Ynamide Click chemistry in development of triazole VEGFR2 TK modulators

Margaréta Vojtičková a, b, Juraj Dobiaš b, Gilles Hanquet a, **, Gabriela Addová c, Rengul Cetin-Atalay d, Deniz Cansen Yildirim e, Andrej Bohač b, f, *

a Université de Strasbourg, Ecole européenne de Chimie, Polyémers et Matériaux (ECPM) Laboratoire de Synthèse et Catalyse (UMR CNRS 7509), 25, rue Becquerel, F-67087 Strasbourg, France
b Comenius University in Bratislava, Faculty of Natural Sciences, Department of Organic Chemistry, Mlynská dolina, 842 15 Bratislava, Slovakia
c Institute of Chemistry, Mlynská dolina, 842 15 Bratislava, Slovakia
d Cancer Systems Biology Laboratory, Graduate School of Informatics, METU, 06800 Ankara, Turkey
e Department of Molecular Biology and Genetics, Faculty of Science, Bilkent University, Ankara, Turkey
f Biomagi, Ltd., Mamateyova 26, 851 04 Bratislava, Slovakia

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Structure novelty, chemical stability and synthetic feasibility attracted us to design 1,2,3-triazole compounds as potential inhibitors of VEGFR2 tyrosine kinase. Novel triazoles T1 – T7 were proposed by oxazole (AAZ) from PDB: 1Y6A)/1,2,3-triazole isosteric replacement, molecular modelling and docking. In order to enable synthesis of T1 – T7 we developed a methodology for preparation of ynamide T2. Compound T2 was used for all Click chemistry reactions leading to triazoles T1 – T3 and T6 – T7. Among the obtained products, T1, T3 and T7 specifically bind VEGFR2 TK and modulate its activity by concentration dependent manner. Moreover predicted binding poses of T1 – T7 in VEGFR2 TK were similar to the one known for the oxazole inhibitor AAZ (PDB: 1Y6A). Unfortunately the VEGFR2 inhibition by triazoles e.g. T3 and T7 is lower than that determined for their oxazole biososters T3-ox and AAZ, resp. Different electronic properties of 1,2,3-triazole/oxazole heterocyclic rings were proposed to be the main reason for the diminished affinity of T1 – T3, T6 and T7 to an oxazole AAZ inhibitor binding site in VEGFR2 TK (PDB: 1Y6A or 1Y6B). Moreover T1 – T3 and T6 were screened on cytotoxic activity against two human hepatocellular carcinoma cell lines. Selective cytotoxic activity of T2 against aggressive Mahlavu cells has been discovered indicating possible affinity of T2 to Mahlavu constitutionally active PI3K/Akt pathway.

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1. Introduction

Anti-angiogenesis agents that target malignant vasculature are of considerable interest due to their perceived potential to target tumour resistance towards chemo- and radiotherapy [1,2]. Vascular endothelial growth factors (VEGFs) and their corresponding family of receptor tyrosine kinases (VEGFRs) are the key proteins modulating angiogenesis, the formation of new vasculature from an existing blood network. These include VEGFR1 (Flt1), VEGFR2 (Kinase Insert Domain Receptor (KDR) or Flk1). The last one, VEGFR3 is specialized for lymphangiogenesis [3]. VEGFR2 is the major positive signal transducer for endothelial cells proliferation and differentiation [4]. There has been considerable evidence, including clinical observations, that the abnormal angiogenesis is implicated in a number of diseases including rheumatoid arthritis, inflammation, degenerative eye conditions and cancer [5,6].

Cancer stem cells (CSCs) represent a small but the most tumourigenic subpopulation from the tumour cells responsible for metastasis, tumour recurrence and drug resistance. CSCs, also called “the roots of cancer”, are considered to be a new promising therapeutic target [7]. VEGFR2 is regarded as an endothelial cell protein but evidences suggest that VEGFRs may be expressed also by cancer cells. Glioblastoma multiforme (GBM) is characterized by
florid vascularisation and aberrantly elevated VEGF. Antiangiogenic therapy with bevacizumab reduces GBM tumour growth. VEGFR2 is expressed on CD133 + human glioma cancer stem cells (GCSCs). VEGF-VEGFR2-Neuropilin 1 (NRPI) axis influences GCSCs viability, self-renewal, and tumourigenicity. GCSCs viability was attenuated by direct inhibition of VEGFR2 TK activity and/or knockdown of VEGFR2 or NRPI. VEGFR2 inhibitors may block the VEGF-VEGFR2-NRP1 pathway [8,9]. VEGF via VEGFR2 stimulates proliferation of glioblastoma multiform CSCs. VEGF stimulates GCSCs tumourigenesis and angiogenesis. Suppression of VEGFR2 signalling is therefore a potential therapeutic strategy in GBM [10]. VEGFR2 plays a key role in ability of glioblastoma CSCs to vasculogenic mimicry (VM) formation, neovascularisation and tumour initiation. Knockdown of VEGFR2 in GCSCs markedly reduced their self-renewal, forming tubules, initiating xenograft tumours, promoting vascularisation and the establishment of VM. VEGFR2 is an essential molecule to sustain the “stemness” of GCSCs, their capacity to initiate tumour vasculature, and direct initiation of tumours [11]. Therefore VEGFR2 inhibitors are important compounds reducing angiogenesis and promising compounds to interfere with CSCs resistance.

The PDB database contains VEGFR2 TK complex 1Y6A possessing N-aryl-5-aryloxazol-2-amine ligand AAZ (Fig. 1) determined as a powerful VEGFR2 inhibitor (IC50: 22 nM). Ligand AAZ was prepared in five steps in a low overall 10% yield mostly due to the problematic oxazole-2-amine fragment formation [12]. Low yields of oxazole-2-amine formation (1–58 %) have been also described [13,14]. Moreover AAZ contains N-aryloxazol-2-amine part that uses to liberate from connection with oxazole by influence of nucleophilic reagent (e.g. amines, alkoxides etc.). Nucleophilic attack on C(2) of AAZ oxazole ring resulting in toxic aniline 26 (Scheme 1). (unpublished results) Low yielding synthesis of N-aryloxazol-2-amines, their problematic stability and potential toxicity resulting from releasing aniline inspired us to develop novel, stable and synthetically more feasible VEGFR2 modulators based on the oxazole/1,2,3-triazole isosteric replacement. (Fig. 1) Replacing the heterocyclic ring in the structure of some inhibitors can provide a novel compounds with improved properties [15].

1,4-Disubstituted 1,2,3-triazoles are stable compounds easily obtainable in high yields from organic alkynes and azides by Cu(I) catalyzed reaction (CuACC Click chemistry) [16]. Click reaction allows rapid preparation of different triazoles that is especially advantageous if one of the reactants is the same in all performed reactions (e.g. ynamide 22a in our case, Schemes 8 and 11). Additionally, Click reaction can selectively provide also 1,5-regioisomeric compounds via Ru (II) catalyzed cycloaddition [17] (Fig. 1).

Only few active 1,2,3-triazole containing VEGFR2 TK inhibitors are described in the literature: 6 inhibitors 1–6 possessing IC50 < 50 nM. (Fig. 2) 10 substances with IC50 < 100 nM and 19 compounds having IC50 < 200 nM [18].

Imidazopyridazine 1 is the most active 1,2,3-triazole containing VEGFR2 inhibitor (IC50: 1.2 nM) [19]. Other active inhibitors are e.g. benzoxazolamine 2 (IC50: 8 nM) [20], quinazolinamines 3 and 4 (IC50: 10 and 30 nM, resp.) [21,22], indazolure 5 (IC50: 45 nM) [23] and ureidopyrimidine-N-oxide 6 (IC50: 49 nM) [24]. All of these VEGFR2 inhibitors (Fig. 2) possess a terminal 1,2,3-triazole fragment. In these cases, triazole group need not contribute to the inhibitor target affinity. Triazole core can be used to improve the ligand pharmacokinetic properties. E.g. the most potent inhibitor 1 (IC50: 1.2 nM) was designed by pyrazole/triazole isosteric replacement in the structure of ligand from PDB complex 3VO3. In case of PDB: 3VO3 a pyrazole ring does not bind the VEGFR2 TK directly. It is exposed towards the solvent accessible part of the protein [19]. Therefore triazole core that replaces a pyrazole ring can have the same position. Compound 7 is a close analogue of sunitinib that is an active base of the drug Sutent (Pfizer Inc.). Sunitinib inhibitory activity IC50 = 39 nM (VEGFR2 TK) was determined [25]. The structure 7 possesses N,1,2,3-triazolethyl group instead of N,N-diethylaminoethyl group present in sunitinib (Fig. 3). Recently an X-ray structure of VEGFR2 TK/sunitinib complex (PDB: 4AGD) appeared [26]. From its analysis is clear that the N,N-diethylaminoethyl group in sunitinib is exposed out of the VEGFR2 protein and represents only a group improving the ligand pharmacokinetic profile.

Although X-ray structure of complex 7 with VEGFR2 kinase is not known, an analogous function can be expected also for the triazole group in 7 (Fig. 3).

Only few VEGFR2 TK inhibitors possessing internal 1,2,3-triazole core were found in the literature. Compounds 8–10 were described as VEGFR2 activity modulators (Ki > 915 nM (VEGFR2)) [27]. The staurosporin-like inhibitor 11 moderately influences the VEGFR2 kinase activity (IC50: 200 nM) [28] (Fig. 4).

Kiselev et al. described the most active VEGFR2 inhibitors 12–14 (IC50: 51–87 nM) possessing an internal 1,2,3-triazole core [29] (Fig. 5).

Our docking results proposed that the triazole fragment in 12–14 directly contributes the binding with VEGFR2 TK. The predicted intermolecular interactions of 12 and 14 are depicted on Fig. 6. Moreover proposed positions of the above triazole inhibitors in VEGFR2 ATP active site is similar to the poses of ligands from PDB complexes 2P2I (IC50: 38 nM), and 3EFL (IC50: 3 nM). (not shown) Both VEGFR2 kinase conformers (PDB: 2P2I and 3EFL) used in docking experiments are VEGFR2 inactive (DFG-out) kinases originating from X-ray structure of VEGFR2 TK/sunitinib complex (PDB: 1Y6A) [12]. The intermolecular interactions for both AAZ conformers present in VEGFR2 TK complex PDB: 1Y6A are depicted on Fig. 7.

Considering the data mentioned above, we decided to prepare VEGFR2 TK modulators T1–T7 possessing an internal 1,2,3-triazole core and determine their VEGFR2 TK inhibition potential.

2. Results and discussion

2.1. Interaction analysis of AAZ conformers

Oxazole VEGFR2 TK inhibitor AAZ was developed by GlaxoSmithKline [12]. The intermolecular interactions for both AAZ conformers present in VEGFR2 TK complex PDB: 1Y6A are depicted on Fig. 7.

Based on the above analysis, we decided to keep the pharmacologically interesting 5-(ethylsulfonil)-2-methoxyphenylamine fragment from AAZ ligand in all predicted triazole structures T1–T7 (Fig. 10). The proposed intermolecular interactions of triazoles T1–T7 and their poses in VEGFR2 kinase were similar to those known for AAZ ligand from PDB complex 1Y6A (e.g. T3 from Fig. 12).
2.2. In Silico predictions

An interaction analyse, molecular modelling and docking were used to identify the skeletons of 1,2,3-triazole derivatives T1 – T7. (Fig. 10) A reduced success of in Silico predictions is often associated with the ligand based target induced fit [30–32]. Therefore, for our docking experiments we selected the kinase variants of VEGFR2 from PDB complexes 1Y6A and 1Y6B possessing structurally the most relative oxazole ligands (AAZ and AAX, resp.). These ligands represent Type I, ATP competitive VEGFR2 inhibitors that bind to an exceptional inactive VEGFR2 tyrosine kinase possessing an opened activation loop as was discovered by us recently [14]. Because the structure similarity between AAZ (AAX) and proposed triazoles (e.g. T7, T6, T3 and T5) we did not expect strong influence of triazole ligand based induced fit. On the other hand, it was not easy to find triazole structures possessing AAZ-like score and pose by docking. The initial in Silico experiments were performed by an older DOCK software version with a kinase taken from PDB complex 1Y6B [33].

Fig. 2. The structures of VEGFR2 TK inhibitors 1–6.
Fig. 3. **On the Left:** The structure of sunitinib and its VEGFR2 TK inhibitory activity. **In the middle:** The PDB: 4AGD complex of VEGFR2 TK with sunitinib ligand that projects Et2NCH2CH2- group out of the protein into the solvent accessible area. (Juxtamembrane part Leu802-Pro812, part of VEGFR2 activation loop: Asp1046–Asp1056 and water molecules were omitted for clarity). **On the right:** The structure of 1,2,3-triazole containing inhibitor 7 (mimicking sunitinib).

![Fig. 3](image)

Fig. 4. VEGFR2 modulators 8–11 possessing an internal 1,2,3-triazole core.

![Fig. 4](image)

Fig. 5. Potent VEGFR2 inhibitors 12–14 with an internal 1,2,3-triazole ring.

![Fig. 5](image)

Fig. 6. Structures, VEGFR2 TK IC50 activities, predicted intermolecular interactions and docking scores of 12 and 14 obtained in VEGFR2 TK variants from PDB: 2P2I and 3EFL, resp. In both cases a triazole fragment in 12 and 14 directly contributes to the ligand/receptor binding (+II: induced dipole, HB: hydrogen bond, III: stacked interaction). However the position of the triazole ring in 12 and 14 is conformationally blocked by an intermolecular HB with neighbouring NH group.
Some 1,4-disubstituted triazole skeletons that fulfilled both required score (AAZ: −53.6 kcal/mol) and pose conditions were selected: e.g. T3 (score = −57.2), T5 (−52.9), T6 (−51.3), T2 (−51.3), T4 (−49.2). The triazole core of T1−T7 retains predicted hydrogen bond interaction with the kinase backbone amino acid residue Cys917 an important interaction known from AAZ oxazole ring in PDB: 1Y6A (Fig. 7). Pharmacokinetic properties of triazoles T1−T7 predicted by Molinspiration toolkit supported their drug-likeness [34–38] (see supporting material). Proposed properties for 1,4-regioisomeric triazoles T1−T7 were promising. Therefore we decided to perform their synthesis. 1,5-Regioisomeric triazoles (Fig. 1) of azides [39,40] were also docked by the same conditions. All these compounds were less advantageous possessing lower docking scores and several of them did not retain expected AAZ-like pose in VEGFR2 TK complex (not shown). Therefore 1,5-regioisomers were omitted.

For the synthesis of all 1,4-disubstituted 1,2,3-triazoles T1−T7 the pharmacophoric ynamide 22 (in modifications 22a−22d) was required. (Schemes 8, 9 and 11) Its synthesis was developed starting from aniline derivative 26. (Scheme 1)

### 2.3. Synthesis

In order to obtain compounds T1−T7 (Fig. 10), we started synthesis their precursors azides 15−21 and ynamides 22a−22d (Fig. 8).

#### 2.3.1. Preparation of ynamides 22a,b

Ynamides are stabilized equivalents of the corresponding reactive ynamines [39,40]. Diminishing the electron-donating ability of ynamine nitrogen by its protection with an electron-withdrawing group (EWG) leads to more stable ynamides. Since the ynamine nitrogen by its protection with an electron-withdrawing group (EWG) leads to more stable ynamides. Since the ynamine nitrogen by its protection with an electron-withdrawing group (EWG) leads to more stable ynamides. Since the ynamine nitrogen by its protection with an electron-withdrawing group (EWG) leads to more stable ynamides. Since the ynamine nitrogen by its protection with an electron-withdrawing group (EWG) leads to more stable ynamides. Since the ynamine nitrogen by its protection with an electron-withdrawing group (EWG) leads to more stable ynamides. Since the ynamine nitrogen by its protection with an electron-withdrawing group (EWG) leads to more stable ynamides. Since the ynamine nitrogen by its protection with an electron-withdrawing group (EWG) leads to more stable ynamides. Since the ynamine nitrogen by its protection with an electron-withdrawing group (EWG) leads to more stable ynamides. Since the ynamine nitrogen by its protection with an electron-withdrawing group (EWG) leads to more stable ynamides. Since the ynamine nitrogen by its protection with an electron-withdrawing group (EWG) leads to more stable ynamides. Since the ynamine nitrogen by its protection with an electron-withdrawing group (EWG) leads to more stable ynamides. Since the ynamine nitrogen by its protection with an electron-withdrawing group (EWG) leads to more stable ynamides. Since the ynamine nitrogen by its protection with an electron-withdrawing group (EWG) leads to more stable ynamides. Since the ynamine nitrogen by its protection with an electron-withdrawing group (EWG) leads to more stable ynamides. Since the ynamine nitrogen by its protection with an electron-withdrawing group (EWG) leads to more stable ynamides. Since the ynamine nitrogen by its protection with an electron-withdrawing group (EWG) leads to more stable ynamides. Since the ynamine nitrogen by its protection with an electron-withdrawing group (EWG) leads to more stable ynamides. Since the ynamine nitrogen by its protection with an electron-withdrawing group (EWG) leads to more stable ynamides. Since the ynamine nitrogen by its protection with an electron-withdrawing group (EWG) leads to more stable ynamides. Since the ynamine nitrogen by its protection with an electron-withdrawing group (EWG) leads to more stable ynamides. Since the ynamine nitrogen by its protection with an electron-withdrawing group (EWG) leads to more stable ynamides. Since the ynamine nitrogen by its protection with an electron-withdrawing group (EWG) leads to more stable ynamides. Since the ynamine nitrogen by its protection with an electron-withdrawing group (EWG) leads to more stable ynamides. Since the ynamine nitrogen by its protection with an electron-withdrawing group (EWG) leads to more stable ynamides. Since the ynamine nitrogen by its protection with an electron-withdrawing group (EWG) leads to more stable ynamides. Since the ynamine nitrogen by its protection with an electron-withdrawing group (EWG) leads to more stable ynamides. Since the ynamine nitrogen by its protection with an electron-withdrawing group (EWG) leads to more stable ynamides.

Corey–Fuchs alkylation proved to be ineffective in our hands. (Scheme 1, Path A) In fact, application of the Brückner procedure [43] on protected formamides 24, which have to be protected with an EWG protecting group prior to their formylation, was successful only in the case of the corresponding dichlorovinyl intermediate 23c (tosyl protecting group) in a very modest yield (9%), and therefore this path was abandoned. The Bestmann–Ohira alkylation (Scheme 1, Path B) was ineffective with 24c and only starting material was recovered [44]. A transformation of N-trichloroacetate 28c to the corresponding ynamide 22c according to the methodology of Spezie and Smith has been tested [45]. (Scheme 1, Path C and Scheme 2) Treatment of N-trichloroacetate 28c obtained from N-tosyl-aniline 31c with PPh3 in refluxing toluene led to a mixture of uncharacterized products. (Scheme 2)

An exciting expansion in ynamide chemistry [46,47] has been initiated by the pioneer work of Stang and Kitamura who prepared ynamides by reaction of alkyl ylidonium triflates [48] or tosylates [49] with lithium amides. Unfortunately, this methodology applied to amides 31a,b and iodonium triflate 30 failed. (Scheme 1, Path D) Inspired by Buchwald’s copper-catalyzed N-alkylamylides [50], practical cross-coupling between amides and alkylene bromides have been developed using copper salts (CuSO4·5 H2O [51], CuI, KHMDS) [49] or tosylates [48] or tosylates with PPh3 in re.

#### 2.3.2. Preparation of azides 15−21

The synthesis of azides 15−19 and 21 was performed by a Suzuki–Miyaura cross coupling conditions followed by an aromatic substitution (Scheme 4).

According to this strategy, azides 15, 16, 17 and 19 were prepared in 57 and 45% yield over two steps for 15 and 17, 33% over three steps for 16 and 37% yield over seven steps for 19 starting from p-nitroaniline 37. (Scheme 5) Palladium catalyzed coupling of 2-bromopyridine with boronic acid 33 in the presence of sodium carbonate in a mixture of water/ethanol and DME at 75 °C within 16 h [54] afforded biaryl bromide 34 which was treated with sodium azide and copper(I) iodide according to the procedure of Liang and co-workers to deliver azide 15 [55]. Subsequently a palladium-catalyzed acetoxylaition of arne C–H bond at ortho position to the pyridine-2-yl substituent has been performed by phenylidinium diacetate (PhI(OAc)2) in acetic anhydride [56]. These conditions led regioselectively to azide 16 in 57% yield. Biaryl bromide 36 was obtained from the Suzuki–Miyaura cross coupling

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**Fig. 7.** The intermolecular interactions for both AAZ conformers present in VEGFR2 TK complex PDB: 1Y6A.
between pinacolboronic ester \(35\) and \(1,3\)-dibromobenzene and converted to azide \(17\) using the Liang’s protocol. (Scheme 5)

Preparation of azide \(19\) started with ortho-iodination \[58\] of \(p\)-nitroaniline \(37\), followed by protection of amino group of the resulting iodide \(38\) as acetamide \(39\) \[59\] and subsequent Suzuki–Miyaura coupling with 1-naphtylboronic acid \[60\] to deliver biarylic compound \(40\) in 82% yield over three steps. The transformation of nitro derivative \(40\) to the corresponding azide \(42\) was performed in 2 steps via aniline \(41\) \[61,62\]. Deacetylation of \(42\) was performed under basic conditions. Finally, urea derivative \(19\) was prepared from unstable amino azide \(43\) by trichloroacetyl

TIPS: \^{\text{t-Pr}}\text{Si}; Tos: tosyl; Piv: pivaloyl

**Fig. 8.** Structures of required azides \(15–21\) and ynamides \(22a–22d\).

**Scheme 2.** An attempt for preparation of ynamide \(22c\) via trichloroaetate \(28c\).

**Scheme 3.** Successful synthesis of ynamides \(22a,b\) via copper-mediated amide alkynylation.

**Scheme 4.** General strategy towards azides \(15–19\) and \(21\).
isocyanate in dry dichloromethane followed by basic treatment [63] (Scheme 5).

Preparation of azides 18 and 21 following the same pathway proved to be unsuccessful with exception of 18b that was prepared in 44% yield over two steps (Scheme 6).

Biarylic bromophenols 46a,b were obtained by coupling reaction of pinacolboronic ester 35 with protected iodophenols 44a,b, prepared from para-bromoanisol respectively in one (98% yield) or three steps (87% overall yield), and subsequent deprotection of the resulted phenol ethers 45a,b in 23 and 49% two step yield, respectively. Unfortunately zwitterionic bromophenol 46 was insoluble and its transformation into the azide 18 was impossible. For this reason we decided to perform the Huisgen cycloaddition prior HO-deprotection step in order to prepare triazole T5 (Scheme 8). Methoxymethyl protected phenol 18b was obtained in 44% yield through two steps from 44b (Scheme 6). Preparation of pyrrole boronic ester 48 from commercially available 3-bromo-N-triisopropylsilylpyrrole 47 has been performed using pinacolborane in the presence of a catalytic amount of bis(acetonitrile)palladium dichloride and S-Phos (dicyclohexyl[2,6-di-methoxybiphenyl]-2-yl)phosphine) [64]. The Suzuki–Miyaura cross coupling [63,65,66] between 48 and the dihalogenated phenol 49 led only to 16% yield of biarylic compound 50 (Scheme 6). This low yield led us to consider another pathway and to perform the coupling reaction

Scheme 5. Synthesis of azides 15—17 and 19.

Scheme 6. Attempts for the synthesis of azides 18 and 21.
Preparation of 18b.
after the Click cycloaddition in order to prepare triazole T1 (Scheme 11).

The pyrimidine azide 20 was prepared through pyrimidine core construction in 41% yield over three steps. Compound 53 was obtained from condensation of benzamidine hydrochloride 51 with β-ketoester 52 in 56% yield according to the literature [67] (Scheme 7). Treatment of 53 in refluxing POCl3 and PCl5 within 3 h delivered 54 in 85% yield. Transformation of chloride 54 to azide 20 was performed using sodium azide and catalytic amount of tetra-n-butylammonium bromide (TBAB) [68] (Scheme 7).

2.3.3. Click reactions and preparation of target compounds

We tried to perform the synthesis of predicted 1,2,3-triazoles T2 – T7 via cycloaddition between azides 15 – 20 and ynamide 22a (Scheme 8). The Click chemistry concept was introduced by Sharpless and co-workers in 2001 [69,70]. All Click reactions were performed under mild conditions using in situ prepared catalytic amount of copper(I) to control the 1,4-regioselectivity (5 mol %

![Scheme 7. Preparation of pyrimidine azide 20.](image1)

![Scheme 8. Preparation of target molecules T2, T3, T6 and T7 via Click chemistry approach.](image2)
CuSO₄·5H₂O/10 mol % sodium ascorbate in a mixture of solvents (t-BuOH/H₂O/CHCl₃) at room temperature. Deprotection of the resulting methyl carbamates 55a–59a was performed in 1 M methanolic solution of KOH to give triazoles T2, T3, T6, T7 in good yields (67–82%). Unfortunately, pyrimidine triazole T4 was unstable in these conditions and only decomposition products were observed. Cycloaddition of 22a with 22a and its crude mixture deprotection resulted in a mixture of products containing the expected triazole T5 which was impossible to isolate (Scheme 8).

In order to get pyrimidine triazole T4, different conditions for deprotection of 59a have been tested (1 M KOH in MeOH or 0.5 M KOH ethylene glycol both at rt or by reflux) and gave only products of decomposition. In order to circumvent these difficulties, we prepared also N-Boc protected triazole 59b by Click reaction of ynamide 22b and azide 20. Then we tried different conditions for deprotection of cycloadduct 59b (Scheme 9) and found that 12 M HCl in EA or TFA in THF led to T4 with 80 and 90% conversion [71,72] (Scheme 9). Unfortunately, we were unable to isolate the pure triazole T4 in reasonable amounts as it was unstable on silica or alumina gel. Trituration or crystallization of the crude mixture was also unsuccessful in our hands.

Since the preparation of azide 18 failed due to the high insolubility of its precursor 46 (Scheme 6), we tried a Suzuki–Miyaura coupling between triazole derivative 60 and pinacol boronic ester 61 [54] or the boronic acid 61 [73,74] but also in these cases only insoluble material had been obtained (Scheme 10).

Finally, the triazole T1 has been prepared by the Click reaction prior to the biaryl formation (Scheme 11). The synthesis started with the preparation of the functionalized azides 65 and 68 by nitration of 1-iodo phenol 62 using a 70% solution of nitric acid giving nitrophenol 63. Part of 63 was acetylated to ester 66 and a subsequent reduction of both nitro compounds 63 and 66 using SnCl₂ provided anilines 64 and 67, resp. [75] that were transformed into azides 65 and 68 by diazotation and reaction with NaH. Cycloaddition between ynamide 22a and azides 65 or 68 using the Click chemistry conditions furnished triazole derivatives 69 or 70 in 80 and 92% yield respectively. The latter was submitted to Suzuki–Miyaura coupling with pinacolboronic ester 54 to afford N-protected triazole T1. Compound T1 was deprotected under basic conditions to give triazole T1 in 5% yield over 6 steps (via azide 65) or 8% yield over 7 steps (via azide 68) both started from 2-iodophenol 62 (Scheme 11).

2.4. Inhibition of VEGFR2 TK activity

Prepared compounds T1–T3 and T6–T7 were screened on their ability to inhibit VEGFR2 kinase activity. Their IC₅₀ values were determined by radiometric protein kinase assay in 10 semi-logarithmic concentrations [25]. Compounds T1, T3 and T7 bind specifically to VEGFR2 tyrosine kinase and resulting typical concentration dependent enzymatic activity sigmoid curves. (e.g. Fig. 9).

The structures of AAZ and 1,2,3-triazoles T1–T7 together with their docking score [76] and obtained biological activity (IC₅₀, VEGFR2 TK) are depicted on Fig. 10.

The compounds T1–T3, T6–T7 exhibited different inhibitory properties: from inactive compounds T2 and T6 (IC₅₀ > 1E-4 M), through weakly active T7, T1 (IC₅₀: 42 and 40 μM, resp.) to moderately active inhibitor T3 (IC₅₀: 69.6 μM). Triazole compounds T1–T3 and T6–T7 possess the same pharmacophoric 5-(ethylsulfonyl)-2-methoxyphenylamine moiety therefore the observed activities are dependent on the remaining aryltriazole fragments.

2.5. Re-docking experiments

In initially performed docking experiments (an older DOCK software) triazoles T2–T6 showed better or similar scores as their oxazole precursor AAZ (vide supra, see part 2.2 In Silico predictions). These results do not correlate well with obtained IC₅₀ activities determined from the biological assay. Therefore an additional docking experiment was performed with newer version of the docking software (DOCK 3.6) on more calibration reliable VEGFR2 kinase taken from PDB: 1Y6B [76]. By these conditions all triazole structures although still retaining AAZ-like pose in VEGFR2 TK, showed worse score values compared to their oxazole analogue AAZ (Figs. 10 and 11).

A similar result, disfavouring 1,2,3-triazoles compare to oxazoles, was obtained from docking experiment of isosteric pairs
Predicted VEGFR2 TK interactions of the most active 1,2,3-triazole and its oxazole bioisostere are depicted on Fig. 12. The activity of T3-ox inhibitor (IC\textsubscript{50}: 12.8 nM, VEGFR2 TK) was published by us recently\cite{77}.

2.6. Influence of isosteric oxazole/triazole replacement

The above re-docking experiment of 1,2,3-triazoles T1 – T7 resulted lower score than that obtained for their oxazole analogues. Triazoles T3 and T7 exhibit much less inhibitory activity against VEGFR2 kinase compared to their known oxazole bioisosters T3-ox and AAZ (T3/T3-ox, IC\textsubscript{50}: 6950 nM/12.8 nM and T7/AAZ, IC\textsubscript{50}: 42 400 nM/22 nM). Therefore we proposed that different electronic properties of oxazole/triazole core (the size and dipole moment orientation) could be responsible for disfavouring of 1,2,3-triazole derivatives in VEGFR2 TK AAZ binding site. The structures and dipole moments depicted on Fig. 13 were performed by Discovery Studio 3.5 Visualizer\cite{78}.

VEGFR2 kinase surrounds the oxazole core of AAZ ligand (PDB: 1Y6A) by seven nonpolar amino acid residues: Phe\textsubscript{916}, Val\textsubscript{914}, Val\textsubscript{897}, Ala\textsubscript{864}, Leu\textsubscript{838}, Leu\textsubscript{1033}, and Cys\textsubscript{917}. This lipophilic pocket is less favourable to accommodate more polar 1,2,3-triazole ring. Therefore triazoles T1 – T7 are less suitable to inhibit VEGFR2 kinase compare to their bioisosteric oxazole analogues.

2.7. Cytotoxic activity of triazoles on hepatocellular carcinoma cell lines

Four of developed 1,4-triazole compounds T1 – T3 and T6 were
screened on their cytotoxic activity against well and poorly-differentiated aggressive human hepatocellular carcinoma cell lines (Huh-7 and Mahlavu). Huh-7 is a well-differentiated (epithelial-like) hepatocellular carcinoma cell line commonly used studying liver cancer potential therapies. Huh7 expresses mutated but functional p53 protein. Mahlavu is a poorly-differentiated (mesenchymal-like) hepatocellular carcinoma cell line. This cell line is associated with the loss of PTEN protein expression leading to the constitutive activation of PI3K/Akt pathway involved in cell survival and anti-apoptotic signalling. Comparative analysis of both cell lines with lower IC50 values for Mahlavu cells are preferred for the reason that the inhibitor may acts on its hyperactive PI3K/Akt pathway. Additionally selective inhibitor can be further analysed for its possible specific target in PI3K/Akt pathway.

Results indicate that three of four tested triazole molecules were able to inhibit growth of both tumour cell lines by half in indicated concentrations. (Table 1).

All triazoles T1–T3 and T6 display lower IC50 values in mesenchymal-like Mahlavu cells. Even though no enzymatic modulation of T2 on VEGFR2 TK was obtained, triazole T2 is highly selective inhibitor of growth of Mahlavu cells. The mechanism of its action as possible inhibitor of PI3K/Akt pathway could be further investigated at molecular level.

3. Conclusions

Seven 1,2,3-triazole compounds T1–T7 derived from oxazole VEGFR2 TK inhibitor AAZ were designed. A methodology for the synthesis of the pharmacophoric ynamides 22a,b was developed. Ynamide 22a was used as a joined precursor for the synthesis of all proposed compounds T1–T7. Cu(I) catalyzed Click reaction performed from 22a with different azides (yields: 68–92 %) confirmed the synthetic reliability of this still “exotic” ynamide reagent. Five triazole compounds T1–T3, T6–T7 were prepared (Schemes 8 and 11) and screened in the radiometric VEGFR2 kinase assay. Within concentration limits of used biological test, two compounds T2, T6 showed to be inactive (IC50 > 1E-4 M) and other three triazoles modulate VEGFR2 tyrosine kinase activity: T7 (IC50: 42.0 μM), T1 (IC50: 40.1 μM) and T3 (IC50: 6.96 μM). (Fig. 10) The activities of new compounds were significantly lower than the ones obtained for their oxazole bioisosters (e.g. T3/T3-ox in Fig. 12 and T7/AAZ in Fig. 10, resp.). Despite the diminished activity, the triazole modulators T1, T3 and T7 inhibit VEGFR2 kinase by concentration dependent manner. Concerning the identical substructure, the inhibitory activity of 1,4-triazoles T1–T3, T6–T7 is depending on the decoration of their aryl part joined to N(1) of the triazole core. (Fig. 1) The different electronic properties of 1,2,3-triazole and oxazole fragments (size and orientation of the dipole moments, Fig. 13) were proposed to be responsible for low activities of triazoles T1–T3, T6–T7. The more polar triazole core binds less readily into VEGFR2 TK lipophilic oxazole binding pocket known

![Fig. 9](image9.png)  
An example of diminishing VEGFR2 TK activity (y axis, %) by increasing concentration of triazole T3 (x axis, logarithmic scale) and determining T3 activity (IC50: 0.96 μM).

![Fig. 10](image10.png)  
The structures of AAZ and T1–T7, their docking score (software DOCK3.6, [76] VEGFR2 TK conformer from PDB: 1Y6B [1Y6A], resp.) and obtained IC50 activity (VEGFR2 TK), if not otherwise stated. NA: the compound was not available.
from PDB: 1Y6A or 1Y6B. We can conclude that the 1,2,3-triazole compounds T1, T3 and T7 are weaker VEGFR2 inhibitors than their oxazole analogues.

In addition T1–T3 and T6 were screened on their cytotoxic activity against two human hepatocellular carcinoma cell lines (Huh-7 and Mahlavu). Mahlavu is aggressive hepatocellular carcinoma.

**Table 1**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Tumour cell lines IC50 [uM]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Huh7</td>
</tr>
<tr>
<td>T6</td>
<td>12.8</td>
</tr>
<tr>
<td>T1</td>
<td>18.6</td>
</tr>
<tr>
<td>T3</td>
<td>17.7</td>
</tr>
<tr>
<td>T2</td>
<td>NAa</td>
</tr>
</tbody>
</table>

Data in the table are sorted according to activity against aggressive Mahlavu tumor cells.

a NA: not active.
with constitutive active PI3K/Akt anti-apoptotic signalling. All screened triazole compounds performed low IC50 activity (12–28 µM) against Mahlavu cells (Table 1). Even though compounds T6 and T2 were not active in VEGFR TK assay, both of them showed interesting cytotoxic activity against Mahlavu carcinoma. The high selectivity of triazole T2 to Mahlavu over Huh-7 cell lines indicates its possible affinity to PI3K/Akt pathway that can be further investigated.

4. Experimental

4.1. Molecular docking

Docking experiments were accomplished according the expert self-assessing system DOCK Blaster (University of California, San Francisco) described in the literature [79]. The calculations were performed by an older DOCK version and later on with the DOCK 3.6 version of UCSF DOCK software [33,76].

4.2. Chemistry

All reactions and compounds leading to prepared 1,2,3-triazole products T1 – T3, T6 – T7 are completely described in Supplementary Material in order as they are depicted in the Scheme 12. Beside other compound characteristics the Supplementary material contains also 1H and 13C NMR spectral graphical abstracts. For simplicity, only the synthetic pathway leading to the most active triazole T3 is described here (Scheme 13).

4.2.1. General procedures

THF and Et2O were dried over and distilled from Na/benzophenone under Ar atmosphere. DCM and Et3N were dried over calcium hydride or KOH pellets, resp. and distilled. Commercially available chemicals and solvents were purchased from Sigma–Aldrich company and were used without further purification. The course of the reactions was followed by TLC analysis (Merck Silica gel 60 F254). UV lamp (254 nm) and iodine vapours were used for visualization of TLC spots. Flash column chromatography was performed on silica gel (40–60 mesh). Melting points were determined on a Büchi B-540 apparatus (Büchi Labortechnik, Flawil, Switzerland) and were uncorrected. All 1H-NMR and 13C-NMR spectra were recorded on Bruker instruments (500, 300 MHz for hydrogen and 100 MHz for carbon, Bruker Bioscience, Billerica, MA, USA) with CDCl3, DMSO-d6 or acetone-d6 as solvent. Chemical shifts are given in parts per million (ppm). IR spectra were acquired on FT-IR-ATR REACT IR 1000 (ASI Applied Systems) with diamond probe and MTS detector. ESI Mass spectral data were obtained by Esquire-LC-00075 spectrometer (Bruker Bioscience). Other mass spectra were performed on LC-MS (Agilent Technologies 1200 Series equipped with Mass spectrometer Agilent Technologies 6100 Quadrupole LC-MS). Elemental analyses for carbon, hydrogen and nitrogen were performed with an Eager 300 analyzer. Used abbreviations: EA: ethyl acetate, Boc: tert-butylloxycarbonate, Cy: cyclohexane, DCM: dichloromethane, EWG: electron withdrawing group (e.g. -COOMe, -Boc, -Ts etc.), FLC: Flash liquid chromatography, N,N-DMED: N,N-dimethylmethylenediamine, RT: room temperature, S-Phos: 2-dicyclohexylphosphino-2',6'-dimethoxybiphenyl [CAS 657408-07-6], TIPS: triisopropylsilyl, TLC: thin layer chromatography (SiO2/UV254). Elemental analyses indicated in the experimental part by the symbols of the elements were within ±0.4% of the theoretical values.

4.2.1.1. A: N-alkynylation of protected anilines with bromoalkyne 29 (Skydrup’s protocol). 1-Bromoalkyne (1.1 mol eq) 29 (1.0 M solution in toluene) was added to a mixture of an amide (1.0 mol eq) (EWG protected aniline) 31a–c, (3.0 mol eq) K3PO4, (0.2 mol eq)
CuSO₄, 5H₂O and (0.4 mol eq) 1,10-phenanthroline. The reaction mixture was heated at 65–75 °C (oil bath temperature) for 20 h. Upon completion, detected by TLC, the reaction mixture was cooled to RT, diluted with EA, filtered through Celite and the filtrate concentrated under reduced pressure. The crude product was purified by flash chromatography on silica gel (Scheme 3).

4.2.1.2. B: TIPS group deprotection. A (1.0 mol eq) 1 M solution of KOH in MeOH at RT overnight. The progress of the reaction was monitored by TLC. When the aryl bromide was completely consumed, or when the progress of the reaction had stopped, the reaction mixture was cooled to RT and the crude product purified either by extraction and/or FLC chromatography giving the desired aryl azide (Scheme 5).

4.2.1.3. C: substitution of an aromatic halide to azide. Prepared (1.0 mol eq) aryl bromide, (2.0 mol eq) NaN₃, (0.05 mol eq) sodium ascorbate, (0.1 mol eq) CuI and (0.1 mol eq) CuSO₄ · 5H₂O in 3 mL of H₂O was added. The reaction mixture was heated at 65 °C for 20 h. Upon completion, detected by TLC, the reaction mixture was filtered through Celite and the filtrate, evaporated leading to a desired product (Scheme 6).

4.2.1.4. D: copper-catalyzed Click chemistry reaction. Prepared (1.0 mol eq) ynamide (1.0 mol eq) 22a and (1.0 mol eq) of corresponding azide 22a in dry THF. After stirring overnight the reaction mixture was diluted with 10 mL H₂O and extracted with EA (3 × 3 mL) was refluxed under Ar atmosphere. Progress of the reaction was monitored by TLC. When the ynamide was completely consumed, or when the progress of the reaction had stopped, the reaction mixture was cooled to RT and the crude product purified either by extraction and/or FLC chromatography giving the desired azide (Scheme 7).

4.2.1.5. E: deprotection of methoxycarbonyl group. Protected amniotriazole derivative (2.0 mmol) was stirred in 5 mL of mixture tert-butanol : H₂O (7 : 3) was re refluxed under Ar atmosphere. The syntheses of triazole T₃ (marked in blue), its intermediates and yields. Starting compounds are marked green, ynamide 22a and azides (15–16) are marked in red and brown, resp. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

CuSO₄, 5H₂O and (0.4 mol eq) 1,10-phenanthroline. The reaction mixture was heated at 65–75 °C (oil bath temperature) for 20 h. Upon completion, detected by TLC, the reaction mixture was cooled to RT, diluted with EA, filtered through Celite and the filtrate concentrated under reduced pressure. The crude product was purified by flash chromatography on silica gel (Scheme 3).

**Scheme 3.** The syntheses of triazole T₃ (marked in blue), its intermediates and yields. Starting compounds are marked green, ynamide 22a and azides (15–16) are marked in red and brown, resp. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

CuSO₄, 5H₂O and (0.4 mol eq) 1,10-phenanthroline. The reaction mixture was heated at 65–75 °C (oil bath temperature) for 20 h. Upon completion, detected by TLC, the reaction mixture was cooled to RT, diluted with EA, filtered through Celite and the filtrate concentrated under reduced pressure. The crude product was purified by flash chromatography on silica gel (Scheme 3).

**Scheme 5.**

| (26) | 72% | 7% |
| (33) | 68% | 15% |

**Scheme 6.**

| (31a) | 97% |
| (32) | 97% |

| (Scheme 3) |

| (26) | 72% | 97% |
| (33) | 68% | 15% |

**Scheme 8.**

| (T₃ (-OH, -py-2-yl)) | 72% |
| (26) | 97% |

**Scheme 13.** The syntheses of triazole T₃ (marked in blue), its intermediates and yields. Starting compounds are marked green, ynamide 22a and azides (15–16) are marked in red and brown, resp. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

**Scheme 13.** The syntheses of triazole T₃ (marked in blue), its intermediates and yields. Starting compounds are marked green, ynamide 22a and azides (15–16) are marked in red and brown, resp. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

**Scheme 13.** The syntheses of triazole T₃ (marked in blue), its intermediates and yields. Starting compounds are marked green, ynamide 22a and azides (15–16) are marked in red and brown, resp. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
4.3.4. Synthesis of 2-(3-bromophenyl)pyridine (34)

A degassed mixture of 2.4 mL (24.9 mmol, 1.00 mol eq) 2-bromopyridine, 5.75 g (54.8 mmol, 2.20 mol eq) Na2CO3, Ac2O. The reaction vial was sealed with a Te(aq) cation: Flash chromatography (SiO2, eluent: Cy/EA, 1/1). Colourless liquid.

1H-NMR (75 MHz, CDCl3): δ 8.56 (ddd, 1H, J(B2,B6) = 5.0 Hz, J(B1,B4) = 1.8 Hz, J(B6,B4) = 1.0 Hz, H-C(6)), 7.88 (ddd, 1H, J(A1,A8) = 7.9, J(A2,A8) = 1.8 Hz, H-C(2)), 7.88 (ddd, 1H, J(A2,A8) = 1.8 Hz, H-C(2)), 7.88 (ddd, 1H, J(B1,B4) = 1.8 Hz, H-C(6)), 7.69 (ddd, 1H, J(B1,B4) = 7.9 Hz, J(B6,B4) = 7.2 Hz, J(B6,B4) = 1.8 Hz, H-C(4)), 7.63 (ddd, 1H, J(B1,B4) = 7.9 Hz, J(B6,B4) = 1.3 Hz, J(B6,B4) = 1.0 Hz, H-C(3)), 7.51 (ddd, 1H, J(A2,A8) = 8.0 Hz, J(A2,A8) = 1.8 Hz, J(A2,A8) = 1.3 Hz, H-C(4)), 7.30 (ddd, 1H, J(A2,A8) = 8.0 Hz, J(A2,A8) = 1.8 Hz, J(A2,A8) = 1.3 Hz, H-C(4)), 7.20 (ddd, 1H, J(B1,B4) = 7.2 Hz, J(B6,B4) = 5.0 Hz, J(B6,B4) = 1.3 Hz, H-C(5)).

13C-NMR (75 MHz, CDCl3): δ 155.8 (s, C(2)), 149.8 (d, C(6)), 141.4 (s, C(1)), 136.9 (d, C(4)), 131.9, 130.0, 130.3 and 125.4 (4 d, C(2)), C(4), C(5) and C(6)), 123.1 (s, C(3)), 122.7 (d, C(5)), 120.6 (d, C(3)).


16 was isolated in form of pale yellow oil.

4.3.7. Synthesis of 4-((4-(5-(ethylsulfonyl)-2-methoxyphenyl)amino)-1H-1,2,3-triazol-1-yl)-2-(pyridin-2-yl)phenyl acetate (56)

Compound 56a was prepared according to the general procedure D. Yield: 72%. Purification: Flash chromatography (SiO2, eluent: Cy/EA, 1:3). M. Vojtěch et al. / European Journal of Medicinal Chemistry 103 (2015) 105–122

1H-NMR (300 MHz, CDCl3): δ 8.72 (ddd, 1H, J(D1,D5) = 4.8 Hz, J(D1,D5) = 1.8 Hz, J(D1,D5) = 0.8 Hz, H-C(6)), 8.46 (br s, 1H, H-C(5)), 8.10 (d, 1H, J(A1,A8) = 2.3 Hz, H-C(2)), 7.92 (dd, 1H, J(C5,C6) = 8.6 Hz, J(C2,C6) = 2.2 Hz, H-C(6)), 7.88 (d, 1H, J(C5,C6) = 2.2 Hz, J(H-C(2)), 7.84 (dd, 1H, J(A1,A8) = 8.7 Hz, J(A1,A8) = 2.7 Hz, H-C(4)), 7.77 (ddd, 1H, J(D1,D5) = 7.9 Hz, J(D1,D5) = 1.8 Hz, H-C(4)), 7.61 (ddd, 1H, J(D1,D5) = 7.9 Hz, J(D1,D5) = 0.8 Hz, H-C(3)), 7.33 (d, 1H, J(C5,C6) = 8.6 Hz, H-C(5)), 7.29 (ddd, 1H, J(D1,D5) = 7.7 Hz, J(D1,D5) = 1.8 Hz, J(D1,D5) = 0.8 Hz, H-C(3)), 7.15 (d, 1H, J(A1,A8) = 8.7 Hz, H-C(3)), 3.88 (s, 1H, CH2(C2OCH3)), 3.77 (3H, -NCOOCH3), 3.13 (q, 2H, J(CH2CH3) = 7.5 Hz, -SO2CH2CH3), 2.21 (2H, -COCH3), 1.31 (t, 3H, J(CH2CH2CH2) = 7.5 Hz, -SO2CH2CH3).

13C-NMR (75 MHz, CDCl3): δ 169.0 (s, -COCH3), 154.2 and 153.6 (2 × s, -NCOOCH3 and C(2)), 159.8 (s, C(2)) 149.9 (d, C(6)), 148.0 (s, C(4)), 136.6 (d, C(4)), 135.2, 134.5, 131.2, 130.0, 130.3 and 125.4 (4 d, C(2)), C(4), C(5) and C(6)), 123.1 (s, C(3)), 122.7 (d, C(5)), 120.6 (d, C(3)).
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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version, at http://dx.doi.org/10.1016/j.ejmech.2015.08.012. These data include physico-chemical characteristics, spectra and graphical abstracts of prepared compounds depicted in Scheme 12 (also those not included in this printed version), MOL files and InChiKeys of the most important compounds described in this article.

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Further reading