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RESEARCH ARTICLE

Identification of Gli-mediated transcription inhibitors through synthesis and evaluation of taepeenin D analogues†

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The Hedgehog pathway has emerged as a favourable target to address drug resistance in cancer stem cells. Inhibitors of Gli1, the terminal effector of the pathway, are anticipated to evade drug resistance and to display fewer side effects than inhibitors targeting Smo. Inspired by the Gli-mediated transcription inhibitor taepeenin D, synthesis and evaluation of a series of second generation analogues has led to the enrichment of available structure-activity relationships for this natural product and has identified an oxazole analogue as an improved lead compound.

1. Introduction

Cancer stem cells (CSCs)[1,2] are a subset of cancerous cells that are endowed with properties usually associated with normal stem cells,[3] such as the ability to self-renew and differentiate. They are thought to lead tumour initiation and to be responsible for its relapse after treatment.[1,2] Among the pathways responsible for drug resistance in CSCs, the Hedgehog (Hh) pathway has emerged as a favourable target for pharmaceutical intervention since its aberrant activation has been linked to several human cancers.[4,5] A natural product, the steroidal alkaloid cyclopamine (Fig. 1),[6,7] has inaugurated the quest for small molecule modulators of this pathway that has led to the identification of several inhibitors of synthetic or natural origin.[5,8] The majority of them, including vismodegib (GDC-0449; Fig. 1) and sonidegib (NVP-LDE225; Fig. 1) that have been approved by the United States Food and Drug Administration for the treatment of basal cell carcinoma (BCC), target the membrane receptor smoothened (Smo) that controls canonical pathway activation.[9,10] However, recent evidence suggests that targeting glioma-associated oncogene 1 (Gli1), which is the terminal effector of the pathway, might be more advantageous target.[5] Gli1-mediated transcription inhibitors are anticipated not only to evade the potential emergence of drug resistance associated with Smo inhibitors (either through mutation of Smo or through compensating cross-talk of the Hh pathway with other signalling pathways) but also to display fewer side effects.[5]

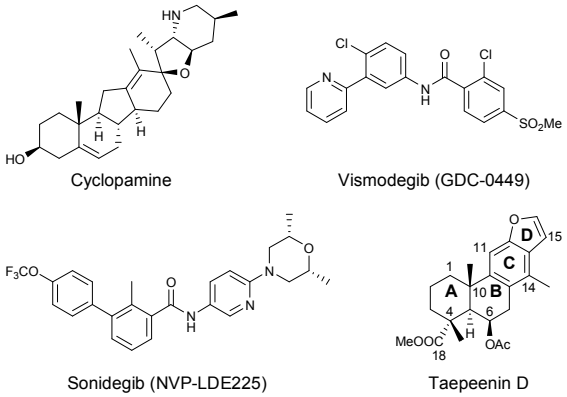

Fig. 1 Structures of some Hh pathway inhibitors.

In this context, we were attracted[11,12] to taepeenin D (Fig. 1), a cassane-type furano-diterpenoid isolated from *Caesalpinia crista*[13] that was later identified as a constituent of *Acacia pennata* with Gli-mediated transcription inhibitory activity (IC_{50} = 1.6 μM) and selective cytotoxicity against cancer cells with increased Hh signalling levels (IC_{50} = 3.2–3.4 μM). Furthermore, this natural product reduces the exogenous Gli1 protein level in HaCaT and PANC1 cells.[14]

In a previous communication we have disclosed an expedient entry to 14-desmethyl taepeenin derivatives from readily available abietic acid (**1**, Scheme 1) that has allowed the establishment of some essential structural features.[11] Thus, while a methyl group at position 14 does not seem to be crucial for activity, substitution at position 6 and an aromatic D ring are. Derivatives **2a** (IC_{50} ≈ 16 μM) and **2b** (IC_{50} ≈ 13 μM)

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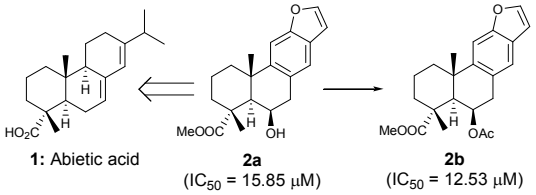
were identified as potential new leads for further optimization (Scheme 1).



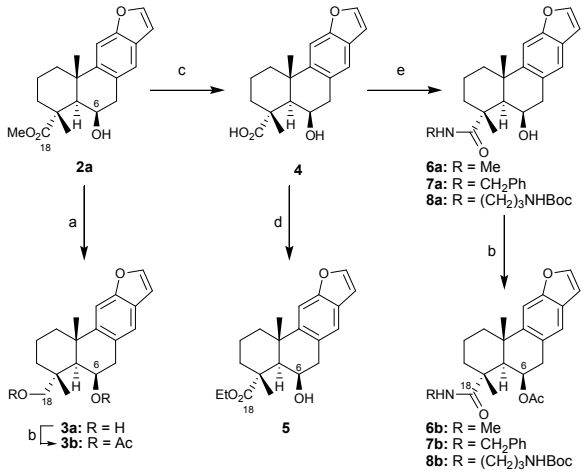
Scheme 1 Rosin acid derived first generation 14-desmethyl-taepeenin analogues.

The aim of the present study was to explore further the structure-activity relationship (SAR) and to identify more potent Gli-mediated transcription inhibitors based on this novel scaffold.

2. Results and discussion

2.1. Chemistry

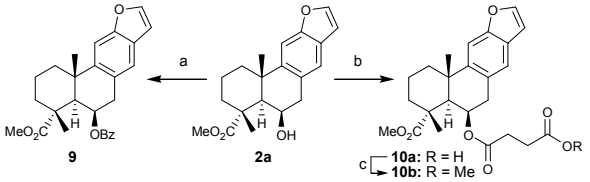
Preparation of the new taepeenin D analogues was based on the previously established synthetic route.[11] Thus, hydroxyester **2a** was exploited as an entry to C18 (Scheme 2) as well as C6 derivatives (Scheme 3). For the former, it was reduced to the corresponding diol (**3a**), and thence converted to diacetate **3b** (Scheme 2). The same substrate (**2a**) could be hydrolysed to hydroxyacid **4**, and subsequently converted either to ethyl ester **5** or to a series of amide derivatives (**6a**–**8a**). Acetylation of the latter led to derivatives **6b**–**8b**.



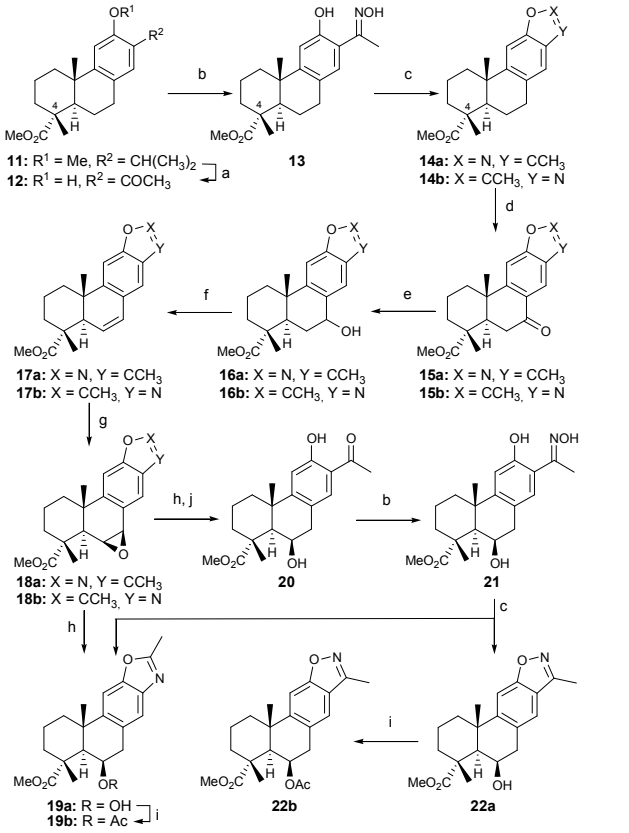
Scheme 2 Reagents and conditions: (a) $LiAlH_4$, THF, 0 °C, 85%; (b) Ac_2O, DMAP, CH_2Cl_2, 0 °C (65% for **3b**, 81% for **6b**, 78% for **7b**, 84% for **8b**); (c) ^tBuOK, DMSO, rt, 82%; (d) EtI, $NaHCO_3$, DMF, 0 °C to rt, 70%; (e) PyBOP, $^iPr_2EtN$, RNH_2, CH_2Cl_2, rt (68% for **6a**, 75% for **7a**, 66% for **8a**).

On the other hand, esterification of the hydroxyl moiety of **2a** with benzoyl chloride or succinic anhydride provided the corresponding C6 derivatives **9** and **10b** (Scheme 3).

Analogues **8a**,**b** and **10a**,**b** were designed having in mind to investigate, at a later stage, the activity of dimeric or hybrid derivatives.[15-17] Derivatives **19a**,**b** and **22a**,**b** (Scheme 4) were targeted seeking to substitute the oxidation prone[18,19] benzofuran moiety of the prototype with a more robust heteroaromatic ring system. To this end, Friedel–Crafts *ipso*-acylation of dehydroabietate derivative **11**[11] led to acetophenone **12** (Scheme 4).[20] Its oxime (**13**) was converted to a separable mixture of isoxazole **14a** and oxazole **14b** in analogy to the chemistry developed by Beresford and his coworkers for related podocarpic acid derivatives (i.e., C4 epimers).[21] Subsequently, these intermediates were converted to epoxides **18a** and **18b** through benzylic oxidation, reduction, dehydration, treatment with NBS in THF/H_2O, and subsequent exposure of the mixture of bromohydrins thus obtained to potassium *tert*-butoxide at low temperature (Scheme 4).[11] Mindful of the lability of the corresponding benzofuran



Scheme 3 Reagents and conditions: (a) benzoyl chloride, pyridine, DMAP, 0 to 70 °C, 88%; (b) succinic anhydride, pyridine, DMAP, CH_2Cl_2, 0 to 60 °C; (c) $TMSCH_2N_2$, toluene/MeOH, rt, 32% from **2a**.



Scheme 4 Reagents and conditions: (a) AcCl, $AlCl_3$, CH_2Cl_2, 0 °C to rt then $AlCl_3$, CH_2Cl_2, reflux, 96%; (b) $HONH_2{\cdot}HCl$, pyridine, MeOH, reflux (93% for **13**, 80% for **21**); (c) TsCl, pyridine, rt (43% for **14a** and 40% for **14b** from **13**; 30% for **22a** and 24% for **19a** from **21**); (d) CrO_3, $AcOH/H_2O$, 10–15 °C (92% for **15a**, 78% for **15b**); (e) $NaBH_4$, MeOH, 0 °C to rt; (f) $TsOH{\cdot}H_2O$, benzene, reflux (80% for **17a** from **15a**, 85% for **17b** from **15b**); (g) NBS, THF/H_2O, 0 °C; ^tBuOK, THF, −78 to 0 °C; (h) H_2, 5% $Pd/BaSO_4$, $MeOH/N(CH_2CH_2OH)_2$ (75% from **17b**); (i) Ac_2O, DMAP, CH_2Cl_2, 0 °C, (82% for **19b**, 81% for **22b**); (j) Montmorillonite K-10, $MeOH/H_2O$, rt, 31% from **17a**.

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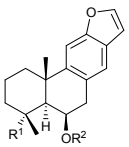
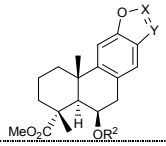
Table 1 Gli-luc reporter inhibition by second generation taepeenin D derivatives

Compound	Scaffold	Substituents	Gli-luc reporter IC_{50} (μM)[a,b]
3a		$R^1 = CH_2OH$, $R^2 = H$	>25
3b		$R^1 = CH_2OAc$, $R^2 = Ac$	>25
5		$R^1 = CO_2Et$, $R^2 = H$	**0.74±0.29[b], 0.86±0.42[c]**
6a		$R^1 = CONHMe$, $R^2 = H$	>25
6b		$R^1 = CONHMe$, $R^2 = Ac$	>25
7a		$R^1 = CONHBn$, $R^2 = H$	5.38±1.02
7b		$R^1 = CONHBn$, $R^2 = Ac$	8.98±2.90
8a		$R^1 = CONH(CH_2)_3NHBoc$, $R^2 = H$	>25
8b		$R^1 = CONH(CH_2)_3NHBoc$, $R^2 = Ac$	>25
9		$R^1 = CO_2Me$, $R^2 = COPh$	>25
10b		$R^1 = CO_2Me$, $R^2 = CO(CH_2)_2CO_2Me$	12.34±2.84
19a		X = CCH_3, Y = N, $R^2 = H$	**0.64±0.22[b], 1.09±0.73[c]**
19b		X = CCH_3, Y = N, $R^2 = Ac$	>25
22a		X = N, Y = CCH_3, $R^2 = H$	>25
22b		X = N, Y = CCH_3, $R^2 = Ac$	>25
Cyclopamine[d]			5[22]

[a] Average of triplicate separate determinations. [b] 100 nM of SAG used. [c] 10 nM of SAG used. [d] Used as positive control.

epoxide,[11] isoxazole/oxazole epoxides **18a**/**18b** were subjected without rigorous purification to hydrogenolysis over 5% $Pd/BaSO_4$ in the presence of triethanolamine.[11] The desired chemoselective reduction proceeded uneventfully in the case of **18b** to yield alcohol **19a**, which was subsequently converted to acetate **19b**. However, in the case of **18a** it was accompanied by hydrogenolysis of the isoxazole ring to provide, after hydrolysis of the intermediate imine, acetophenone **20**. The hydroxy-isoxazole derivative **22a** and the corresponding acetate **22b** could be obtained from **20** by submitting its oxime derivative (**21**) to the same conditions (i.e., tosyl chloride in pyridine) that were initially employed to establish the heteroaromatic rings.

2.2. Biological evaluation

A well established cell-based Gli-luciferase reporter gene assay[4] was employed in order to test the ability of the new taepeenin D analogues to inhibit the activation of the Hh pathway. In particular, their ability to reduce firefly luciferase activity in Shh-LIGHTII cells, a clonal mouse fibroblast cell line (NIH 3T3) stably transfected with Gli-dependent firefly luciferase and constitutive *Renilla* luciferase reporters, was evaluated in the presence of a known Smo agonist, SAG (100 nM).[7,23] All compounds were initially screened at two different concentrations (5 μM and 25 μM) using cyclopamine (8 μM) as a positive control. Subsequently, IC_{50} values were calculated for the ones exhibiting inhibition by evaluating their activity at concentrations 0.05−50 μM (Table 1).

Reduction of the C18 methyl ester functionality to a hydroxymethyl one (analogues **3a**, **3b**) leads to loss of activity. On the other hand, substitution of the methyl for an ethyl ester (i.e., **2a** vs. **5**) results in a marked improvement in Hh pathway inhibitory potency (IC_{50} = 15.85 μM vs. 0.74 μM, respectively). Amides bearing short (**6a**,**b**) or (**8a**,**b**) longer alkyl chains were found not active. Interestingly, the corresponding benzyl amides (**7a** and **7b**) demonstrated slightly improved inhibitory activity (IC_{50} = 5.38 μM and 8.98 μM, respectively).

Regarding the impact of the introduction of aromatic vs. unbranched aliphatic moieties on inhibitory activity, the opposite trend was observed upon variation of the ester functionality at position C6. Thus, it appears that a benzoate (i.e. analogue **9**) at this position is not tolerated while the aliphatic succinate (**10b**) retains the inhibitory activity (IC_{50} = 12.34 μM) of the related acetate (**2b**; IC_{50} = 12.53 μM).[11]

Contrary to the isoxazole derivatives (**22a**,**b**) that have abolished the inhibitory activity exhibited by their furan counterparts (i.e., **2a**,**b**), the oxazole derivative **19a** exhibited significant enhancement in potency (IC_{50} = 0.64 μM). Interestingly, acetylation of the C6 hydroxyl in the case of this new scaffold led to an inactive compound (**19b**). This marked

difference in activity suggests that hydrolysis of acetate **19b** to alcohol **19a** does not occur under the assay conditions.

Although the assay employed cannot per se distinguish between Smo and non-Smo inhibitors, a decrease in the observed inhibitory potency at higher agonist concentration is accepted as a sign of Smo-targeted inhibitors ("Gli shift" assay).[24,25] Thus, in order to gain some insight on the molecular target of the two most potent derivatives (i.e., **5** and **19a**), their activity was also evaluated in the presence of a lower (10 nM) agonist concentration. They exhibited similar activities [for **5**: IC_{50} = 0.86 μM (10 nM SAG), IC_{50} = 0.74 μM (100 nM SAG); for **19a**: IC_{50} = 1.09 μM (10 nM SAG), IC_{50} = 0.64 μM (100 nM SAG)] indicative of inhibition downstream of Smo.

Finally, both **5** and **19a** at concentrations up to 100 μM were proven non-toxic towards a human breast cancer cell line (MCF-7) and human umbilical vein endothelial cells (HUVEC).

3. Conclusions

Natural products are a rich source of novel pharmacophoric scaffolds.[26,27] The natural product taepeenin D offers a new structural motif in the quest for novel Hh pathway inhibitors.[14] The synthesis and evaluation of a series of related second generation analogues has led to the enrichment of available structure-activity relationships for this Gli-mediated transcription inhibitor and has led to the identification of an oxazole analogue as a new lead compound. Studies aimed to further improve its inhibitory potency as well as to pinpoint its molecular target downstream of Smo are planned and their results will be reported in due course.

Experimental section

Synthetic protocols and analytical data for all new compounds, procedures and methods for *in vitro* assays are available in the ESI.†

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Abietic acid derivatives related to taepeenin D were identified as new Hh pathway inhibitors that operate downstream of Smo.