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Research paper

# ALK5 kinase inhibitory activity and synthesis of 2,3,4-substituted 5,5-dimethyl-5,6-dihydro-4*H*-pyrrolo[1,2-*b*]pyrazoles

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ABSTRACT

A series of 2,3,4-substituted 5,5-dimethyl-5,6-dihydro-4*H*-pyrrolo[1,2-*b*]pyrazoles (DPPs) was synthesized and evaluated for their ALK5 inhibition activity. The most potent compounds displayed submicromolar $IC_{50}$ values for ALK5. Preliminary profiling of one of the most active compounds in a panel of 50 protein kinases revealed its selectivity for ALK5. In cells, the compounds caused dose-dependent dephosphorylation of SMAD2, a well-established substrate of ALK5. In addition, the compounds blocked translocation of SMAD2/3 to nuclei of cells stimulated with TGFβ and the protein remained predominantly in cytoplasm, further confirming their molecular target. Therefore, novel DPP derivatives proved to be active as ALK5 inhibitors.



## 1. Introduction

The human genome encodes more than 30 proteins that belong to the transforming growth factor β (TGFβ) superfamily [1]. In humans, three highly homologous isoforms of TGFβ exist: TGFβ1, TGFβ2 and TGFβ3. They share a receptor complex and signal in similar ways, but their expression depends on the tissue type. TGFβ ligands are synthesized as inactive precursors, held as homodimers in complexes with other proteins in the extracellular matrix [2]. Their activation includes proteolytic cleavage of latency-associated peptide (LAP) and a latent TGFβ-binding protein (LTBP), followed by the release of TGFβ that are free to activate surface cellular receptors which are heteromeric complexes of two related transmembrane type I and type II serine/threonine kinase receptors (these receptors are also referred to as activin receptor-like kinases - ALKs). Binding of TGFβ to the type I and type II receptors induces changes in conformation of the intracellular kinase domains of the receptors, allowing the phosphorylation and activation of the type I receptor [3]. The activation of TGFβ receptor type I (TGFBR1, ALK5) leads to phosphorylation of SMAD2 and SMAD3 proteins [4]. Phosphorylated SMAD2/3 dimers translocate to the nucleus, interact with other transcription factors, bind to promoters and regulate gene expression.

The physiological roles of TGFβ are pleiotropic, starting from embryonal development to adult tissue homeostasis. On a cellular level, TGFβ ligands regulate expression of genes involved in proliferation, differentiation, migration, extracellular matrix production and apoptosis [2,5].

Aberrant expression and activity of TGFβ and hyperactivation of downstream signaling molecules are associated with developmental defects and human diseases, including cancer, fibrosis and inflammation [2,6]. Due to mentioned deregulation of TGFβ signaling in several diseases, the components of the pathway have been studied as possible drug targets for several different therapeutic uses, including cancers, myelodysplastic syndrome, fibroses,

*Abbreviations:* ALK5, activin A receptor type II-like kinase; DPP, 5,5-dimethyl-5,6-dihydro-4*H*-pyrrolo[1,2-*b*]pyrazole; TGFβ, transforming growth factor beta; TGFBR1, transforming growth factor beta receptor I.

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Marfan syndrome, scleroderma, and restenosis after coronary artery bypass [2,7,8]. Various approaches have been described, including the use of small molecule inhibitors of the intracellular kinase domain of ALK5 [2].

Several small molecule ALK5 inhibitors have been developed, such as SB-525334, GW6604, SD-208, IN-1130 and LY-2157299 (galunisertib), illustrated in Fig. 1 [9–15]. All these compounds are ATP competitors and proved to specifically inhibit the SMAD signaling and block TGFβ-mediated tumor progression and metastasis. Some even enhanced immune response in the mouse model of prostate cancer [16].

Although many inhibitors demonstrated pronounced efficacy in animal models of cancer and fibrosis, there were some drawbacks that limit their further development as clinical candidates, including especially limited pharmacokinetic properties (oral availability) and kinase selectivity. Despite these limitations, galunisertib has progressed to phase II trials for hepatocellular carcinoma, recurrent or refractory non-small cell lung cancer, glioma, and advanced refractory solid tumors. Other molecules are still optimized and evaluated in preclinical experiments [17].

In our previous study [18] we described the synthesis of a small library of DPPs bearing diverse substitution at C2 and C3 and these compounds revealed in most cases selective inhibition of ALK5 kinase. Since the synthesis is straightforward, has a low step count and is high yielding, we endeavoured to continue the structure-activity studies and focused on derivatives substituted at position C4. This position of the DPP scaffolding has not been previously explored and therefore we chose to prepare novel derivatives and study the impact of this modification on biochemical activity. From a synthetic point of view, an essential motif of our compounds is a dimethylated carbon C5, but we clearly showed tolerance towards ALK5 inhibition and even a 5,5-dimethyl homologue of galunisertib showed reasonable activity ($IC_{50}$ value of 0.603 μM).

## 2. Results and discussion

### 2.1. Synthesis

The synthesis of DPP derivatives consists of three general steps, from protected hydrazone **1** through nonsymmetrical homoallenyl azine **2** to molecule **3** containing a DPP core (Supplementary data). The latter served as reagents for final derivatization, for which we utilized the Suzuki cross-coupling reaction, C-H activation/functionalization sequence and an *N*-oxide protective pathway depending on the quality of the side aromatic substituent at C2 of DPP. Inspired by our recent paper [18] we chose only the most potent examples as models and the relevant substitution was introduced to all of the novel compounds. Specifically, the best hits contain 4-pyridyl and 4-hydroxyphenyl moieties at C3. Although prepared sulfonamides showed reasonable activity as ALK5 inhibitors either, these compounds were not selective with significant inhibition of ABL kinase and had increased cytotoxicity (see below).

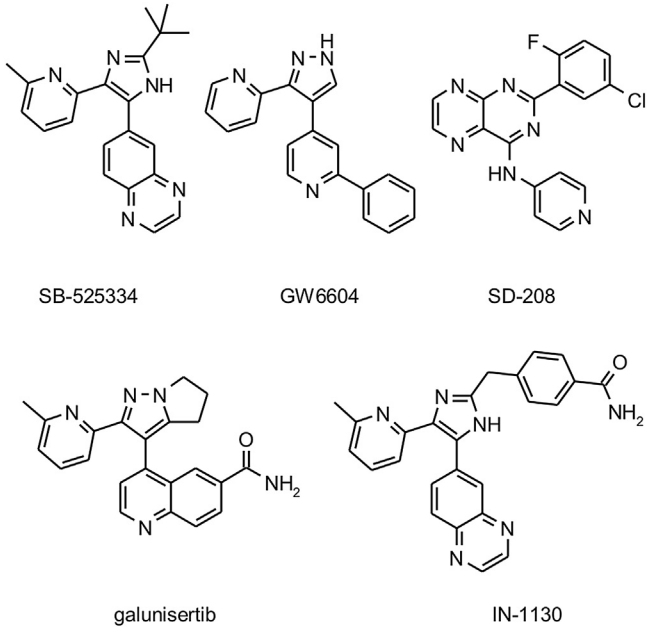


**Fig. 1.** Examples of known ALK5 inhibitors.

The first set of four final products **4a-h** was synthesized via C-H activation in one step and in good yields (Scheme 1). This appeared to be the optimal way for reaction with electron-poor aryl halides.

Recently, we have demonstrated one important highlight where we observed unwillingness of DPPs to undergo the direct C-H activation reaction with electron-rich aryl halides (very low conversions) [18]. This problem was cleanly bypassed with the protection of pyridine nitrogen atom by the transformation to the *N*-oxide. Suzuki cross-coupling reaction of brominated *N*-oxides **7a** or **7b** with (4-methoxyphenyl)boronic acid provided otherwise inaccessible products **4i** and **4j** in good yields (Scheme 2). We also explored a different approach with initiative oxidation to **6a** and **6b** followed by bromination, but with slightly lower overall yields.

DPPs **4i** and **4j** exhibit reasonable inhibition activities and selectivities toward ALK5 kinase. However, to our delight, we obtained even better results after simple demethylation of methoxy group to products **4k** and **4l** and these two compounds revealed the highest potency towards ALK5 inhibition in our study. Moreover, formation of the stereogenic centre at methylated C4 stimulated us to separate both methyl- and ethyl-possessing racemates to enantiomers and investigate their individual activities compared to a racemic form. Using chiral HPLC we succesfully obtained both pure isomers (Fig. 2). In both cases, the *R* isomers were approximately two-fold more potent than their respective *S* isomers. However, both enantiomer pairs showed slightly lower activities than racemates, which could be explained by contamination during chiral separation with an unknown impurity.

The next set of four final products **4m-p** was synthesized via bromination of cyano-DPPs **3c** and **3d** (Supplementary data) and subsequent Suzuki cross-coupling reaction providing high yields (Scheme 3). The cyano group in these products was supposed to imitate interaction between the nitrogen of the pyridin-2-yl substituent with amino acids in the active site of ALK5, as seen in the two available crystal structures of ALK5 with competitive inhibitors (PDB ID: 3HMM, 1RW8, see Supplementary data) [19,20]. This modification has been found to be functional, albeit not in all prepared molecules (see below).

For SAR studies it was obviously necessary to explore more than a simple methyl substitution at the C4 position. Thus, we had to start almost from the beginning and we applied the hydroxymethylation reaction as adequate substitution (longer chain) on the propargyl(vinyl)ether **8** [21] to alcohol **9** with subsequent methylation to product **10** and Claisen rearrangement to methoxymethyl substituted homoallenyl aldehyde **11** (see Supplementary data). Fortuitously, such reactions proceeded in high three-step overall yield of 54% followed by homoallenyl azine **2e** formation in 79% yield (see Supplementary data). This azine provided DPP **3e** with methoxymethyl substitution at C4 in very good 87% yield (Scheme 4). Then, we applied the same conditions for C-H activation as described in Scheme 1 and obtained DPP **4q** (82%) and after demethylation of methoxy group alcohol **4r** (85%).