Small molecules, LLL12 and FLLL32 inhibit STAT3 and exhibit potent growth suppressive activity in oOsteosarcoma cells and tumor growth in vivo.

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ABSTRACT

Constitutive activation of Signal Transducers and Activators of Transcription 3 (STAT3 signaling) is frequently detected in most types of cancer, including human osteosarcoma ~~cells~~, hence configuring its potential as a novel cancer therapeutic agent. To evaluate the therapeutic potential of targeting STAT3 in human osteosarcoma, we tested two non-peptide, cell-permeable, small molecular STAT3 inhibitors, termed as LLL12 and FLLL32 that were recently developed in our laboratory.

Our work demonstrated that LLL12 and FLLL32 effectively inhibit STAT3 phosphorylation and the expression of STAT3 downstream target genes in human osteosarcoma cell lines. IL-6 is an interleukin that is well known to potentiate STAT3 phosphorylation, and this stimulating effect was inhibited by LLL12 and FLLL32. The inhibition of STAT3 resulted in the induction of apoptosis as evidenced by cleaved caspase-3 and FITC Annexin V staining using ~~Flow~~ flow cytometry. We also showed that LLL12 and FLLL32 are potent to inhibit cell viability in human osteosarcoma cells and have lower IC50 values than five other previously reported JAK2 and STAT3 inhibitors. The inhibition of STAT3 by LLL12 and FLLL32 also resulted in the reduction of ~~Plating~~ plating efficiency and migration in osteosarcoma cells. Furthermore, we demonstrated that LLL12 and FLLL32 inhibit tumor growth in mice xenografts using SJSA and OS-33 osteosarcoma cell lines. Our results provided evidences that constitutive STAT3 signaling is required for osteosarcoma survival and migration *in vitro* and tumor growth *in vivo*. Blocking persistent STAT3 signaling by LLL12 and FLLL32 may be a novel therapeutic approach for human osteosarcoma treatment.

1. Introduction

Osteosarcoma is the most common malignant bone tumor in children and young adults with approximately 750 to 900 new cases diagnosed annually in the United States, and 400 of these arise in children and adolescents younger than 20 years of age. It generally presents as a painful mass that has a mixed lytic and sclerotic appearance on radiography and is characterized histologically by the presence of malignant cells that produce osteoid or immature bone (1-3). The treatment of osteosarcoma has not changed significantly over the last 20 years and consists of a combination of chemotherapy and aggressive surgical resection of the primary tumor and all sites of metastatic disease. Unfortunately, the improvement in outcome seen for many other cancers has not been demonstrated in osteosarcoma, with 5-year disease free survival rates of 40-60% for patients with localized disease and less than 20% for patients with metastasis at diagnosis(5). Also, because the chemotherapy regimens consist of high doses of anthracycline and cisplatin they carry a significant risk of long term toxicities including cardiotoxicity and ototoxicity. It is clear that there is a need for new and more effective therapy for osteosarcoma to help improve outcomes for these patients.

The Signal Transducers and Activators of Transcription (STAT) proteins family is a group of related proteins that play a role in relaying signals from cytokines and growth factors [1; 2; 3]. Ligand dependent activation of STAT3 regulatory cascade is often associated with modulation of cell growth and differentiation; hence, ~~accordling;~~ the abnormal activation of STAT proteins is becoming more frequently associated with un-restricted cell growth and malignant transformation [4] .Constitutively activated STAT3 has been described in human and canine osteosarcoma cell lines [5; 6]

STAT3 has been classified as a proto-oncogene because an activated form of STAT3 can mediate oncogenic transformation in cultured cells and tumor formation in nude mice (Bromberg et al, 1999; Bowman et al, 2000). Constitutive STAT3 signaling may participate in oncogenesis by stimulating cell proliferation, promoting angiogenesis, mediating immune evasion, and conferring resistance to apoptosis induced by conventional therapies [7].

STAT3 activation occurs when the Tyrosine 705 (Tyr705) residue is phosphorylated, leading to dimerization and translocation from the cytoplasm to the nucleus [8; 9; 10]. In the nucleus, STAT3 binds to the promoters of the downstream target genes and induces the transcription and up regulation of proliferation and anti-apoptotic associated proteins [2; 8; 11; 12]. Therefore, constitutive STAT3 signaling is involved in stimulating cell cycle progression and preventing apoptosis which both contribute to malignant progression [2; 8]. In addition, persistently activated STAT3 plays a role in impairing both innate and adaptive immune responses by enhancing immunologic tolerance and enabling cancer cells to evade immune surveillance [13]. Further, the survival of these tumors appears to depend on the presence of STAT3 signaling [1; 14].

The implications of constitutive STAT3 signaling in tumors have presented it as a possible target for cancer treatment. Experiments aimed at blocking STAT3 signaling using dominant-negative STAT3, RNA interference, and STAT3 antisense oligonucleotides have provided further evidence of the potential of STAT3 as a target for treating cancer [1; 3; 15] . Inhibiting STAT3 using the stated approaches have been successful, resulting in an inhibition of growth and the

induction of death in tumors. It was also determined that in normal cells, blocking STAT3 is neither harmful nor toxic to the cells [1; 14]. Given the oncogenic functions of STAT3 and the promise of inhibiting it, directly targeting STAT3 signaling represents a potential therapeutic approach to treating cancer.

We aim at demonstrating that LLL12 and FLLL32 inhibit STAT3 phosphorylation (Tyr705) and STAT3 activities, down-regulate STAT3 downstream targets, inhibit proliferation, colony formation, cell migration, and induce apoptosis in osteosarcoma cells. Tumor xenografts were are also used to demonstrate the anti-tumor growth effects of LLL12 and FLLL32 in mice models.

2. Materials and Methods

2.1. Chemical Reagents.

Chemicals among which were MTT, Tris, glycine, Nacl, SDS, Creamphor, Solubilization solution (N, N-dimethylformamide) and DMSO were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO).

2.2. Inhibitors.

LLL12, a STAT3 inhibitor, and FLLL32, a JAK2/STAT3 inhibitor, were synthesized in Dr. Pui-Kai Li's and Dr James Fuchs's laboratory (College of Pharmacy, The Ohio State University). Curcumin was obtained from Sigma-Aldrich (St. Louis MO). The powder was dissolved in sterile dimethyl sulfoxide (DMSO) to make a 20mM stock solution. Aliquots of the stock solution were stored at -20˚C.

2.3. Cell lines/Culture

Human osteosarcoma cancer cell lines (U2O~~0~~S, SAOS2 and SJSA) were purchased from the American type culture collection (ATCC - Manassas, VA). OS-33 xenograft was kindly provided by Dr Houghton's laboratory at the children's cancer research institute, Nationwide children's Hospital; and is a xenograft from an ~~Osteosarcoma~~ osteosarcoma patient. All the cell lines were maintained in Dulbecco's Modified Eagle Medium supplemented with 10% FBS, 4.5 g/L L-glutamine, sodium pyruvate, and 1% penicillin/streptomycin. All cell lines were stored in a humidified 37^{o}C incubator with 5% CO_2.

2.4. Cell viability assay/MTT assay

Osteosarcoma cell lines (U2OS~~0s~~, SAOS2, and SJSA) were seeded in 96-well plates (3,000 cells/well) and treated with 0.5-5 µmol/L of LLL12 and FLLL32 in triplicates. The cells were incubated at 37oC for 72 hours. 25 µl of MTT was added to each sample and incubated for 3.5 hours. After this, 100 µl of N, N-dimethylformamide solubilization solution was added to each well. The absorbance at 450 nm was read the following day. Half-~~Maximal~~ maximal inhibitory concentrations (IC_{50}) were determined using Sigma Plot 9.0 software (Systat Software Inc - Chicago, IL).

2.5. FITC Annexin V Apoptosis Detection

Human osteosarcoma ~~cancer~~ cell lines (U2O~~0~~S, SAOS2 and SJSA) were treated for 24 hours with LLL12 and FLL~~l~~32 (5-10 µM). The cells were then harvested and stained with FITC dye and PI dye (FITC V Annexin staining precedes the loss of membrane integrity which accompanies the latest stages of cell death resulting from either apoptotic or necrotic process, therefore staining with FITC Annexin V is typically used in conjunction with a vital dye such as propidium iodide (PI) to allow early identification of early apoptotic cells (PI negative Annexin V positive)

The cells were then passed through flow cytometry to detect FITC positive PI negative cells.

2.6. Western blotting

Human osteosarcoma cell lines (U2O~~0~~S, SAOS2 and SJSA) were treated with LLL12 (5µM or 10µM) or DMSO at 70-90% confluence in presence of 10% FBS for 24 hours, and then lysed in cold RIPA lysis buffer containing protease inhibitors and subjected to SDS-PAGE. Membranes

were probed with a 1:1000 dilution of antibodies (Cell Signaling Tech –Danvers, MA.) against phospho-specific STAT3 (Tyrosine 705), phospho-specific ERK1/2 (Threonine 202/Tyrosine 204), cleaved Poly (ADP-ribose) polymerase (PARP), cleaved caspase-3, cyclin D, Bcl-2, survivin, and GAPDH. The membrane was developed in ECF solution and then the membranes were analyzed using a film developer.

2.7. Reverse transcription PCR analysis

RNA was collected from U2O~~0~~S, SAOS2 & SJSA cells with RNeasy Kits (Qiagen - Valencia CA) following 24 hours of treatment with LLL12 and FLLL32. cDNA was generated from 500ng sample RNA using Omniscript RT (Qiagen - Valencia, CA). Primer sequences and source information can be found in supplemental Table 1.

2.8. IL-6 induction of STAT3 phosphorylation

SJSA osteosarcoma ~~cancer~~ cells were seeded in 10cm plates and allowed to adhere overnight. The following night, the cells were serum starved. The cells were then left untreated or were treated with LLL12 and FLLL32 (0.5µM - 20µM) or DMSO. After 2 hours, the untreated and LLL12 treated cells were stimulated by IL-6 (50ng/mL). The cells were harvested at 30 min and analyzed by ~~western~~ Western blot.

2.9. Wound healing/Cell Migration Assay

U2OS, SAOS2 and SJSA o~~O~~steosarcoma cells were seeded in a six-well plate. Approximately 24 hours later, when the cells were 100% confluent, the monolayer was scratched using a 1 mL pipette tip, and washed once to remove non-adherent cells. New medium containing LLL12 and

FLLL32 (2.5-10μM) or DMSO was added. The treatments were removed after 4 hours and fresh medium was added. After an additional 20 hours without treatment the cells were observed under the microscope. When the wound in the control was closed, the inhibition of migration was assessed by using a microscope.

2.10. Colony formation assay

Osteosarcoma ~~cancer~~ cells with 10% FBS in DMEM were treated for 5 hours with LLL12 and FLLL32 (2.5μM-10μM) or DMSO. The cells were then plated at a density of 500 cells per dish. The cells were maintained at 37^{o}C and allowed to grow for two weeks. The colonies were stained using crystal violet dye (5ml per plate). Pictures of the colonies were taken using a Leica MZ 16FA inverted microscope (Leica Microsystems- Deerfield, IL) with a 7.4 Slider Camera (Diagnostic Instruments Inc- Sterling Heights MI). The colonies were scored by counting and numbers were normalized as a percentage of colonies formed in DMSO.

2.11. Mouse xenografts

All animal studies were conducted in accordance with the principles and standard procedures approved by IACUC of the Research Institute at Nationwide Children's Hospital. SJSAcells ($1x10^{7}$) in Matrigel (BD Science Franklin Lakes, NJ) were implanted subcutaneously into the flank region of 4-5-wk-old female athymic nude mice. After tumors developed, the mice were randomized into two groups and treated with 5 mg/kg LLL12; 50mg/kg FLLL32 or vehicle intraperitoneally on a daily basis. The inhibitors were combined with Cremaphor, DMSO and 5% Dextrose water to enhance delivery and limit toxicity encountered with DMSO only as the

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mixing base. OS-33 cells were transplanted into female athymic mice. Following tumor engraftment the mice were randomized into two groups and treated with 5 mg/kg LLL12; 50mg/kg FLLL32 or vehicle intraperitoneally on a daily basis. ~~The inhibitors were combined with Cremaphor, DMSO and 5% Dextrose water to enhance delivery and limit toxicity encountered with DMSO only as the mixing base.~~ Tumor growth was determined by measuring the major (L) and minor (W) diameter with a caliper. The tumor volume was calculated according to the formula: Tumor volume= 0.5236 x L x W^2.

2.11.1. Statistical analysis

Statistical analysis was performed using ~~Excel 2007~~ independent t-test (Microsoft Excel 2007). The probability (p) values less than 0.05 were considered statistically significant.

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3. Results

3.1. LLL12 and FLLL32 inhibit constitutive STAT3 phosphorylation in ~~Osteosarcoma~~ osteosarcoma cell lines.

STAT3 activity is ultimately dependent upon phosphorylation of residue Y705. The ability of LLL12 and FLLL32 (Fig.1) to inhibit STAT3 activation in osteosarcoma cells was investigated. Western blot analysis with a phospho-Y705-specific STAT3 antibody ~~using~~was used in ~~Osteosarcoma~~ osteosarcoma cell lines ~~U20S~~U2OS, SAOS2 and SJSA. LLL12 and FLLL32 effectively reduced levels of Y705-phosphorylated STAT3 (hereafter referred to as P-STAT3) in ~~U20S~~U2OS, SAOS2 and SJSA osteosarcoma cell lines in a dose-dependent fashion (Fig. 2A, 2B and 2C) .The expression of ~~STAt3~~ STAT3 downstream target genes such as ~~Cyclin~~ cyclin D1(4,34,35) were likewise downregulated (Fig.2A) Decreases in P-STAT3 were concomitant with increased cleavage of pro-caspase-3 and poly-ADP ribose polymerase (PARP), one of the immediate substrates of this effector caspase.

3.2. LLL12 and FLLL32 induce apoptosis in osteosarcoma cell lines and inhibit cell viability

In apoptotic cells, the membrane phospholipid phosphatidylserine (PS) is translocated from the inner to the outer leaflet of the plasma membrane, thereby exposing PS to the external cellular membrane. Annexin V conjugated to fluorochromes including FITC has a high affinity for PS

and binds to cells with exposed PS; and is used in ~~conjuction~~conjunction with PI dye to identify early apoptotic cells (PI negative Annexin V positive) via flow cytometry. A significant population of osteosarcoma cell lines treated for 24 hours with LLL12 and FLLL32 showed early apoptosis when stained with staining detected via flow cytometry (Fig. 2D)

LLL12 and FLLL32 show a lower half-maximal inhibitory concentration (IC_{50}) than other STAT3/JAK2 small molecule inhibitors (table 1). This demonstrates that LLL12 and FLLL32 exhibit potent suppressive activity in cell viability of osteosarcoma cells (~~U20S~~U2OS, SAOS2, and SJSA) when compared with LLL3, FLLL101, Stattic, S31-201 and AG490.

3.3. LLL12 and FLLL32 are potent inhibitors of STAT3-mediated gene transcription

STAT3 activation is known to regulate the expression of various gene products involved in cell survival, proliferation, angiogenesis and chemoresistance (45). Several STAT3 target genes, such as ~~Cyclin~~ cyclin D1, Bcl-2, Bcl-xL~~l~~, ~~Survivin~~ survivin (4, 34,35) expression was mostly down regulated by LLL12 and FLLL32 compared to a DMSO control in ~~Osteosarcoma~~ osteosarcoma cell lines (Figure 3A, 3B and 3C)

3.4. LLL12 and FLLL32 suppress IL-6 induced STAT3 phosphorylation

Activation of STAT3 can be induced by IL-6 [9; 10]. Osteosarcoma cells (SJSA) which express lower levels of constitutively phosphorylated STAT3, were used to determine if LLL12 is capable of inhibiting IL-6 induced STAT3 phosphorylation. We found that the treatment with LLL12 inhibited the induction of STAT3 phosphorylation by IL-6 (Fig. 4). FLLL32 was also able to inhibit the induction of STAT3 phosphorylation by IL-6, and its action was found to be more potent than curcumin (Fig.4)

3.5. LLL12 and FLLL32 suppress colony formation/plating efficiency in oOsteosarcoma cell lines

To determine the colony formation activity of cells transduced with LLL12 and FLLL32, a plating efficiency assay was performed. Malignant cells have been shown to have a higher plating efficiency than normal cells. U20SU2OS, SAOS2 and SJSA cells were treated for 5 hours with LLL12 and FLLL32 and compared to the DMSO control, led to a decrease of over 95% in colony formation (Fig. 5A and 5B)

3.6. LLL12 and FLLL32 inhibit wound healing that occurs via cell migration

Cell migration is important in physiologic processes, such as wound healing and tumor metastasis. Wound healing and cancer are both characterized by cell proliferation, remodeling of extracellular matrix, cell invasion and migration, new blood vessel formation, and modulation of blood coagulation (98). It has been shown that STAT3 regulates a common set of genes involved in wound healing and cancer (99). To assess the affect of LLL12 and FLLL32 on cell migration a wound-healing assay was done. Following the creation of a wound, cells were treated with various concentrations of LLL12 and FLLL32. The treatment was removed after 4 hours. Cells were allowed to migrate into the denuded area for 24 hours. Treatment with LLL12 and FLLL32

at a concentration of 2.5 µM or higher caused a significant decrease in cell migration in ~~U20S~~U2OS, SAOS2 and SJSA (Fig. 6A and 6B)

3.7. LLL12 and FLLL32 suppress tumor growth in vivo

Mouse xenograft experiments were then performed to gauge the anti-tumor effects of our compounds in an *in vivo* system. Two groups of 12 athymic nude mice were obtained for tumor xenografts with SJSA & OS-33 cell line. After seeding and allowing the tumors to develop for 7 days, six mice from each group were given daily intraperitoneal doses of 50 mg/kg FLLL32 and 5mg/kg LLL12, whereas the other twelve served as controls. The administration of LLL12 and FLLL32 resulted in significantly reduced tumor burdens in the SJSA and OS-33 xenografted mice relative to their DMSO-treated counterparts (Fig. 7A and 8A). Western blots performed with tumor tissue samples harvested from these mice also showed decreases in total levels of P-STAT3 (Fig. 7B)

4. Discussion

The response to therapy in metastatic osteosarcoma continues to be dismal. The disease free response in standard response patients remains only 40-60% .This shows that there is a need to optimize therapy, and STAT3 inhibitors can play a role here.~~.~~ STAT3, a member of the STAT family of transcription factors, is an oncogenic protein that is frequently activated in many types of cancer [1; 2; 3]. Chen et al 2007 showed that STAT3 phosphorylation levels were elevated in ~~Osteosarcoma~~osteosarcoma, rhabdomyosarcoma and other soft- tissue sarcoma tissues and cell lines. In its active form, it is found predominantly in the cytoplasm. Phosphorylation at Tyr-705 causes it to dimerise and translocate to the nucleus where it binds to specific promoter sequences on its target genes. It has been clearly shown that phosphorylation of tyrosine 705 is important for the activation of STAT3.The mechanisms underlying the elevated STAT3 phosphorylation in sarcoma tissues is not clear and might be due to constant upstream activation by cytokines and growth factors, down regulation of counter balancing signal transduction pathways, such as SOCS1, or both. STAT3 activation results in the expression of downstream genes which promote cell proliferation and provide resistance to apoptosis, such as ~~Cyclin~~ cyclin D1 and Bcl-2 respectively [2; 8; 11; 12]. STAT3 has been classified as an oncogene because activated STAT3 can mediate oncogenic transformation in cultured cells and tumor formation in nude mice [11]. In contrast, STAT3 deficient fibroblasts were shown to be resistant to transformation by a variety of oncogenes [14; 16]. Constitutive STAT3 signaling participates in oncogenesis by stimulating

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cell proliferation, mediating immune evasion, promoting angiogenesis, and conferring resistance to apoptosis induced by conventional therapies [1; 12; 13; 17]. Studies have shown that inhibition of STAT3 activation through various methods such as RNA interference, double-stranded decoy oligodeoxynucleotides, and small molecule inhibitors results in decreased viability and apoptosis of a variety of human tumor cell lines including those derived from sarcomas. Despite a critical role in embryogenesis, the presence of STAT3 is dispensable in the majority of tissue types, presumably owing to functional redundancies in other signaling pathways. As such, there is a potentially large therapeutic index for targeting STAT3 in cancer therapy [18].

In this study we evaluated the inhibitory efficacy of LLL12 and FLLL32 in human osteosarcoma cells. We have shown that treatment of osteosarcoma cells with LLL12 and FLLL32 inhibits STAT3 activities, inhibits cell viability and induced apoptosis in these cell lines. Also LLL12 and FLLL32 were shown to inhibit tumor growth in both SJSA and OS-33 mice xenograft. OS-33 is a xenograft obtained for osteosarcoma of the right humerus in a 7 year old Caucasian female. It did not undergo sequencing, RT-PCR showed it was mutant, so it is difficult to state if this finding was really post transcriptional or in reality a mutation. LLL12 and FLLL32 should be a suitable agent for targeting cancer cells with constitutively activated STAT3 due to its ability to inhibit STAT3 phosphorylation and its potent growth suppressive activity. We have also observed that LLL12 and FLLL32 are potent inhibitors of STAT3 phosphorylation and DNA binding activity in cancer cells.

LLL12 and FLLL32 show potential as a cancer therapeutic and further exploration of its use as a potential agent in the treatment of cancer is warranted.

Figure Legends

Figure 1. The chemical structures of LLL12 and FLLL32.

Figure 2. Western Blot analysis of osteosarcoma cells treated with LLL12 and FLLL32. ~~Cancer~~ Osteosarcoma cell lines expressing constitutively active STAT3, (**A**) U2OS (**B**) SAOS2, (**C**) SJSA, exhibit a decrease in the levels of expression of STAT3 phosphorylation after treatment with LLL12 and FLLLL32.~~—~~ Apoptosis is indicated by the induction of cleaved PARP, cyclin D1 ~~and~~ or cleaved caspase-3. **(D)** LLL12 and FLLL32 induce apoptosis shown via Annexin V dye staining/Flow cytometry

Figure 3. LLL12 and FLLL32 have an inhibitory effect on STAT3 dependent transcriptional activity. (**A, B, C**) Transcription of STAT3-regulated genes, Bcl-x~~X~~L, Bcl-2, and ~~Cyclin~~ cyclin D, survivin is inhibited by LLL12 and FLLL32. Reverse transcriptase PCR reveals decreased expression of STAT3 target genes over a DMSO control and Negative preparation (did not contain RNA) following treatment with LLL12 and FLLL32.

Figure 4. (A) LLL12 and FLLL32 inhibit STAT3 phosphorylation induced by IL-6 in SJSA ~~Osteosarcoma cancer~~ cells with minimal STAT3 phosphorylation. The cells were serum starved overnight, and then were treated with LLL12/FLLL32/Curcumin (5µM - 20µM) or DMSO. After 4 hours the untreated and LLL12 treated cells were stimulated by IL-6 (25ng/mL). The cells were harvested at 30 min and analyzed by ~~western~~ Western blot. STAT1 IL-6 induced phosphorylation was not inhibited by LLL12 and FLLL32 **(B)**.

Figure 5. (**A, B)** Treatment with LLL12 and FLLL32 greatly decreased the ability of SAOS2, SJSA and ~~U20S~~ U2OS Osteosarcoma cells to form colonies in comparison to a DMSO control.

Figure 6. (**A, B**) LLL12 and FLLL32 inhibit cell migration in SAOS2, SJSA and U2OS ~~Osteosarcoma cancer~~ cells. A wound healing assay reveals that LLL12 and FLLL32 have a significant impact on SAOS2, SJSA and U2OS ~~Osteosarcoma~~ osteosarcoma cell migration. The ability of the cells to migrate is increasingly inhibited by an increase in dose of LLL12 and FLLL32.

Figure 7. (A, B, C) Effect of LLL12 and FLLL32 on tumor growth in SJSA mouse xenografts

Figure 8. (A, B, C) Effect of LLL12 and FLLL32 on tumor growth in OS-33 mouse xenografts. After the tumor development, the mice were given daily intraperitoneal dosages of 5mg/kg LLL12 and 50mg/kg FLLL32 or control.

Table 1. The half-maximal inhibitory concentrations (IC_{50}) obtained for STAT3 inhibitors in Osteosarcoma cells. All values reflect concentrations calculated following 72 hour of treatment in an MTT viability assay.

5. Acknowledgements

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