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Original article

Inhibitors of the RET tyrosine kinase based on a 2-(alkylsulfanyl)-4-(3-thienyl) nicotinonitrile scaffold

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ABSTRACT

In an approach to optimize 2-(4-fluorobenzylsulfanyl)-4-(2-thienyl)-5,6,7,8-tetrahydroquinoline-3-carbonitrile (**1a**), a weak inhibitor of the cancer-related tyrosine kinase RET originating from a screening campaign, analogues with 3-thienyl substitution were prepared. Among the novel derivatives, 2-amino-6-{[2-(4-chlorophenyl)-2-oxoethyl]sulfanyl}-4-(3-thienyl)pyridine-3,5-dicarbonitrile (**13g**) was identified as a submicromolar RET inhibitor, displaying 3- and 100-fold selectivity versus ALK and ABL kinases, respectively. The novel inhibitor exhibited antiproliferative activity in the micromolar concentration range against both RET-dependent and RET-independent cancer cell lines. Docking experiments suggest a binding mode of the new inhibitors in the ATP binding pocket of the target kinase, explaining the observed structure–activity relationships.

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1. Introduction

The RET (rearranged during transfection) protooncogene encodes for a membrane-bound receptor-like tyrosine kinase that is mainly expressed in the central and peripheral nervous system and in the developing urogenital tract [1,2]. The RET tyrosine kinase is essential for the early development of the enteric nervous system and the kidney [3,4]. The extracellular part of RET contains a cadherin-like domain probably responsible for interaction with the ligand and a cysteine-rich region that is important for the tertiary structure and the dimerization of two RET proteins [5,6]. The transmembrane domain is followed by the tyrosine kinase domain on the cytoplasmic side [7,8]. RET can be activated by the four members of the glial cell line-derived neurotrophic factor (GDNF) family of ligands in complex with a ligand-specific coreceptor (GFRα1-4, GDNF receptor α) [6]. Binding of the complex is followed by dimerization of RET and autophosphorylation of tyrosine residues in the intracellular kinase domain, which initiates signaling cascades regulating survival, differentiation, proliferation, migration and chemotaxis [7]. Constitutively activated RET evolving from mutations is associated with the development of several forms of endocrine tumors. Somatic chromosomal rearrangements result in chimeric *RET/PTC* oncogenes that encode for constitutively activated RET. These chimeric oncogenes have been identified in 5–30% of sporadic and 60–70% of radiation-induced papillary thyroid carcinomas (PTC) that arise from thyroid follicular cells [9]. Germline point mutations of the *RET* protooncogene lead to multiple endocrine neoplasia type 2 (MEN 2) which includes MEN 2A, MEN 2B and familial medullary thyroid carcinoma (FMTC), which differ in the clinical manifestation [10–12]. FMTC is characterized only by MTC (medullary thyroid carcinoma, a tumor arising from the C cells), whereas MEN 2A also involves pheochromocytoma and hyperplasia of the parathyroid. MEN 2B patients have a more complex phenotype including MTC, pheochromocytoma, ganglioneuromatosis of the gastroenteric mucosa and marfanoid habitus [13]. The constitutively activated RET tyrosine kinase has been suggested as a suitable target for therapeutic intervention for distinct thyroid neoplastic diseases [14]. Along these lines, both established kinase inhibitors and novel small molecular entities have been found to block RET kinase activity, e.g. PP1 [15], PP2 [16], vandetanib (**14**) [17], sorafenib [18], semaxanib (SU5416) [19], RPI-1 [14], imatinib [20], the indolocarbazole CEP-751 [21], and beta-carbolin-1-ones [22]. Several of these compounds constitute potential agents in treatment of thyroid carcinomas. It has been pointed out that, similar to other cancer

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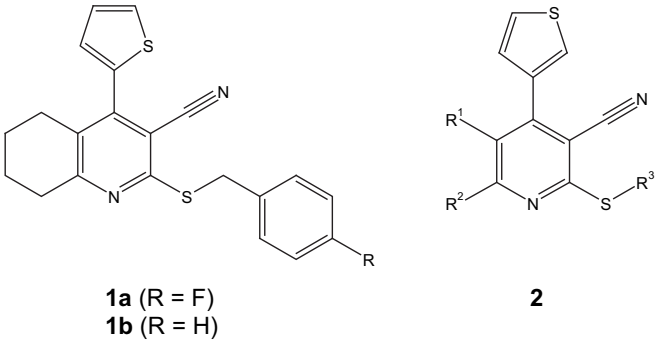


Fig. 1. Hit structures **1a** and **1b** and core structure **2** present in the test compounds **7** and **13**. Compounds **1a** and **1b** were identified in a screening campaign for RET kinase inhibitors ($IC_{50} = 4.8$ μM). In the basic structure **2** present in derivatives **7** and **13** the thiophene substituent is rotated counter clockwise by one position.

therapies based on protein kinase inhibitors, clinical resistance caused by mutations of the targeted oncogene is conceivable also for RET inhibitors. For this reason, a broad array of structurally different inhibitors would be desirable to circumvent such resistances [23]. Consequently, there is still an urgent need for additional RET inhibitors with proven antiproliferative activity. In this study we present the development of a novel RET inhibitor chemotype based on a nicotinonitrile scaffold. The investigations were started with a screening campaign using a collection of commercially available compounds, which identified the substituted 4,5,6,7-tetrahydroquinoline-3-carbonitrile **1a** as a moderate in vitro inhibitor of RET, exhibiting an IC_{50} of 4.8 μM. Unfortunately, **1a** failed to inhibit the growth of RET-dependent TPC1 thyroid carcinoma cells. Analogue **1b** showed a slightly increased RET inhibition, but was also not antiproliferative for cultivated cancer cell lines in vitro (refer to Table 3). In the context of our interest in thiophene derivatives as antiproliferative compounds, we were interested in optimizing the kinase inhibitory and antiproliferative activity within this compound class and in extending the study towards entities of higher structural novelty. In this regard, we synthesized and evaluated derivatives of the general structure **2** in which the thienyl substituent is rotated by one position related to **1**. Examples of published compounds with the 2-(alkylsulfanyl)-4-(3-thienyl) nicotinonitrile partial structure present in **2** are rare in the literature [24,25] and have not been mentioned as kinase inhibitors before (Fig. 1).

2. Chemistry

The syntheses of the 2-(alkylsulfanyl)-4-(3-thienyl)-5,6,7,8-tetrahydroquinoline-3-carbonitriles **7a**–**e** and the 6-methyl-6-aza-analogue **7f** are depicted in Scheme 1. Reaction of the Knoevenagel product **3** with either cyclohexanone (**4a**) or *N*-methylpiperidone (**4b**) in the presence of piperidine yielded thiolactams **5** or **6**, respectively. Without further purification, intermediate **5** or **6** was then *S*-alkylated by reaction with an appropriate alkyl halide in DMF. For the latter reaction, it is important to restrict the amount of the base potassium hydroxide to one equivalent in order to avoid a subsequent Ziegler–Thorpe ring closure reaction [26], which might lead to thieno[2,3-*b*]pyridine derivates **8** as undesired byproducts depicted in Scheme 2.

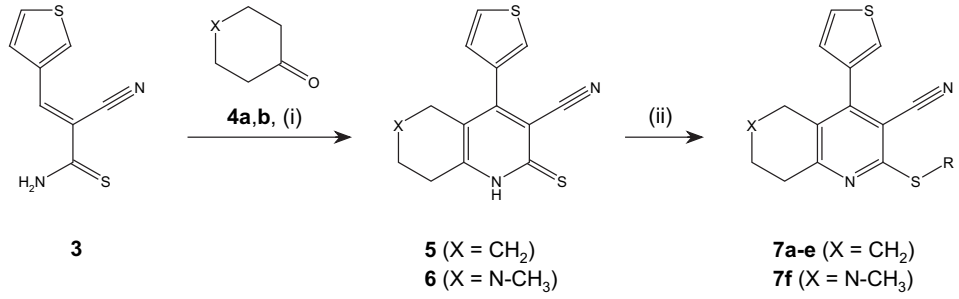
Scheme 3 illustrates the preparation of the more polar derivatives **13a**–**l** of the general structure **2**. For the synthesis of the intermediate thiolactam **12** [27], the three components thiophene-3-carbaldehyde (**9**), malononitrile (**10**), and cyanothioacetamide (**11**) were refluxed in ethanol in the presence of piperidine as basic catalyst. Without further purification, the resulting **12** was alkylated at the sulphur atom by treatment with an appropriate alkyl halide in DMF in the presence of 1 equiv potassium hydroxide. The 6-(alkylsulfanyl)-2-amino-4-(3-thienyl)pyridine-3,5-dicarbonitriles **13a**–**l** produced by this method are listed in Table 2.

3. Biological evaluation and discussion

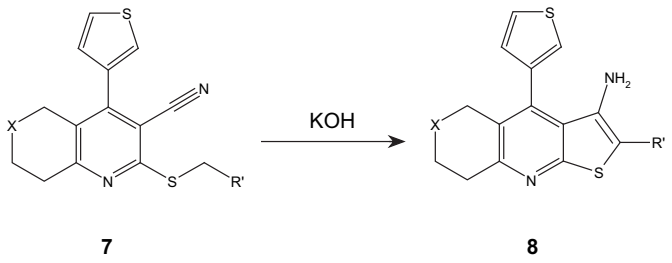
3.1. Results of in vitro RET kinase assays

All synthesized derivatives **7a**–**f** and **13a**–**l** were evaluated for inhibitory activity of recombinant RET kinase by an ELISA assay described recently [28]. Derivatives **7a** and **7b**, which are structurally closest related to the hit structures **1a** and **1b**, showed a decreased RET inhibitory activity. Interestingly, formal insertion of a carbonyl group in the side chain attached to the exocyclic sulphur as realized in compound **7c** improved the activity. However, this structure modification turned out to be rather sensitive against enlargement of the side chain as demonstrated by both derivatives **7d** and **7e**, which are clearly inferior to **7c**. The insertion of an ionisable nitrogen into the parent ring system, which was undertaken to improve the water solubility within the series, led to a definite loss of RET inhibitory activity (compound **7f**, $IC_{50} = 27$ μM, refer to Table 1 for kinase inhibition results).

In compounds **13a**–**l**, the hydrophobic cyclohexene fusion as present in series **7** is exchanged for a pair of polar substituents, namely, a cyano and an amino group. Testing of these compounds showed that similar to our findings with compounds **7a** and **7b** substituted or unsubstituted *S*-benzyl side chains (**13a**–**13e**) were less active than the hit structures **1a** and **1b**. Again, insertion of a carbonyl group improved the RET kinase inhibitory activity (**13f**). In contrast to **7d**, compounds **13f**–**l** tolerate substituents at the *para* position of the phenacyl residue to a certain extent (**13h**, **13i** and **13k**). The *p*-chloro derivative **13g** showed the highest RET



Scheme 1. Synthesis of 2-(alkylsulfanyl)-4-(3-thienyl)-5,6,7,8-tetrahydroquinoline-3-carbonitriles **7a**–**e** and analogue **7f**. Reagents and conditions: (i) dioxane, piperidine, air, 80 °C, 5 h (→**5**) or EtOH, piperidine, air, reflux, 1 h (→**6**); (ii) appropriate alkyl halide, 1 equiv. KOH, DMF, room temperature, 30 min. For substituents R, refer to Table 1.



Scheme 2. Possible Ziegler–Thorpe cyclization in the presence of excess KOH as a side reaction in the synthesis of target compounds **7**.

inhibitory potency reported in this study. Of note, a *para*-cyano group (**13j**) or a meta-chloro substituent (**13l**) are detrimental for kinase inhibition, suggesting that also in this series the substitution pattern at the phenyl ring is important for biological activity (Table 2).

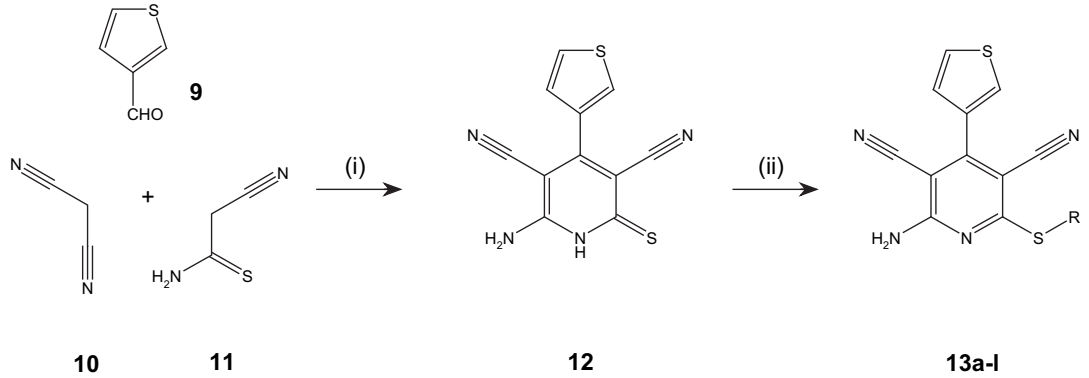
3.2. Kinase selectivity and antiproliferative activity

To obtain a preliminary impression of the kinase selectivity profile within this new class of inhibitors, the three derivatives with highest inhibitory potency (**7c**, **13f** and **13g**) were tested in an analogous ELISA assay for inhibition of ALK (anaplastic lymphoma kinase) and ABL (Abelson leukemia kinase) (Table 3). In contrast to **1b**, the new derivatives **7c**, **13f** and **13g** affected RET with highest potency. However, a modest selectivity for the inhibition of RET compared to ALK was observed. In contrast, a selectivity index of one to two orders of magnitude was found for the RET/ABL comparison.

To check whether **7c**, **13f** and **13g** are able to penetrate cell membranes the properties of the three compounds in a cellular assay were studied (Table 3). When incubated with TPC1, which is a human papillary thyroid carcinoma cell line growth-dependent on the constitutively activated RET/PTC1 fusion kinase, both **7c** and **13g** exhibited clear antiproliferative activity with IC_{50} values in the low two-digit micromolar concentration range. Interestingly, **13g** was even more active against the RET-negative carcinoma cell lines LS174 T (colon) and ARO (thyroid). As a speculative explanation for this observation, it can be assumed that other cancer-related kinases besides RET, which are important for the growth of LS174 T and ARO, are inhibited by **13g** as well. Compound **7c** showed the expected selectivity in the cancer cell line assays, since it inhibited the thyroid carcinoma cell line in lower concentrations compared to the two RET-independent carcinoma cell lines. The *p*-unsubstituted analogue **13f** was clearly inferior to the *p*-chloro derivative **13g** regarding its antiproliferative activity for cancer cell lines. Of note, the fully optimized RET inhibitor vandetanib (**14**) exhibited a more than 100-fold higher antiproliferative potency when compared with **13g** in the proliferation assay with TPC1 cells.

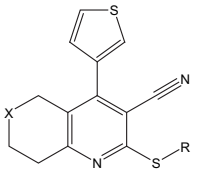
3.3. Docking experiments

For a rationalization of the observed structure–activity relationships in series **7** and **13,** docking studies were carried out applying the docking program FlexX. As template, the X-ray structure of a complex of RET with the inhibitor vandetanib (**14**) (pdb-file 2ivu [29]) was used. Docking of **7c** and **13g** as representatives of highest inhibitory activity from each series produced poses in which the inhibitors occupy a comparable area in the ATP binding site of the RET kinase (Fig. 2). In the vandetanib/RET X-ray structure [29], the quinazoline core element of the inhibitor is located in the center of the pocket where it is kept in position by a hydrogen bond to Ala807 of the hinge element. Furthermore, vandetanib is connected via two water molecules to Ser891 at the bottom of the pocket by hydrogen bonds. While the *N*-methylpiperidine ring protrudes out of the ATP binding pocket towards the solvent, the 4-bromo-2-fluorophenyl substituent is buried in a hydrophobic pocket bordered by Glu775, Leu779, Ile788, Leu802 and Val804 (Fig. 2B). The depicted docking solution of **7c** shows its tetrahydroquinoline ring in the plane occupied by the quinazoline ring of vandetanib (Fig. 2C). Compound **7c** addresses the same amino acids as vandetanib: The nitrile group of **7c** is hydrogen bonded to Ala807, and a direct hydrogen bond is connecting the carbonyl group of **7c** and the hydroxyl function of Ser891. Compared to vandetanib, the phenyl substituent is located deeper in the hydrophobic pocket which explains the observed loss of activity upon enlargement of this moiety (e.g. **7d** and **7e**). Similar to the *N*-methylpiperidine ring of vandetanib, the thiophene ring of **7c** is directed out of the ATP binding pocket in the depicted docking solution, suggesting that further molecular modifications should be possible in this part of the molecule without loss of activity [30]. The depicted docking pose of **13g** resembles the orientation of **7c** on first sight (Fig. 2D). However, **13g** is not located as deeply in the binding pocket as **7c**, and **13g** uses its carbonyl oxygen to address Ala807 by a hydrogen bond. The depicted docking solution illustrates why in this series certain phenyl *para* substituents are tolerated (refer to **13g**, **13h**, **13i** and **13k**) and why the ketone moiety is important for the kinase inhibitory activity (compare with **13a**–**e**). On the other hand, the poses fail to explain why

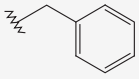
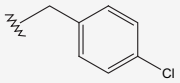
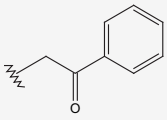
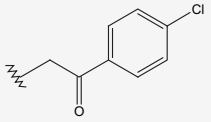
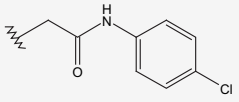
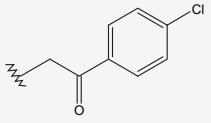


Scheme 3. Synthesis of 6-(alkylsulfanyl)-2-amino-4-(3-thienyl)pyridine-3,5-dicarbonitriles **13a**–**l**. Reagents and conditions: (i) EtOH, piperidine, air, reflux, 3 h; (ii) appropriate alkyl halide, 1 equiv. KOH, DMF, room temperature, 30 min. For substituents R, refer to Table 2.

Table 1
Structures and in vitro inhibition of RET kinase by 2-(alkylsulfanyl)-4-(3-thienyl)-5,6,7,8-tetrahydroquinoline-3-carbonitriles **7a**–**e** and by the 6-methyl-6-aza-analogue **7f**.



7a-e (X = CH_2)
7f (X = N-CH_3)

	R	IC_{50} RET (μM)[a]
7a		8.0 ± 1.1
7b		10 ± 2
7c		2.3 ± 0.2
7d		8.6 ± 0.5
7e		14 ± 4
7f		27 ± 5

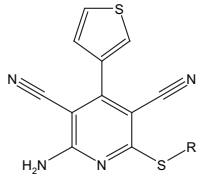
[a] Results are mean values of at least three experiments ± SEM.

compounds **13** are superior to analogues of series **7**. Presumably, water molecules which were not considered in the docking routine are involved in the binding of inhibitors **13**, adding favourable contacts.

4. Conclusion

The molecular modification of the screening hits **1a** and **1b** identified two series **7** and **13** as inhibitors of the cancer-related tyrosine kinase RET. Both novel inhibitor series are structurally based on a nicotinonitrile partial structure. The most potent representatives of the series, namely, **7c** and **13g**, displayed a modest degree of selectivity against ALK and pronounced selectivity against ABL. Both compounds inhibited the growth of TPC1 cancer cells which are dependent on activated RET kinase. Docking experiments suggested a binding mode comparable to the structurally unrelated established RET inhibitor vandetanib. The docking solutions suggest meaningful molecular modifications for future studies directed to improve RET inhibitors belonging to the series.

Table 2
Structures and in vitro inhibition of RET kinase by 6-(alkylsulfanyl)-2-amino-4-(3-thienyl)pyridine-3,5-dicarbonitriles **13a**–**l**.



13

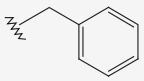
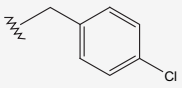
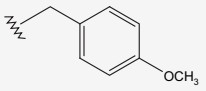
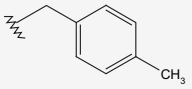
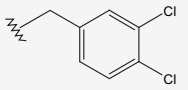
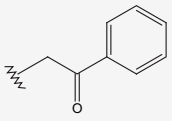
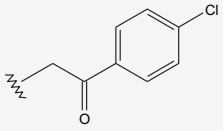
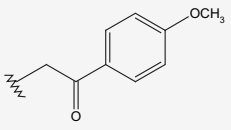
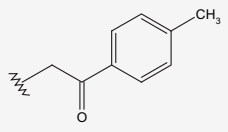
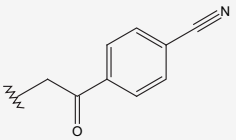
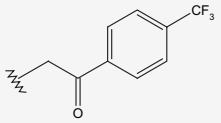
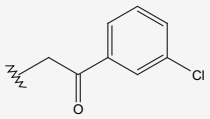
	R	IC_{50} RET (μM)[a]
13a		19 ± 2
13b		8.3 ± 2.1
13c		13 ± 1
13d		11 ± 2
13e		9.2 ± 1.9
13f		3.4 ± 0.2
13g		0.69 ± 0.01
13h		3.6 ± 0.4
13i		5 ± 0.1
13j		39 ± 10

Table 2 (*continued*)

	R	IC_{50} RET (μM)[a]
13k		3.6 ± 0.6
13l		26 ± 6

[a] Results are mean values of at least three experiments ± SEM.

5. Experimental protocols

5.1. Chemistry

5.1.1. General

Melting points (mp) were determined on an electric variable heater (Electrothermal IA 9100). Infrared spectra were recorded using KBr pellets on a Thermo Nicolet FT-IR 200 spectrometer. Nuclear magnetic resonance spectra were recorded on a Bruker Avance DRX-400 or a Bruker Avance II-600 (Bruker, Billerica, MA, spectra recorded at NMR laboratories of the Chemical Institutes of the Technische Universität Braunschweig) using DMSO-d_6 as solvent and tetramethylsilane as internal standard. NMR signals are reported in ppm on a δ scale. C, H, N analyses were performed with a CE Instruments FlashEA® 1112 Elemental Analyzer (Thermo Quest, San Jose, CA). Analyses indicated by the symbols of elements were within ±0.4% of the theoretical values. Mass spectra were recorded on a Finnigan-MAT 95 instrument (Finnigan-MAT GmbH, Bremen, Germany, performed at department of mass spectrometry of the Chemical Institutes of the Technische Universität Braunschweig). The HPLC purity analyses were carried out using a Merck Hitachi LaChrom Elite system (pump: L-2130, DAD detector: L-2450; autosampler: L-2200; column: Merck LiChroCART 125-4, LiChrospher 100 RP-18 (5 μm); eluent:acetonitrile/water mixtures, or acetonitrile/buffer mixtures (preparation of the buffer: triethylamine (20 mL) and sodium hydroxide (242 mg) are dissolved in distilled water (1 L). The solution is adjusted to pH 2.5 by the addition of concentrated sulphuric acid); elution rate 1.000 mL/min; detection wavelength: 254 and 280 nm; overall run time: 15 min); t_M = hold-up time, t_N = net retention time.

Starting materials **4a**, **4b**, **9** and **11** were purchased from Acros Organics (Geel, Belgium); **10** was purchased from Sigma-Aldrich (St. Louis, MO). With the exception of **9**, which was purified by washing with saturated sodium carbonate solution, all starting materials were used as supplied.

5.1.2. 2-Cyano-3-(3-thienyl)-2-propenthioamide (*3*)

Cyanothioacetamide (11) (1.00 mmol, 100 mg) was dissolved in EtOH (2 mL). Thiophene-3-carbaldehyde (**9**, 87.7 μL, 1.00 mmol) and triethylamine (one drop) were added successively and the mixture was stirred for 30 min at 40–50 °C. After cooling to room temperature, the precipitate was collected and crystallized from EtOH (96%) to yield yellow crystals (59%); mp 151–155 °C (151 °C [31]); IR (KBr): 3361, 3263 and 3139 cm^{-1} (NH), 2207 cm^{-1} (C≡N); ^{1}H NMR (DMSO-d_6, 400 MHz): δ (ppm) = 7.80 (d, 2H, J = 2.0 Hz, thiophene-H), 8.15 (s, 1H, CH), 8.40 (t, 1H, J = 2.0 Hz, thiophene-H), 9.51 (s, 1H, NH), 10.03 (s, 1H, NH); ^{13}C NMR (DMSO-d_6, 100.6 MHz): δ (ppm) = 131.8, 134.1, 141.0, 146.3 (tert. C); 115.3, 122.0, 139.5, 197.5 (quat. C); Anal ($C_8H_6N_2S_2$) C, H, N.

5.1.3. 4-(3-Thienyl)-2-thioxo-1,2,5,6,7,8-hexahydroquinoline-3-carbonitrile (*5*)

To a mixture of cyclohexanone (**4a**) (104 μL, 1.00 mmol), 1,4-dioxane (2 mL) and piperidine (1 drop) **3** (194 mg, 1.00 mmol) was added in small portions. Before the addition of a new portion of **3**, completeness of the reaction was ensured by tlc monitoring. The reaction required 5 h overall. After cooling to room temperature, the precipitate was collected to yield a yellow powder (46%) which was used for the subsequent syntheses without further purification. mp 258–259 °C; ^{1}H NMR (DMSO-d_6, 400 MHz): δ (ppm) = 1.56–1.73 (m, 4H, 2× CH_2), 2.20 (t, 2H, J = 6.3 Hz, CH_2), 2.77 (t, 2H, J = 6.3 Hz, CH_2), 7.21 (dd, 1H, J = 5.1/1.3 Hz, thiophene-H), 7.74 (dd, 1H, J = 5.1/3.0 Hz, thiophene-H), 7.80 (dd, 1H, J = 3.0/1.3 Hz, thiophene-H), 13.91 (br s, 1H, NH).

5.1.4. 6-Methyl-4-(3-thienyl)-2-thioxo-1,2,5,6,7,8-hexahydro[1,6]naphthyridine-3-carbonitrile (*6*)

A mixture of **3** (194 mg, 1.00 mmol), 1-methyl-4-piperidone (**4b**, 116 μL, 1.00 mmol), EtOH (3 mL) and piperidine (1 drop) was refluxed for 1 h. After cooling to room temperature, the precipitate was collected and washed with petrol ether to yield a yellow powder (18%) which was used for the synthesis of **8** without further purification; mp 235–247 °C (dec.); IR (KBr): 3429 cm^{-1} (NH), 2222 cm^{-1} (C≡N); ^{1}H NMR (DMSO-d_6, 600 MHz): δ (ppm) = 2.24 (s, 3H, N-CH_3), 2.60 (t, 2H, J = 5.7 Hz, CH_2), 2.85 (t, 2H, J = 5.8 Hz, CH_2), 3.01 (s, 2H, CH_2), 7.24 (dd, 1H, J = 4.9/1.3 Hz, thiophene-H), 7.77 (dd, 1H, J = 4.9/3.0 Hz, thiophene-H), 7.86 (dd, 1H, J = 2.8/1.3 Hz, thiophene-H), 14.06 (br s, 1H, NH); ^{13}C NMR (DMSO-d_6, 150.9 MHz): δ (ppm) = 27.4 (CH_2), 44.9 (N-CH_3), 49.5, 53.4 (2× CH_2); 127.0, 127.4, 127.4 (tert. C); 113.6, 116.4, 118.7, 133.3, 150.0, 151.1, 175.8 (quat. C).

Table 3
In vitro kinase inhibition and antiproliferative activity of compounds **1a**, **1b**, **7c**, **13f**, **13g** and the standard vandetanib (**14**).

Entry	IC_{50} RET (μM)[a]	IC_{50} ALK (μM)[a]	IC_{50} ABL (μM)[a]	IC_{50} TPC1[b] (μM)[a]	IC_{50} LS174 T[c] (μM)[a]	IC_{50} ARO[d] (μM)[a]
1a	4.8 ± 0.7	35 ± 4	>150	>300	>300	9.0 ± 3.3
1b	1.6 ± 0.4	1.7 ± 0.3	0.29 ± 0.02	>300	>300	>300
7c	2.3 ± 0.2	9.7 ± 1.4	60 ± 2	28 ± 6	92 ± 23	159 ± 42
13f	3.4 ± 0.2	4.0 ± 0.4	74 ± 22	101 ± 48	>300	>300
13g	0.69 ± 0.01	2.1 ± 0.7	72 ± 8	17 ± 1	6.2 ± 1.2	8.5 ± 2.0
14	0.097 ± 0.006	NT[f]	NT[f]	0.116 ± 0.037	NT[f]	NT[f]

[a] Results are mean values of at least three experiments ± SEM.
[b] Human papillary thyroid carcinoma cell line expressing the RET/PCT1 fusion kinase.
[c] RET-negative colon carcinoma cells.
[d] RET-negative thyroid carcinoma cells.
[f] NT: not tested.

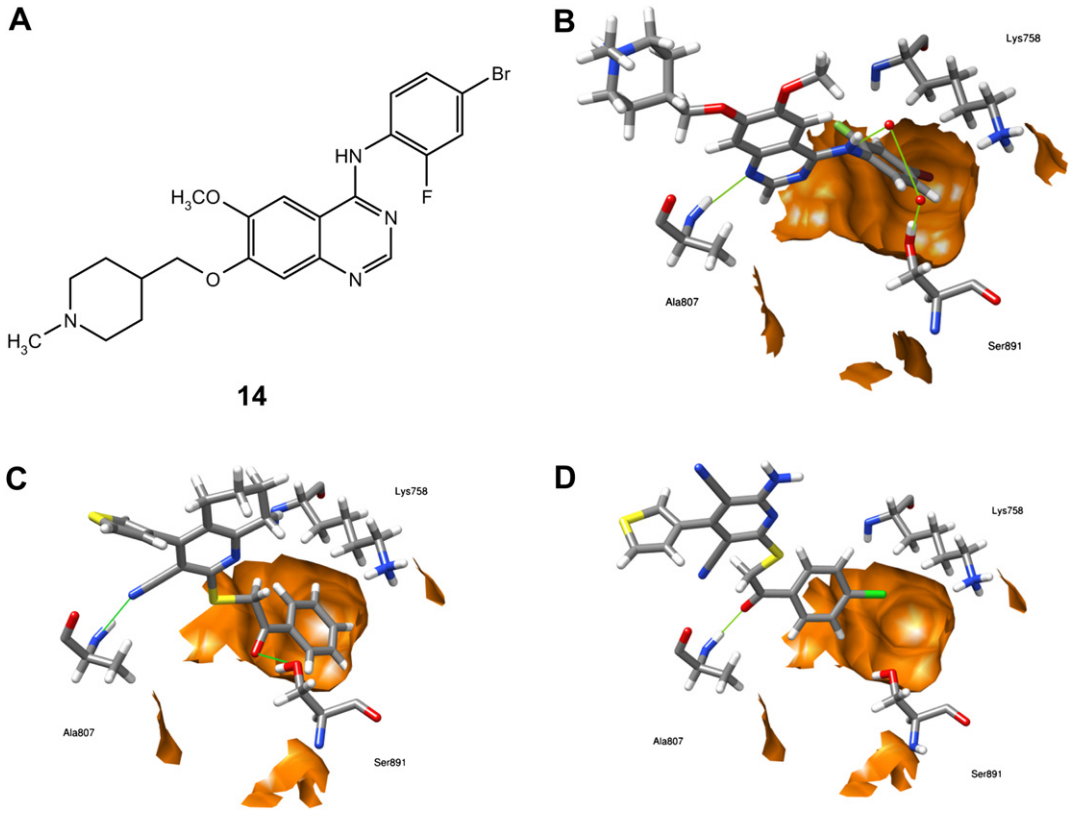


Fig. 2. Inhibitors in the ATP binding pocket of the RET protein kinase. *Legend:* Upper row: structure of vandetanib **14** (A); vandetanib in the ATP binding site (pdb-file 2ivu [29]) (B); Lower row: compound **7c** (C) and **13g** (D) docked to the ATP binding site of RET. A hydrophobic pocket lined by the amino acids Glu775, Leu779, Ile788, Leu802 and Val804 is depicted in orange colour. Water molecules are depicted as red balls and hydrogen bonds are depicted green.

*5.1.5. 6-Amino-4-(3-thienyl)-2-thioxo-1,2-dihydropyridine-3, 5-dicarbonitrile (**12**)*

A mixture of thiophene-3-carbaldehyde (438 μL, 5.00 mmol), malonodinitrile (330 mg, 5.00 mmol), 2-cyanothioacetamide (501 mg, 5.00 mmol), piperidine (50 μL), and EtOH (4 mL) was refluxed for 3 h. Subsequently, the solvent was evaporated. Acetic acid (six drops), water (10 mL) and CH_2Cl_2 (2 mL) were added. After shaking the mixture for 2 min at room temperature, it was left standing overnight at 2–5 °C. The precipitate was collected to yield a yellow powder (47%) which was used for the subsequent syntheses without further purification. Mp 234–238 °C; IR (KBr): 3477, 3384 and 3324 cm^{-1} (NH), 2214 cm^{-1}, 2201 cm^{-1} (C≡N); ^1H NMR (DMSO-d_6, 400 MHz): δ (ppm) = ^1H NMR (DMSO-d_6, 600 MHz): δ (ppm) = 7.34 (dd, 1H, J = 5.1/1.3 Hz, thiophene-H), 7.45–7.93 (br s, 2H, NH_2), 7.74 (dd, 1H, J = 5.1/2.8 Hz, thiophene-H, superimposed by br s at 7.45–7.93), 8.00 (dd, 1H, J = 2.8/1.3 Hz, thiophene-H), 12.87 (br s, 1H, NH).

*5.1.6. General procedure for the synthesis of **7a–f 8**, and **13a–l***

Compound **5**, **6**, or **12** (0.400 mmol), respectively, is dissolved in DMF (0.5 mL). Ten percent aqueous potassium hydroxide solution (224 μL) is added. After stirring the mixture for 1 min, the appropriate alkyl halide is added and stirring is continued for 0.5 h. Water (5 mL) is added. The precipitate is filtered off and washed successively with water and petrol ether.

*5.1.6.1. 2-(Benzylsulfanyl)-4-(3-thienyl)-5,6,7,8-tetrahydroquinoline-3-carbonitrile (**7a**).* Prepared from **5** and benzylbromide. Grey–-green crystals (48%); mp 132–133 °C (EtOH); IR (KBr): 2218 cm^{-1} (C≡N); ^1H NMR (DMSO-d_6, 400 MHz): δ (ppm) = 1.63–1.69 (m, 2H, CH_2), 1.79–1.85 (m, 2H, CH_2), 2.45 (t, 2H, J = 6.3 Hz, CH_2), 2.98 (t, 2H, J = 6.3 Hz, CH_2), 4.51 (s, 2H, CH_2), 7.22 (dd, 1H, J = 4.8/1.3 Hz, thiophene-H), 7.23–7.26 (m, 1H, ArH), 7.29–7.33 (m, 2H, ArH), 7.43–7.47 (m, 2H, ArH), 7.74 (dd, 1H, J = 4.8/2.8 Hz, thiophene-H), 7.77 (dd, 1H, J = 2.8/1.3 Hz, thiophene-H); ^{13}C NMR (DMSO-d_6, 100.6 MHz): δ (ppm) = 21.6, 21.8, 26.1, 32.8, 33.2 (sec. C); 126.4, 127.0 (2 C), 127.8, 128.3 (2 C), 129.1 (2 C) (tert. C); 103.9, 115.2, 127.1, 134.4, 137.8, 149.2, 157.2, 161.1 (quat. C); Anal. ($C_{21}H_{18}N_2S_2$) C, H, N; HPLC: 98.6% at 254 nm and 97.9% at 280 nm, t_N = 3.83 min, t_M = 1.08 min (ACN/H_2O; 80:20).

*5.1.6.2. 2-[(4-Chlorobenzyl)sulfanyl]-4-(3-thienyl)-5,6,7,8-tetrahydroquinoline-3-carbonitrile (**7b**).* Prepared from **5** and 4-chlorobenzylbromide. Light yellow powder (16%); mp 108–109 °C (EtOH); IR (KBr): 2218 cm^{-1} (C≡N); ^1H NMR (DMSO-d_6, 600 MHz): δ (ppm) = 1.64–1.68 (m, 2H, CH_2), 1.80–1.84 (m, 2H, CH_2), 2.45 (t, 2H, J = 6.4 Hz, CH_2), 2.97 (t, 2H, J = 6.4 Hz, CH_2), 4.49 (s, 2H, CH_2), 7.22–7.23 (m, 1H, ArH), 7.36–7.39 (m, 2H, ArH), 7.48–7.50 (m, 2H, ArH), 7.74–7.75 (m, 1H, ArH), 7.77–7.79 (m, 1H, ArH); ^{13}C NMR (DMSO-d_6, 150.9 MHz): δ (ppm) = 21.6, 21.8, 26.1, 32.3, 32.8 (sec. C); 126.6, 127.1, 127.8, 128.2 (2C), 131.0 (2C) (tert. C); 103.9, 115.3, 127.1, 131.6, 134.3, 137.2, 149.2, 156.9, 161.2 (quat. C); Anal. ($C_{21}H_{17}ClN_2S_2$) C, H, N; HPLC: 99.3% at 254 nm and 98.6% at 280 nm, t_N = 5.17 min, t_M = 1.06 min (ACN/H_2O; 80:20).

*5.1.6.3. 2-[(2-Oxo-2-phenylethyl)sulfanyl]-4-(3-thienyl)-5,6,7,8-tetrahydroquinoline-3-carbonitrile (**7c**).* Prepared from **5** and 2-bromo-1-phenylethanone. Grey–green powder (88%); mp 123–124 °C; IR (KBr): 2218 cm^{-1} (C≡N), 1685 cm^{-1} (C=O); ^1H NMR ($CDCl_3$, 400 MHz): δ (ppm) = 1.61–1.68 (m, 2H, CH_2), 1.71–1.78 (m, 2H, CH_2), 2.45 (t, 2H, J = 6.3 Hz, CH_2), 2.64 (t, 2H, J = 6.6 Hz, CH_2), 4.64 (s, 2H, CH_2), 7.06 (dd, 1H, J = 5.1/1.3 Hz, thiophene-H), 7.36 (dd, 1H, J = 3.0/1.3 Hz, thiophene-H), 7.45 (dd, 1H, J = 5.1/3.0 Hz, thiophene-H), 7.49–7.54 (m, 2H, ArH), 7.60–7.64 (m,

1H, ArH), 8.07–8.10 (m, 2H, ArH); ^{13}C NMR (DMSO-d_6, 150.9 MHz): δ (ppm) = 20.7, 20.9, 25.3, 31.7, 36.2 (sec. C); 125.8, 126.4, 127.1, 127.4 (2C), 127.9 (2C), 132.6 (tert. C); 102.9, 114.7, 126.3, 133.6, 135.7, 148.3, 156.0, 160.2, 192.9 (quat. C); Anal. ($C_{22}H_{18}N_2OS_2$) C, H, N; HPLC: 99.1% at 254 nm and 98.4% at 280 nm, t_N = 4.71 min, t_M = 1.04 min (ACN/H_2O; 70:30).

*5.1.6.4. 2-{[2-(4-Chlorophenyl)-2-oxoethyl]sulfanyl}-4-(3-thienyl)-5,6,7,8-tetrahydroquinoline-3-carbonitrile (**7d**).* Prepared from **5** and 2-bromo-1-(4-chlorphenyl)ethanone. Light yellow powder (66%); mp 179–180 °C (EtOH); IR (KBr): 2216 cm^{-1} (C≡N), 1702 cm^{-1} (C = O); ^{1}H NMR (DMSO-d_6, 400 MHz): δ (ppm) = 1.55-1.61 (m, 2H, CH_2), 1.65–1.70 (m, 2H, CH_2), 2.41 (t, 2H, J = 6.3 Hz, CH_2), 4.80 (s, 2H, CH_2), 7.23 (dd, 1H, J = 5.1/1.3 Hz, thiophene-H), 7.66 (m, 2H, ArH), 7.75 (dd, 1H, J = 5.1/2.8 Hz, thiophene-H), 7.80 (dd, 1H, J = 2.8/1.3 Hz, thiophene-H), 8.11 (m, 2H, ArH), 2 H (5,6,7,8-tetrahydroquinoline) are overlapped by DMSO-Peak; ^{13}C NMR (DMSO-d_6, 150.9 MHz): δ (ppm) = 21.4, 21.7, 26.0, 32.4, 36.8 (sec. C); 126.6, 127.2, 127.8, 128.8 (2C), 130.1 (2C) (tert. C); 103.6, 115.4, 127.0, 134.3, 135.1, 138.2, 149.1, 156.6, 160.9, 192.9 (quat. C); Anal. ($C_{22}H_{17}ClN_2OS_2$) C, H, N; HPLC: 99.3% at 254 nm and 98.7% at 280 nm, t_N = 3.51 min, t_M = 1.07 min (ACN/H_2O; 75:25).

*5.1.6.5. N-(4-Chlorophenyl)-2-{[3-cyano-4-(3-thienyl)-5,6,7,8-tetrahydroquinolin-2-yl]-sulfanyl}acetamide (**7e**).* Prepared from **5** and 2-chloro-*N*-(4-chlorphenyl)acetamide. Yellow needles (61%); mp 204–205 °C (EtOH); IR (KBr): 3284 and 3251 cm^{-1} (NH), 2215 cm^{-1} (C≡N), 1673 cm^{-1} (C = O); ^{1}H NMR (DMSO-d_6, 600 MHz): δ (ppm) = 1.60–1.64 (m, 2H, CH_2), 1.72–1.76 (m, 2H, CH_2), 2.44 (t, 2H, J = 6.4 Hz, CH_2), 2.80 (t, 2H, J = 6.4 Hz, CH_2), 4.16 (s, 2H, CH_2), 7.25 (dd, 1 H, J = 5.1/1.3 Hz, thiophene-H), 7.38–7.40 (m, 2H, ArH), 7.62-7.64 (m, 2H, ArH), 7.77 (dd, 1H, J = 5.1/2.8 Hz, thiophene-H), 7.81 (dd, 1H, J = 2.8/1.3 Hz, thiophene-H), 10.49 (s, 1H, NH); ^{13}C NMR (DMSO-d_6, 150.9 MHz): δ (ppm) = 21.5, 21.7, 26.0, 32.8, 34.8 (sec. C); 120.4 (2C), 126.6, 127.1, 127.8, 128.6 (2C) (tert. C); 103.6, 115.3, 126.7, 127.0, 134.3, 137.9, 149.0, 157.1, 161.0, 166.2 (quat. C); Anal. ($C_{22}H_{18}ClN_3OS_2$) C, H, N; HPLC: 98.9% at 254 nm and 98.5% at 280 nm, t_N = 3.71 min, t_M = 1.03 min (ACN/H_2O; 70:30).

*5.1.6.6. 2-{[2-(4-Chlorophenyl)-2-oxoethyl]sulfanyl}-6-methyl-4-(3-thienyl)-5,6,7,8-tetrahydro[1,6]naphthyridine-3-carbonitrile (**7f**).* Prepared from **6** and 2-bromo-1-(4-chlorophenyl)ethanone. Orange powder (60%); mp 194–195 °C (EtOH); IR (KBr): 2212 cm^{-1} (C≡N), 1671 cm^{-1} (C = O); ^{1}H NMR (DMSO-d_6, 600 MHz): δ (ppm) = 2.25 (s, 3H, CH_3), 2.60 (s, 4H, 2× CH_2), 3.23 (s, 2H, CH_2), 4.83 (s, 2H, CH_2), 7.28 (dd, 1H, J = 4.9/1.3 Hz, thiophene-H), 7.65–7.67 (m, 2H, ArH), 7.79 (dd, 1H, J = 4.9/2.8 Hz, thiophene-H), 7.88 (dd, 1H, J = 2.8/1.3 Hz, thiophene-H), 8.10–8.12 (m, 2H, ArH); ^{13}C NMR (DMSO-d_6, 150.9 MHz): δ (ppm) = 32.1, 36.9, 50.9, 54.2 (sec. C); 45.1 (CH_3); 127.0, 127.5, 127.7, 128.8 (2C), 130.1 (2C) (tert. C); 103.5, 115.2, 124.7, 133.2, 135.0, 138.2, 147.2, 157.8, 158.4, 192.8 (quat. C); Anal. ($C_{22}H_{18}ClN_3OS_2$) C, H, N; HPLC: 99.4% at 254 nm and 99.4% at 280 nm, t_N = 2.06 min, t_M = 1.03 min (ACN/buffer; 40:60).

*5.1.6.7. 2-Amino-6-(benzylsulfanyl)-4-(3-thienyl)pyridine-3,5-dicarbonitrile (**13a**).* Prepared from **12** and benzylbromide. Light beige powder (40%); mp 207–208 °C (EtOH); IR (KBr): 3440, 3326 and 3216 cm^{-1} (NH), 2219 cm^{-1}, 2207 cm^{-1} (C≡N); ^{1}H NMR (DMSO-d_6, 400 MHz): δ (ppm) = 4.50 (s, 2H, CH_2), 7.23–7.28 (m, 1H, ArH), 7.30–7.34 (m, 2H, ArH), 7.37 (dd, 1H, J = 5.1/1.5 Hz, thiophene-H), 7.49–7.52 (m, 2H, ArH), 7.76 (dd, 1H, J = 5.1/2.8 Hz, thiophene-H), 8.03 (dd, 1H, J = 2.8/1.5 Hz, thiophene-H), 8.10 (br s, 2H, NH_2, superimposed by dd at 8.03 ppm); ^{13}C NMR (DMSO-d_6, 150.9 MHz): δ (ppm) = 33.0 (CH_2); 127.2, 127.3, 127.6, 128.3 (2C), 128.7, 129.3 (2C) (tert. C); 85.3, 92.7, 115.3, 115.4, 133.3, 137.5, 153.0, 159.6, 166.3 (quat. C); Anal. ($C_{18}H_{12}N_4S_2$) C, H, N; HPLC: 99.6% at 254 nm and 99.4% at 280 nm, t_N = 7.35 min, t_M = 1.03 min (ACN/H_2O; 50:50).

*5.1.6.8. 2-Amino-6-[(4-chlorobenzyl)sulfanyl]-4-(3-thienyl)pyridine-3,5-dicarbonitrile (**13b**).* Prepared from **12** and 4-chlorobenzylbromide. Beige crystals (48%); mp 217–219 °C (EtOH); IR (KBr): 3468, 3352 and 3214 cm^{-1} (NH), 2210 cm^{-1} (C≡N); ^{1}H NMR (DMSO-d_6, 400 MHz): δ (ppm) = 4.48 (s, 2H, CH_2), 7.35–7.38 (m, 3H, ArH, superimposed by thiophene-H), 7.54–7.58 (m, 2H, ArH), 7.76 (dd, 1H, J = 5.1/3.0 Hz, thiophene-H), 8.03 (dd, 1H, J = 3.0/1.3 Hz, thiophene-H), 8.10 (br s, 2H, NH_2, superimposed by dd at 8.03 ppm); ^{13}C NMR (DMSO-d_6, 100.6 MHz): δ (ppm) = 32.3 (CH_2); 127.3, 127.7, 128.2 (2C), 128.7, 131.2 (2C) (tert. C); 85.5, 92.9, 115.3, 115.4, 131.8, 133.4, 136.9, 153.1, 159.6, 166.1 (quat. C); Anal. ($C_{18}H_{11}ClN_4S_2$) C, H, N; HPLC: 99.6% at 254 nm and 99.8% at 280 nm, t_N = 4.01 min, t_M = 1.03 min (ACN/H_2O; 60:40).

*5.1.6.9. 2-Amino-6-[(4-methoxybenzyl)sulfanyl]-4-(3-thienyl)pyridine-3,5-dicarbonitrile (**13c**).* Prepared from **12** and 4-methoxybenzylbromide. Light grey powder (74%); mp 208–209 °C; IR (KBr): 3439 3348 and 3238 cm^{-1} (NH), 2212 cm^{-1} (C≡N); ^{1}H NMR (DMSO-d_6, 400 MHz): δ (ppm) = 3.73 (s, 3H, OCH_3), 4.45 (s, 2H, CH_2), 6.85–6.89 (m, 2H, ArH), 7.37 (dd, 1H, J = 5.1/1.3 Hz, thiophene-H), 7.41–7.45 (m, 2H, ArH), 7.76 (dd, 1H, J = 5.1/3.0 Hz, thiophene-H), 8.03 (dd, 1H, J = 3.0/1.3 Hz, thiophene-H), 8.08 (br s, 2H, NH_2, superimposed by dd at 8.03 ppm); ^{13}C NMR (DMSO-d_6, 100.6 MHz): δ (ppm) = 32.7 (CH_2); 54.9 (OCH_3); 113.7 (2 C), 127.3, 127.6, 128.7, 130.5 (2 C) (tert. C); 85.2, 92.7, 115.4, 115.4, 129.2, 133.3, 152.9, 158.4, 159.6, 166.5 (quat. C); Anal. ($C_{19}H_{14}N_4OS_2$) C, H, N; HPLC: 99.2% at 254 nm and 98.7% at 280 nm, t_N = 2.50 min, t_M = 1.03 min (ACN/H_2O; 60:40).

*5.1.6.10. 2-Amino-6-[(4-methylbenzyl)sulfanyl]-4-(3-thienyl)pyridine-3,5-dicarbonitrile (**13d**).* Prepared from **12** and 4-methylbenzylbromide. Beige needles (46%); mp 204–206 °C (EtOH); IR (KBr): 3459, 3339 and 3227 cm^{-1} (NH), 2218 cm^{-1} and 2208 cm^{-1} (C≡N); ^{1}H NMR (DMSO-d_6, 400 MHz): δ (ppm) = 2.27 (s, 3H, CH_3), 4.45 (s, 2H, CH_2), 7.12 (d, 2H, J = 7.8 Hz, ArH), 7.36 (dd, 1H, J = 5.1/1.3 Hz, thiophene-H), 7.38 (d, 2 H, J = 7.8 Hz, ArH, superimposed by dd at 7.36 ppm), 7.76 (dd, 1H, J = 5.1/3.0 Hz, thiophene-H), 8.03 (dd, 1H, J = 3.0/1.3 Hz, thiophene-H), 8.06 (br s, 2H, NH_2, superimposed by dd at 8.03 ppm); ^{13}C NMR (DMSO-d_6, 100.6 MHz): δ (ppm) = 20.7 (CH_3); 33.0 (CH_2); 127.3, 127.7, 128.7, 128.9 (2 C), 129.2 (2 C) (tert. C); 85.4, 92.9, 115.4, 115.4, 133.4, 134.3, 136.5, 153.0, 159.6, 166.5 (quat. C); Anal. ($C_{19}H_{14}N_4S_2$) C, H, N; HPLC: 98.7% at 254 nm and 99.0% at 280 nm, t_N = 3.77 min, t_M = 1.04 min (ACN/H_2O; 60:40).

*5.1.6.11. 2-Amino-6-[(3,4-dichlorobenzyl)sulfanyl]-4-(3-thienyl)pyridine-3,5-dicarbonitrile (**13e**).* Prepared from **12** and 3,4-dichlorobenzylbromide. Grey powder (52%); mp 215–218 °C (EtOH); IR (KBr): 3469, 3348 and 3223 cm^{-1} (NH), 2214 cm^{-1} (C≡N); ^{1}H NMR (DMSO-d_6, 400 MHz): δ (ppm) = 4.48 (s, 2H, CH_2), 7.37 (dd, 1H, J = 5.1/1.5 Hz, thiophene-H), 7.53–7.58 (m, 2H, ArH), 7.76 (dd, 1H, J = 5.1/3.0 Hz, thiophene-H), 7.86 (d, 1H, J = 1.3 Hz, ArH), 8.03 (dd, 1H, J = 3.0/1.5 Hz, thiophene-H), 8.11 (br s, 2H, NH_2, superimposed by dd at 7.76 ppm, d at 7.86 ppm and dd at 8.03 ppm); ^{13}C NMR (DMSO-d_6, 150.9 MHz): δ (ppm) = 31.6 (CH_2); 127.3, 127.6, 128.7, 129.8, 130.3, 131.3 (tert. C); 85.5, 92.7, 115.3, 115.3, 129.7, 130.6, 133.3, 139.3, 153.0, 159.5, 165.7 (quat. C); Anal. ($C_{18}H_{10}Cl_2N_4S_2$) C, H, N; HPLC: 98.2% at 254 nm and 98.4% at 280 nm, t_N = 5.78 min, t_M = 1.04 min (ACN/H_2O; 60:40).

*5.1.6.12. 2-Amino-6-[(2-oxo-2-phenylethyl)sulfanyl]-4-(3-thienyl)pyridine-3,5-dicarbonitrile (**13f**).* Prepared from **12** and 2-bromo-1-phenylethanone. Light grey powder (65%); mp 265–268 °C (EtOH);

IR (KBr): 3471, 3340 and 3212 cm^{-1} (NH), 2210 cm^{-1} (C≡N), 1676 cm^{-1} (C=O); ^1H NMR (DMSO-d_6, 400 MHz): δ (ppm) = 5.01 (s, 2H, CH_2), 7.41 (dd, 1H, J = 5.1/1.3 Hz, thiophene-H), 7.57–7.61 (m, 2H, ArH), 7.69–7.73 (m, 1H, ArH), 7.79 (dd, 1H, J = 5.1/3.0 Hz, thiophene-H), 7.92 (br s, 2H, NH_2), 8.07–8.09 (m, 3H, ArH); ^{13}C NMR (DMSO-d_6, 150.9 MHz): δ (ppm) = 37.5 (CH_2); 126.9, 127.2, 127.9 (2C), 128.3 (2C), 128.4, 133.2 (tert. C); 84.9, 92.4, 114.8, 115.0, 132.8, 135.0, 152.4, 159.1, 165.5, 192.2 (quat. C); Anal. ($C_{19}H_{12}N_4OS_2$) C, H, N; HPLC: 99.4% at 254 nm and 98.9% at 280 nm, t_N = 2.87 min, t_M = 1.03 min (ACN/H_2O; 55:45).

*5.1.6.13. 2-Amino-6-{[2-(4-chlorophenyl)-2-oxoethyl]sulfanyl}-4-(3-thienyl)pyridine-3,5-dicarbonitrile (**13g**).* Prepared from **12** and 2-bromo-1-(4-chlorophenyl)ethanone. Light grey powder (57%); mp 243–244 °C (EtOH); IR (KBr): 3438, 3334 and 3224 cm^{-1} (NH), 2208 cm^{-1} (C≡N), 1687 cm^{-1} (C=O); ^1H NMR (DMSO-d_6, 400 MHz): δ (ppm) = 4.97 (s, 2H, CH_2), 7.41 (dd, 1H, J = 5.1/1.3 Hz, thiophene-H), 7.64–7.67 (m, 2H, ArH), 7.78 (dd, 1H, J = 5.1/3.0 Hz, thiophene-H), 7.90 (br s, 2H, NH_2), 8.07–8.10 (m, 3H, ArH); ^{13}C NMR (DMSO-d_6, 100.6 MHz): δ (ppm) = 37.9 (CH_2); 127.4, 127.7, 128.9, 128.9 (2C), 130.3 (2C) (tert. C); 85.6, 92.9, 115.3, 115.5, 133.3, 134.3, 138.6, 153.0, 159.6, 165.9, 191.8 (quat. C); Anal ($C_{19}H_{11}ClN_4OS_2$) C, H, N; HPLC: 99.2% at 254 nm and 98.6% at 280 nm, t_N = 4.66 min, t_M = 1.03 min (ACN/H_2O; 55:45).

*5.1.6.14. 2-Amino-6-{[2-(4-methoxyphenyl)-2-oxoethyl]sulfanyl}-4-(3-thienyl)pyridine-3,5-dicarbonitrile (**13h**).* Prepared from **12** and 2-bromo-1-(4-methoxyphenyl)ethanone. Light grey powder (71%); mp 250–252 °C (EtOH); IR (KBr): 3390, 3321 and 3217 cm^{-1} (NH), 2210 cm^{-1} (C≡N), 1672 cm^{-1} (C=O); ^1H NMR (DMSO-d_6, 600 MHz): δ (ppm) = 3.87 (s, 3H, OCH_3), 4.95 (s, 2H, CH_2), 7.09–7.10 (m, 2H, ArH), 7.41 (dd, 1H, J = 5.1/1.3 Hz, thiophene-H), 7.79 (dd, 1H, J = 5.1/3.0 Hz, thiophene-H), 7.93 (br s, 2H, NH_2, superimposed by dd at 7.79, m at 8.05–8.06 and dd at 8.08), 8.05–8.06 (m, 2H, ArH), 8.08 (dd, 1H, J = 3.0/1.3 Hz, thiophene-H); ^{13}C NMR (DMSO-d_6, 150.9 MHz): δ (ppm) = 37.5 (CH_2); 55.5 (OCH_3); 113.9 (2 C), 127.3, 127.6, 128.8, 130.8 (2C) (tert. C); 85.4, 92.9, 115.3, 115.5, 128.3, 133.3, 152.9, 159.5, 163.5, 166.1, 191.0 (quat. C); Anal. ($C_{20}H_{14}N_4O_2S_2$) C, H, N; HPLC: 99.7% at 254 nm and 99.7% at 280 nm, t_N = 4.83 min, t_M = 1.01 min (ACN/H_2O; 50:50).

*5.1.6.15. 2-Amino-6-{[2-(4-methylphenyl)-2-oxoethyl]sulfanyl}-4-(3-thienyl)pyridine-3,5-dicarbonitrile (**13i**).* Prepared from **12** and 2-bromo-1-(4-methylphenyl)ethanone. Light yellow needles (70%); mp 234–235 °C (EtOH); IR (KBr): 3423, 3325 and 3221 cm^{-1} (NH), 2210 cm^{-1} (C≡N), 1681 cm^{-1} (C=O); ^1H NMR (DMSO-d_6, 600 MHz): δ (ppm) = 2.41 (s, 3H, CH_3), 4.97 (s, 2H, CH_2), 7.38–7.40 (m, 2H, ArH), 7.41 (dd, 1H, J = 5.1/1.5 Hz, thiophene-H, superimposed by m at 7.38–7.40), 7.79 (dd, 1H, J = 5.1/3.0 Hz, thiophene-H), 7.91 (br s, 2H, NH_2, superimposed by dd at 7.79, m at 7.97–7.99 and dd at 8.08), 7.97–7.99 (m, 2H, ArH), 8.08 (dd, 1H, J = 3.0/1.5 Hz, thiophene-H); ^{13}C NMR (DMSO-d_6, 150.9 MHz): δ (ppm) = 21.1 (CH_3); 37.7 (CH_2); 127.3, 127.6, 128.5 (2 C), 128.8, 129.3 (2 C) (tert. C); 85.4, 92.9, 115.3, 115.5, 133.0, 133.3, 144.1, 152.9, 159.5, 166.0, 192.2 (quat. C); Anal. ($C_{20}H_{14}N_4OS_2$) C, H, N; HPLC: 99.8% at 254 nm and 99.8% at 280 nm, t_N = 3.77 min, t_M = 1.02 min (ACN/H_2O; 55:45).

*5.1.6.16. 2-Amino-6-{[2-(4-cyanophenyl)-2-oxoethyl]sulfanyl}-4-(3-thienyl)pyridine-3,5-dicarbonitrile (**13j**).* Prepared from **12** and 4-(bromoacetyl)benzonitrile. Light yellow powder (65%); mp 264 °C (dec.; EtOH); IR (KBr): 3434, 3330 and 3213 cm^{-1} (NH), 2213 cm^{-1} (C≡N), 1695 cm^{-1} (C=O); ^1H NMR (DMSO-d_6, 600 MHz): δ (ppm) = 5.00 (s, 2H, CH_2), 7.41 (dd, 1H, J = 4.9/1.3 Hz, thiophene-H), 7.79 (dd, 1H, J = 4.9/2.8 Hz, thiophene-H), 7.89 (br s, 2H, NH_2, superimposed by dd at 7.79 and m at 8.06–8.08), 8.06–8.08 (m, 3H, ArH), 8.20–8.22 (m, 2H, ArH); ^{13}C NMR (DMSO-d_6, 150.9 MHz): δ (ppm) = 38.1 (CH_2); 127.4, 127.6, 128.8, 128.9 (2C), 132.7 (2C) (tert. C); 85.5, 92.8, 115.2, 115.4, 115.4, 118.1, 133.2, 138.8, 152.9, 159.5, 165.7, 192.1 (quat. C); Anal. ($C_{20}H_{11}N_5OS_2$) C, H, N; HPLC: 99.3 at 254 nm and 99.3 at 280 nm, t_N = 3.99, t_M = 1.02 min (ACN/H_2O; 50:50).

*5.1.6.17. 2-Amino-6-({2-oxo-2-[4-(trifluoromethyl)phenyl]ethyl}sulfanyl)-4-(3-thienyl)pyridine-3,5-dicarbonitrile (**13k**).* Prepared from **12** and 2-bromo-1-[4-(trifluoromethyl)phenyl]ethanone. Light yellow crystals (50%); mp 236–237 °C (EtOH); IR (KBr): 3409, 3323 and 3231 cm^{-1} (NH), 2217 cm^{-1} (C≡N), 1697 cm^{-1} (C=O); ^1H NMR (DMSO-d_6, 600 MHz): δ (ppm) = 5.02 (s, 2H, CH_2), 7.41 (dd, 1H, J = 5.0/1.3 Hz, thiophene-H), 7.79 (dd, 1H, J = 5.0/3.0 Hz, thiophene-H), 7.90 (br s, 2H, NH_2, superimposed by dd at 7.79, d at 7.96 and dd at 8.08), 7.96 (d, 2H, J = 8.1 Hz, ArH), 8.08 (dd, 1H, J = 3.0/1.3 Hz, thiophene-H), 8.27 (d, 2H, J = 8.1 Hz, ArH); ^{13}C NMR (DMSO-d_6, 150.9 MHz): δ (ppm) = 38.1 (CH_2); 125.7 (q, 2 C, $^3J_{C,F}$ = 3.6 Hz), 127.4, 127.6, 128.8, 129.2 (2 C) (tert. C); 85.5, 92.8, 115.2, 115.4, 123.7 (q, $^1J_{C,F}$ = 273 Hz), 132.7 (q, $^2J_{C,F}$ = 31.8 Hz), 133.3, 138.8, 152.9, 159.5, 165.7, 192.1 (quat. C); Anal. ($C_{20}H_{11}F_3N_4OS_2$); HPLC: 99.9 at 254 nm and 99.9 at 280 nm, t_N = 5.37, t_M = 1.01 min (ACN/H_2O; 55:45).

*5.1.6.18. 2-Amino-6-{[2-(3-chlorophenyl)-2-oxoethyl]sulfanyl}-4-(3-thienyl)pyridine-3,5-dicarbonitrile (**13l**).* Prepared from **12** and 2-bromo-1-(3-chlorphenyl)ethanone. Light yellow crystals (60%); mp 224–225 °C (EtOH); IR (KBr): 3471 and 3353 cm^{-1} (NH), 2210 cm^{-1} (C≡N), 1685 cm^{-1} (C=O); ^1H NMR (DMSO-d_6, 600 MHz): δ (ppm) = 4.99 (s, 2H, CH_2), 7.41 (dd, 1H, J = 5.1/1.3 Hz, thiophene-H), 7.63 (t, 1H, J = 7.9 Hz, ArH), 7.77–7.79 (m, 1H, ArH), 7.79 (dd, 1H, J = 5.1/3.0 Hz, thiophene-H, superimposed by m at 7.77–7.79), 7.93 (brs, 2H, NH_2), 8.02–8.04 (m, 1H, ArH), 8.08 (t, 1H, J = 1.7 Hz, ArH), 8.09 (dd, 1H, J = 3.0/1.3 Hz, thiophene-H); ^{13}C NMR (DMSO-d_6, 150.9 MHz): δ (ppm) = 38.0 (CH_2); 127.0, 127.4, 127.7, 128.0, 128.8, 130.7, 133.3 (tert. C); 85.4, 92.8, 115.3, 115.5, 133.3, 133.6, 137.3, 152.9, 159.5, 165.8, 191.6 (quat. C); Anal. ($C_{19}H_{11}ClN_4OS_2$) C, H, N; HPLC: 99.7 at 254 nm and 99.7 at 280 nm, t_N = 4.48, t_M = 1.01 min (ACN/H_2O; 55:45).

5.2. Biological assays

5.2.1. In vitro kinase assays

Recombinant kinase domains of human RET (aa 700–1020, NP_066124), ABL (aa 230–517, isoform a, NP_005148) and ALK (aa 1103–1459, NP_004295) were expressed in Sf9 cells with an amino terminal histidine tag and purified as described [28].

The ELISA kinase assay was performed as described [28] using approximately 1 pmol recombinant enzyme and 1 nmol peptide substrate. In brief, the enzyme was incubated in reaction buffer (25 mM Hepes pH 7, 1 mM $MnCl_2$, 5 mM $MgCl_2$) with inhibitors and 300 μM ATP, in a 96-well plate pre-coated with peptide substrate, for 15 min at 30 °C. After five washes with wash buffer (0.05% Tween in PBS), the plate was incubated with anti-phosphotyrosine primary antibody (clone 4G10, Millipore) and HRP-conjugated anti-mouse secondary antibody. A colorimetric signal was developed by addition of TMB substrate (Pierce) and stopped with 0.1 M H_2SO_4. Absorbance was read at 450 nm with a 96-well microplate reader (Bio-Rad). As a substrate, an ALK activation loop-derived peptide was used [32]. Each data point was done in duplicate and experiments were repeated at least three times.

The IC_{50} values were calculated as the inhibitor concentrations that caused 50% inhibition of the kinase activity.

5.2.2. Cell growth assay

TPC1 is a human papillary thyroid carcinoma cell line expressing the RET/PTC1 fusion kinase. These cells were grown in DMEM medium supplemented with 10% fetal calf serum, 100 U/mL penicillin, 100 mg/mL gentamicin, and 2 mM glutamine. RET-negative colon carcinoma cells LS174 T were cultured in RPMI-1640 with 10% serum and antibiotics.

Cell proliferation was measured using the tritiated thymidine (^{3}H-T) incorporation assay. Serial dilutions of the inhibitors were prepared directly in 96-well plates in DMSO as 100× concentrated. The cells (10^4/well) were then added to the wells and incubated for 72 h. The cells were pulsed with ^{3}H-T (1 μCi/well) during the last 8 h of culture and then harvested onto glass fiber filter paper. Cell-associated radioactivity was measured by liquid scintillation. Percent inhibition of ^{3}H-T uptake at each inhibitor dose relative to control cells without treatment was calculated. Data points were done in triplicate. Data are presented as the mean of two independent experiments.

5.3. Docking experiments

Compounds **7c** and **13g** were docked into the ATP binding site of 2ivu using the docking programme FlexX 3.0 (BiosolveIT, 53757 St. Augustin, Germany). The active site was defined as all residues surrounding the original ligand to about 6.5 Å. The ligands and all water molecules were deleted from the experimental protein structure prior to docking. Compounds **7c** and **13g** were constructed in SYBYL (SYBYL 8, Tripos International, 1699 South Hanley Rd., St. Louis, MO, 63144, USA), AM1 charges were calculated and forcefield minimisations were done using the Tripos forcefield. Docking was performed using FlexX with default parameters except the usage of the SIS mode of FlexX for base fragment placement. Fifty poses were saved for each docking run and clustered by rms deviations for subsequent binding mode analysis.

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References

[1] T. Attié-Bitach, M. Abitbol, M. Gérard, A.-L. Delezoide, J. Augé, A. Pelet, J. Amiel, V. Pachnis, A. Munnich, S. Lyonnet, M. Vekemans, Am. J. Med. Genet. 80 (1998) 481–486.
[2] V. Avantaggiato, N.A. Dathan, M. Grieco, N. Fabien, D. Lazzaro, A. Fusco, A. Simeone, M. Santoro, Cell Growth Differ. 5 (1994) 305–311.
[3] H. Enomoto, P.A. Crawford, A. Gorodinsky, R.O. Heuckeroth, E.M. Johnson Jr., J. Milbrandt, Development 128 (2001) 3963–3974.
[4] A. Schuchardt, V. D'Agati, L. Larsson-Blomberg, F. Costantini, V. Pachnis, Nature 367 (1994) 380–383.
[5] J. Anders, S. Kjaer, C.F. Ibánez, J. Biol. Chem. 276 (2001) 35808–35817.
[6] E. Arighi, M.G. Borrello, H. Sariola, Cytokine Growth Factor Rev. 16 (2005) 441–467.
[7] J.W.B. de Groot, T.P. Links, J.T.M. Plukker, C.J.M. Lips, R.M.W. Hofstra, Endocrinol. Rev. 27 (2006) 535–560.
[8] M. Takahashi, G.M. Cooper, Mol. Cell. Biol. 7 (1987) 1378–1385.
[9] S.M. Jhiang, Oncogene 19 (2000) 5590–5597.
[10] K.M. Carlson, S. Dou, D. Chi, N. Scavarda, K. Toshima, C.E. Jackson, S.A. Wells Jr., P.J. Goodfellow, H. Donis-Keller, Proc. Natl. Acad. Sci. U. S. A. 91 (1994) 1579–1583.
[11] R.M.W. Hofstra, R.M. Landsvater, I. Ceccherini, R.P. Stulp, T. Stelwagen, Y. Luo, B. Pasini, J.W.M. Höppener, H.K. Ploos van Amstel, G. Romeo, C.J.M. Lips, C.H.C. M. Buys, Nature 367 (1994) 375–376.
[12] L.M. Mulligan, J.B.J. Kwok, C.S. Healey, M.J. Elsdon, C. Eng, E. Gardner, D. R. Love, S.E. Mole, J.K. Moore, L. Papi, M.A. Ponder, H. Telenius, A. Tunnacliffe, B.A.J. Ponder, Nature 363 (1993) 458–460.
[13] F. Marini, A. Falchetti, F. Del Monte, S. Carbonell Sala, I. Tognarini, E. Luzi, M. L. Brandi, Orphanet J. Rare Dis 1 (2006) 45.
[14] C. Lanzi, G. Cassinelli, G. Cuccuru, N. Zaffaroni, R. Supino, S. Vignati, C. Zanchi, M. Yamamoto, F. Zunino, Cell. Mol. Life Sci. 60 (2003) 1449–1459.
[15] F. Carlomagno, D. Vitagliano, T. Guida, M. Napolitano, G. Vecchio, A. Fusco, A. Gazit, A. Levitzki, M. Santoro, Cancer Res. 62 (2002) 1077–1082.
[16] F. Carlomagno, D. Vitagliano, T. Guida, F. Basolo, M.D. Castellone, R.M. Melillo, A. Fusco, M. Santoro, J. Clin. Endocrinol. Metab. 88 (2003) 1897–1902.
[17] F. Carlomagno, D. Vitagliano, T. Guida, F. Ciardiello, G. Tortora, G. Vecchio, A. J. Ryan, G. Fontanini, A. Fusco, M. Santoro, Cancer Res. 62 (2002) 7284–7290.
[18] I. Plaza-Menacho, L. Mologni, E. Sala, C. Gambacorti-Passerini, A.I. Magee, T. P. Links, R.M.W. Hofstra, D. Barford, C.M. Isacke, J. Biol. Chem. 282 (2007) 29230–29240.
[19] L. Mologni, E. Sala, S. Cazzaniga, R. Rostagno, T. Kuoni, M. Puttini, J. Bain, L. Cleris, S. Redaelli, B. Riva, F. Formelli, L. Scapozza, C. Gambacorti-Passerini, J. Mol. Endocrinol. 37 (2006) 199–212.
[20] J.W.B. de Groot, I. Plaza-Menacho, H. Schepers, L.J. Drenth-Diephuis, J. Osinga, J.T.M. Plukker, T.P. Links, B.J.L. Eggen, R.M.W. Hofstra, Surgery 139 (2006) 806–814.
[21] C.J. Strock, J.-I. Park, M. Rosen, C. Dionne, B. Ruggeri, S. Jones-Bolin, S. R. Denmeade, D.W. Ball, B.D. Nelkin, Cancer Res. 63 (2003) 5559–5563.
[22] R. Cincinelli, G. Cassinelli, S. Dallavalle, C. Lanzi, L. Merlini, M. Botta, T. Tuccinardi, A. Martinelli, S. Penco, F. Zunino, J. Med. Chem. 51 (2008) 7777–7787.
[23] C. Lanzi, G. Cassinelli, V. Nicolini, F. Zunino, Biochem. Pharmacol. 77 (2009) 297–309.
[24] N.M. Evdokimov, A.S. Kireev, A.A. Yakovenko, M.Y. Antipin, I.V. Magedov, A. Kornienko, J. Org. Chem. 72 (2007) 3443–3453.
[25] N.M. Evdokimov, I.V. Magedov, A.S. Kireev, A. Kornienko, Org. Lett. 8 (2006) 899–902.
[26] V. Litvinov, V. Dotsenko, S. Krivokolysko, Adv. Heterocycl. Chem. 93 (2007) 117–178.
[27] L.A. Rodinovskaya, A.M. Shestopalov, Russ. Chem. Bull. Int. Ed. 49 (2000) 348–354.
[28] L. Mologni, E. Sala, B. Riva, L. Cesaro, S. Cazzaniga, S. Redaelli, O. Marin, N. Pasquato, A. Donella-Deana, C. Gambacorti-Passerini, Prot. Expr. Purif. 41 (2005) 177–185.
[29] P. Knowles, J. Murray-Rust, S. Kjaer, R. Scott, S. Hanrahan, M. Santoro, C. Ibáñez, N. McDonald, J. Biol. Chem. 281 (2006) 33577–33587.
[30] C. Kunick, Z. Zeng, R. Gussio, D. Zaharevitz, M. Leost, F. Totzke, C. Schächtele, M.H.G. Kubbutat, L. Meijer, T. Lemcke, ChemBioChem 6 (2005) 541–549.
[31] V.G. Brunton, M.J. Lear, D.J. Robins, S. Williamson, P. Workman, Anticancer Drug Des. 9 (1994) 291–309.
[32] A. Donella-Deana, O. Marin, L. Cesaro, R. Gunby, A. Ferrarese, A. Coluccia, C. Tartari, L. Mologni, L. Scapozza, C. Gambacorti-Passerini, L. Pinna, Biochemistry 44 (2005) 8533–8542.