European Journal of Medicinal Chemistry 121 (2016) 143–157

Contents lists available at ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: http://www.elsevier.com/locate/ejmech

Research paper

Novel pyrazolopyridine derivatives as potential angiogenesis inhibitors: Synthesis, biological evaluation and transcriptome-based mechanistic analysis

Maria Michailidou [a], Vassiliki Giannouli [b], Vasilios Kotsikoris [a], Olga Papadodima [c], Georgia Kontogianni [c], Ioannis K. Kostakis [b], Nikolaos Lougiakis [b], Aristotelis Chatziioannou [c], Fragiskos N. Kolisis [d], Panagiotis Marakos [b], Nicole Pouli [b, **], Heleni Loutrari [a, *]

[a] GP Livanos and M Simou Laboratories, 1st Department of Critical Care Medicine & Pulmonary Services, Evangelismos Hospital, Faculty of Medicine, National and Kapodistrian University of Athens, 3 Ploutarchou St., 106 75, Athens, Greece
[b] Department of Pharmaceutical Chemistry, Faculty of Pharmacy, National and Kapodistrian University of Athens, Panepistimiopolis-Zografou, Athens, 15771, Greece
[c] Metabolic Engineering and Bioinformatics Group, Institute of Biology, Medicinal Chemistry and Biotechnology, National Hellenic Research Foundation, 48 Vassileos Constantinou Ave., 116 35, Athens, Greece
[d] School of Chemical Engineering, National Technical University of Athens, 9 Iroon Polytechneiou, Zografou, 157 80, Athens, Greece

ARTICLE INFO

Article history:
Received 13 April 2016
Received in revised form
15 May 2016
Accepted 18 May 2016
Available online 20 May 2016

Keywords:
Pyrazolo[3,4-*c*]pyridines
Receptor tyrosine kinase inhibitors
Angiogenesis
Vascular endothelial growth factor
Microarray
Gene ontology

ABSTRACT

Modified purine derivatives exemplified by pyrazolopyrimidines have emerged as highly selective inhibitors of several angiogenic receptor tyrosine kinases. Herein, we designed and synthesized a new series of substituted pyrazolopyridines and explored their ability to influence crucial pro-angiogenic attributes of endothelial cells. Four of the synthesized compounds, possessing analogous substitution pattern, were found able to inhibit at low micromolar concentrations endothelial cell proliferation, migration and differentiation, constitutively or in response to Vascular Endothelial Growth Factor (VEGF) and to attenuate VEGF-induced phosphorylation of VEGF receptor-2 and downstream kinases AKT and ERK1/2. Administration of effective compounds in mice delayed the growth of syngeneic Lewis lung carcinoma transplants and reduced tumor microvessel density, without causing toxicity. Genome-wide microarray and gene ontology analyses of treated endothelial cells revealed derivative **18c** as the most efficient modulator of gene expression and "mitotic cell cycle/cell division" along with "cholesterol biosynthesis" as the most significantly altered biological processes.

Abbreviations: VEGF, Vascular Endothelial Growth Factor; VEGFR, Vascular Endothelial Growth Factor receptor(s); EC, endothelial cells; RTK, receptor Tyr kinase; FDA, United States Food and Drug Administration; HUVEC, human umbilical vein endothelial cells; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; LLC, Lewis lung carcinoma; GO, gene ontology; qRT-PCR, real-time quantitative reverse PCR; HMGCS, hydroxy-3-methylglutaryl-CoA synthase 1; MSMO1, methylsterol monooxygenase; INSIG1, insulin induced gene 1; SQLE, squalene epoxidase; UBE2C, ubiquitin-conjugating enzyme E2C; CCNB2, cyclin B2; CCNA2, cyclin A2; CDC20, cell division cycle 20; HMOX1, heme oxygenase 1; Pddba, bis(dibenzylideneacetone)palladium; X-Phos, 2-dicyclohexylphosphino-2′,4′,6′-triisopropylbiphenyl; Et_2O, diethyl ether; NIS, *N*-iodosuccinimide.

* Corresponding author.
** Corresponding author.
E-mail addresses: mmichail@med.uoa.gr (M. Michailidou), v_giannouli@yahoo.gr (V. Giannouli), vasiliskotsikoris@yahoo.gr (V. Kotsikoris), opapadod@eie.gr (O. Papadodima), gkontogianni@eie.gr (G. Kontogianni), ikkostakis@pharm.uoa.gr (I.K. Kostakis), nlougiak@pharm.uoa.gr (N. Lougiakis), achatzi@eie.gr (A. Chatziioannou), kolisis@chemeng.ntua.gr (F.N. Kolisis), marakos@pharm.uoa.gr (P. Marakos), pouli@pharm.uoa.gr (N. Pouli), elloutrar@med.uoa.gr (H. Loutrari).

http://dx.doi.org/10.1016/j.ejmech.2016.05.035
0223-5234/

1. Introduction

Angiogenesis, the formation of new blood vessels from pre-existing vasculature, has become an important therapeutic target because of its crucial role in several pathologies, characteristically in tumor progression and metastasis [1]. As a result the discovery of new compounds possessing potent anti-angiogenic activity has been the focus of intense current research [2–6]. Numerous growth factors are involved in tumor angiogenesis such as Vascular Endothelial Growth Factor (VEGF) [7], epidermal growth factor [8], platelet-derived growth factor [9], and basic fibroblast growth factor [10]. Yet, the members of VEGF family including VEGF-A, B, C, D, E and placenta growth factor are considered to be the most critical endothelium-specific mediators of angiogenesis [7]. VEGF exerts its biological effects by binding to and activating VEGF receptors (VEGFR) which are almost exclusively expressed in endothelial cells (EC). There are three types of VEGFR, namely, VEGFR1 (Flt-1), VEGFR2 (KDR/Flk-1) and VEGFR3. Among them, VEGFR2 plays a major role in VEGF-A-mediated vascular cell biology [11,12]. Binding of VEGF-A to VEGFR2 results in the activation of several signal transduction events. These involve receptor dimerization and autophosphorylation at specific Tyr residues followed by phosphorylation of downstream proteins including PKC, phospholipase Cγ, PI3K, 3-phosphoinositide-dependent kinase 1, AKT, focal adhesion kinase, ERK1/2, Src and p38MAPK [13–16]. More specifically, activation of PI3K–AKT cascade by VEGFR2 has been shown to play a causal role in VEGF-stimulated survival, migration and sprouting of endothelial cells in vitro and angiogenesis in vivo, whereas VEGF-induced ERK1/2 and p38MAPK signaling have been mostly associated with regulation of EC proliferation and motility, respectively [11,12]. Consequently, VEGF and VEGFR2 signaling system is recognized as an attractive target for anti-angiogenic intervention and a variety of approaches are currently being assessed in cancer clinical trials. These include the use of soluble VEGFR, monoclonal antibodies against VEGF or VEGFR and small molecule inhibitors of VEGFR Tyr kinase activity [17–19].

In recent years targeted inhibition of several oncogenic receptor Tyr kinases (RTK) by small molecules that compete with the ATP for binding in the catalytic domain has been introduced as a systemic treatment strategy for cancer. Actually, an array of RTK inhibitors are being studied in clinical trials whereas several of them such as sorafenib and sunitinib (Chart 1) have already been approved by the United States Food and Drug Administration (FDA) for indication of specific types of cancer [20]. Nevertheless limitations in the use of these targeted therapies, mostly associated with clinical resistance and toxicity [21], prompt the need for developing new drug candidates with improved bioactivity and a more favorable safety profile. Pyrazolo[3,4-*d*]pyrimidines through their selectivity toward different RTK have proved to be a very promising chemical class in the discovery of new lead compounds to treat cancer and pathological angiogenesis [22,23].

Focusing on VEGFR inhibitors, a great number of ATP site-targeted ligands have been produced using either analogue synthesis approaches or a combination of more sophisticated methods, including structure-based drug design and fragment-based strategies. Among them, some disubstituted 6-aminopurines [24] and trisubstituted pyrazolo[4,3-*d*]pyrimidines [25] inhibit tumor angiogenesis and cell migration, whereas benzothiopyrano[4,3-*d*]pyrimidines [5] and 3-aminopyrazolo[3,4-*b*]pyridine ureas [26] target kinases of the VEGF pathway. Closely related to the later, pyrrolo[3,2-*d*]pyrimidine derivatives display strong inhibitory activities against both VEGFR and fibroblast growth factor receptor kinases [27] and possess anti-proliferative activity against VEGF-stimulated human umbilical vein endothelial cells (HUVEC) [28]. In this respect the study of bioisosters provides an important tool for the complete investigation of the bioactivity and the discovery of new/modified derivatives with improved properties.

As part of our involvement in the design and synthesis of new potentially bioactive purine analogues [29–31], we report here the synthesis and evaluation of a number of novel pyrazolo[3,4-*c*]pyridine derivatives as angiogenesis inhibitors. To this end we initially examined the ability of this class of compounds to affect essential pro-angiogenic functions of EC, constitutively or upon stimulation with VEGF in cell-based assays and next to modulate tumor angiogenesis and growth in a mouse cancer model. Finally, we analyzed the global effect of best candidates on EC transcriptome in an effort to uncover potential action mechanisms. Our study may provide a basis for further evaluation of the most promising derivative(s) in anti-angiogenic therapeutic interventions, especially for cancer treatment.

2. Results and discussion

2.1. Chemistry

The target compounds were prepared using 2-amino-5-nitro-4-picoline (**1**, Scheme 1) [32] as starting material. This compound was diazotized and the resulting pyridinone **2** [33] was treated with phosphorus oxychloride and converted to the chloropicoline **3** [34].

The nitroderivative **3** was then reduced using stannous chloride as the reducing agent to give the aminopyridine **4** which was acetylated to the acetamide **5**. This acetamide was then heated at reflux in benzene with isoamyl nitrite, in the presence of acetic anhydride [35,36]. This furnished a mixture of the corresponding 1- and 2-acetylpyrazolo[3,4-*c*]pyridines through a rearrangement of the intermediate *N*-nitroso compound. These isomers were not isolated, but the acetyl groups were easily cleaved upon treatment with methanolic ammonia to provide the pyrazolopyridine **6** [36]. Compound **6** was subsequently treated with 4-methoxybenzyl chloride in the presence of sodium hydride to provide derivative **7**, together with an amount of the corresponding *N*2 regio-isomer. From a brief study of the reaction conditions we resulted that the use of a polar solvent (DMF) at room temperature favors the formation of *N*1 over *N*2 isomer, which was thus prepared in 2:1 ratio. Both isomers were separated and identified using NOE spectroscopic data. More precisely, in the case of the *N*1-isomer we recorded a clear cross-coupling of both the benzylic and *o*-phenyl protons with H-7. Compound **7** was then converted to the corresponding *N*-oxide **8**, using *m*-CPBA as oxidizing agent. The rearrangement of the *N*-oxide in the presence of phosphorous oxychloride, produced the 5,7-dichloropyrazolopyridine **9**, which was used for the nucleophilic substitution of the 7-chloro group using suitable secondary amines, in order to provide compounds **10a-c**. The chlorides **10** were converted to the target derivatives **11a-d** through a Suzuki-type coupling with aniline or 4-(4-methylpiperazin-1-yl)aniline, in the presence of cessium carbonate, using bis(dibenzylideneacetone)palladium (Pddba) [37] as catalyst and 2-dicyclohexylphosphino-2′,4′,6′-triisopropylbiphenyl (X-Phos) as ligand [38]. ^{1}H and ^{13}C NMR spectra of target compounds **11a-d** are provided in Supplementary data.

The corresponding 3-phenyl analogues (**18a-c**, Scheme 2), were prepared from the intermediate chloride **6**, which was successively converted to the iodide **12** upon treatment with *N*-iodosuccinimide and then to the 4-methoxybenzyl derivative **13** [39]. This iodide provided the 3-phenylanalogue **14**, through a Suzuki-type coupling using phenylboronic acid in the presence of tetrakis(-triphenylphosphine)palladium (0) and sodium bicarbonate. Following a synthetic procedure analogous to the above mentioned one, analogue **14** was converted to the target derivatives **18a-c**. ^{1}H and ^{13}C NMR spectra of target compounds **18a-c** are provided in