives

Calc ... ects. permeable to live cells; cellular esterase in live cells cleaves acetyl group off the fluorescent probe, which results in the emission of green fluorescence. In contrast, PI is impermeable to live cells and is commonly used for staining dead cells. Based on the cell viability assay, a compound **BS11** was selected as a protective agent against glutamate toxicity in HT22 cells. The other compounds show less or no protective effect in indicated concentration or slight cytotoxicity at high concentration (Figure 3).

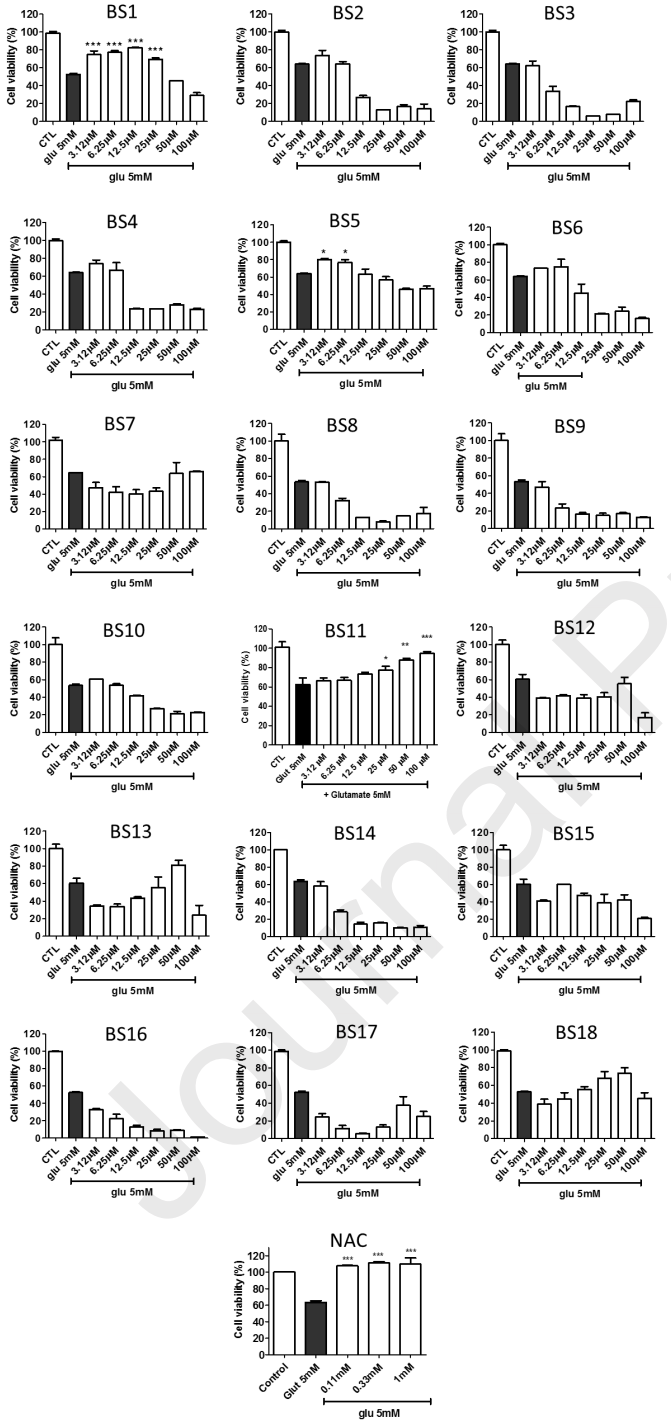


Figure 3. The protective effects of isoliquiritigenin (ISL) derivatives were evaluated in glutamate induced HT22 cell death. The indicated concentration of compounds were treated to HT22 cells in the presence of 5 mM of glutamate for 24 h. Cell viability was determined by image analysis using calcein AM. BS1 is ISL. NAC is N-acetylcustein.

High concentration (50 μM to 100 μM) of BS1 showed cytotoxicity. However, a compound **BS11** showed neuroprotective effects in high concentration. The compounds (BS2 and BS3) with *p*-methoxy phenyl and *m*-, *p*-dimethoxy phenyl group shows almost no neuroprotection effects. The compounds BS5 with *m*-, *p*-dimethoxy, *o*-bromo phenyl group shows neuroprotective effects at 3 μM to 6 μM. A compound BS16 with *m*-hydroxy phenyl group, which has similar structure with ISL shows no neuroprotective effects. The compound BS18 with indole group shows potent neuroprotective effects.

We next investigated whether and how the synthesized compound **BS11** could play a role in protecting HT22 cells against excessive oxidative stress. As shown in Figure 4, glutamate treatment significantly decreased cell viability, while the additional compound **BS11** under the same experimental condition increases survival ratio of HT22 cells. An additional experiment was performed using MTT. In the MTT assay data, compound **BS11** treated HT22 cells exhibited dramatically increased cell viability against oxidative stress (Figure 4).

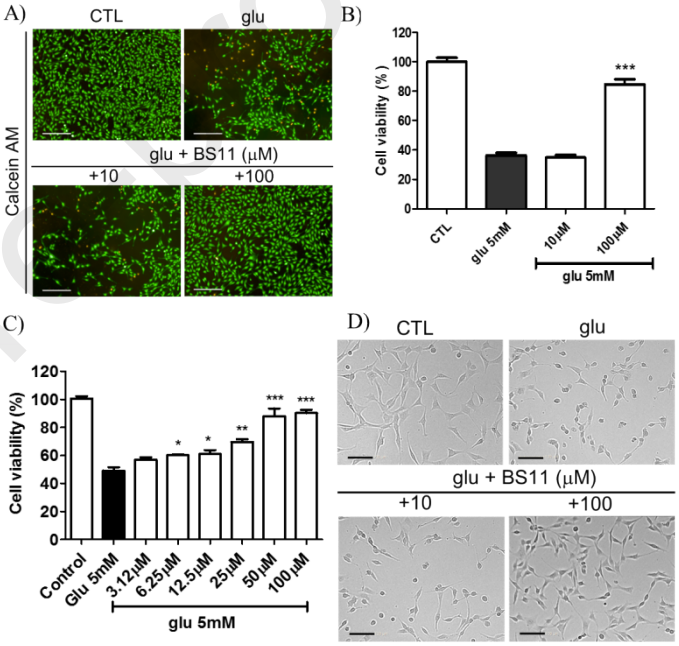


Figure 4. Neuroprotection effect of compound **BS11** on glutamate induced cell death on HT22 cells. HT22 cells were treated with 5 mM of glutamate for 24h with or without compound **BS11**. A) Cell viability was determined by calcein AM and propidium iodide (PI). Live cells were stained with green color fluorescence and dead cells were stained with yellow color fluorescence. Scale bar 200 μm. B) The protective effect of compound BS11 was determined using live and dead assay. 5 mM of glutamate treatment induced neurotoxicity. 100 μM of BS11 treatment shows neuroprotective effect against glutamate induced neurotoxicity. C) Cell viability was measured by MTT. BS11 shows neuroprotective effects between 25 μM to 100 μM range. D) Microscopic images show neuroprotective effect of BS11. 5 mM of glutamate treatment induced cytotoxicity in HT22 hippocampal neuronal cells. 100 μM of BS11 treatment shows neuroprotective effects against glutamate induced neurotoxicity. Scale bar 100 μm.

To confirm whether a compound **BS11** could reduce the level of cellular ROS, we utilized a DCFDA fluorescent probe for FACS analysis of glutamate-treated HT22 cells. DCFDA is usually non-fluorescent at lower ROS levels. ROS oxidize DCF

which treatment increased fluorescence intensity. However, the compound **BS11** treated HT22 cells exhibited decreased fluorescent intensity in both FACS and image-based analysis (Figure S1). Thus, our data demonstrated that compound **BS11** can prevent excessive cellular ROS in HT22 cells.

Because glutamate-induced neuronal cell death was dramatically suppressed by treatment with compound **BS11**, it is expected that compound **BS11** would decrease Ca^{2+} influx, which is an upstream signal of apoptosis. HT22 cells were stained with Fluo-3 AM to measure intracellular Ca^{2+}. Compared with control cells, 5mM glutamate exhibited increased green fluorescence intensity. On the contrary, cells cotreated with compound **BS11** and glutamate (5mM) exhibited decreased calcium concentration by 50%, indicated that compound **BS11** blocks Ca^{2+} influx (Figure 5).

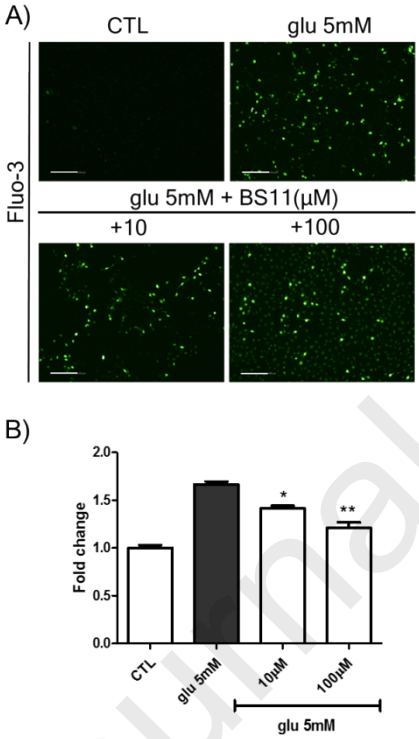


Figure 5. Compound **BS11** blocked cellular Ca^{2+} influx induced by glutamate. A) HT22 cells were treated with compound **BS11** with or without glutamate for 8h and then Flu-3 was treated to determine cellular Ca^{2+} concentration. The images were obtained by fluorescent microscope. Scale bar 200 μm. B) The fluorescent intensity was quantitatively analyzed and presented as a fold change comparing with the control.

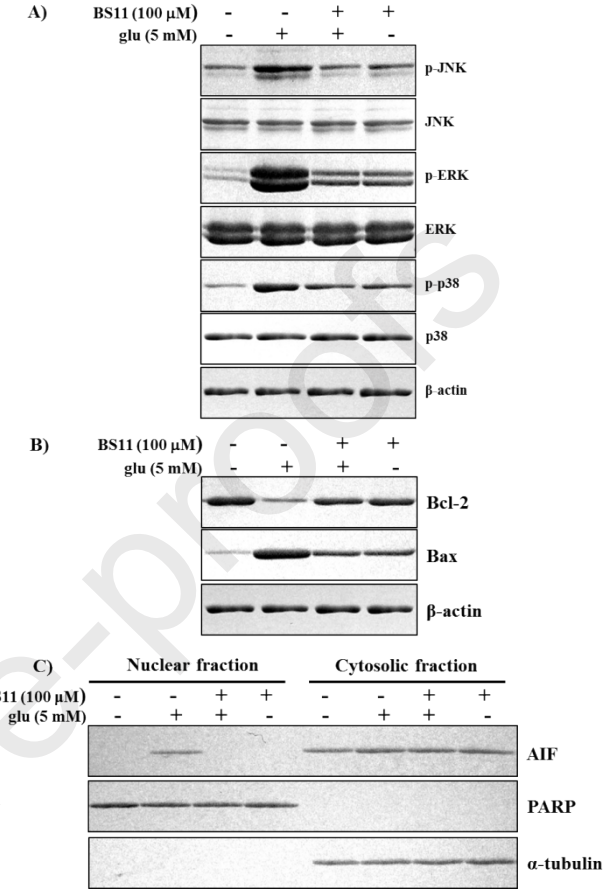


Figure 6. The effect of compound **BS11** on MAPK, Bcl-2, Bax, and AIF translocation in HT22 with glutamate induced cell death. A) Protein expression level of p-JNK, p-ERK, and p-p38 was increased by glutamate. Compound **BS11** blocked phosphorylation of JNK, ERK, and p38. B) Glutamate decreases Bcl-2 level and increases Bax level. However, compound **BS11** treatment increases Bcl-2 level and decreases Bax level. C) Translocation of AIF induced by glutamate was blocked by compound **BS11**.

To gain insight into the mechanism of protective effect of compound **BS11**, we investigated the effects of compound **BS11** treatment on MAPKs and apoptosis signaling pathway proteins using western blotting (Figure 6A).[37] MAPKs and apoptosis signaling pathways are mainly involved in the neuronal and epithelial cell death caused by ROS.[38] We therefore decided to analyze cellular levels of JNK, p-JNK, ERK, p-ERK, p38, and p-p38. In Figure 6A, glutamate treatment increased p-JNK, p-ERK, and p-p38 in HT22 cells, which resulted in the activation of MAPK signaling. In contrast, compound treatment followed by 5mM glutamate treatment decreased the level of p-JNK, p-ERK, and p-p38 in the densitometry analysis. Therefore, we assumed that compound **BS11** could ameliorate oxidative stress-induced HT22 cell death. In the additional western blot analysis, glutamate treatment increased Bax level and decreased Bcl-2 level in HT22 cells. The addition of compound **BS11** dramatically decreased level of Bax, pro-apoptotic protein. Furthermore, the level of the anti-apoptotic protein Bcl-2 was

rest… addition, translocation of AIF to the nucleus is significantly induced by 5mM glutamate treatment, and cotreatment with compound **BS11** decreased translocation of AIF to the nucleus (Figure 6C).

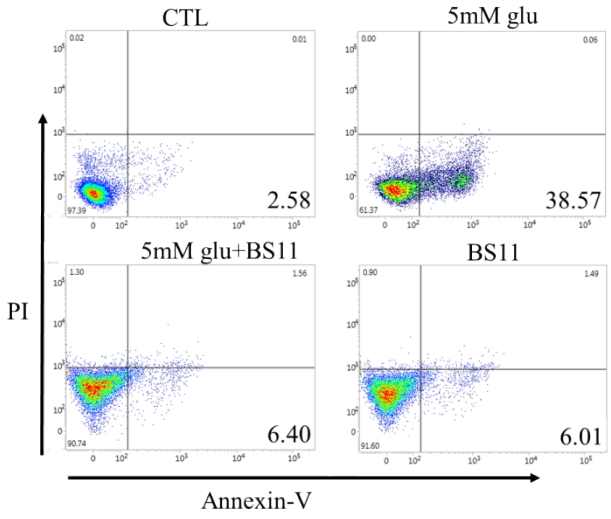


Figure 7. Compound **BS11** prevents apoptotic cell death induced by glutamate in HT22 cells. HT22 cells were exposed with glutamate in the presence or in the absence of compound **BS11** for 8h and then stained with annexin V and propidium Iodide (PI). Glutamate treated cells increased the number of apoptotic or dead cells. Cotreatment of compound **BS11** reduced the number of apoptotic or dead cells.

…we performed FACS analysis using annexin V and PI staining. In the process of apoptosis, phospho-lipids inside the membrane are moved to the outer membrane and it can be stained by annexin V. PI can permeate dead cells. Glutamate-treated cells showed a large population of apoptotic and dead cells. However, compound **BS11** treated cells showed a reduction in the number of apoptotic and dead cells (Figure 7).

In conclusion, we measured the protective effects of isoliquiritigenin analogs against glutamate-induced HT22 hippocampal neuronal cell death. In cell-based assays, we identified compound **BS11** as the most potent protective agent. In addition, western blotting analysis revealed that treatment with compound **BS11** attenuated glutamate-induced HT22 hippocampal neuronal cell death by blocking MAPKs signaling. The MAPKs signaling pathway is involved in modulating inflammation, differentiation, proliferation, and cell death. MAPK signaling pathway is activated by ROS, which can cause cell death. N-acetyl cysteine (NAC), a known antioxidant, can also reduce activation of the MAPK signaling pathway and prevent cell death by ROS. A recent report demonstrated that treatment with known chemical MAPK inhibitor shows protective effects in glutamate-treated HT22 cells.[39] In addition, natural compounds also shows protective effects by inhibiting MAPK signal cascades.[37,40,41] In previous research, Jeong and et. al. reported that NRF2/ARE pathway is involved in protecting cells.[42] However, BS11 showed no NRF2 activation effects (Figure S2). As the efforts to develop more potent protective agent for neuronal cells, further structure-activity relationship studies are underway.

Acknowledgments

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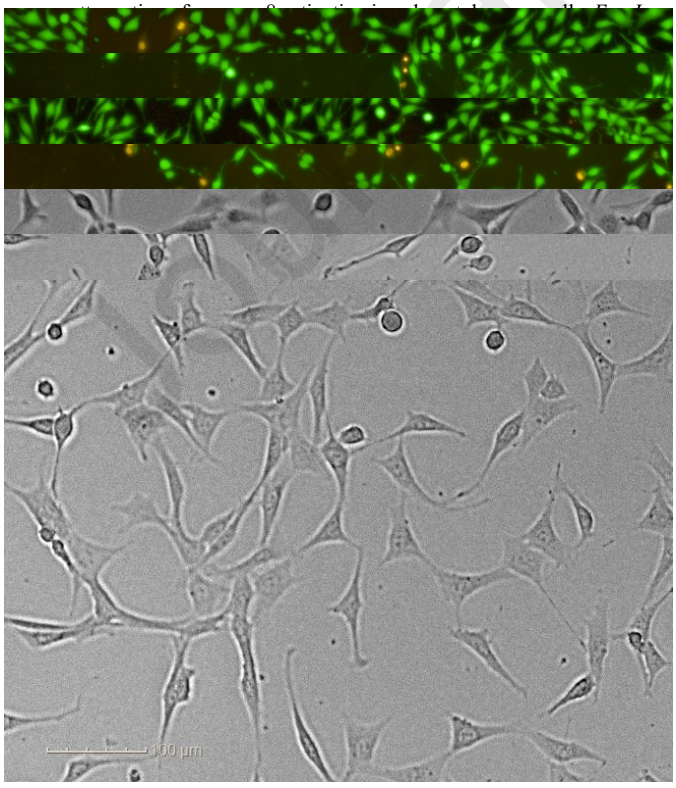

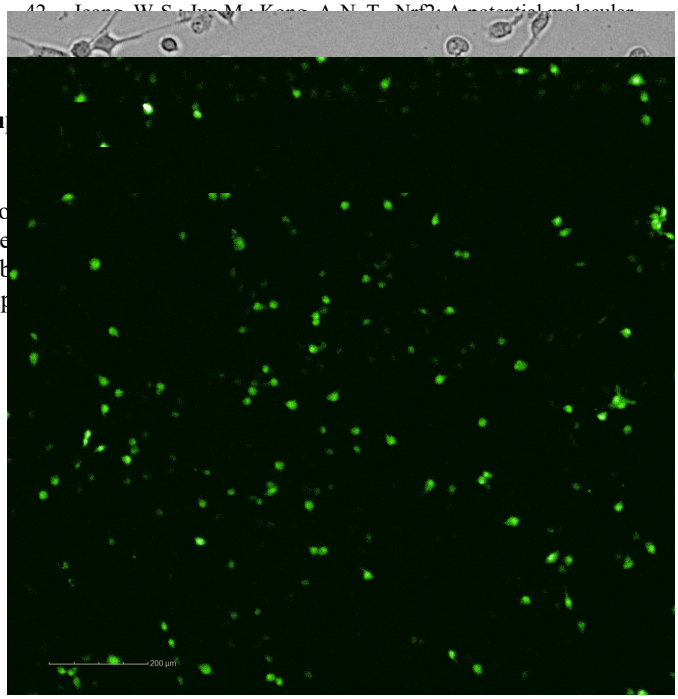

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