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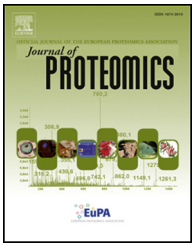

Quantitative proteomic analysis of global effect of LLL12 on U87 cell's proteome: An insight into the molecular mechanism of LLL12

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ABSTRACT

Glioblastoma multiforme (GBM) is one of the most devastating and dreadful WHO grade IV brain tumors associated with poor survival rate and limited therapeutics. Signal transducer and activator of transcription factor 3 (STAT3) is persistently active in several cancers, including gliomas, and STAT3 inhibitors hold great promise for treatment of glioma. LLL12, a curcumin derivative, inhibits STAT3 functions, thereby reduces growth of GBM. However, the global effects of targeting STAT3 using LLL12 have not been studied well. To shed light on this aspect, we performed quantitative proteomic analyses using differential in-gel electrophoresis (2D-DIGE) and isobaric tags for relative and absolute quantitation (iTRAQ) as well as label-free mass spectrometric analysis with 0.5 μM (IC_{50}) concentration of LLL12. Through this approach, we identified a total dataset of 1012 proteins with 1% FDR, of which 143 proteins were differentially expressed associated with various cellular functions. Results suggest that LLL12 influences central cellular metabolism and cytoskeletal proteins, in addition to its apoptosis inducing and anti-angiogenic activities, which altogether contribute to its anti-tumorigenic function. Interestingly, triose phosphate isomerase (TPI), phosphoglycerate mutase 1 (PGAM1), adaptor molecule (CRK2), protein DJ-1 (PARK7) and basic transcription factor 3 (BTF3) were found to be down-regulated and can be studied further to understand their therapeutic potential in gliomas. TPI1 and PGAM1 protein expressions were validated using immunoblot. Conclusively, our results suggest the therapeutic potential of LLL12 and it can be investigated further for a significant role in glioma treatment.

Biological significance
LLL12 holds great promise for therapeutic development in gliomas with constitutive expression of STAT3. This study investigated the global effect of LLL12 on the proteome of U87 glioma cells using complementary proteomic approaches, and our findings suggest that LLL12 influences central metabolism, translation, transport processes, and cytoskeleton of a cell in addition to its anti-angiogenic and apoptosis inducing functions which altogether contributes to anti-tumorigenic activity of LLL12. This study leads to the identification of several proteins which may serve as prognostic or predictive markers in GBM. We identified

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TPI1, PGAM1, CRK and BTF3 as potential therapeutic targets and further investigations on these candidates may facilitate therapeutic development.

1. Introduction

Glioblastoma (WHO grade IV) is the most common and severe form of malignant brain tumors [1]. The standard treatment of glioma, which includes surgical resection of tumor followed by radiation and chemotherapy, is relatively ineffective and most of the patients die around 6–12 months after diagnosis because of the infiltrative and aggressive nature of GBM [2–4]. Therefore, recent research mainly focuses on understanding the signaling pathways implicated in glioblastoma progression. This is a challenging task because of the involvement of several signaling pathways in the progression of GBM, such as phosphoinositide-3 kinase, AKT, Ras, and mitogen-activated protein kinase pathways (MAPKs), pathways mediated through receptor tyrosine kinases (RTKs) etc. All these pathways seem to contribute strongly to the growth and promotion of glioblastoma through some transcription factors [3,5]. Dysfunction of STAT3 protein, which is a transcription factor, has been shown in several cancer including gliomas [6,7]. It has been shown that STAT3 protein is activated by JAKs and other kinases, cross-talk with other cellular proteins and regulate functions of downstream molecules. Identification of their interacting partners and downstream molecules will therefore help in understanding the role of the STAT3 protein in various cellular processes.

Function of STAT3 as regulator of transcriptional expression of genes responsible for proliferation, suppressor of apoptosis, and inducer of angiogenesis is well known [8]. Besides having a core role as a transcription factor in several cancers, recent studies have also shed light on the importance of STAT3 as a regulator of the cellular metabolism. STAT3 regulates the cell metabolism in primary fibroblasts and in STAT3-dependent tumor cell lines by inducing the aerobic glycolysis and by down-regulating mitochondrial activity in an HIF-1α-dependent and HIF-1α-independent manner, respectively [9]. This mitochondrial function of STAT3 requires phosphorylation at the S727 residue. Similar evidences for mitochondrial activity of STAT3 have been reported in another study where decreased activities of complex I and complex II of electron transport chain was observed in $STAT3^{-/-}$ cells [10]. STAT3 regulates various cellular functions in cancer cells and seems to be a potential target for therapeutic interventions and hence several natural and synthetic compounds have been identified and studied in literature as inhibitors of STAT3. However, the effect of these inhibitors on the proteome of these cells has not been studied earlier which could help us to understand the therapeutic promise of such potential inhibitors. Till date numerous small molecules have been reported to inhibit STAT3 functions. Curcumin, a natural product, inhibits STAT3 but also has been identified as an inhibitor of numerous other signaling pathways. LLL12, derived from curcumin, has been reported to inhibit STAT3 dimerization and thus further downstream signaling. This compound has been shown to inhibit growth of several cancer cells including GBM cells [11,12].

Several studies have been performed using mass spectrometric based proteomics approach to understand the molecular patho-physiology of glioma as well as for biomarker discovery [13–17]. In this study, our major objective was to elucidate the effect of LLL12 treatment on the proteome of U87 glioma cells. This study may also help us to gain insight into the molecular mechanism of action of these inhibitors. Here, we used labeled (gel-based and gel-free) as well as label-free complementary approaches in order to increase proteome coverage, considering the advantages and limitations of these approaches [18,19]. Proteomic analysis using differential in-gel electrophoresis (2D-DIGE), isobaric tags for relative and absolute quantitation (iTRAQ) and nano-LC-MS/MS revealed 143 significant differentially expressed proteins in response to LLL12 in U87 glioma cells. Biological significance of some of these important proteins and associated signaling pathways are discussed. PGAM1, in addition to STAT3, was identified as a possible predictive marker for therapeutic interventions in GBM. Adaptor molecule (CRK2) and protein DJ-1 which play essential roles in disease progression and have therapeutic potential are also discussed in this report. With proteomic analysis, new insight may be obtained about functions of STAT3 and the molecular mechanism of LLL12 that ultimately may lead to the identification of predictive marker for GBM.

2. Materials and methods

2.1. Cell culture

Glioma cell lines U87 and U373 were received from Dr. Neelam Shirsat's laboratory, ACTREC, Kharghar (India). U87 cells were maintained in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin. All cell lines were kept in a humidified 37 °C incubator with 5% CO_2.

2.2. STAT3 inhibitor and chemicals

LLL12 (5-hydroxy-9,10-dioxo-9,10-dihydroanthracene-1-sulfonamide) was purchased from BioVision Inc., USA. Cell culture grade chemicals as Dulbecco's modified Eagle medium (DMEM), penicillin/streptomycin, trypsin, phosphate buffer saline (PBS), fetal bovine serum (FBS), dimethyl sulfoxide (DMSO) were purchased from HiMedia. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reagent was purchased from Sigma Aldrich, India. Trizol reagent was purchased from Invitrogen.

2.3. Cell viability assay

Glioblastoma cell line U87 was seeded in 96-well plate at a density of 3000 cells per well in 100 μL of media. The cells were