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Original Article

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Abstract

Prostate cancer (PC) is one of the most common cancers affecting men in the western world. Androgen deprivation was the most common approach for treatment of metastatic PC. Unfortunately, this strategy is accompanied with a several side effects such as diabetes and cardiovascular diseases. Moreover, a tumor relapse was indicated in 80-90% of cases, after 1-3 years of treatment, with a life span 1-2 year due to the development of androgen resistant tumors. This study aimed to identify metabotropic glutamate receptor 1 (mGR1) as a possible target for PC therapy. Androgen sensitive PC cell line LNcap were cultured in presence and absence of glutamate (Glu) release inhibitor Riluzole and mGR1 antagonist BAY36-7620. Functional validation of the effect of Glu deprivation on LNcap cell proliferation, motility, invasiveness was conducted using MTT, wound healing and invasion assays, respectively. Molecular validation of Glu signaling and cell apoptosis was conducted using western blot approach. The data revealed a significant effect of glutamate downmodulation on inhibiting LNcap cell proliferation, motility and invasiveness at concentration of 25µM of Riluzole and BAY36-7620. Molecular analysis showed a marked effect of Glu inhibitors on induction of pro-apoptotic markers c-PARP, c-caspases 3, 7 and 9 and inhibition of Akt dependent cell proliferation. In conclusion, in addition to Androgens, Glu plays an essential role in PC cell proliferation, motility, invasiveness and survival.

1. Introduction

Glutamate (Glu) is a non essential amino acid that plays a fundamental role, as one of the central nervous system main neurotransmitters, in brain rapid excitatory synaptic signaling **(Speyer *et al.*, 2014)**. It is responsible for various physiological processes such as individual behavior, learning and/or memory. High levels of Glu implicated in several neurodegenerative diseases related pathological changes such as hypoxia, epilepsy and/or stroke **(Lin *et al.*, 2012;Lewerenz and Maher, 2015)**. The action of Glu were exerted through two main families of receptors ionotropic receptor, which is a ligand gated ion channel activated by glutamate, and metabotropic receptors which is a type of G protein coupled receptors activated by binding of two molecules of glutamate **(Stepulak *et al.*, 2014)**.

Metabotropic glutamate receptor 1 (mGR1) is a member of group I of Glu receptors that exerts its actions through activation of the coupled phospholipase C **(Prickett and Samuels, 2012)**. Studies on mGR1 knocked out mice showed a significant sign of severe depression and motor dysfunctions. The expression of mGR1 in brain endothelial cells suggested a role that mGR1 may play in regulation of endothelial cells proliferation and angiogenesis**(Stepulak *et al.*, 2014)**. Recent reports, illustrated the involvement of mGR1 in signaling for cancer progression. Treatment of non-small cell lung cancer cell line A549 with mGR1 specific antagonist BAY36-7620 resulted in a reduction at cell proliferation and at the expression of bcl-2, HIF-1α, and VEGF proteins indicating a role of mGR1 in signaling for cancer dependent angiogenesis **(Xia *et al.*, 2016)**.

Targeted inhibition of mGR1 in glioma in vitro and in vivo models using small interfering RNA and/or mGR1 specific antagonist BAY36-7620 and glutamate inhibitor Riluzole significantly induced cell apoptosis via activation of caspases and attenuated glioma cell line U87 capability for cell migration and invasion via targeting PI3k/Akt/mTOR signaling pathway **(Zhang *et al.*, 2015a)**. Furthermore, another report illustrated the increase at glutamate secretion and mGR1 expression

in the progression of herpesvirus infection associated Kaposi's sarcoma **(Veettil *et al.*, 2014)**. A survey of serum glutamate levels in normal, primary PC and metastatic castrate-resistant PC correlated its dependent expression of mGR1 to aggressiveness of PC **(Koochekpour *et al.*, 2012)**.

This study aimed to assess the inhibitory effect of Glu deprivation on molecular signaling via which mGR1 is implicated in promoting PC LNcap cell line proliferation, motility, invasion and survival.

2. Materials and Methods

Cell culture

PC LNcap cell line were cultured using RPMI 1640 medium nourished with 10% FBS and in presence of 1% penicillin/ streptomycin (Gibco, US). Cultured cells were incubated at 37^{o}C and 5% Co_2 at humidified atmosphere.

Cell proliferation assay

To evaluate the effect of mGR1 down-modulation after glutamate release inhibition with its release inhibitor Riluzole and in presence of mGR1 antagonist BAY36-7620 on cell proliferation (Tocris, UK), LNCaP cells were seeded in cell culture 96 well Plates (500 cells/well), in complete medium and incubated for one day before starting the Riluzole and BAY36-7620 treatment. After wards, Cells were washed twice with 1 x phosphate buffer saline pH7.4 (PBS) then incubated, in 8 replicates, in the presence and absence of 1, 10, 25, 50 and 100 µg Riluzole and BAY36-7620 for 2, 4 and 6 days. Number of cells was measured using MTT assay cell proliferation kit according to manufactures' protocol (promega, USA).

Cell motility (Wound healing assay)

PC cell line LNcap were cultured in six well plates ($3x10^5$cells/well). After 24 hr, cells were kept overnight in 2% FBS nourished medium for cell synchronization, then full medium were added and cell were kept till reach confluency. A scratch with 10 µL pipette tip were induced at the middle of each plate, then Cells were washed twice with PBS to remove the detached cells. Afterwards, cells were cultured in complete medium in presence of 0, 1, 10, 25 µM of Riluzole and BAY36-7620 (Tocris. UK), photographs of the wound size were taken after 1, 2, 3 and 4 days of treatment.

Cell invasion assay

The effect of mGR1 inhibition on cell invasion was performed using 8-µm transwell filters (Co star, Corning, NY). For the invasion assay, the upper compartment was coated with 50 µg Matrigel (BD Biosciences, San Jose, CA) to form a matrix barrier. A suspension of LNcap cells (2×10^4) were seeded in a basal medium (low nourished medium) containing 0.1% BSA into the upper compartment in presence and absence of 12.5 and 25µM of Riluzole (Tocris, UK). The lower compartment was filled with 400 µl of complete medium containing 5% FBS as chemoattractant. After 24 hr, the non-migratory cells on the upper surface were removed by a cotton swab and the invaded cells to the lower compartment were photographed and counted using ocular microscope.

Protein extraction and Western blot

PC LNcap cells were cultured in duplicates in six well plates in presence of 0, 1, 10 and 25 µM of Riluzole for 48 hrs. Cell lysates were obtained by adding 200 µL of Ripa lysis buffer (Santa Cruz, USA) to harvested cells. 25 µg of total protein extract were separated using SDS-PAGE in 12 % precast gel, separated proteins were then transferred into nitrocellulose membrane (Invitrogen, USA). Western blot analysis of protein expression was conducted by probing the membranes with primary antibodies of polyclonal rabbit antihuman-mGR1 antibody (at 1:500, Sigma, USA), Polyclonal rabbit anti human C-PARP, c-caspase 3, 7, 9 , PI3K and P-AKT (308) (at 1:1000, Cell signal, USA), followed by an incubation with goat anti-rabbit, IgG–horseradish peroxidase conjugated, secondary antibody (at 1:5000, cell signal, USA). Protein levels were detected using enhanced chemiluminescence (ECL) detection system (GE Healthcare Biosciences, UK).

3. Results

Effect of Riluzole and BAY36-7620 on cell proliferation

LNcap cells were cultured in presence of 0, 1, 10, 25, 50 and 100 µM concentrations of Riluzole (glutamate release inhibitor) and BAY36-7620 (mGR1 specific antagonist). The effect of glutamate deprivation on LNcap cell proliferation was evaluated using MTT assay after 2, 4 and 6 days of Rliuzole and BAY36-7620 treatment. The data revealed that both glutamate signaling inhibitors showed significant attenuation of LNcap cells ability to proliferate with a significance started at concentration of 25µM. However, Riluzole effect was more significant in diminishing the proliferation of LNcap cells than BAY36-7620 did after 4 days of treatment compared to control cells (0µM). Meanwhile, the effect of 50 and 100 µM doses seems to be a result of cell toxicitywhich leads to sudden cell death upon exposure to both inhibitors at day 2 of treatment. Low doses of 1 and 10 µM showed a non-significant effect on cell proliferation (Fig.1).

Effect of glutamate signaling deprivation on cell motility

Cells were cultured till confluence and the ability of Riluzole and BAY36-7620 treated cells to migrate into the empty space of the induced scratch were monitored

using wound healing assay approach. Cells were treated with 0,1,10 and 25 µM of both inhibitors for several days, capability of cell motility were estimated tothe time point when control cells (0µM) migrated into and covered the empty scratch as a sign of the complete healing of the induced wound.

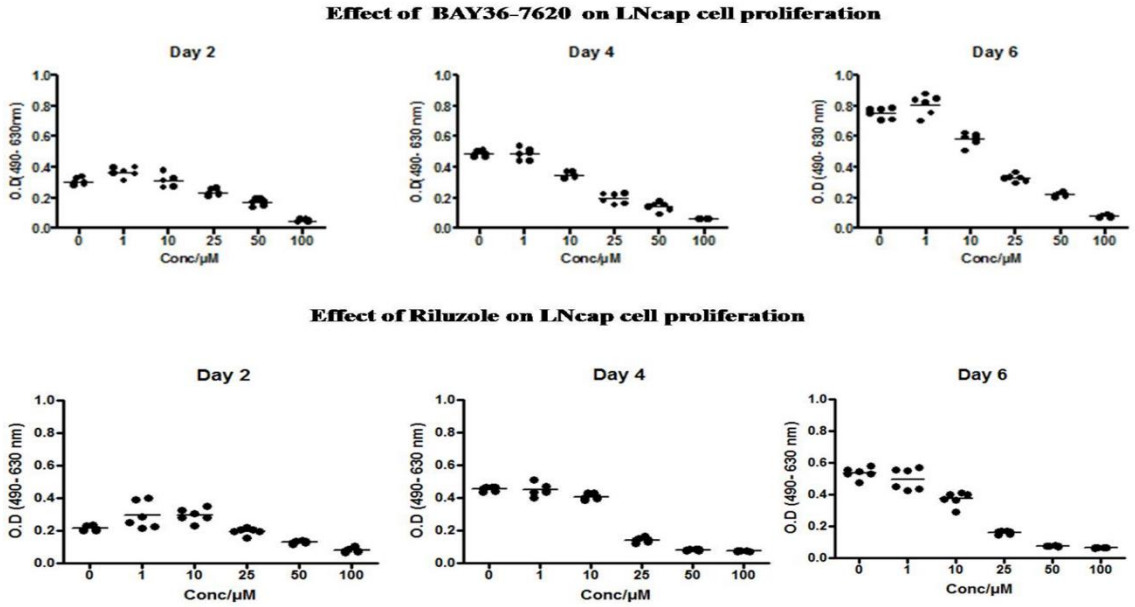

Figure 1. Effect of mGR1 antagonists on LNcap cell proliferation. (Upper panel) Rliluzole treatment at 0, 1,10, 25,50 and 100 µM concentration showed a significant effect on diminishing cell proliferation at 25 µM. **(Lower Panel)** Cells treatment with mGR1 antagonist BAY36-7620 showed a less significant effect in diminishing LNcap cell proliferation.

The resulting data illustrated a significant dose and time dependent effect of Riluzole on inhibiting cell motility, wherea dose of 25 µM of Riluzole significantly diminished the LNcap cells motilitywith a sings of cell apoptosis after 4 days of treatment (Fig. 2a). Similarly, LNcap cell treatment with BAY36-7620resulted in demolishment of cell motility at concentration of 25 µM (Fig. 2b). Similarly, to cell proliferation data, a low dose of 1, 10 µM of both inhibitors showed a non and/or less marked effect on cell motility, respectively (Fig 2).

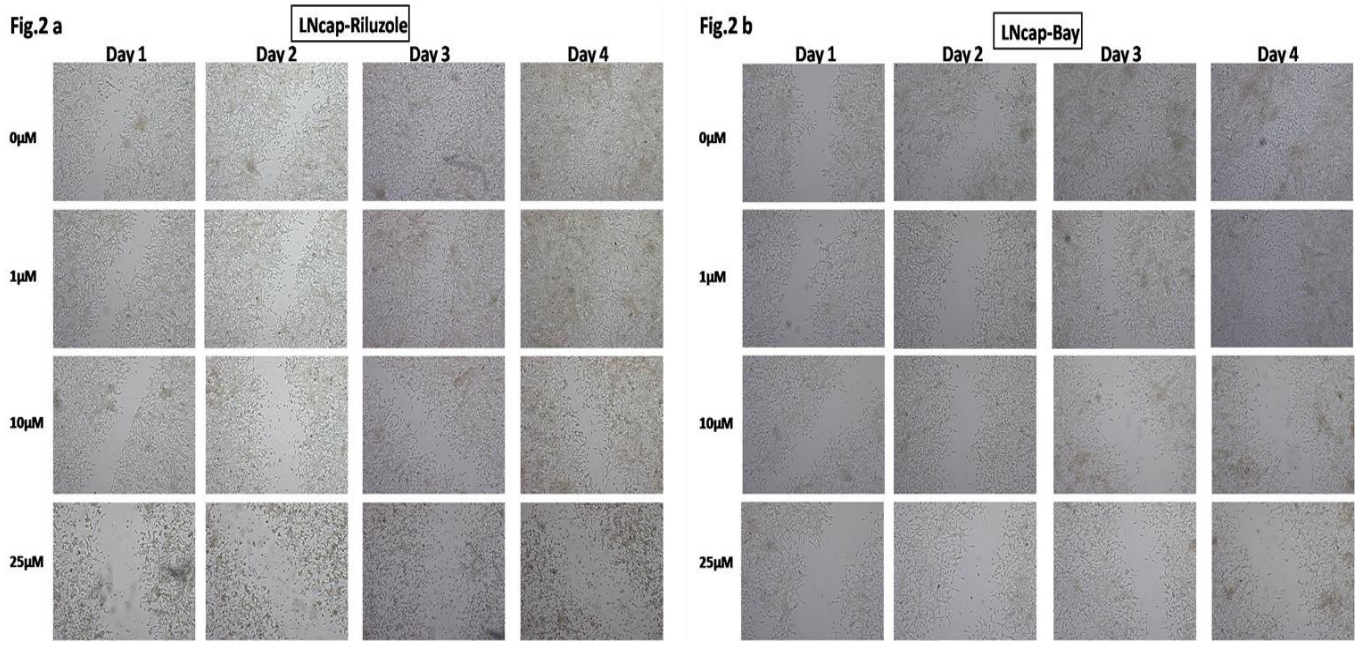

Figure 2. Effect of mGR1 antagonists on LNcap cell motility. (a.) Rliluzole treatment at 0, 1, 10 and 25 µM concentration showed a marked effect on diminishing cell motility capability to heal the induced wound at 10 and 25 µM after 4 days of treatment. **(b.)** BAY36-7620 treated cells showed a similar results to that of Riluzole in attenuating the cells from healing the induced scratch at the same concentrations after 4 days of treatment.

Effect of Riluzole on LNcap cell invasiveness.

The effect of Riluzole dependent inhibition of glutamate release on LNcap cell invasiveness were indicated by counting the number of cells that succeed in invading the Matrigel coated Boyden chambers perforated bottom into highly nourished medium. Glutamate deprivation resulted in a significant dose dependent reduction at the number of invaded cells, starting a dose of 12.5 and 25 µM of Riluzole (Fig. 3).

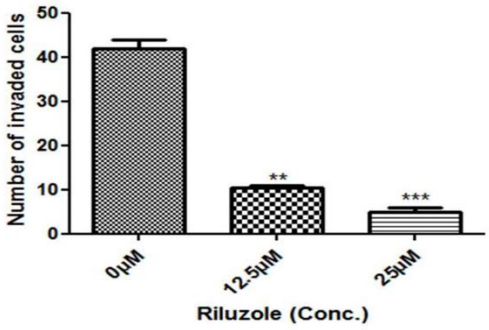

Figure 3. Effect of Riluzole on LNcap cell invasiveness. The number of invaded cells, though matrigel covered Boyden chambers, upon Riluzole treatment indicated a significant dose dependent effect of glutamate signaling deprivation on lessen the LNcap cells invasiveness. Data were presented as mean ± SEM and significance was denoted as *<0.05,**<0.001,***<0.000.1 as compared to 0µM.

Molecular signaling cascade of Riluzole dependent glutamate deprivation

Molecular analysis of the glutamate signaling for induction of cell proliferation using western blot approach pointed out that glutamate deprivation with Riluzole resulted in marked downregulation of mGR1 expression. This downregulation leads to a PI3k independent downregulation of P-Akt (308) expression. Moreover, a dose of 25 µM of Riluzole markedly resulted in reduction at p-Mek and p-Erk expression. On the other hand, in order to assess the effect of Riluzole in induction of LNcap cell apoptosis, the expression of apoptotic markers such as cleaved poly ADP-ribose polymerase (c-PARP), c-caspases 3, 7 and 9 were assessed. The data indicated a marked Riluzole dependent induction of the apoptotic marker expression at dose of 25µM in order to activate cell apoptosis (Fig. 4).

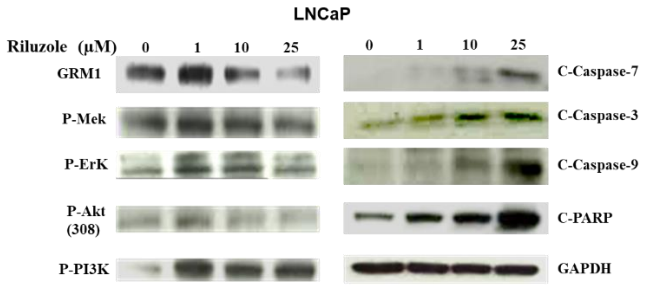

Figure 4. Western blot data showed effect of Riluzole on inhibiting Glu signaling of cell proliferation and survival. Data revealed marked downregulation of mGR1, P-Akt 308, P-Mek, P-Erk expression at 25 µM of Riluzole. Meanwhile, marked upregulation of pro-apoptotic c-Caspase-3,-7,-9 and C-PARP after 48 hr of LNcap cell treatment with 25 µM of Riluzole**.**

4. Discussion

Even though, Glutamate, as neurotransmitter, for a long term was concerned in neurodegenerative diseases evolvement, recent reports pointed out the implication of glutamate expression in lung, glioma and breast cancer progression **(Prickett and Samuels, 2012;Willard and Koochekpour, 2013;Fazzari *et al.*, 2015)**. This study aimed to determine the possible implication of glutamate signaling in promoting PC cell proliferation, migration, invasion and survival.

The inhibition of glutamate release via Riluzole and/or demolishing of its signaling using mGR1 antagonist BAY36-7620 resulted in a significant reduction of LNcap cells ability to proliferate after 2 days of treatment (Fig. 1). The impact of Glutamate signaling in promoting glioma, sarcoma, breast and ovarian cancer cell proliferation was recently addressed **(Vanhoutte and Hermans, 2008;Veettil *et al.*, 2014;Banda *et al.*, 2014;Yuan *et al.*, 2015)**. Furthermore, the effect of glutamate on LNcap cell motility was determined using wound healing assay. The inhibition of Glu signaling via either Riluzole and/or BAY36-7620 resulted in a marked reduction at the cell ability to migrate after 4 days of treatment (Fig 2a&b). Cells exposed to higher doses of 50 and 100 µM of both inhibitors showed a marked signs of cell death after 1 day of treatment (data not shown). Inhibition of mGR1 expression in U87 glioma cell line attenuated the cell migration and invasion capability **(Zhang *et al.*, 2015b)**. Treatment of the triple negative breast cancer cell line MCF10A with mGR1 antagonist lead to a significant inhibition at cell proliferation **(Banda *et al.*, 2014)**.

Functional validation of the effect of Glu deprivation on cell invasiveness using *in vitro* invasion assay illustrated the pivotal role of Glu in signaling for promotion of PC capability to invade the extracellular matrix to metastasize into distant organs. The data revealed a significant dose dependent effect of Glu expression demolishment via Riluzole in downregulating the number of invaded cells into the nourished medium (Fig. 3). Recently, the implication of Glu signaling on promotion of melanoma and breast cancer cell invasiveness was well established **(Song *et al.*, 2012;Banda *et al.*, 2014)**. The effect of Glu deprivation on inhibition of cell proliferation was further confirmed by molecular evaluation of the role of Glu in stimulation of Akt phosphorylation. The effect of Riluzole in inhibition of Glu signaling was confirmed by the

resulting in a PI3K independent reduction at phosphorylated Akt (Thr 308) (Fig 4). The role of Akt, as carcinogen, signaling in promotion of cancer

cell proliferation is an indeed fact that was elucidated by several reports **(Garcia *et al.*, 2006;Xu *et al.*, 2012;Wang *et al.*, 2016)**.

Molecular validation of the effect of Glu deficiency on induction of cell apoptosis was elucidated via assessment of the expression levels of several pro-apoptotic markers in Riluzole treated LNcap cells. A dose dependent effect of Riluzole in induction of c-PARP, C-caspase-3,-7 and -9 expression was illustrated after 48 hr of treatment (Fig. 4). PARP is DNA repairing enzymes that been activated dependent to P53 signaling. In case of cell apoptosis, active caspase -3 and -7 induced PARP cleavageinto two fragments of 89 and 24 kDa in a process that been considered pinpointing of functional caspase activation **(Bressenot *et al.*, 2009)**.

In summary, targeting of Glu signaling represents a promising therapeutic approach in PC treatment which was illustrated by the pivotal role of Glu deprivation in induction of cell apoptosis and ceasing the androgen sensitive PC cell line LNcap ability to proliferate, migrate and invasiveness.

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