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Indeno[1,2-*d*]pyrido[1,2-*a*]pyrimidines: A New Class of Receptor Tyrosine Kinase Inhibitors

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Receptor tyrosine kinases (RTKs) are important mediators of signal transduction and play critical roles in cell growth, differentiation, metabolism, and apoptosis, and are deeply involved in oncogenesis. Binding of growth factors to the extracellular domains of RTKs leads to their dimerization, resulting in activation of the intracellular kinase domain, which eventually enables autophosphorylation at specific tyrosine residues. This family is also represented in oncogenic fusion proteins such as the Bcr-Abl protein of the Philadelphia chromosome.

RTKs are organized into different families based on sequence homology and structural characteristics. There are more than 90 known protein kinase genes; 58 encode transmembrane receptor TKs distributed in 20 subfamilies, and 32 encode cytoplasmic non-receptor TKs distributed in 10 subfamilies.[1,2]

Tyrosine kinases have been validated as suitable pharmacological targets for anticancer drugs, and several TK inhibitors have shown promising results in preclinical in vitro and in vivo models; others have been approved for the treatment in patients with cancer. This is best exemplified by the tyrosine kinase inhibitor imatinib (Gleevec), which has shown impressive activity against chronic myelogenous leukemia (CML) and has also been successful in the treatment of gastrointestinal stromal tumors, other leukemias, and solid tumors.[3]

The majority of the recently developed RTK inhibitors,[4,5] including imatinib, target multiple mechanisms and are known as multiplex or multi-target tyrosine kinase inhibitors (MTKIs). A common characteristic of most of these drugs is that they are inhibitors of angiogenesis and also target additional receptors located on the surface of the cancer cell. Representative examples include the already approved and registered sunitinib[6] and sorafenib,[7] as well as AMG 706.[8]

Considering the observed development of tumor resistance[9] to tyrosine kinase inhibitors and in order to circumvent drug-associated side effects, the discovery of new templates and their subsequent elaboration to clinically useful RTK inhibitors continues to be an important issue. Herein we report the discovery of a new class of cell-permeable RTK inhibitors belonging to the indeno[1,2-*d*]pyrido[1,2-*a*]pyrimidine-11,12-dione class of compounds.

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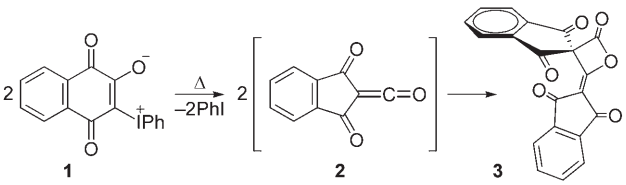
Chemistry

Recently, we reported the isolation of the unusual tetraoxo-oxetanone **3**, obtained in quantitative yield from the dimerization of the highly reactive α,α'-dioxoketene **2**.[10] The latter results from the thermal decomposition of the phenyliodonium ylide of 2-hydroxy-1,4-naphthoquinone **1** (Scheme 1).

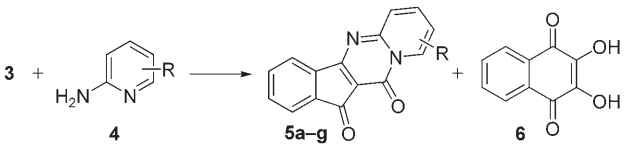


Scheme 1. Formation of tetraoxo-oxetanone **3** from dimerization of intermediate **2**.

The interesting reactivity of tetraoxo-oxetanone **3** towards amines and anilines[11] prompted us to investigate the possibility of the reaction of **3** with compounds bearing two nucleophilic nitrogen groups in the same molecule. Indeed, the reaction of **3** with 2-aminopyridines and 2-aminopyrimidines offered easy access to novel fused nitrogen ring systems. The addition of one equivalent of substituted 2-aminopyridines **4a–g** to a suspension of tetraoxo-oxetanone **3** in dichloromethane afforded the indenopyridopyrimidinediones **5a–g** and the dihydroxyquinone **6** in good yields (Scheme 2).

Dihydroxyquinone **6**[12] was isolated by crystallization from the reaction solution, and compounds **5** by column chromatography. The structures were characterized based on their spectroscopic data and NOE experiments.

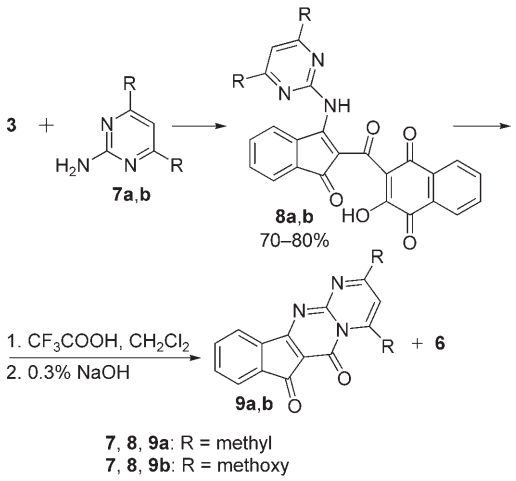
Analogous products were obtained from the reaction of 2-aminopyrimidines **7a,b** with tetraoxo-oxetanone **3**. In this case, the intermediate enamino esters **8** were isolated, as they crystallized out from the reaction solution with yields in the



4a: R = H
4b: R = 3-methyl
4c: R = 4-methyl
4d: R = 6-methyl
4e: R = 4,6-dimethyl
4f: R = 5-chloro
4g: R = 3-hydroxy

Scheme 2. Formation of compound **5** from tetraoxo-oxetanone **3**.

range of 70–80% (Scheme 3). Purification of these intermediate enamino esters **8** is difficult, as they are insoluble labile compounds and their decomposition starts as soon as they go into solution. For these reasons, no acceptable ^{1}H NMR and ^{13}C NMR spectra could be obtained, but HRMS data verify their formation.



Scheme 3. Generation of compounds **9**.

As mentioned, enamino esters **8** are labile, insoluble in many solvents, and can react further with water and other nucleophiles. To avoid such decomposition and to obtain the final fused products **9**, it is important to proceed with the cyclization of these esters as soon as possible after their isolation. Addition of 10 equivalents of trifluoroacetic acid (TFA) in a stirred suspension of **8** in dichloromethane followed by the addition of a dilute aqueous solution of NaOH (0.3%) afforded the desired products **9a,b** in respective overall yields of 45 and 41%, based on the amount of dimer **3** (Scheme 3). The alkaline conditions result in the decomposition of any pyrimidinium trifluoroacetates that may be formed, and also cause dissolution of the formed dihydroxyquinone **6**, thus facilitating the isolation of **9**.

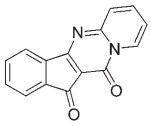
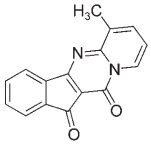
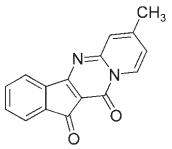
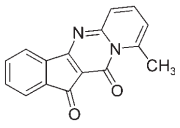
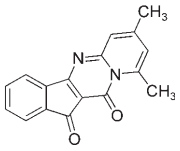
Biology

The synthesized compounds were screened for potential inhibitory action against the following RTKs: epidermal growth factor receptor (EGFR), vascular endothelial growth factor receptor 2 (VEGFR-2, KDR), Flt-4, fibroblast growth factor receptor (FGFR), insulin-like growth factor receptor (IGFR), and Tie2 receptor.

Indenopyridopyrimidinediones **5a**–**e** are inhibitors of EGFR, KDR, and Flt-4 kinases (Table 1). Compound **5d**, with a methyl group at position 9 next to the fused ring nitrogen atom, shows similar effects to the parent compound **5a** and inhibits the same kinases. The dimethyl derivative **5e** appears to be more selective and inhibits only the kinases KDR and Flt-4. Interestingly, the monomethyl derivatives **5b** and **5c** are selective against KDR, whereas the hydroxy and chloro derivatives **5f** and **5g** are completely inactive. Also inactive are the indeno[1,2-*d*]pyrimido[1,2-*a*]pyrimidinediones **9a** and **9b**. Furthermore, we did not observe any inhibitory activity against FGFR, insulin-like growth factor receptor type 1 (IGF1R) and Tie2 receptor. Interestingly, derivatives **9a** and **9b**, which are structurally very similar to the active derivative **5e**, were completely inactive against the tyrosine kinases tested.

Next we wanted to study these inhibitors for their ability to influence endothelial cell proliferation. The results from the proliferation assays are summarized in Table 2 and indicate that the inhibitors are cell-permeable. The observed IC_{50} value of the selective KDR inhibitor **5b** in the proliferation assay is below its IC_{50} value against KDR in the in vitro assay, indicating that there are possibly further intracellular targets for this inhibitor. We also tested the toxicity of derivatives **5a**–**d** using

Table 1. Inhibition of receptor tyrosine kinases by indenopyridopyrimidinediones and indenopyrimidopyrimidinediones.

Compound		IC_{50} [μM] EGFR	KDR	Flt-4
5a		1.17 ± 0.23	3.50 ± 0.22	4.72 ± 0.19
5b		NA[a]	1.48 ± 0.79	NA
5c		NA	0.64 ± 0.04	NA
5d		0.76 ± 0.17	0.34 ± 0.05	2.49 ± 0.29
5e		NA	0.68 ± 0.06	13.26 ± 2.19

[a] NA: $IC_{50} > 50$ μM.

Table 2. Inhibition of HUVE cell proliferation by the indenopyridopyrimidinedione derivatives **5a–e**.

Compound	IC_{50} [μM]
5a	10.14 ± 2.5
5b	0.45 ± 0.04
5c	14.6 ± 2.0
5d	0.9 ± 0.2
5e	9.5 ± 5.0

the WST-1 assay. All these derivatives proved to be nontoxic up to a concentration of 30 μM.

Lastly, we investigated the mode of action of one of our RTK inhibitors: increased ATP concentration decreased the inhibitory potency of compound **5d** (Table 3). This indicates that derivative **5d** acts in an ATP-competitive manner.

Table 3. ATP dependence of the IC_{50} value of inhibitor **5d**.

ATP concentration [μM]	IC_{50} [μM]
25	0.34 ± 0.05
50	1.31 ± 0.12
250	3.51 ± 0.20

In conclusion, we have developed a new class of RTK inhibitors based on the new indenopyridopyrimidinedione scaffold. Derivatives **5b** and **5c** showed significant inhibitory activities against KDR and are selective, as they do not inhibit other selected tyrosine kinases. The indenopyridopyrimidinediones **5b** and **5c** can be regarded as valuable agents for the inhibition of the angiogenesis process. Further detailed SAR studies are necessary in order to gain additional insight regarding the interaction of this class of compounds with the corresponding RTKs.

Experimental Section

Chemistry general

All commercially available reagents were obtained from Acros, Aldrich, and Fluka, and used without further purification. Melting points were measured with a Koffler hot stage and are uncorrected. IR spectra were obtained with a PerkinElmer 1310 instrument. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AM 300 (300 MHz for ^{1}H NMR; 75 MHz for ^{13}C NMR) and Bruker Avance-DRX 400 (400 MHz for ^{1}H NMR; 100 MHz for ^{13}C NMR). The residual solvent peak was used as an internal reference. HRMS data were obtained on a Bruker Daltonics APEX II (for ESI). Analyses were performed with a PerkinElmer 2400-II analyzer. Column chromatography was performed on silica gel (Merck 60, 70-230 Mesh) and analytical TLC was carried out on pre-coated silica gel plates (Merck 60 F_{254}, 0.25 mm).

General procedure for the reaction of tetraoxo-oxetanone 3 with 2-aminopyridines 4a–g.

A solution of the appropriate aminopyridine **4a–g** (0.3 mmol) in CH_2Cl_2 (3 mL) was added to a stirred suspension of tetraoxo-oxetanone **3** (0.3 mmol) in CH_2Cl_2 (4 mL). A clear solution resulted after 5–10 min, and stirring was continued for 5 h, during which time the red crystals of dihydroxyquinone **6** were deposited. The crystals of quinone **6** were filtered off, and the filtrate was concentrated and separated by column chromatography (silica gel, hexanes/EtOAc (3:1) → pure EtOAc) to afford the indenopyridopyrimidinedione derivatives **5a–g** as bright-yellow crystals. The isolated derivatives **5a–g** were sufficiently pure, but analytical samples were obtained by recrystallization from CH_2Cl_2/hexanes or EtOAc/Et_2O.

In the case of 3-hydroxy-2-aminopyridine (**4g**), the reaction was stirred for 5 h, and the resulting suspension was concentrated to a small volume (~2 mL), and thereafter MeOH (1 mL) and hexanes (0.2 mL) were added. The product **5g** was isolated by filtration.

Reaction with 2-aminopyridine (4a); indeno[1,2-*d*]pyrido[1,2-*a*]pyrimidine-11,12-dione (5a). Yield 81%; mp: 300–302 °C; IR (KBr) $\tilde{\nu}$ = 1747, 1677 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz) δ = 9.39 (d, J = 6.8 Hz, 1H), 8.06 (dt, J_1 = 6.8 Hz, J_2 = 1.6 Hz, 1H), 7.93 (d, J = 7.6 Hz, 1H), 7.90 (d, J = 6.8 Hz, 1H), 7.78 (d, J = 7.2 Hz, 1H), 7.61 (dt, J_1 = 7.2 Hz, J_2 = 1.2 Hz, 1H), 7.56 (dt, J_1 = 7.6 Hz, J_2 = 0.8 Hz, 1H), 7.40 ppm (dt, J_1 = 6.8 Hz, J_2 = 1.6 Hz, 1H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ = 189.2, 172.1, 155.2, 151.4, 140.8, 139.9, 136.6, 133.8, 133.0, 130.8, 127.4, 123.4, 122.2, 117.8, 103.0 ppm; EI-MS *m/z* (%) 248 ([M^+], 100), 220 (92), 192 (27); Anal. calcd for $C_{15}H_8N_2O_2$: C 72.58, H 3.25, N 11.28, found: C 72.27, H 3.28, N 11.36.

Reaction with 3-methyl-2-aminopyridine (4b); 6-methylindeno-[1,2-*d*]pyrido[1,2-*a*]pyrimidine-11,12-dione (5b). Yield 61%; mp: 298–302 °C; IR (KBr) $\tilde{\nu}$ = 1740, 1670 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz) δ = 9.27 (d, J = 6.4 Hz, 1H), 7.92 (d, J = 6.8 Hz, 1H), 7.88 (d, J = 6.4 Hz, 1H), 7.77 (d, J = 7.2 Hz, 1H), 7.59 (t, J = 6.8 Hz, 1H), 7.54 (t, J = 7.2 Hz, 1H), 7.27 (t, J = 6.4 Hz, 1H), 2.76 ppm (s, 3H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ = 189.5, 171.0, 154.4, 152.0, 140.3, 140.1, 136.7, 136.6, 133.6, 132.8, 128.7, 123.2, 122.0, 117.2, 102.6, 18.3 ppm; ESI-HRMS *m/z* calcd for $C_{16}H_{10}N_2O_2$+Na ([M + Na$^+$]) 285.06345, found 285.06343; Anal. calcd for $C_{16}H_{10}N_2O_2$: C 73.27, H 3.84, N 10.68, found: C 72.91, H 4.05, N 11.01.

Reaction with 4-methyl-2-aminopyridine (4c); 7-methylindeno-[1,2-*d*]pyrido[1,2-*a*]pyrimidine-11,12-dione (5c). Yield 82%; mp: 263–265 °C; IR (KBr) $\tilde{\nu}$ = 1738, 1670 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz) δ = 9.19 (d, J = 7.0 Hz, 1H), 7.82 (d, J = 6.9 Hz, 1H), 7.74 (d, J = 6.9 Hz, 1H), 7.64 (s, 1H), 7.58 (t, J = 6.9 Hz, 1H), 7.53 (t, J = 6.9 Hz, 1H), 7.19 (dd, J_1 = 7.0 Hz, J_2 = 1.8 Hz, 1H), 2.58 ppm (s, 3H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ = 189.1, 172.0, 154.7, 154.0, 151.4, 139.8, 136.6, 133.5, 132.6, 129.9, 126.1, 123.0, 121.8, 120.1, 110.1, 21.7 ppm; ESI-HRMS *m/z* calcd for $C_{16}H_{10}N_2O_2$+Na ([M + Na$^+$]) 285.06345, found 285.06382; Anal. calcd for $C_{16}H_{10}N_2O_2$: C 73.27, H 3.84, N 10.68, found: C 72.87, H 4.14, N 10.93.

Reaction with 6-methyl-2-aminopyridine (4d); 9-methylindeno-[1,2-*d*]pyrido[1,2-*a*]pyrimidine-11,12-dione (5d). Yield 66%; mp: 261–263 °C; IR (KBr) $\tilde{\nu}$ = 1745, 1681 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz) δ = 7.85 (d, J = 6.7 Hz, 1H), 7.82–7.69 (m, 3H), 7.60 (t, J = 6.7 Hz, 1H), 7.55 (t, J = 6.7 Hz, 1H), 6.97 (d, J = 6.7 Hz, 1H), 3.21 ppm (s, 3H); ^{13}C NMR ($CDCl_3$+[D_6]DMSO, 75 MHz) δ = 188.1, 170.1, 157.2, 154.9, 148.1, 139.4, 138.4, 135.8, 132.6, 131.7, 125.5, 121.8, 121.3, 121.0, 103.0, 24.3 ppm; ESI-HRMS *m/z* calcd for $C_{16}H_{10}N_2O_2$+Na ([M + Na$^+$]) 285.06345, found 285.06378.

Reaction with 4,6-dimethyl-2-aminopyridine (4e); 7,9-dimethylindeno[1,2-*d*]pyrido[1,2-*a*]pyrimidine-11,12-dione (5e). Yield 89%; mp: 265–267 °C; IR (KBr) $\tilde{\nu}$ = 1733, 1668 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ = 7.82–7.69 (m, 2H), 7.61–7.49 (m, 2H), 7.41 (s, 1H), 6.72 (s, 1H), 3.11 (s, 3H), 2.41 ppm (s, 3H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ = 188.9, 171.0, 157.6, 155.7, 152.5, 148.1, 139.2, 136.9, 133.1, 132.3, 125.2, 124.0, 122.7, 121.6, 106.6, 24.9, 21.1 ppm; EI-MS *m/z* (%) 276 ([M^+], 91), 261 (20), 248 (100), 219 (29); Anal. calcd for $C_{17}H_{12}N_2O_2$: C 73.90, H 4.38, N 10.14, found: C 73.54, H 4.44, N 9.91.

Reaction with 5-chloro-2-aminopyridine (4f); 8-chloroindeno-[1,2-*d*]pyrido[1,2-*a*]pyrimidine-11,12-dione (5f). Yield 64%; mp: > 310 °C; IR (KBr) $\tilde{\nu}$ = 1738, 1673 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ = 9.38 (d, J = 2.0 Hz, 1H), 7.97 (dd, J_1 = 9.1 Hz, J_2 = 2.0 Hz, 1H), 7.92–7.87 (m, 2H), 7.79 (d, J = 6.6 Hz, 1H), 7.63 (t, J = 7.0 Hz, 1H), 7.58 ppm (t, J = 6.6 Hz, 1H); ^1H NMR ($CDCl_3$+TFA, 300 MHz) δ = 9.50 (d, J = 2.2 Hz, 1H), 8.58 (dd, J_1 = 9.4 Hz, J_2 = 2.2 Hz, 1H), 8.42 (d, J = 8.8 Hz, 1H), 8.07–8.02 (m, 1H), 7.94–7.87 (m, 1H), 7.85–7.79 ppm (m, 2H); ^{13}C NMR ($CDCl_3$+TFA, 75 MHz) δ = 188.6, 165.0, 148.2, 148.1, 146.4, 136.8, 136.4, 134.3, 132.8, 132.6, 131.0, 125.8, 125.1, 121.9, 101.7 ppm; ESI-HRMS *m/z* calcd for $C_{15}H_7ClN_2O_2$+Na ([M + Na^+]) 305.00883, found 305.00892.

Reaction with 3-hydroxy-2-aminopyridine (4g); 6-hydroxyindeno[1,2-*d*]pyrido[1,2-*a*]pyrimidine-11,12-dione (5g). Yield 62%; mp: 300–301 °C (dec); IR (KBr) $\tilde{\nu}$ = 1732, 1672 cm^{-1}; ^1H NMR ($CDCl_3$+TFA, 300 MHz) δ = 9.09 (d, J = 6.7 Hz, 1H), 8.34–8.27 (m, 1H), 8.14 (d, J = 7.9 Hz, 1H), 7.95–7.89 (m, 1H), 7.87–7.78 ppm (m, 3H); ^{13}C NMR ($CDCl_3$+TFA, 75 MHz) δ = 188.4, 163.6, 148.7, 147.7, 138.7, 136.5, 136.1, 134.2, 132.6, 130.0, 125.5, 125.4, 123.7, 122.9, 101.3 ppm; ESI-HRMS *m/z* calcd for $C_{15}H_8N_2O_3$+Na ([M + Na^+]) 287.04271, found 287.04280.

General procedure for the reaction of tetraoxo-oxetanone 3 with 2-aminopyrimidines 7a,b.

A solution of the appropriate aminopyrimidine **7a,b** (0.3 mmol) in CH_2Cl_2 (3 mL) was added to a stirred suspension of tetraoxo-oxetanone **3** (0.3 mmol) in CH_2Cl_2 (4 mL). After a few minutes a precipitate began to form, and stirring was continued until TLC showed the disappearance of **3** (1–2 h). Hexanes (4–5 mL) were added to the solution to complete precipitation, and the resulting enamino esters **8a,b** were collected by filtration and washed on the filter with CH_2Cl_2/hexanes. The proper ester **8** (calculated 0.2 mmol) was suspended in CH_2Cl_2 (7 mL); CF_3COOH (2 mmol) was added, and the resulting clear solution was stirred for 4 h. After that time the solution was diluted with CH_2Cl_2 (10 mL) and extracted with a solution of NaOH (0.3%, 3×30 mL). The organic layer was washed with water, dried under Na_2SO_4, and concentrated to dryness. The residue was crystallized with CH_2Cl_2/hexanes to afford **9a,b**.

Reaction with 4,6-dimethyl-2-aminopyrimidine (7a); 4,6-dimethyl-3-hydroxy-1,4-dioxo-1,4-dihydronaphthalen-2-yl-1-oxo-3-(pyrimidin-2-ylamino)-1*H*-indene-2-carboxylate (8a). IR (KBr) $\tilde{\nu}$ = 1671, 1618 cm^{-1}, 1594; ESI-HRMS *m/z* calcd for $C_{26}H_{17}N_3O_6$ ([M + H^+]+MeOH) 500.14523, found 500.14504 and after treatment with TFA and NaOH, **2,4-dimethylindeno[1,2-*d*]pyrimido[1,2-*a*]pyrimidine-6,7-dione (9a)** was obtained. Yield 45% (overall yield based on **3**): mp: 225–230 °C (dec); IR (KBr) $\tilde{\nu}$ = 1739, 1676 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ = 7.96 (d, J = 6.9 Hz, 1H), 7.75 (d, J = 7.0 Hz, 1H), 7.64–7.53 (m, 2H), 6.86 (s, 1H), 3.19 (s, 3H), 2.70 ppm (s, 3H); ^1H NMR ($CDCl_3$+TFA, 300 MHz) δ = 8.06–7.98 (m, 1H), 7.82–7.70 (m, 3H), 7.36 (s, 1H), 3.21 (s, 3H), 2.76 ppm (s, 3H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ = 188.7, 174.5, 174.1, 158.0, 157.8, 155.5, 139.2, 136.4, 133.8, 133.1, 123.1, 122.5, 118.1, 104.6, 25.1, 24.7 ppm; ^{13}C NMR ($CDCl_3$+TFA, 75 MHz) δ = 86.7, 179.4, 165.0, 160.9, 151.7, 151.5, 136.2, 135.5, 134.0, 132.0, 125.0, 122.3, 103.0, 24.9, 24.0 ppm; ESI-HRMS *m/z* calcd for $C_{16}H_{11}N_3O_2$+H ([M + H^+]) 278.09240, found 278.09238.

Reaction with 4,6-dimethoxy-2-aminopyrimidine (7b); 4,6-dimethoxy-3-hydroxy-1,4-dioxo-1,4-dihydronaphthalen-2-yl-1-oxo-3-(pyrimidin-2-ylamino)-1*H*-indene-2-carboxylate (8b). IR (KBr) $\tilde{\nu}$ = 1669, 1622, 1598 cm^{-1}; ^1H NMR ($CDCl_3$+TFA, 300 MHz) δ = 8.29–8.12 (m, 2H), 7.98–7.76 (m, 5H), 7.70–7.58 (m, 1H), 6.09 (s, 1H), 4.06 ppm (s, 6H); ESI-HRMS *m/z* calcd for $C_{26}H_{17}N_3O_8$+Na ([M + Na^+]) 522.09070, found 522.09093. After treatment with TFA and NaOH, **2,4-dimethoxyindeno[1,2-*d*]pyrimido[1,2-*a*]pyrimidine-6,7-dione (9b)** was obtained. Yield 41%; mp: 215–218 °C (dec); IR (KBr) $\tilde{\nu}$ = 1750, 1675 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ = 7.95 (dd, J_1 = 6.4 Hz, J_2 = 1.9 Hz, 1H), 7.73 (dd, J_1 = 6.4 Hz, J_2 = 2.3 Hz, 1H), 7.61–7.53 (m, 2H), 5.93 (s, 1H), 4.21 (s, 3H), 4.12 ppm (s, 3H); ^1H NMR ($CDCl_3$+TFA, 300 MHz) δ = 7.96 (dd, J_1 = 6.4 Hz, J_2 = 1.8 Hz, 1H), 7.86–7.73 (m, 3H), 6.29 (s, 1H), 4.24 (s, 3H), 4.21 ppm (s, 3H); ^{13}C NMR ($CDCl_3$+TFA, 75 MHz) δ = 187.8, 172.9, 166.7, 164.8, 151.9, 149.6, 136.4, 135.8, 133.7, 131.6, 125.4, 125.1, 102.9, 84.5, 59.8, 57.8 ppm; ESI-HRMS *m/z* calcd for $C_{16}H_{11}N_3O_4$+Na ([M + Na^+]) 332.06418, found 332.06445.

Biology

For assays of inhibitory activity, the kinase-catalyzed phosphorylation of poly(Glu-Tyr) in the presence of varying concentrations of inhibitor was determined as described previously.[13] Kinases were employed as fusion proteins of glutathione-*S*-transferase (GST; Biomol, Germany) and the respective kinase domain. The relative amount of phosphorylated substrate was quantified by an anti-phosphotyrosine enzyme-linked immunosorbent assay (ELISA), using an anti-phosphotyrosine antibody conjugated to horseradish peroxidase (POD). The bound antibody was detected by light emission after the addition of a chemiluminescent substrate for POD. All IC_{50} values were calculated from at least four independent determinations.

Cell proliferation was measured using the Cell Proliferation ELISA, (BrdU (5-bromo-2′-deoxyuridine) Assay Kit, Roche, Cat. No. 11669915001). Assays were performed according to the manufacturer's instructions. Briefly, HUVE cells (PromoCell HUVEC-c C-12200) were cultured in 25-cm^2 flasks at 37 °C in a humid atmosphere with 5% CO_2. For each assay, ~5000 cells were seeded into each well of a 96-well microplate (white, clear bottom) and cultured in a final volume of 100 μL $well^{-1}$. After 24 h, the medium was replaced, and the cells were incubated with the compounds (100 μL $well^{-1}$) for 48 h, keeping the DMSO concentration < 0.5% (*v*/*v*).

In the presence of the inhibitors, cells were incubated with BrdU labeling solution, leading to the incorporation of BrdU only into mitotically active cells for 4 h. After removal of the solution, the cells were fixed and incubated with an anti-BrdU–POD solution. Finally, the cells were washed several times and incubated with the substrate solution. The bottom of the microplate was sealed with white cover foil to measure the chemiluminescence using an Orion Microplate Luminometer (Berthold). For determination of IC_{50} values, the relative luminescence units per second (RLU s^{-1}) were plotted against inhibitor concentrations. Each point of the curves represents three independent measurements.

Cytotoxicity was measured using the Cell Proliferation Reagent WST-1 (Roche, Cat. No. 13396300). The assays were performed according to the manufacturer's instructions. The cells were cultured as described above. Cells (~7500 per well) were exposed to the inhibitors at various concentrations for 24 h before adding the WST-1 reagent. After 4 h and again after 24 h the absorbance was measured using an Optimax UV/Vis spectrophotometer (λ: 440 and 650 nm).

Keywords: anticancer agents · inhibitors · kinases · pyrimidines · receptors

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