Received Date : 15-Jul-2016
Revised Date : 31-Aug-2016
Accepted Date : 18-Sep-2016
Article type : Research Article

In Silico Prediction and In Vitro and In Vivo Validation of Acaricide Fluazuron as a Potential Inhibitor of FGFR3 and a Candidate Anticancer Drug for Bladder Carcinoma

Kunbin Ke[1,3], Hongjian Li[2,4], Hong Yao[5], Xi-Nan Shi[1,6], Chao Dong[1,7], Ying Zhu[1,8], Xu Liu[1], Ling Li[1], Kwong-Sak Leung[4*], Man-Hon Wong[4], Xiao-Dong Liu[3], Hsiang-fu Kung[1,9], Marie Chia-mi Lin[10*]

[1]Biomedical Engineering Research Center, Kunming Medical University, Kunming, Yunnan, China.

[2]Institute of Future Cities, Chinese University of Hong Kong, Hong Kong, China.

[3]Department of Urology, The 1st Affiliated Hospital of Kunming Medical University, Kunming, China.

[4]Department of Computer Science and Engineering, Chinese University of Hong Kong, Hong Kong, China.

[5]The Cancer Biotherapy Institute of Jiangsu Province, Xuzhou Medical College, Xuzhou, China.

[6]Department of Pathophysiology, School of Basic Medical Sciences, Yunnan University of TCM, Kunming, China.

[7]Department of the Second Medical Oncology, The 3rd Affiliated Hospital of Kunming Medical University, Yunnan Tumor Hospital, Kunming, China.

[8]Department of Cadre Medical Branch, The 3rd Affiliated Hospital of Kunming Medical University, Kunming, China.

[9]School of Biomedical Sciences, Chinese University of Hong Kong, Hong Kong, China.

[10]Shenzhen Key Lab of Translational Medicine of Tumor, School of Medicine, Shenzhen University, Shenzhen, China.

*ksleung@cse.cuhk.edu.hk (KSL); mcmlin@163.com (MCML)

Keywords: fluazuron; FGFR3 inhibition; bladder carcinoma；cancer therapy; drug repurposing

Abstract

Bladder carcinoma (BC) is the 9th most common cause of cancer worldwide. Surgical resection and conventional chemotherapy and radiotherapy will ultimately fail due to tumor recurrence and resistance. Thus, the development of novel treatment is urgently needed. Fibroblast growth factor receptor 3 (FGFR3) is an important and well-established target for BC treatment. In this study, we utilized the free and open-source protein-ligand docking software idock to prospectively identify potential inhibitors of FGFR3 from 3167 worldwide approved small-molecule drugs using a repositioning strategy. Six high-scoring compounds were purchased and tested in vitro. Among them, the acaricide drug fluazuron exhibited the highest anti-proliferative effect in human BC cell lines RT112 and RT4. We further demonstrated that fluazuron treatment significantly increased the percentage of apoptosis cells, decreased the phosphorylation level of FGFR3 and its downstream proteins FRS2-α, AKT and ERK. We also investigated the anticancer effect of fluazuron in vivo in BALB/C nude mice subcutaneously xenografted with RT112 cells. Our results showed that oral treatment with fluazuron (80mg/kg) significantly inhibited tumor growth. These results suggested for the first time that fluazuron is a potential inhibitor of FGFR3 and a candidate anticancer drug for the treatment of BC.

Introduction

Bladder carcinoma (BC) is the second most common urologic cancer in terms of incidence and mortality (1). BCs can be divided into two subgroups: muscle-invasive BC (MIBC) and nonmuscle-invasive BC (NMIBC) (2). Transurethral resection of the bladder tumor combined with intravesical chemotherapy or intravesical immunotherapy with Bacillus Calmette-Guerin (BCG) are recommended for the treatment of NMBIC (3). However, about 50–70% of NMIBCs will recur and approximately 10–20% will progress to MIBC (T2–4) (4). The current standard of treatment in patients with MIBC T2-T4a N0-Nx M0 is radical cystectomy combined with neoadjuvant chemotherapy when possible (5). In patients with metastatic MIBC, the standard of care is radiotherapy and/or chemotherapy. MIBC patients have a higher cancer-specific mortality than NMIBC patients, with a 5-year survival rate below 50% (6). Therefore, the development of novel therapies against BC is urgently needed.

The cause of BC involves multiple pathways, with the fibroblast growth factor receptor 3 (FGFR3) pathway playing an important role (7). FGFR3 is a member of the FGFR family which has highly conserved transmembrane receptor tyrosine kinases (8). Amplification or mutation of FGFR3 can constantly activate the FGFR3 pathway (7). Hence, FGFR3 is an attractive therapeutic target in cancer treatment.

To date, a number of FGFR3 inhibitors (Table 1) have been reported (9-18). However, due to their drug toxicity or low selectivity, only the nintedanib is clinically used in combination with docetaxel for the treatment of locally advanced, metastatic or locally recurrent non-small cell lung cancer (NSCLC) after first line chemotherapy (18). Unfortunately, nintedanib is expensive, and finding an affordable drug for the treatment of BC motivated us to conduct the present study.

Given the obstacle that developing a new drug de novo is a laborious and costly endeavor, repurposing well-studied old drugs for new indications is gradually becoming a favorable strategy. Previously, we have successfully used this powerful synergy of drug repositioning combined with in silico structure-based virtual screening (19, 20), where, by targeting cyclin-dependent kinase 2 (CDK2), two FDA-approved drugs fluspirilene and adapalene have been rediscovered as anticancer agents in vitro and in vivo for the treatment of hepatocellular and colorectal carcinomas, respectively. Inspired by these successful recent cases, in this study we further improved the same overall computational and experimental workflow to search for potential inhibitors of FGFR3 and thus novel treatments of bladder carcinoma.

We used the free and open-source protein-ligand docking software idock (21, 22) together with the binding affinity prediction software RF-Score-v3 (23) to virtually screen and rank worldwide approved small-molecule drugs (including but not limited to those approved by US FDA) with potential ability to inhibit FGFR3, and then used the molecular visualization tool iview (24) to inspect and analyze putative interactions. Among the high-scoring compounds shortlisted computationally, six were purchased for experimental validation in vitro in BC cell lines RT112 and RT4 via cell viability assays, cell apoptosis assays, western blotting and immune-precipitation experiments. Consequently, the acaricide drug fluazuron was successfully identified as a potential inhibitor of FGFR3. In vivo experiments in nude mice xenografted with RT112 cells showed that fluazuron exhibited strong anti-tumor activity. Next, we analyzed the predicted binding conformation of fluazuron and revealed critical intermolecular interactions that possibly govern fluazuron binding to FGFR3. Finally, we described the safety profile of fluazuron on animals and discussed its potential and novel anticancer application on humans. Taken together, in this study, we demonstrated for the first time that fluazuron is a potential FGFR3 inhibitor to treat bladder carcinoma.

Results

Structure-based virtual screening provided candidate inhibitors to test

At the very first step, FGFR3 was selected as the target protein, as it has been shown to be a therapeutic target in the regulation of BC and a hallmark of cancer. Totally 3167 drugs that have been approved for clinical use by worldwide authorities constituted a library of compounds to screen. They were individually docked to the ATP binding pocket of FGFR3, and then sorted in the ascending order of their predicted binding free energy. The docking results with molecular visualization are freely available at http://istar.cse.cuhk.edu.hk/idock/iview/?4K33-dbap+fda+npc. The high-scoring compounds were manually examined based on in silico estimations of binding strength, appropriate molecular weight and other drug-like properties, complementary matching of molecular shape, plus some sense of intuition from a computational chemist's experience. Finally, six high-scoring compounds (Table 2) were shortlisted and purchased for subsequent wet-lab investigations.

Fluazuron decreased cell viability of bladder carcinoma

We first evaluated the anticancer effect of the six compounds by MTT assay (Fig 1). All the six compounds decreased cell viability in RT112 and RT4 cells, but had discrepant cytotoxicity at different concentrations. Among them, fluazuron exhibited the highest cytotoxicity (IC_{50} = 4.142μM for RT112 and 6.481μM for RT4) compared to the control with statistical significance (p<0.05). Such inhibitory effect was dose- and time-dependent. Marked inhibition was observed at 3μM, 10μM and 30μM.

Fluazuron treatment induced cell apoptosis

We also investigated whether fluazuron could induce cell apoptosis (Fig 2). Fluazuron treatment at 3μM concentration significantly increased the percentage of apoptosis in RT112 and RT4 cell lines compared to the control (p<0.05).

Fluazuron treatment inhibited FGFR3 phosphorylation and downstream signaling

Since activation of FGFR3 can cause cell proliferation, differentiation and tumorigenesis by its downstream proteins such as fibroblast receptor substrate 2-α (FRS2-α), AKT and ERK1/2 (25-27), we investigated the effect of fluazuron on the expression of critical proteins involved in FGFR3 signal network in RT112 and RT4 cells by western blotting and immune-precipitation (Fig 3). Fluazuron treatment mainly reduced the phosphorylation level of FGFR3, FRS2-α, AKT and ERK. In contrast, the expression level of FGFR3, FRS2, AKT and ERK remained mostly unchanged. These results are consistent with what are expected from a FGFR3 inhibitor (9, 10, 16, 28).

Daily oral fluazuron treatment reduced tumor growth in vivo

We evaluated the effect of fluazuron on the growth of bladder carcinoma in vivo in BALB/C nude mice subcutaneously injected with RT112 cells (Fig 4). On day 21 after treatment, fluazuron (80 mg/kg) resulted in significant reduction of tumor weight and volume compared to the control (p<0.05), while making no significant change to the body weight. This result suggested for the first time that fluazuron is a potential FGFR3 inhibitor and a candidate anticancer drug for the treatment of human bladder carcinoma.

Structural analysis of the predicted binding conformation of fluazuron revealed critical interactions

Fig 5 (A) plots the 2D structure of fluazuron. Its binding free energy predicted by idock (21, 22) was -11.28 kcal/mol and its binding affinity predicted by RF-Score-v3 (23) was 7.92 pKd. Rendered using iview (24), its predicted binding conformation in complex with FGFR3 is shown in Fig 5 (B) and (C). Putatively, it was observed to establish three hydrogen bonds with LYS508, a hydrogen bond with GLY484, and a hydrophobic contact with PHE483.

Discussion

Bladder carcinoma (BC) is a lethal urologic cancer. Superficial BC was often found to have activating mutations of FGFR3 (29-31). Moreover, BC, especially in high grade and stage, was found to have over-expression of wild-type FGFR3 (7). Activation of FGFR3 can mediate cell proliferation, differentiation and tumorigenesis through fibroblast receptor substrate 2-α (FRS2-α), phospholipase C-γ, MAPKs, ERK1/2, signal transducers, activators of transcriptions and PI3K (25-27). Therefore, FGFR3 is an important target for cancer therapy.

Although some FGFR3 inhibitors have been discovered (9-18), few of them are used in clinical practice except nintedanib (18). Given that the market price of nintedanib is high for ordinary patients, an alternative but affordable inhibitor of FGFR3 for BC therapy is desirable. Realizing that developing a new drug de novo is far beyond the capability of small research groups like us, we decided to adopt the drug repositioning methodology to quickly identify new therapeutic uses of approved drugs.

Indeed, approved drugs represent an attractive library of candidate compounds to screen, as they have been experimentally proven to be safe for use in humans, and are often well tested and well annotated. Identifying novel therapeutic indications for already approved drugs is referred to as repurposing or repositioning, the rationale of which is that a drug typically exhibits activities on more than one target, some of which might be previously unknown, due to promiscuous interactions explaining drug efficacy or side effects. This approach is substantially faster and cheaper with a lower attrition rate than developing new drugs, as the majority of safety tests prior to phase II clinical trial could be possibly bypassed or easily passed again given the previous successful test results of the original indication.

A straightforward, rapid implementation of drug repositioning is virtual screening, where a database of drugs is computationally evaluated and ranked. Virtual screening can be broadly classified into two types: ligand-based and structure-based. Structure-based virtual screening uses molecular docking to predict the binding conformation and binding affinity of small molecules to a target macromolecule. In real life it has led to a substantial amount of successful cases of protein inhibitor discovery. What especially caught our attention is two recent applications (19, 20) where two FDA-approved drugs have been repurposed as anticancer agents in vitro and in vivo for the treatment of hepatocellular and colorectal carcinomas. Given the similar nature, we felt confident to conduct this work using the same overall computational and experimental approach.

Regarding the computational part, there are some key improvements over the previous two studies. Here we did not limit the scope of drugs to those approved by US FDA only, but also incorporated small-molecule drugs approved in UK, EU, Japan and Canada. Such a worldwide drug dataset would probably lead us to more novel findings, as the FDA-approved drugs have already been intensively studied. Another key improvement is the utilization of the accurate scoring function RF-Score-v3 (23), which was recently developed using the machine-learning algorithm of random forest (RF) and trained on more than 3700 protein-ligand complexes with 42 numerical descriptors. We observed that the docking software idock (21, 22) often gave correct prediction of binding poses of ligands but not so accurate prediction of their binding strength, including RF-Score-v3 into the computational workflow as an alternative classifier would help to select a small subset of assumingly active compounds for us to concentrate. As can be seen in Table 2, compounds having a good idock score did not necessarily have a good RF score, and vice versa, so decisions should be made by comprehensively considering different estimations of binding strength, appropriate molecular weight, as well as complementary shape matching through molecular visualization. The use of the convenient web-based visualizer iview (24) aided this selection process. It automatically detected putative intermolecular interactions between the target and the drug, and rendered them in a 3D canvas for the users to interactively analyze the binding conformation.

Based on the in silico results, six compounds were purchased and validated experimentally. Among them, fluazuron was selected for further investigations because its IC_{50} was less than 10 μmol/L as determined by MTT assay. Fluazuron is a benzoyl phenylurea compound. It is a FDA-approved drug currently used as acaricide for controlling rhipicephalus (boophilus) microplus (32). By topical pour-on route fluazuron can inhibit chitin synthesis of these parasites, probably by restraining of specific enzymes involved on the ticks' ecdysis (33). Fluazuron was used in many animals at dosage from 2.5mg/kg to 80mg/kg (34, 35). Two studies reported its use by oral dosage. Woodrats were given 40mg dosage of fluazuron per bait cube (36). Weaner pigs were given fluazuron 10mg/kg/day for 7 days. It was found that absorption of fluazuron was quick with a rapid rise in blood levels 24 hours after oral administration. Fluazuron remained detectable in the blood for 28 days after beginning of treatment (37). No adverse effect was reported in the above studies.

On the other hand, fluazuron is a known p53 activator. p53 is a tumor suppressor protein and is often activated by DNA damage and other cellular stresses. The activation of p53 controls cell destiny by inducing DNA repair, cell cycle arrest, apoptosis, inhibition of angiogenesis, or cellular senescence. Fluazuron was found to activate the p53 signaling pathway by quantitative high throughput screening (qHTS) assay in the U.S. Tox21 Program (PubChem (38) AID 651631). The qHTS assay used the p53RE-bla HCT-116 cell line which contained a stably combined beta-lactamase reporter gene under control of the p53 response elements. The cell line was used to screen the Tox21 compound library to find agonists that activate the p53 signaling pathway. Interestingly, the AC50 (concentration at which compound exhibits half-maximal efficacy) of fluazuron was 2.371μM, roughly the same potency as we tested fluazuron's efficacy in BC cancer cell lines RT112 and RT4. Given these experimental evidences, we believe the anticancer effect of fluazuron might be due to a polypharmacophoric mechanism by simultaneously acting as a p53 activator as well as a FGFR3 inhibitor. However, in previous studies, fluazuron was never investigated in vitro in BC cell lines, nor in vivo in nude mice. Its role as a potential FGFR3 inhibitor has remained unknown until now.

In this study, we reported for the first time that fluazuron is a potential inhibitor of FGFR3, and oral administration of fluazuron (80mg/kg) exhibited significant and strong anticancer efficacy in vivo in nude mice xenografted with bladder carcinoma RT112 cells. Meanwhile, we did not observe significant change in body weight for 21 days (Fig 4), suggesting that oral administration of fluazuron is relatively safe. These findings suggest that fluazuron could be the first FGFR3 inhibitor to be used for the treatment of BC and other types of cancers.

Conclusions

This study presents the first prospective application of our in-house structure-based virtual screening tool idock in identifying FGFR3 inhibitors from small-molecule drugs using a repurposing strategy in silico. We showed that fluazuron, currently used as the application of acaricide, exhibited anticancer effect in human bladder carcinoma RT112 and RT4 cells in vitro. Consistent with the expected properties of FGFR3 inhibitors, fluazuron treatment significantly increased the percentage of apoptosis cells and decreased the phosphorylation level of FGFR3 and its downstream proteins FRS2-α, AKT and ERK. Oral fluazuron treatment significantly inhibited tumor growth in vivo. These results suggested for the first time that fluazuron is a potential FGFR3 inhibitor and a candidate anticancer drug for the treatment of human bladder carcinoma.

Acknowledgements

The present study was supported by grants from the Hsiang-fu Kung academician workstation of Kunming Medical University (no. NSFC 81272549), the Key Lab project of Shenzhen of the P.R. China (no. ZDSY 20130329101130496), the Scientific Research Fund project of Department of Education of Yunnan Province (no. 2015J051), the Direct Grant from the Chinese University of Hong Kong and the GRF Grant from the Research Grants Council of Hong Kong SAR (project references 414413, 772910 and 470911).

Conflict of Interest

The authors have no conflict of interest to declare.

Methods and Materials

Molecular docking and compound selection

From Protein Data Bank (PDB) (39), only one X-ray crystallographic structure of FGFR3 bearing the ATP-binding site was found (PDB code: 4K33). The FGFR3 structure was manually extracted from the complex with water and ion molecules removed, and then converted from PDB format to PDBQT format using AutoDockTools (40) for use by the docking software. The cubic search space was placed at the geometrical center of the bound ligand, with the length, width and height set to be 30% greater than that of the bound ligand, based on the observation that the geometry of the binding site is often proportional to that of the bound ligand. The search space was then further expanded by 4 Å in all three dimensions to spare sufficient room for the ligand to translate and rotate within.

The structures of approved drugs worldwide were obtained from three catalogs of the ZINC database (41), which are DrugBank-approved (42), FDA-approved drugs (via DSSTOX), and the NCGC Pharmaceutical Collection (NPC) (43). These, having been filtered and curated, constituted a non-redundant set of 3167 drugs that have been approved for clinical use by US (FDA), UK (NHS), EU (EMA), Japanese (NHI), and Canadian (HC) authorities. Similarly, these compounds in Mol2 format were also converted to PDBQT format.

The free and open-source docking software idock (21, 22) v2.2.1 was executed to predict the binding conformations and the binding affinities of the 3167 compounds upon docking against the FGFR3 structure. Program settings were tuned to make the conformational searching procedure more exhaustive than the default settings. Specifically, for each protein structure, grid maps of free energy with a fine granularity of 0.08 Å were constructed in parallel; and for each compound, 256 Monte Carlo conformational optimization tasks were run in parallel across multiple CPU cores.

After docking, up to nine putative conformations were outputted for each input compound. The docked conformation with the best idock score was selected because it had been previously shown to be most likely closest to the crystal conformation with a redocking success rate of over 50% on three different benchmarks. The compounds were sorted in the ascending order of their predicted binding free energy. Moreover, the more accurate scoring function RF-Score v3 (23) was used to re-score all the compounds and thus provided an additional and more reliable estimation of intermolecular binding strength, given the assumption that the compounds were correctly docked. Therefore, the top-scoring compounds would be those with both a low idock score (in terms of binding free energy) and a high RF score (in terms of binding affinity). Then the high-scoring compounds were visually examined using the

convenient web-based visualizer iview (24) in the context of FGFR3. Finally, commercially available compounds were purchased and subsequently validated in vitro.

Cell lines, cell culture, chemicals, antibodies

Bladder tumor cell lines RT112 and RT4 were obtained respectively from the Leibniz Institute DSMZ (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) and American Type Culture Collection, Manassas, Virginia, USA. These cell lines were cultured respectively in RPMI 1640 and McCOY's 5A medium containing 10% fetal bovine serum (FBS) (Invitrogen, Rockville, Maryland, USA), 100 U/ml penicillin and 0.1 mg/ml streptomycin at 37°C in 5% CO2.

Tioconazole were purchased from Selleck Chemicals, Shanghai, China. Latamoxef sodium and mizolastine were purchased from Dalian Meilun Biology Technology, Guangzhou, Guangdong, China. Bentiromide was purchased from Santa Cruz Biotechnology, Shanghai, China. Imidocarb and fluazuron were purchased from Toronto Research Chemicals, Toronto, Ontario, Canada. Anti-FGFR-3 (C-15) and FRS2 were purchased from Santa Cruz Biotechnology, Inc. Santa Cruz, CA, USA. Anti-Phospho-FRS2-α (Tyr196), Akt (pan), Phospho-Akt (Ser473), Erk1/2, Phospho-Erk1/2 (Thr202/Tyr204), 4G10 antiphosphotyrosine and β-actin were obtained from Cell Signaling Technology, Inc., Danvers, Massachusetts, USA.

MTT assay

7×10^3 cells per well were plated in 96-well plates and allowed to attach for 24 hours before adding testing chemicals. Cells were treated with testing chemicals for 24, 48 and 72 hours. After treatment, 10µl 5mg/ml 3-(4,5-methylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide(MTT) was added and further incubated for 4 hours. The medium was then discarded and the precipitate dissolved in DMSO. The absorbance was measured at 570 nm with a Synergy 2 microplate reader (Bio-Tek Instruments, Inc., Winooski, VT, USA) according to the standard protocol. The IC_{50} values were calculated by GraphPad Prism 5.

Cell apoptosis analysis

RT112 and RT4 cells were seeded in 24-well plates, allowed to attach to growth surface for 24 hours and then cultured with 10% FBS medium containing 3µM fluazuron for 72 hours. According to the manufacturer's instructions to quantify the apoptotic cells, the occurrence of apoptosis was determined by staining cells with Annexin V-FITC/propidium iodide (PI) (Beijing 4A Biotech Co., Ltd, Beijing, China). Briefly, cells were trypsinized with 0.25% trypsin in the absence of ethylenediamine tetraacetic acid (EDTA). The cells were washed with 4°C phosphate-buffered saline (PBS) twice and resuspended in 250µl of binding buffer at a concentration of 10^6 cells/ml. 5µl of Annexin V-FITC and 10µl 20µg/ml of PI were added to the 100µl suspension followed by 5 to 15 minutes of incubation in the dark. The cells were then analyzed by flow cytometry (CyFlow Space/Partec, Germany).

Western blotting and immune-precipitation

RT112 and RT4 cells were seeded in 6-well plates, allowed to attach to growth surface for 24 hours and then with 10% FBS medium containing fluazuron at concentrations of 3, 10, 30µM. Cells were harvested after 24 hours of incubation. Cells were lysed with RIPA buffer containing 1 mM PMSF and protease inhibitor cocktail at 4°C for 30 minutes. After centrifugation at 13,000 rpm for 15 minutes, the supernatants were recovered and the protein concentration was measured by BCA Protein Assay Kit

(Thermo). Immune-precipitation of FGFR3 was carried out at 4°C. Lysates were incubated with rotation overnight with FGFR3 antibody and protein A/G-beads (Millipore, Billerica, MA). After the mixture was centrifuged at 3000g, 4°C for 5 min, the precipitate was washed three times by 1×RIPA buffer and boiled with 2×SDS sample buffer for 10 min. Proteins were resolved in 10% SDS-PAGE and transferred onto nitrocellulose membranes (Sigma). After blocking, the membranes were incubated sequentially with the appropriate diluted primary and secondary antibodies. Proteins were detected by the enhanced chemiluminescence detection system (Amersham, Piscataway, New Jersey, USA). To ensure equal loading of the samples, the membranes were re-probed with an anti-β-actin antibody (Cell Signalling Technologies).

Fluazuron treatment in vivo

Male BALB/C nude mice, 5 to 6 weeks old from Department of Animal Experiment, Kunming Medical University, were kept under specific pathogen-free conditions. For the xenografted tumor growth assay, 1×10^6/0.2ml PBS RT112 cells were injected subcutaneously into the right flank of the mice. Three days after inoculation when the tumors grew to a volume of 80 to 100 m^3, the mice were randomly divided into groups of 5 mice, and fed by oral gavage with 0.5% CMC-NaCl containing fluazuron (80mg/kg) for 21 days. Tumor volumes were measured every 3 to 4 days after tumor appearance. Tumor volume was calculated by the equation $V=ab^2/2$, where a is the longest axis and b is shortest axis. The mice were then sacrificed by cervical dislocation. This study was approved by the laboratory animal ethics committee of Kunming Medical University.

Statistical analysis

Data were obtained from at least three different experiments and expressed as mean ± SEM (standard error of the mean). Statistical analysis was performed by one-way ANOVA (Analysis Of Variance) and differences were considered to be statistically significant if $p < 0.05$. Statistically significant results are marked with the asterisk symbol in the figures.

References

1. Ferlay J, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, et al. Cancer incidence and mortality worldwide: Sources, methods and major patterns in GLOBOCAN 2012. International journal of cancer Journal international du cancer. 2014.
2. Burger M, Catto JW, Dalbagni G, Grossman HB, Herr H, Karakiewicz P, et al. Epidemiology and risk factors of urothelial bladder cancer. European urology. 2013;63(2):234-41.
3. Babjuk M, Burger M, Zigeuner R, Shariat SF, van Rhijn BW, Comperat E, et al. EAU guidelines on non-muscle-invasive urothelial carcinoma of the bladder: update 2013. European urology. 2013;64(4):639-53.
4. Rubben H, Lutzeyer W, Fischer N, Deutz F, Lagrange W, Giani G. Natural history and treatment of low and high risk superficial bladder tumors. The Journal of urology. 1988;139(2):283-5.
5. Witjes JA, Comperat E, Cowan NC, De Santis M, Gakis G, Lebret T, et al. EAU guidelines on muscle-invasive and metastatic bladder cancer: summary of the 2013 guidelines. European urology. 2014;65(4):778-92.
6. Parekh DJ, Bochner BH, Dalbagni G. Superficial and muscle-invasive bladder cancer: principles of management for outcomes assessments. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2006;24(35):5519-27.
7. Tomlinson DC, Baldo O, Harnden P, Knowles MA. FGFR3 protein expression and its relationship to mutation status and prognostic variables in bladder cancer. The Journal of pathology. 2007;213(1):91-8.

8. Hart KC, Robertson SC, Donoghue DJ. Identification of tyrosine residues in constitutively activated fibroblast growth factor receptor 3 involved in mitogenesis, Stat activation, and phosphatidylinositol 3-kinase activation. Molecular biology of the cell. 2001;12(4):931-42.
9. Guagnano V, Furet P, Spanka C, Bordas V, Le Douget M, Stamm C, et al. Discovery of 3-(2,6-dichloro-3,5-dimethoxy-phenyl)-1-{6-[4-(4-ethyl-piperazin-1-yl)-phenylamin o]-pyrimidin-4-yl}-1-methyl-urea (NVP-BGJ398), a potent and selective inhibitor of the fibroblast growth factor receptor family of receptor tyrosine kinase. Journal of medicinal chemistry. 2011;54(20):7066-83.
10. Gavine PR, Mooney L, Kilgour E, Thomas AP, Al-Kadhimi K, Beck S, et al. AZD4547: an orally bioavailable, potent, and selective inhibitor of the fibroblast growth factor receptor tyrosine kinase family. Cancer research. 2012;72(8):2045-56.
11. Zhao G, Li WY, Chen D, Henry JR, Li HY, Chen Z, et al. A novel, selective inhibitor of fibroblast growth factor receptors that shows a potent broad spectrum of antitumor activity in several tumor xenograft models. Molecular cancer therapeutics. 2011;10(11):2200-10.
12. Motzer RJ, Porta C, Vogelzang NJ, Sternberg CN, Szczylik C, Zolnierek J, et al. Dovitinib versus sorafenib for third-line targeted treatment of patients with metastatic renal cell carcinoma: an open-label, randomised phase 3 trial. The Lancet Oncology. 2014;15(3):286-96.
13. Nakanishi Y, Mizuno H, Sase H, Fujii T, Sakata K, Akiyama N, et al. ERK Signal Suppression and Sensitivity to CH5183284/Debio 1347, a Selective FGFR Inhibitor. Molecular cancer therapeutics. 2015;14(12):2831-9.
14. Pan BS, Chan GK, Chenard M, Chi A, Davis LJ, Deshmukh SV, et al. MK-2461, a novel multitargeted kinase inhibitor, preferentially inhibits the activated c-Met receptor. Cancer research. 2010;70(4):1524-33.
15. Krejci P, Murakami S, Prochazkova J, Trantirek L, Chlebova K, Ouyang Z, et al. NF449 is a novel inhibitor of fibroblast growth factor receptor 3 (FGFR3) signaling active in chondrocytes and multiple myeloma cells. The Journal of biological chemistry. 2010;285(27):20644-53.
16. Lamont FR, Tomlinson DC, Cooper PA, Shnyder SD, Chester JD, Knowles MA. Small molecule FGF receptor inhibitors block FGFR-dependent urothelial carcinoma growth in vitro and in vivo. British journal of cancer. 2011;104(1):75-82.
17. Miyake M, Ishii M, Koyama N, Kawashima K, Kodama T, Anai S, et al. 1-tert-butyl-3-[6-(3,5-dimethoxy-phenyl)-2-(4-diethylamino-butylamino)-pyrido[2,3 -d]pyrimidin-7-yl]-urea (PD173074), a selective tyrosine kinase inhibitor of fibroblast growth factor receptor-3 (FGFR3), inhibits cell proliferation of bladder cancer carrying the FGFR3 gene mutation along with up-regulation of p27/Kip1 and G1/G0 arrest. The Journal of pharmacology and experimental therapeutics. 2010;332(3):795-802.
18. Reck M, Kaiser R, Mellemgaard A, Douillard JY, Orlov S, Krzakowski M, et al. Docetaxel plus nintedanib versus docetaxel plus placebo in patients with previously treated non-small-cell lung cancer (LUME-Lung 1): a phase 3, double-blind, randomised controlled trial. The Lancet Oncology. 2014;15(2):143-55.
19. Shi XN, Li H, Yao H, Liu X, Li L, Leung KS, et al. In Silico Identification and In Vitro and In Vivo Validation of Anti-Psychotic Drug Fluspirilene as a Potential CDK2 Inhibitor and a Candidate Anti-Cancer Drug. PloS one. 2015;10(7):e0132072.
20. Shi XN, Li H, Yao H, Liu X, Li L, Leung KS, et al. Adapalene inhibits the activity of cyclin-dependent kinase 2 in colorectal carcinoma. Molecular medicine reports. 2015;12(5):6501-8.
21. Li H, Leung KS, Ballester PJ, Wong MH. istar: a web platform for large-scale protein-ligand docking. PloS one. 2014;9(1):e85678.
22. Li H LK, Wong MH. idock: A multithreaded virtual screening tool for flexible ligand docking. In:Proceedings of the 2012 IEEE Symposium on Computational Intelligence in Bioinformatics and Computational Biology (CIBCB). 2012:77–84.

23. Li H LK, Wong MH and Ballester PJ. Improving AutoDock Vina using Random Forest: the growing accuracy of binding affinity prediction by the effective exploitation of larger data sets. Molecular Informatics. 2015;34(2-3):115-26.
24. Li H LK, Nakane T and Wong MH. iview: an interactive WebGL visualizer for protein-ligand complex. BMC Bioinformatics. 2014;15(1):56.
25. Ong SH, Hadari YR, Gotoh N, Guy GR, Schlessinger J, Lax I. Stimulation of phosphatidylinositol 3-kinase by fibroblast growth factor receptors is mediated by coordinated recruitment of multiple docking proteins. Proceedings of the National Academy of Sciences of the United States of America. 2001;98(11):6074-9.
26. Ong SH, Guy GR, Hadari YR, Laks S, Gotoh N, Schlessinger J, et al. FRS2 proteins recruit intracellular signaling pathways by binding to diverse targets on fibroblast growth factor and nerve growth factor receptors. Molecular and cellular biology. 2000;20(3):979-89.
27. L'Hote CG, Knowles MA. Cell responses to FGFR3 signalling: growth, differentiation and apoptosis. Experimental cell research. 2005;304(2):417-31.
28. Chell V, Balmanno K, Little AS, Wilson M, Andrews S, Blockley L, et al. Tumour cell responses to new fibroblast growth factor receptor tyrosine kinase inhibitors and identification of a gatekeeper mutation in FGFR3 as a mechanism of acquired resistance. Oncogene. 2013;32(25):3059-70.
29. Billerey C, Chopin D, Aubriot-Lorton MH, Ricol D, Gil Diez de Medina S, Van Rhijn B, et al. Frequent FGFR3 mutations in papillary non-invasive bladder (pTa) tumors. The American journal of pathology. 2001;158(6):1955-9.
30. Cappellen D, De Oliveira C, Ricol D, de Medina S, Bourdin J, Sastre-Garau X, et al. Frequent activating mutations of FGFR3 in human bladder and cervix carcinomas. Nature genetics. 1999;23(1):18-20.
31. Sibley K, Stern P, Knowles MA. Frequency of fibroblast growth factor receptor 3 mutations in sporadic tumours. Oncogene. 2001;20(32):4416-8.
32. Santos TR, Mendonca RP, Lopes WDZ, Lima RCA, Sackamoto CA, Silva HM. Atividade anti-ixodídica de uma formulação contendo Fluazuron 3,0%+Abamectina 0,5% em bovinos experimentalmente infestados por Rhipicephalus (Boophilus) microplus. A Hora Veterinária2010. p. 18–21.
33. Kemp DH, Hughes S, Binnington KC, Bird PE, Nolan J. Mode of action of CGA 157419 on the cattle tick Boophilus microplus. Bull Soc Jn Parasitol. 81990. p. 1048.
34. Gomes LV, Lopes WD, Cruz BC, Teixeira WF, Felippelli G, Maciel WG, et al. Acaricidal effects of fluazuron (2.5 mg/kg) and a combination of fluazuron (1.6 mg/kg) + ivermectin (0.63 mg/kg), administered at different routes, against Rhipicephalus (Boophilus) microplus parasitizing cattle. Experimental parasitology. 2015;153:22-8.
35. Calligaris IB, De Oliveira PR, Roma GC, Bechara GH, Camargo-Mathias MI. Action of the insect growth regulator fluazuron, the active ingredient of the acaricide Acatak(R), in Rhipicephalus sanguineus nymphs (Latreille, 1806) (Acari: Ixodidae). Microscopy research and technique. 2013;76(11):1177-85.
36. Slowik TJ, Lane RS, Davis RM. Field trial of systemically delivered arthropod development-inhibitor (fluazuron) used to control woodrat fleas (Siphonaptera: Ceratophyllidae) and ticks (Acari: Ixodidae). Journal of medical entomology. 2001;38(1):75-84.
37. Pasay C, Rothwell J, Mounsey K, Kelly A, Hutchinson B, Miezler A, et al. An exploratory study to assess the activity of the acarine growth inhibitor, fluazuron, against Sarcoptes scabei infestation in pigs. Parasites & vectors. 2012;5:40.
38. Kim S, Thiessen PA, Bolton EE, Chen J, Fu G, Gindulyte A, et al. PubChem Substance and Compound databases. Nucleic Acids Res. 2016;44(D1):D1202-13.
39. Berman HMaW, John and Feng, Zukang and Gilliland, Gary and Bhat, T. N. and Weissig, Helge and

Shindyalov, Ilya N. and Bourne, Philip E. The Protein Data Bank. Nucleic Acids Research. 2000.
40. Morris GMaH, Ruth and Lindstrom, William and Sanner, Michel F. and Belew, Richard K. and Goodsell, David S. and Olson, Arthur J. AutoDock4 and AutoDockTools4: Automated docking with selective receptor flexibility. Journal of Computational Chemistry. 2009;30(16):2785-91.
41. Irwin JJaS, Teague and Mysinger, Michael M. and Bolstad, Erin S. and Coleman, Ryan G. ZINC: A Free Tool to Discover Chemistry for Biology. Journal of Chemical Information and Modeling. 2012;52(7):1757-68.
42. Law VaK, Craig and Djoumbou, Yannick and Jewison, Tim and Guo, An Chi and Liu, Yifeng and Maciejewski, Adam and Arndt, David and Wilson, Michael and Neveu, Vanessa and Tang, Alexandra and Gabriel, Geraldine and Ly, Carol and Adamjee, Sakina and Dame, Zerihun T. and Han, Beomsoo and Zhou, You and Wishart, David S. DrugBank 4.0: shedding new light on drug metabolism. Nucleic Acids Research. 2014;42(D1):D1091-D7.
43. Huang RaS, Noel and Wang, Yuhong and Yasgar, Adam and Shinn, Paul and Jadhav, Ajit and Nguyen, Dac-Trung and Austin, Christopher P. The NCGC Pharmaceutical Collection: A Comprehensive Resource of Clinically Approved Drugs Enabling Repurposing and Chemical Genomics. Science Translational Medicine. 2011;3(80):80ps16.

Table 1. FGFR3 inhibitors in the literature.

Name	Research institution	Ref.
BGJ398	Novartis	9
AZD4547	AstraZeneca	10
LY2874455	Eli Lilly and Company	11
Dovitinib	ovartis	12
CH5183284	Chugai Pharmaceutical	13
MK-2461	Merck	14
NF449	Calbiochem	15
SU5402	Calbiochem	16
PD173074	Pfizer	17
Nintedanib	Boehringer Ingelheim	18

Table 2. The six high-scoring compounds purchased and tested in vitro.

ZINC ID	idock score (kcal/mol)	RF-Score v3 (pKd)	Molecular weight (g/mol)	Popular name	Clinical indication
2570819	-11.28	7.92	506.214	Fluazuron	Acaricide
13831810	-10.03	7.95	433.511	Mizolastine	Perennial allergic rhinitis
3831157	-9.54	6.08	518.464	Latamoxef sodium	Antibiotic
608204	-9.47	7.51	403.414	Bentiromide	Assessment of pancreatic exocrine function
73661	-9.19	8.12	350.426	Imidocarb	Babesia ovis infection
608101	-8.06	7.62	388.727	Tioconazole	Antifungal medication

The idock score is an in silico estimation of free energy of binding in kcal/mol units. A more negative value translates to a higher positive value of predicted binding affinity. The RF-Score is an in silico estimation of binding affinity in pKd units, i.e. negative logarithmic scale of dissociation constant.

Figure Legends

Fig 1. Comparison of the effect of six candidate FGFR3 inhibitors on the viability of RT112 and RT4 bladder cancer cells.

(A) The six compounds had discrepant cytotoxicity to RT112 and RT4 cell lines at different concentrationas, with fluazuron exhibiting the highest cytotoxicity compared to the control ($p<0.05$). (B) Fluazuron exhibited dose- and time-dependent inhibition on cell viability in RT112 and RT4 cell lines compared to the control ($p<0.05$).

Fig 2. Effects of fluazuron on cell apoptosis in RT112 and RT4 bladder cancer cells.

Fluazuron treatment at 3μM concentration for 72 hours significantly increased the percentage of apoptosis in RT112 and RT4 cell lines compared to the control ($p<0.05$).

Fig 3. Effects of fluazuron treatment on the expressions of important proteins involved in the FGFR3 signaling. The prefix p- means phosphorylated. Fluazuron treatment decreased the phosphorylation levels of FGFR3, FRS2-α, AKT and ERK.

Fig 4. Effects of oral fluazuron treatment on tumor growth in vivo in nude mice xenografted with RT112 cells. The anti-tumor activity of oral fluazuron (80mg/kg) significantly reduced tumor growth in vivo compared to the control ($p<0.05$).

Fig 5. The predicted binding conformation of fluazuron in complex with FGFR3 (PDB ID: 4K33). (A)2D structure of

fluazuron (ZINC ID: 02570819). (B) FGFR3 is shown in line representation colored by atom type. Fluazuron is rendered as sticks colored by atom type. Intermolecular interacting atoms and residues are labeled. The cyan and green dashed lines indicate hydrogen bonds and hydrophobic contacts, respectively. (C) Same as (B), except that the receptor was shown in molecular surface representation to better appreciate the binding of fluazuron in the cavity.

Fig 1. Comparison of the effect of six candidate FGFR3 inhibitors on the viability of RT112 and RT4 bladder cancer cells.

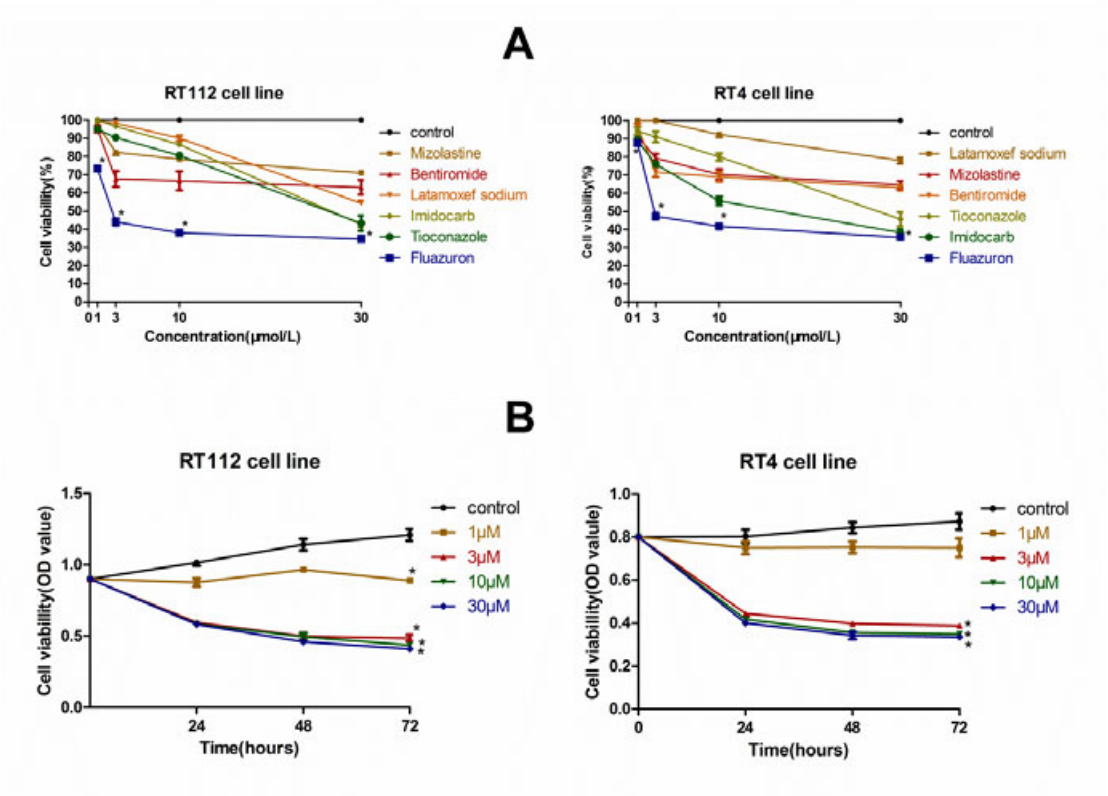

Fig 2. Effects of fluazuron on cell apoptosis in RT112 and RT4 bladder cancer cells.

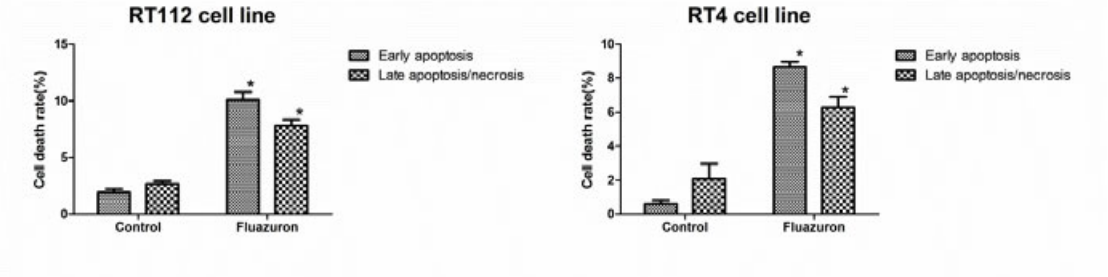

Fig 3. Effects of fluazuron treatment on the expressions of important proteins involved in the FGFR3 signaling.

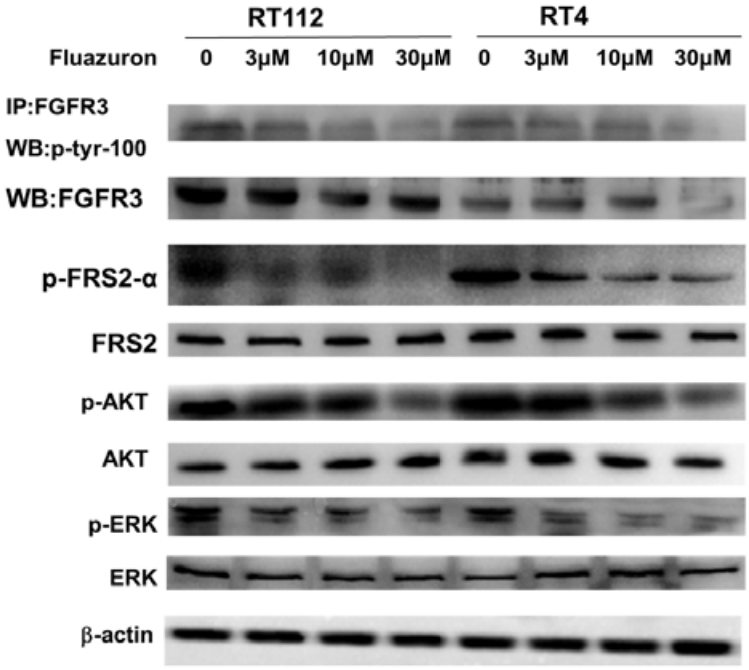

Fig 4. Effects of oral fluazuron treatment on tumor growth in vivo in nude mice xenografted with RT112 cells.

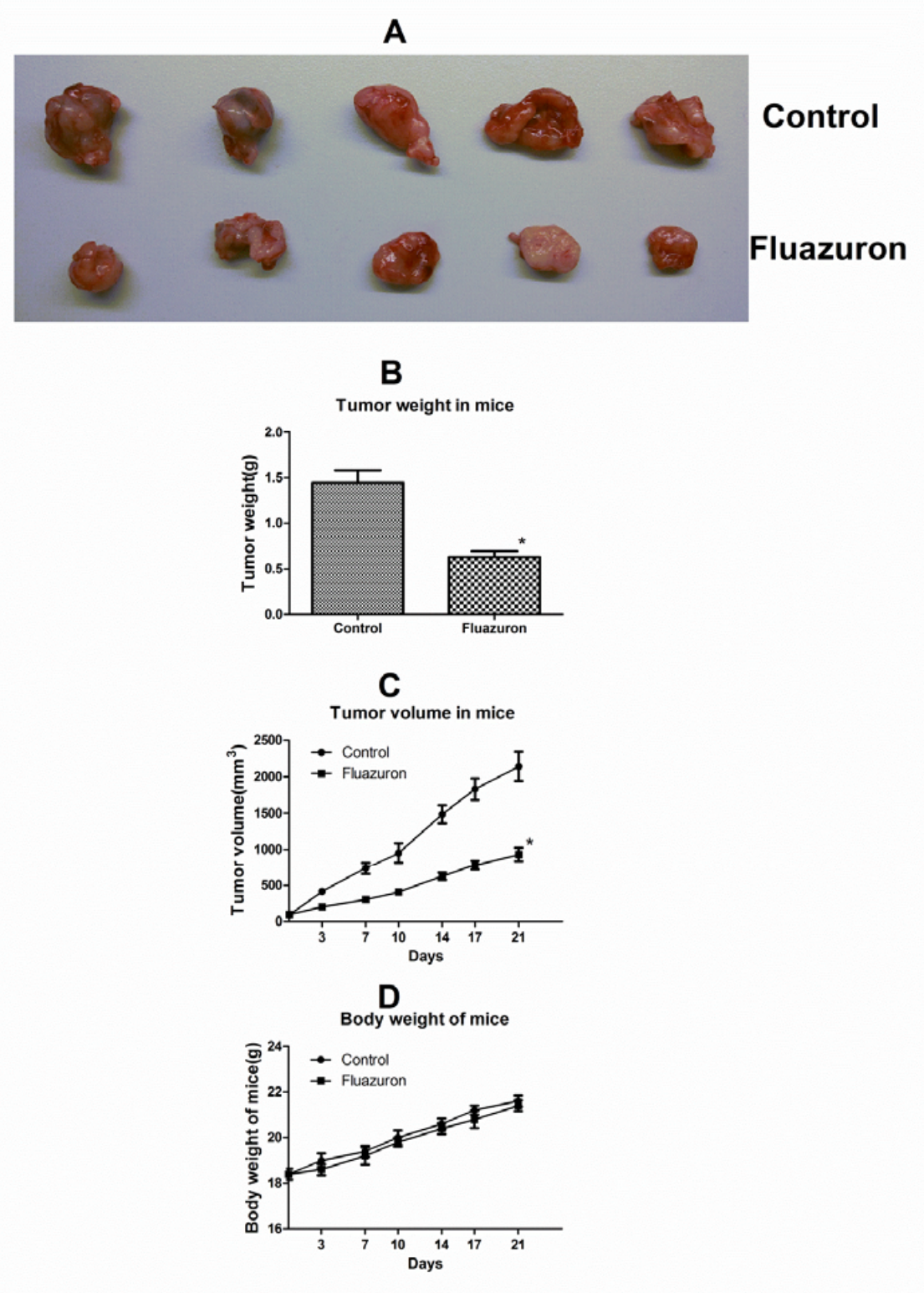

Fig 5. The predicted binding conformation of fluazuron in complex with FGFR3 (PDB ID: 4K33).