**Regular Article**

2,4,5-Trichloro-6-((2,4,6-trichlorophenyl)amino)isophthalonitrile, Exerts Anti-bladder Activities through IGF-1R/STAT3 Signaling

Jiayuan Jiao, Wanqu Wang, Haihong Guan, He Lin, Yanxin Bu, Yunhua Wang, Yi Bi, Baoshan Chai, and Zhaojin Ran

*Pharmaceutical Research Laboratory, Shenyang Research Institute of Chemical Industry Co., Ltd.; Shenyang 110021, China: and Safety Evaluation Center, Shenyang Research Institute of Chemical Industry Co., Ltd.; Shenyang 110021, China.

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2,4,5-Trichloro-6-((2,4,6-trichlorophenyl)amino)isophthalonitrile (SYD007) is a small molecule compound that was synthesized according to the structure of diarylamine. In this study, we evaluated the anti-bladder activities of SYD007, and determined its cytotoxic mechanism. We found that SYD007 exerted cytotoxicity to bladder cancer cells. Furthermore, SYD007 induced bladder cancer cell early apoptosis and arrested cell cycle. Mechanically, SYD007 suppressed phosphorylated signal transducer and activator of transcription 3 (p-STAT3) (Tyr705) level in parallel with increases of p-extracellular signal-regulated kinase (ERK) and p-AKT. SYD007 significantly inhibited insulin-like growth factor 1 (IGF-1)-induced STAT3 activation through down-regulation of total IGF-1R level. No dramatic changes in IGF-1R mRNA levels were observed in SYD007-treated cells, suggesting that SYD007 acted primarily at a posttranscriptional level. Using molecular docking analysis, SYD007 was identified as an IGF-1R inhibitor. In summary, we reported that SYD007 exerted anti-bladder activities, and these effects were partially due to inhibition of IGF-1R/STAT3 signaling.

Key words small molecule compound; insulin-like growth factor 1; molecular docking; signal transducer and activator of transcription 3, anti-bladder activity

**Introduction**

Bladder cancer is a major clinical problem worldwide, whose incidence continues to rise each year. The most recent cancer statistic has estimated numbers of new cases with more patient with cancer estimated deaths in the United States. Bladder tumours show widely differing histopathological and clinical behavior, and this is a key problem in the management of bladder tumours. The 5-year survival rate for patients afflicted with muscle-invasive bladder cancer is approximately 48%. Noteworthy, the surgical treatment of bladder cancer is unfavorable, even after radical cystectomy for localized disease. Much work has therefore focused on identifying clinical and molecular factors that show potential as novel therapeutic targets, aiming to reduce recurrence and progression in superficial tumours and to improve the outlook for patients with advanced disease.

Signal transducer and activator of transcription 3 (STAT3) is emerging as a therapeutic target in bladder cancer. In bladder cancer cells, STAT3 regulates various target genes, which are involved in cell proliferation, angiogenesis, metastasis and inhibition of apoptosis. Importantly, activation of STAT3 signaling is a negative prognostic factor in human bladder cancer. STAT3 is associated with cytokines (interleukin 6 (IL-6), interferon-alpha (IFN-α)), growth factors (epidermal growth factor (EGF), insulin-like growth factor 1 (IGF-1) and platelet-derived growth factor (PDGF), etc.) and non-receptor tyrosine kinases (Src and all the Janus kinase (JAK) family proteins), moreover can be activated by them. IGF-1 is one of these growth factors that plays an essential role in transformation by developing cell growth and preventing multiple tumors from apoptosis. The biological actions of IGF-1 are mediated through the ligand-induced activation of IGF-1 receptor (IGF-1R), a transmembrane tyrosine kinase linked to mitogen-activated protein kinase (MAPK), phosphatidylinositol-3 kinase/Akt (PI3K/AKT), and STAT3 signal-transduction cascades. IGF-1R is made up of two alpha subunits and two beta subunits. The α chains are located extracellularly, while the β subunit spans the membrane and is responsible for intracellular signal transduction upon ligand stimulation. The gene of IGF-1R is a proto-oncogene with potent simulative tumor progression activities. It has been reported that expression of IGF-1R is up-regulated in bladder cancer, furthermore the expression level of IGF-1R decreases, which causes tumor growth arrest and apoptosis. Collectively, these results present a better understanding of the IGF-1R and suggest that IGF-1R may be vital for bladder cancer cells.

SYD007 (2,4,5-trichloro-6-((2,4,6-trichlorophenyl)amino)isophthalonitrile) is a small molecule compound that was a novel diarylamine derivative having multiple chlorine atoms. The compound was synthesized via the straightforward reaction of chlorothalonil and different substituted phenylamines in the presence of Na2CO3 and N,N-dimethylformamide (DMF). Previous studies have shown that multiple chlorines of diarylamines derivatives have favorable anticancer activities, especially for bladder cancer. Therefore, we evaluated the anti-bladder cancer activities of SYD007 in further, and determined the cytotoxic mechanism through IGF-1R/STAT3 pathway.

**Experimental**

**Reagents and Cell Lines** 3-(4,5-Dimethylthiazol-2-yl)-
samples were then centrifuged at 10000 rpm for 20 min at 4°C. Samples were then smeared with a homogenizer in an ice bath. The anesulfonyl fluoride (PMSF). After these procedures, the cell lysates were harvested and were isolated on ice immediately. The cell lysates were analyzed by flow cytometry (BD Biosciences) after incubation for a period of time. These fixed cells were washed with PBS, buffered saline (PBS) and fixed with ice-cold 70% ethanol for 5 min. Finally, MB was removed, the cells were washed twice with distilled water and images were photographed.

**Apoptosis Assay** Cell apoptosis was detected by PI and annexin V staining according to the manufacturer's instructions. Briefly, after designed treatment, cells were harvested and stained with annexin V and PI. Then stained cells were analyzed by flow cytometry (BD FACS Calibur, BD Biosciences).

**Cell Cycle Assay** PI staining analysis of cell cycle was performed using the primers for IGF-1R (sense primer, AGG CTG AAT ACC GCA AAG TC; anti-sense primer, TGT GAA AGG CCG AAG GTT AG). Quantitative real-time PCR was performed according to the manufacturer’s protocol.

**Western Blot Analysis** For Western blotting, 10% and 8% SDS-polyacrylamide gels were prepared. The equivalent amount of protein (50 µg) was separated on SDS-polyacrylamide gel for 2h and then was transferred onto a polyvinylidene difluoride (PVDF) membrane. The protein membranes were blocked with 5% bovine serum albumin or 5% skim milk for 2h at room temperature and were subsequently incubated with the primary monoclonal antibody overnight at 4°C. The primary antibodies against STAT3, p-STAT3, p-ERK, p-AKT, IGF-1Rβ and GAPDH were diluted to 1:1000 and β-actin to 1:3000. All of the membranes were washed with Tris-buffered saline supplemented with 0.1% Tween 20 (TBST) (3 × 10 min) and were incubated with the secondary antibody for 2h at room temperature. The membranes were then washed with TBST for three times (3 × 10 min). The signals were measured using an ECL Western blotting detection system (Beyotime Institute of Biotechnology, Haimen, China). Relative intensities were normalized using β-actin as an internal standard.

**Molecular Docking Analysis** Molecular docking analysis was performed to assess the interaction of SYD007 with the IGF-1R. Before docking, the fully minimized energy conformation for SYD007 was generated by the MMFF94 energy minimization protocol in Chem 3D Ultra 12.0 and the crystalization of IGF-1R protein as well as its co-crystallized ligand equipped with spatial coordinates was downloaded from Protein Data Bank (PDB ID of IGF-1R crystal was 3O23). The docking study was processed by CDOCKER,18) a CHARMM equipped with spatial coordinates was downloaded from Protein Data Bank (PDB ID of IGF-1R crystal was 3O23). The docking study was processed by CDOCKER,18) a CHARMM equipped with spatial coordinates was downloaded from Protein Data Bank (PDB ID of IGF-1R crystal was 3O23). The docking study was processed by CDOCKER,18) a CHARMM equipped with spatial coordinates was downloaded from Protein Data Bank (PDB ID of IGF-1R crystal was 3O23). The docking study was processed by CDOCKER,18) a CHARMM equipped with spatial coordinates was downloaded from Protein Data Bank (PDB ID of IGF-1R crystal was 3O23). The docking study was processed by CDOCKER,18) a CHARMM equipped with spatial coordinates was downloaded from Protein Data Bank (PDB ID of IGF-1R crystal was 3O23). The docking study was processed by CDOCKER,18) a CHARMM equipped with spatial coordinates was downloaded from Protein Data Bank (PDB ID of IGF-1R crystal was 3O23). The docking study was processed by CDOCKER,18) a CHARMM equipped with spatial coordinates was downloaded from Protein Data Bank (PDB ID of IGF-1R crystal was 3O23).
72 h) significantly induced cytotoxicity to human bladder cells (T24) in a time and dose-dependent manner. The IC₅₀ of SYD007 for 24, 48 and 72 h was 3.37, 1.4 and 1.3 µmol/L in T24 cells, respectively. The IC₅₀ value of SYD007 on human embryonic kidney cell (293T) was 0.14 µmol/L, while the IC₅₀ value for monkey peripheral blood mononuclear cells (PBMC) was 60.67 µmol/L. It is suggested that the cytotoxicity of SYD007 is selective, but the selectivity of SYD007 is limited. The mechanism of selectivity is not clear and need further study.

SYD007 Induced Bladder Cancer Cells Apoptosis To determine whether SYD007 would induce apoptosis in T24 cells, the percentages of Annexin V⁻/PI⁻ (early apoptotic cells), Annexin V⁻/PI⁺ (late apoptotic cells) and Annexin V⁺/PI⁻ (necrotic cells) were measured by flow cytometry (Fig. 2). SYD007 treatment (0.5, 1.25, 2.5, 5.0 and 10.0 µmol/L) for 72 h significantly increased both early and late apoptosis in T24 cells, as compared with vehicle control group (p < 0.01). Previous studies [19–21] indicated mitomycin-C (MMC) can significantly induce apoptosis. The effect of pro-apoptosis SYD007 compared with MMC suggest that SYD007 significantly induced early apoptosis on T24.

SYD007 Arrest of Bladder Cancer Cells Cycle In order to further understand the mechanism of anti-proliferative activity of SYD007, we then used flow cytometry to examine cell cycle progression. It was revealed that SYD007 (5, 10, 20 and 40 µmol/L, 24 h) induced the accumulation of S and G2/M phase of T24 cells, especially the accumulation of cells in the G2/M phase in dose-dependent manner, accompanied by a decrease in the percentage of cells in the G1 phase, which indicated dominating G2/M phase arrest induced by SYD007 in T24 cells (Fig. 3). In contrast, MMC treatment did not show G2/M arrest effect, but induced the G1 phase accumulation of T24 cells. These results suggested that the mechanism of
blocking cell proliferation of SYD007 by arresting G2/M cells, thereby induced cell apoptosis.

SYD007 Inhibited IGF-1-Induced STAT3 Activation in Bladder Cancer Cells  It has been known that the constituent phosphorylation/activation of STAT3 leads to the occurrence and development of bladder cancer. Therefore, we dissected whether SYD007 inhibited STAT3 activation. We found that SYD007 treatment (1.25, 2.5, 5.0 and 10µmol/L) for 24h suppressed the phosphorylation at Tyr705 of STAT3 in a dose-dependent manner in T24 cells. The expression level
of total STAT3 was invariant after SYD007 treatment in T24 (Fig. 4A). Activation of STAT3 is regulated by IGF-1.23–25 In order to determine whether SYD007 treatment inhibited IGF-1-induced STAT3 activation, we assessed the expression of p-STAT3 (Tyr705) by Western blotting. Briefly, T24 cells were pretreated with or without SYD007 (2.5, 5.0 and 10 µmol/L) for 2 h, then infected by IGF-1 (50 ng/mL) for 1 h, before above operation cells were grown in serum-free culture for 24 h. A significant augment of STAT3 phosphorylation at Tyr705 was observed after stimulating of IGF-1 while the augment was downregulated by SYD007 (Fig. 4B). Videlicet, SYD007 significantly inhibited IGF-1-induced STAT3 activation in T24 cells.

**SYD007 Reduced the Expression Levels of Total IGF-1R in Bladder Cancer Cells** To further demonstrate the effect of SYD007 on IGF-1R signaling, T24 were treated with different concentrations of SYD007 (1.25, 2.5, 5.0 and 10 µmol/L) for 24 h, then the expression levels of total IGF-1R were determined by Western blot. Results showed that SYD007 treatment markedly reduced the expression levels of total IGF-1R (Fig. 5).

**SYD007 Slightly Inhibited IGF-1R Transcription in Bladder Cancer Cells** To determine whether the suppression IGF-1R by SYD007 at transcriptional level, we performed real-time PCR assay to detected IGF-1R mRNA expression from T24 cells of treated with SYD007 (5, 10, 20 and
40 \mu mol/L) for 24 h. The real-time PCR data indicated that SYD007 40 \mu mol/L treatment slightly reduced IGF-1R mRNA levels in T24 cells, while MMC (0.5, 1.0, 1.5 and 3.0 \mu mol/L) can alter the levels of IGF-1R mRNA in dose dependent manner (Fig. 6). Analysis of the expression level of IGF-1R by Western blotting indicated that the IGF-1R was significantly reduced when treated with SYD007 of 1.25 \mu mol/L. In contrast, only a slight decrease of IGF-1R mRNA was induced in the 40 \mu mol/L SYD007 treatment. Summing up the above, suggested that SYD007 acted primarily at post-transcriptional level.

**SYD007 Activated AKT and ERK Signaling in Bladder Cancer Cells**

ERK and AKT pathways have shown the efficacy for constitutively activated pro-survival in bladder cancer cells. Therefore, we investigated whether SYD007 would inhibit these two signaling pathways. In contrast to the inhibition of STAT3 phosphorylation at Tyr705 site, SYD007 treatment (1.25, 2.5, 5.0 and 10 \mu mol/L) for 24 h significantly increased AKT (Ser473) and ERK (Thr202/Tyr204) phosphorylation in T24 (Fig. 7).

**SYD007 May Be a Potential Inhibitor of IGF-1R**

Research on small molecule compounds as inhibitors is attracting more and more attention. Therefore, we investigate whether SYD007 is a potential IGF-1R inhibitor by the combined use of molecular docking. The “-CDOCKER INTERACTION ENERGY” based criticism of the stability of ligand-receptor interactions...
complexes were employed to study the interactions between the SYD007 and IGF-1R protein. The co-crystallized ligand in IGF-1R (PDB ID: 3O23) with highest absolute values of docking energies were preserved in Fig. 8A, while Fig. 8B shows the interaction of SYD007 having the highest score with the IGF-1R. The “-CDOCKER INTERACTION ENERGY” showed that SYD007 had moderate level score, lower than the ligand of IGF-1R crystal (PDB ID 3O23). In addition, SYD007 exhibited hydrophobic effects the same as a co-crystalline ligand and ideally matched the hydrophobic property of the IGF-1R active site. These findings suggest that SYD007 may be a potential inhibitor of IGF-1R.

Discussion
Small molecule compounds become well known for its significant role in preventing and treating cancer.27) In the research, the structure of SYD007 is simple in comparison with the previously reported IGF-1R inhibitor. SYD007 is worthy of the small molecule compound. In this study, we firstly demonstrated that SYD007 has anti-cancer effects against bladder cancer cells (T24). Cytotoxicity experiment and methylene blue staining showed that SYD007 induced cytotoxicity to T24 cells in a time- and dose-dependent manner. At the same time, cell apoptosis and cycle experimental results suggested that the mechanism of blocking cell proliferation of SYD007 by arresting G2/M cells, thereby significantly induced early apoptosis on T24.

STAT3 has obtained more and more attention, since it is considered a vital target for the treatment of cancers.28,29) STAT3 is a transcription factor activated by IGF1/IGF1R signaling.30) Phosphorylated STAT3 plays an important role...
since it promotes cell survival and proliferation, cell cycle progression, angiogenesis, and metastasis. In this study, we further found that SYD007 inhibited p-STAT3 (Tyr705) protein which has an important role in oncogenesis, but no change total STAT3. IGF-1 is one of growth factors that can activate STAT3. The relationship of IGF-1R and STAT3 was showed in Fig. 9. IGF-1R is linked to ERK, PI3K/AKT and STAT3 signal-transduction cascades. STAT is the downstream target of IGF-1R, can be activated by IGF-1R. The activation of STAT3 induces cell proliferation, metastasis and other changes. Further analysis showed that SYD007 significantly decreased the expression level of IGF-1R, moreover, SYD007 inhibited IGF-1-induced STAT3 activation in T24 cells. In addition, RT-PCR results revealed that IGF-1R were depletion partially at mRNA levels in T24, indicating that the expression reduction is mainly due to the pro-transcriptional regulation. To further explore the interaction between SYD007 and IGF-1R, we used molecular docking technology to analyze. The binding modes and the interactions between ligands and receptor with the key site seemed to be the more important pacing factors. We found SYD007 had moderate level interaction score, accompanying strong H-bond and π–π interactions with amino acid residues of IGF-1R. In detail, when analyzing interactions between SYD007 and amino acid residues of IGF-1R, it was found that there amino acids of Met1079, Asp1153 and Lys1033 could form H-bond interactions with SYD007, which was in accordance with the positive medicine of action presented in Q23. In addition, the Lys1033 could form π–π conjugation with SYD007, indicating that Met1079, Asp1153 and Lys1033 might play a more important role in IGF-1R inhibitor. These results indicated SYD007 may be a potential IGF-1R inhibitor.

It has been reported that ERK and AKT signaling were involved in bladder cancer cells occurrence and development. It is well-known that the phosphorylated IGF1R induced the activation of AKT and ERK pathways. As downstream proteins of the IGF-1R pathway, our study demonstrated that p-AKT (Ser473) and p-ERK (Thr202/Tyr204) were upregulated in a dose-dependent, activation of these signaling pathways might be caused by IGF-1R activation because the AKT and ERK signaling pathways are downstream targets of IGF-1R, but it is unclear whether SYD007 inhibited IGF-1R phosphorylation can cause sufficient change in AKT and ERK phosphorylation to promote cell death. Therefore, we reasonably assumed that the administration of SYD007 might inhibit the IGF-1R protein, subsequently impacting the expression of downstream proteins. However, the expression level of AKT and ERK are also influenced by the other pathways. The roles of SYD007 on AKT and ERK signaling might be to further investigate.

In conclusion, we demonstrated for the first time that SYD007 is a potent agent against human bladder cancer cells. Mechanistically, through inhibiting IGF-1R activation and reducing p-STAT3 expression, in contrast, SYD007 increased the expression level of p-ERK and p-AKT. Our findings provided novel insights that SYD007 could be explored as a chemo-sensitizer in bladder cancer therapy.

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Conflict of Interest The authors declare no conflict of interest.

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Fig. 9. The Relationship of IGF-1R and STAT3
IGF-1R is linked to ERK, PI3K/AKT and STAT3 signal-transduction cascades.
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