

Pyrazolopyrimidine Derivatives as Antineoplastic Agents: with a Special Focus on Thyroid Cancer.

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Running Title: Pyrazolopyrimidine derivatives as antineoplastic agents.

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Abstract

Tyrosine kinase inhibitors (TKIs) are molecules that compete with ATP on tyrosine kinase receptors (TKRs), blocking tyrosine kinase (TK) activation and then oncogenic pathways; they have been studied, and some of them are right approved for the treatment of many types of cancer.

Among TKIs, one of the most explored chemical template is the pyrazolo[3,4-*d*]pyrimidine (PP) heterocyclic core, which proved to be a useful scaffold for the obtainment of effective compounds. Actually, derivatives belonging to this structural class show a large spectrum of activity, thus standing out as multi-target agents. Different PP compounds have been shown to act as: a) ABL inhibitors and antiproliferative agents against human leukemia cell lines; b) Src kinase inhibitors in neuroblastoma, medulloblastoma and osteosarcoma; c) Phospholipase D inhibitors in different neoplasias; d) Urokinase plasminogen activator inhibitors, in breast cancer.

In thyroid cancer (TC), PP1 and PP2 (inhibitors of RET, Hck, lck, and fynT kinases, and a good inhibitor of c-Src and platelet-derived growth factor receptor) showed antineoplastic activity in human papillary TC cell lines that carry spontaneous RET/PTC1 rearrangements. More recently, new derivatives, (R)-1-phenethyl-N-(1-phenylethyl)-1H-pyrazolo[3,4-*d*]pyrimidin-4-amine, namely, CLM3 and CLM29, have been demonstrated to exert a multiple signal transduction inhibition (including the RET-TK, BRAF, EGFR, and with antiangiogenic activity), showing antineoplastic activity, *in vitro* and *in vivo*, in papillary dedifferentiated, medullary and anaplastic TC.

These data have shown the antineoplastic activity of PP in different neoplasias, opening the way to a future clinical evaluation in human cancers.

Keywords: antiangiogenic inhibitors, CLM29, CLM3, PP1, PP2, pyrazolo[3,4-*d*]pyrimidine, RET inhibitors, tyrosine kinase inhibitors.

1. INTRODUCTION

In the last years several mutations and pathogenic mechanisms leading to onset of tumors, or their dedifferentiation and resistance, were discovered. It was shown the cornerstone role of tyrosine kinases (TKs) and tyrosine kinase receptors (TKRs), like epidermal growth factor receptor (EGFR), vascular endothelial growth factor receptor (VEGFR), RET, BRAF, RAS/RAF/ERK and RAS/PI3K/AKT pathways, that are capable of causing cell transformations, giving mitogenic and survival signals, for example in thyroid cancer (TC) [1-6], and in other neoplasias [7-9].

Furthermore recently, it has been shown the importance of cytokines and chemokines [10] (usually involved in autoimmune disorders [11, 12]) in tumorigenesis of TC [13-15], and in other cancers [16, 17].

TKs and TKRs are actually targets of new antineoplastic therapies. Tyrosine kinase inhibitors (TKIs) are molecules that compete with ATP on TKRs, blocking TK activation and then oncogenic pathways [18].

Recently, some TKIs have been studied [1, 19-24], and some of them are right approved for the treatment of some types of cancer, as imatinib (for chronic myelogenous leukemia [25] and gastrointestinal stromal tumors [26]), sorafenib (for advanced renal cell carcinoma [27], advanced hepatocellular carcinoma [28], and radioactive iodine resistant advanced TC [29]), vandetanib and cabozantinib for medullary thyroid cancer (MTC) [30-33], and others [34].

Among TKIs, pyrazolopyrimidines-derived compounds are produced and tested as useful antineoplastic agents. The pyrazolopyrimidines are constituted by a pyrazole ring fused with the pyrimidine moiety differently from the imidazole moiety in purines [35, 36]. At the beginning, pyrazolopyrimidines were studied as adenosine receptor antagonists [37]. Different chemical compounds with pyrazolopyrimidines as central core were produced [38] and recently Mahajan *et al.* published a paper about ACK1-TK targeted cancer cells inhibition of proliferation, including pyrazolo[3,4-d]pyrimidines derivatives [39]. There are several isomeric forms of pyrazolopyrimidines with various mechanisms of action and possible purposes like antiviral [40], anticoccidials [41], antimicrobial [42-44], antitumor [45, 46], antileukemic [47], tuberculostatic [48], radioprotectant [49], and several other activities [50].

2. ANTINEOPLASTIC ACTIVITY

Pyrazolopyrimidines were evaluated against different targets as RET, VEGFR, EGFR, ABL, SRC, Aurora kinase, and others, each employed in the upset, progression and transformation of many tumors.

2.1. ABL inhibitors

Manetti *et al.* [51] examined the role of the pyrazolo[3,4-d]pyrimidines as ABL inhibitors and their antiproliferative action on human leukemia cell line. The effect of substituting different groups, as halogens and the hydrophobic regions of the ATP binding, was demonstrated by molecular modeling studies, showing its determinant activity on the affinity toward Abl. Pyrazolo[3,4-d]pyrimidines were produced by Radi *et al.* [52, 53] with an inhibitory effect in hypoxic human leukemia cells and the *in vitro* "absorption, distribution, metabolism, and excretion" (ADME) properties and metabolic activities were shown. The abovementioned molecules inhibited the Bcr-Abl kinase, increased caspase-3 action and the cleavage of poly-ADP-ribose-polymerase.

2.2. SRC inhibitors

Tintori *et al.* [54] evaluated the antineoplastic activity against neuroblastoma cell proliferation. A subclass of nonreceptor TKs as target in the treatment of human cancers is the Src-family TKs. Among these, c-Src was found to stimulate cell proliferation, migration, and invasion as well as angiogenesis [55]. Hyperactivation of c-Src leads to aberrant cell activity that contributes to cancer development. Elevated expression levels of c-Src have been shown in different types of cancer and are associated with a poor prognosis with respect to overall survival. Moreover, recent studies suggest that c-Src could be associated with the development of acquired drug resistance [56].

The antiproliferative and proapoptotic effects of pyrazolo[3,4-d]pyrimidines as Src kinase inhibitors in human osteosarcoma cells have been evaluated [57], concluding that they are capable in stimulating apoptosis and decreasing the Src phosphorylation. The inhibitory action of the compounds has been demonstrated to be dose dependent, *via* DNA damage or *via* increasing apoptosis.

New pyrazolo[3,4-d]pyrimidine derivatives as Src kinase inhibitors able to arrest cell cycle at G2/M phase and reduce growth of human medulloblastoma cells were produced [58].

2.3. Aurora kinases inhibitors

In 2012, 1,6-disubstituted-1H-pyrazolo[3,4-d]pyrimidines as dual inhibitors of Aurora kinases and

CDK1 were synthesized [59] and the structure activity relationship (SAR) was reported, revealing to be particularly strong in case of low distribution volumes, elevated clearance rate, satisfying ADME properties, and thus demonstrating to be a good antitumor agent in leukemia cells.

2.4. Phospholipase D (PLD) inhibitors

PLD catalyzes the cleavage of phosphatidylcholine at the ester linkage—releasing choline and phosphatidic acid (PA) [60], that is a second lipid messenger belonging to different essential signaling and metabolic pathways [61], and is involved in the regulation of cellular functions mediating the enhancement of cell migration. The enhanced PLD activity and expression have been shown in different human cancer tissues [62-64]; PLD supports cells in initiating defence mechanisms, and its inhibition diminishes the ability of cells to adhere. Kulkarni *et al.* [65] evaluated the action of aminopyrazolopyrimidines, that have earlier been used as TKI [66] and dual inhibitors of tyrosine and phosphoinositide kinases [67], that were produced and used to initially screen the capability of purified bacterial PLD, strongly homologous to the human PLD.

These inhibitory molecules directly blocked enzyme/vesicle substrate binding. Preliminary activity studies, performed by recombinant human PLDs in *in vivo* cell assays evaluating transphosphatidylation and head-group cleavage, showed inhibition in the mid- to low-nanomolar range in a physiological environment.

2.5. Urokinase plasminogen activator (uPA) inhibitors

uPA converts the circulating plasminogen to the active plasmin, is secreted as an inactive single-chain proenzyme by various cell types and is able to bind to a specific membrane uPA receptor (uPAR), in this way existing in a soluble or cell associated form [68]. uPA is involved in cancer invasion and metastasization, as members of the matrix metalloproteinases family, and takes part in many physiological functions [69]. After binding to uPAR, uPA initiates versatile intracellular signal pathways regulating cell proliferation, adhesion, and migration, interacting with different integrins and vitronectin [70]. Urokinase is involved in many malignancies, in lung, breast, cervix, bladder, kidney, brain, and stomach [71, 72]. The cytotoxicity of several compounds was tested using sulforhodamine B assay [73] in the breast cancer cell line MCF-7, the liver cancer cell line HepG2, and the lung carcinoma cell line A549. Shamroukh *et al.* [74] showed that pyrazole derivatives inhibit the activity of

the urokinase enzyme, that is able to reduce cell proliferation leading to growth inhibition, and exerting an anti-carcinogenic activity in MCF-7 breast and HepG2 liver cancer cells. The SAR of the tested compounds showed that whereas substituted amino group enhanced the activity. However, fusing another ring (oxazine, pyrimidine, or fused pyrimidine) to the pyrazole structure decreased the anticancer activity.

2.6. Generation of reactive oxygen species

The synthesis and anticancer activities of new pyrazolo[3,4-d]pyrimidine derivatives were evidenced by Rashad *et al.* [75] in 2011, as they generate reactive oxygen species (ROS) in human breast adenocarcinoma cells. Tumor cells are more sensitive than normal cells to the elevated levels of ROS present in cancer and increased by the supplementary oxidative stress created by anticancer agents, that causes injury to cellular components leading to cell death. The abovementioned compounds exert their antitumor effect partly owing to the production of H₂O₂.

The pyrazolo[3,4-d]pyrimidines have *in vitro* cytotoxic activity against breast adenocarcinoma [76] producing hydrogen peroxide and other free radicals leading to oxidative distress. The potency of pyrazolo[3,4-d]pyrimidines was higher than the one of pyrazole; anticancer activity was increased if the sulfonyl group was present between pyrazolo[3,4-d]pyrimidine and 4-chlorophenyl moiety.

Imine-pyrazolopyrimidinones have been reported as antitumoral agents [46], by multiple stress pathways in tumoral cells, as increased ROS levels, leading to DNA damage and topoisomerase II inhibition. Its binding interactions with topoisomerase II were investigated, showing knowledges about SAR and performing molecular modeling studies.

2.7. PP1 and PP2

Dysregulation of RET signaling by oncogenic mutation, gene rearrangement, overexpression or transcriptional up-regulation is involved in different human cancers (thyroid, breast, lung, etc) [77-83]. Carlomagno *et al.* [84] found that the 4-amino-5-(4-methylphenyl)-7-(*t*-butyl)pyrazolo[3,4-d]pyrimidine (PP1) inhibited RET-derived oncoproteins with a half maximal inhibitor concentration of 80 nM. Moreover, RET/PTC3-transformed cells lost proliferative autonomy and showed morphological reversion if treated with 5 μ M PP1. PP1 inhibited the growth of two human papillary TC (PTC) cell lines, carrying spontaneously RET/PTC1 rearrangements, and stopped anchorage-

independent growth and tumorigenicity in NIH3T3 fibroblasts from nude mice, expressing the RET/PTC3 oncogene. The obtained results showed that a new treatment strategy for RET-associated neoplasms could be targeting RET oncogenes using PP1 or related compounds.

Even if PP1 has a powerful growth inhibitory activity against human TC cell lines with RET/PTC rearrangements, it is not selective for RET, as it is a strong inhibitor also of Ick, Hck, and FynT kinases, and a good inhibitor of c-Src and platelet-derived growth factor receptor (PDGFR) [85]. For this reason, besides to the direct effect on the RET kinase *in vitro*, indirect effects mediated *in vivo* by the inhibition of other kinases (particularly of c-Src, a crucial downstream RET effector) cannot be excluded [86]. If this hypothesis is correct, a single molecule could be used for “multiple-signal transduction therapy” of RET-dependent tumor formation. Furthermore, PP1 has been suggested to be also an inhibitor of PDGFR and c-Src, to prevent restenosis and vascular remodeling [87].

Another pyrazolopyrimidine (PP2; 4-amino-5-(4-chloro-phenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine) [88] is able to block the enzymatic activity of the isolated RET kinase and RET/PTC1 oncoprotein and the *in vivo* phosphorylation and signaling of the RET/PTC1 oncoprotein. PP2 inhibited the serum-independent growth of RET/PTC1-transformed NIH3T3 fibroblasts and of the two human PTC cell lines (TPC1 and FB2) harboring spontaneously RET/PTC1 rearrangements. Moreover, PP2 stopped the potential of invasion of type I collagen matrix exerted by TPC1 cells. Therefore, pyrazolopyrimidines could be a good novel therapy for the treatment of human cancers supporting oncogenic activation of RET.

2.8. CLM3, CLM29 and CLM94

Recently CLM3, (R)-1-phenethyl-N-(1-phenylethyl)-1H-pyrazolo[3,4-d]pyrimidin-4-amine, proposed for a multiple signal transduction inhibition (including the RET-TK, BRAF, EGFR, and with antiangiogenic activity) was disclosed. CLM3 showed an antiangiogenic effect with a less marked inhibitory activity on human TC cell lines, demonstrating time- and dose-dependent antiproliferative and proapoptotic effects on specific cell lines [89, 90].

Sartini *et al.* [91] evaluated new derivatives of CLM3 in order that they completely comply with pharmacophore requirements of the ATP binding sites of VEGFR2 and RET. The new molecules were tested for their inhibitory properties against the target TKs and for their antiproliferative effect against endothelial and human TC cell lines, revealing a promising antiproliferative profile on endothelial and

MTC cell lines. The tested compounds were more efficient against the target TC cell line TT, expressing mutated RET, with respect to the lead CLM3.

Moreover, the antitumor and antiangiogenic activities of the new “cyclic amide” compound CLM94 [92], as well as those of CLM3 [93] and CLM29 (a pyrazolo[3,4-d]pyrimidine, that inhibits RET, EGFR, VEGFR, and has an anti-angiogenic activity) [89], have been demonstrated in primary anaplastic TC cells. Antonelli *et al.* showed that CLM3 and CLM29 inhibited the migration of papillary dedifferentiated thyroid cancer (DePTC) cells. The inhibitory effect of CLM3 and CLM29 was independent from the presence of V600EBRAF mutation. A DePTC cell line (AL), with V600EBRAF mutation, was produced, which was able to grow in nu/nu mice when inoculated sc. CLM3 and CLM29 increased TSP-1 expression in the AL cell line. The antineoplastic activity of CLM3 and CLM29 may result from the combination of an antiproliferative effect associated with the increase of apoptosis in the tumoral cells and the inhibition of the migration and the neoplastic neovascularization. This last effect has been shown *in vivo*. In fact, a significant reduction of microvessels was observed in the CLM3-treated tumors. In addition, a significant decrease in the percentage of anti-VEGF antibody immunoreactivity in the tumor mass was also observed in the CLM3-treated group of animals. The mechanisms underlying the inhibition of the neoplastic neovascularization are probably related to the up-regulation of the main endogenous inhibitor of the angiogenesis, i.e. TSP-1; in fact, CLM3 and CLM29 increased TSP-1 expression in the AL cell line. TSP-1 has many antiangiogenic effect: 1) inducing apoptosis of endothelial replicating cells [94]; and 2) interacting with many extracellular proteins involved in the angiogenic process, such as VEGF [95, 96].

Ferrari *et al.* [97] reported the *in vitro* antineoplastic and antiangiogenic activities of CLM3 and CLM94 in primary cultures of MTC (pMTC) cells and the MTC cell line TT (harboring a RET C634W activating mutation) and MZ-CRC-1 (carrying the MEN2B RET **M918T** mutation). These compounds inhibited the proliferation of TT and pMTC cells *in vitro*, in part by increasing the level of apoptosis. The inhibitory effect of CLM3 and CLM94 seemed to be independent from the presence of RET mutation in pMTC. These results are in agreement with the possibility that CLM3 and CLM94 seem to be inhibitors of multiple signal transduction pathways (including the RET-TK, EGFR, VEGFR) and have an anti-angiogenic effect. A significant decrease in the gene expression of VEGF-A was also observed in TT cells after CLM3 and CLM94 treatments.

More recently, CLM29 was tested in MTC, both in pMTC cells obtained at surgery, and in TT cells

with the C634W RET mutation [98]. CLM29 (10, 30, 50 μ M) inhibited significantly ($P < 0.001$) the proliferation, and increased the percentage of apoptotic pMTC, TT and human dermal microvascular endothelial cells. The inhibition of proliferation by CLM29 was similar in pMTC cells with/without RET mutation. TT cells were injected sc in CD nu/nu mice, and tumor masses became detectable after 20-30 days from xenotransplantation; CLM29 (50 mg/kg/die) reduced significantly tumor growth and weight, and microvessel density. These data showed the antineoplastic activity of CLM29 in MTC *in vitro*, and *in vivo*, opening the way to a future clinical evaluation [98].

CONCLUSION (Table 1)

TKIs are molecules that compete with ATP on TKRs, blocking TK activation and then oncogenic pathways; they have been studied, and some of them are right approved for the treatment of many types of cancer [99].

Among TKIs, one of the most explored chemical template is the pyrazolo[3,4-*d*]pyrimidine (PP) heterocyclic core, which proved to be a useful scaffold for the obtainment of effective compounds. Actually, derivatives belonging to this structural class show a large spectrum of activity, thus standing out as multi-target agents. Different PP compounds have been shown to act as: a) ABL inhibitors and antiproliferative agents against human leukemia cell lines; b) Src kinase inhibitors in neuroblastoma, medulloblastoma and osteosarcoma; c) Phospholipase D inhibitors in different neoplasias; d) Urokinase plasminogen activator inhibitors, in breast cancer.

In TC, PP1 and PP2 (inhibitors of RET, Hck, lck, and fynT kinases, and a good inhibitor of c-Src and PDGFR) showed antineoplastic activity in human papillary TC cell lines carrying spontaneous RET/PTC1 rearrangements. More recently, the new derivatives, CLM3 and CLM29, have been demonstrated to exert a multiple signal transduction inhibition (including the RET-TK, BRAF, EGFR, and with antiangiogenic activity), showing antineoplastic activity, *in vitro* and *in vivo*, in papillary dedifferentiated, medullary and anaplastic TC. These data have shown the antineoplastic activity of PP in different neoplasias, opening the way to a future clinical evaluation in human cancers.

LIST OF ABBREVIATIONS

absorption, distribution, metabolism, and excretion (ADME)

(R)-1-phenethyl-N-(1-phenylethyl)-1H-pyrazolo[3,4-d]pyrimidin-4-amine (CLM3)

epidermal growth factor receptor (EGFR)

medullary thyroid cancer (MTC)

papillary dedifferentiated thyroid cancer (DePTC)

papillary thyroid cancer (PTC)

phosphatidic acid (PA)

phospholipase D (PLD)

platelet-derived growth factor receptor (PDGFR)

primary cultures of MTC (pMTC)

pyrazolo[3,4-d]pyrimidine (PP)

4-amino-5-(4-methylphenyl)-7-(t-butyl)pyrazolo[3,4-d]-pyrimidine (PP1)

4-amino-5-(4-chloro-phenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine (PP2)

reactive oxygen species (ROS)

structure activity relationship (SAR)

thyroid cancer (TC)

tyrosine kinases (TKs)

tyrosine kinase inhibitors (TKIs)

tyrosine kinase receptors (TKRs)

urokinase plasminogen activator (uPA)

uPA receptor (uPAR)

vascular endothelial growth factor receptor (VEGFR)

CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

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Table 1. Key messages.

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1. The important role of tyrosine kinases (TKs) and tyrosine kinase receptors (TKRs) has been shown, that are capable of causing cell transformations, giving mitogenic and survival signals, in thyroid cancer (TC), and in other neoplasias.

 2. Tyrosine kinase inhibitors (TKIs) are molecules that compete with ATP on TKRs, blocking TK activation and then oncogenic pathways.

 3. Among TKIs, the pyrazolo[3,4-d]pyrimidine (PP) heterocyclic core is a useful scaffold for the obtainment of effective compounds, and derivatives belonging to this structural class show a large spectrum of activity, thus standing out as multi-target agents.

 4. Different PP compounds have been shown to act as: a) ABL inhibitors and antiproliferative agents against human leukemia cell lines; b) Src kinase inhibitors in neuroblastoma, medulloblastoma and osteosarcoma; c) Phospholipase D inhibitors in different neoplasias; d) Urokinase plasminogen activator inhibitors, in breast cancer.

 5. In thyroid cancer (TC), PP1 and PP2 (inhibitors of RET, Hck, Ick, and fynT kinases, and a good inhibitor of c-Src and PDGFR) showed antineoplastic activity in human papillary TC cell lines with spontaneous RET/PTC1 rearrangements.

 6. Recently, the new derivatives, CLM3 and CLM29, have been demonstrated to exert a multiple signal transduction inhibition (including the RET-TK, BRAF, EGFR, and with antiangiogenic activity), showing antineoplastic activity, *in vitro* and *in vivo*, in papillary dedifferentiated, medullary and anaplastic TC.
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