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Synthesis and evaluation of apoptosis induction of thienopyrimidine compounds on KRAS and BRAF mutated colorectal cancer cell lines

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ABSTRACT

Monoclonal antibodies (MoAb) and tyrosine kinase inhibitors (TKI) targeting the EGFR (Epidermal Growth Factor Receptor) pathways are currently used in colorectal cancer treatment. Despite the improvement of median overall survival, resistance is observed notably due to KRAS and BRAF gene mutations. We synthesized four series of thienopyrimidines whose scaffold is structurally close to TKI used in clinical practice. We evaluated apoptosis induced by these compounds using flow cytometry on KRAS and BRAF mutated cell lines. Our results confirm that the mutated cell lines (HCT116 and HT29) are more resistant to apoptosis than the non-mutated cell line (Hela). Interestingly, among the 13 compounds tested, three of them (**5b**, **6b** and **6d**) and gefitinib exhibited a noteworthy pro-apoptotic effect, especially on mutated cell lines with an IC_{50} value between 70 and 110 μM. These three compounds seem particularly attractive for the development of novel treatments for colorectal cancer patients harboring EGFR pathway mutations.

1. Introduction

Colorectal cancer is the third most common cancer in the world. Despite recent progress in treatment with the utilization of new targeted therapies such as anti-EGFR (Epidermal Growth Factor Receptor) or anti-VEGF (Vascular Endothelial Growth Factor) monoclonal antibodies (MoAb), the median overall survival is not longer than 25 to 30 months in the metastatic setting.[1,2] Apart from MoAb, another class of small molecules known as tyrosine kinase inhibitors (TKI) has also undergone clinical trials and is of great promise. These molecules perform ATP-competitive binding on the TK domain of the growth factor receptors, and thus prevent phosphorylation of the receptor and its subsequent activation.

In the particular case of colorectal cancer, EGFR constitutes the main targeted receptor as it is over-expressed in a majority of patients. Blockade of this receptor by MoAbs (namely Cetuximab and Panitumumab) leads to inhibition of the downstream signaling cascades known as the PI3K/Akt pathway, which is involved in cell survival and motility invasion, and the Ras/Raf/MAPK (Mitogen Activated Protein Kinase) pathway, which is implicated in cell proliferation. Despite the encouraging results achieved by anti-EGFR therapy, recent studies have underlined the emergence of a non-negligible portion of patients refractory to treatment. At the molecular level, it seems that this resistance comes from an oncogenic activation of the EGFR downstream effectors rather than overexpression or overactivation of the receptor itself. Changes in EGFR gene copy numbers and somatic mutations are indeed rarely found in non-responsive metastatic colorectal cancer patients.[3]

On the other hand, biological downstream markers of the EGFR pathway such as KRAS and BRAF seem actually to play a prominent role. After activation, the intracellular domain of the EGF receptor is phosphorylated, allowing the activation of RAS via the 'linker' Grb2/sos which binds to the phosphorylated form of the EGFR. Activated RAS will, in turn, phosphorylate RAF which will trigger the activation of the MAP Kinase pathway and thereby cell proliferation. Mutational status of KRAS and BRAF isoforms has been proven to interfere with the action of anti-EGFR cetuximab and panitumumab.

KRAS gene mutations, which occur in 40% of colorectal cancer patients, generate a constitutively active protein that counteracts the effect of anti-EGFR MoAbs and renders cells averse to treatment. More recently, mutations in BRAF, another downstream factor of the MAP Kinase pathway, have also been linked to the inefficacy of anti-EGFR therapy (10–15% of non-responders) and are associated with a poor prognosis.[4] Altogether, these two mutations, which are mutually exclusive, constitute major negative predictors of the efficacy of anti-EGFR monoclonal antibodies

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in colorectal cancer.[5] It seems then of great interest to develop molecules able to interfere with TK domain of growth factor receptors and/or with intracellular signaling pathways such as PI3K/Akt and MAP Kinase pathways. Some have already been approved by the FDA and are currently used in clinical practice in different pathological context such as lung cancer (erlotinib, gefitinib), breast cancer (lapatinib), chronic myeloid leukemia (imatinib, dasatinib) or renal cancer (sunitinib). Many others underwent clinical trials or are under development. In general, they do possess a quinazoline skeleton (erlotinib, gefitinib, lapatinib) or approching structures such as pyrimidine, indolinone or benzopyrazole scaffold (dasatinib, imatinib, sunitinib, axitinib) (Fig. 1).[6–10] Recent studies showed that compounds belonging to these chemical classes or possessing close related molecular structure exhibit plethora of biological effects, including antiviral, antimicrobial, anti-inflammatory and anticancer activities.[11–20] Fused pyrazolopyrimidine derivatives possess, for example, potent antiviral and anticancer properties .[18–20] Thienopyrimidine derivatives, which have been more recently studied, exert not only antiviral (anti-H5N1)[16] and antimicrobial activities,[12,14,15] but also inhibit various protein kinases, such as CK2[13] involved in particular in cancer. Our group has also been interested in thienopyrimidine derivatives for years. We synthesized, in a previous work, two series of thienopyrimidine compounds and, showed that the series possessing an aromatic cycle (series # **6**) was linked to an enhanced antitumoral activity.[21] Here, we synthesized three novel series of compounds (series # **4** ,**5** and **7**) conserving the same pyrimidine skeleton known to be linked to anti-EGFR activity and assessed their biological activity. We tested these four series of compounds (series # **4**, **5** ,**6** and **7**) as well as a standard TKI (gefitinib) on colorectal cancer cell lines harboring mutations on *KRAS* (HCT116 cell line) or *BRAF* (HT29 cell line) genes. A KRAS and BRAF wild-type model cell line was chosen as a control. The ultimate goal was to synthesize and evaluate the properties of these novel compounds on cancer cells with mutations conferring resistance to anti-EGFR antibodies.

2. Results

2.1. Chemistry

The benzo[4,5]thienopyrimidin-4*(3H)*-one intermediates B and B′ (Schemes 1 and 2) were prepared according to a method previously described.[22–26] The compounds were obtained by treatment with phosphorous oxychloride, followed by refluxing in dimethylformamide with the appropriate amine. This led to the final aminosubstituted derivatives of thienopyrimidine (Scheme 1 and Scheme 2). Synthesized compounds are summarized in Table 1.

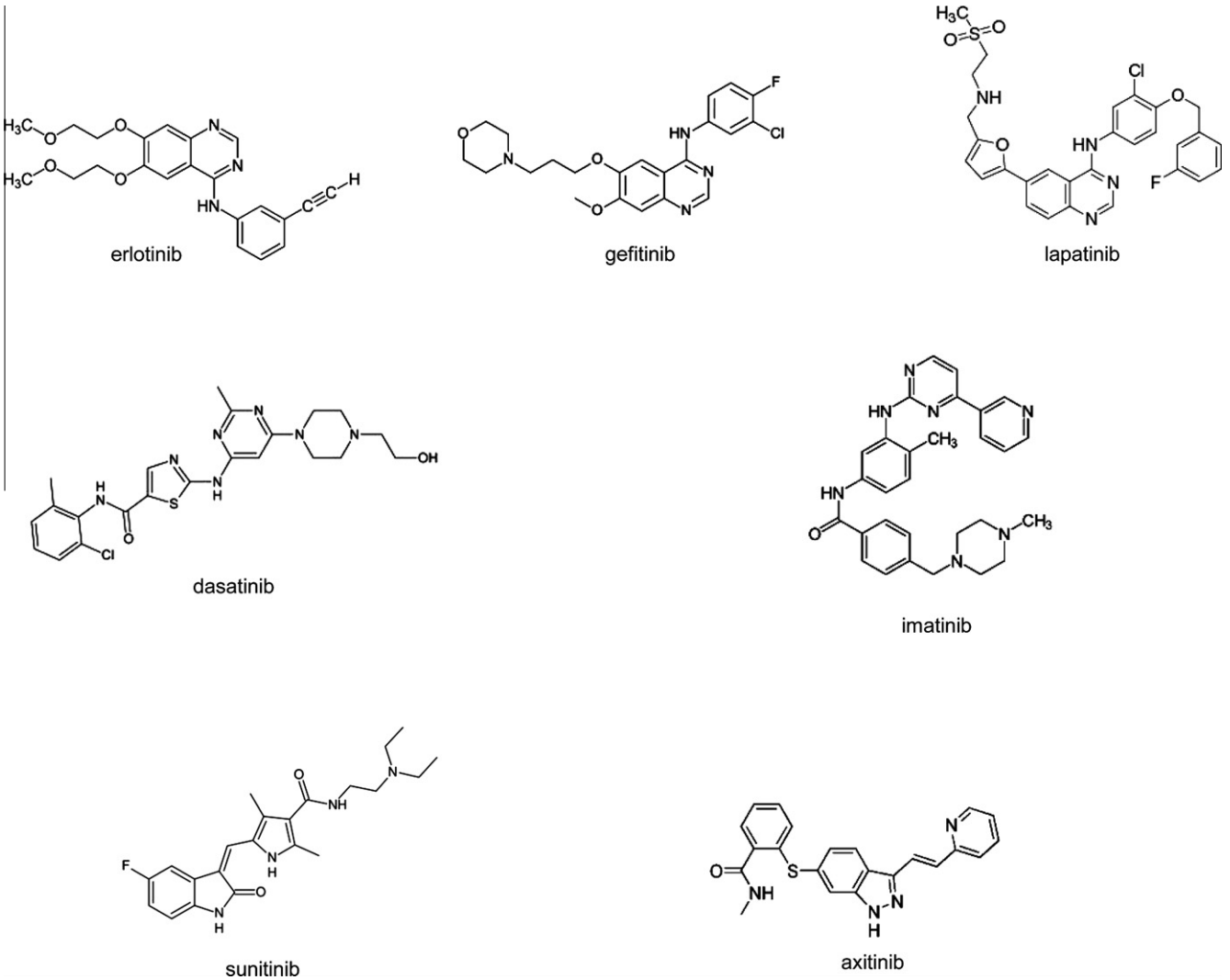
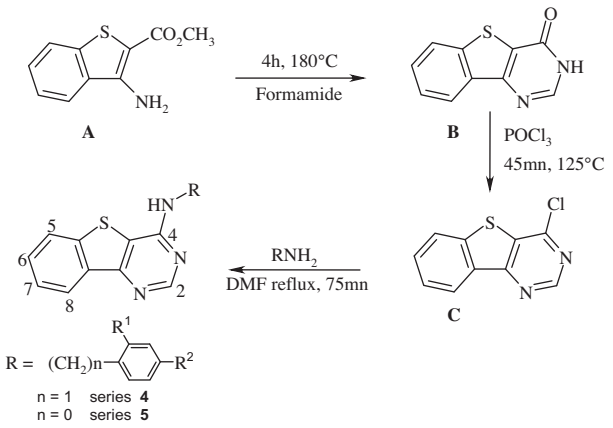
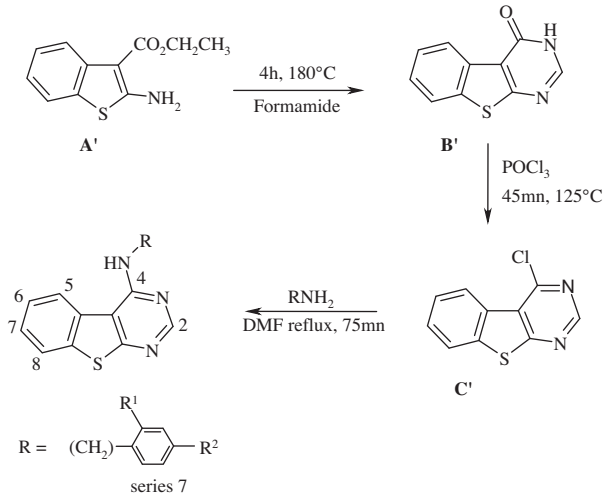


Figure 1. Chemical structures of used tyrosine kinase inhibitors.



Scheme 1. Synthesis of the 4-aminosubstituted thieno[3,2-*d*]pyrimidine derivatives.



Scheme 2. Synthesis of the 4-aminosubstituted thieno[2,3-*d*]pyrimidine derivatives.

2.2. Characterization of cell lines by immunofluorescence

Expression of EGF receptor was assessed by immunofluorescence on HT29, HCT116 and Hela cell lines. We confirmed that the three cell lines exhibit a strong expression of this receptor (Fig. 2). The images show the membrane localization of the receptor, especially on HCT116 and HT29.

2.3. Induction of apoptosis evaluated by flow cytometry

Apoptosis was evaluated by measuring drug-induced depolarization of the mitochondrial membrane assessed by flow cytometry, as described in Experimental protocols. Fourteen drugs including series # **4**, series # **5** ,series # **6** and series # **7** molecules as well as gefitinib (at eight concentrations ranging from 0.1 to 200 μM) were tested on three different cell lines. IC_{50} were calculated from dose-response curves with Prism software. Table 2 shows the IC_{50} of each drug on the three cell lines. Globally, Hela cell line (KRAS wild-type, BRAF wild-type) is more sensitive than HCT116 (KRAS mutated) and HT29 (BRAF mutated), the latter being the most resistant cell line (Table 2, Fig. 3). The anti-EGFR drug gefitinib exerted substantial apoptosis induction on the three cell lines with an IC_{50} of 108.3 ± 8.9 μM on Hela cells, 66.9 ± 16.7 μM on HCT116 cells and 110.3 ± 8.4 μM on HT29 cells. Strikingly, on mutated HCT116 and HT29 cells, IC_{50} were either not reached or quite elevated for the majority of the drugs except for **5b, 6b**, **6d** compounds. In these cases, IC_{50} were comprised between 70–120 μM which seems relatively similar to the ones obtained with gefitinib.

2.4. Evaluation of caspase activation measured on HT29 cells

To ascertain the therapeutic potential of series # **6** compounds, we then tested the capacity of these lead molecules to induce apoptosis using the Differential Anchorage Probes (DAP) technique, as described in Experimental protocols. This new tool allowed us to specifically measure caspase 3 activation induced by these drugs in the HT29 cell line. Experiments were performed using the reference drug gefitinib and # **6** series at eight concentrations ranging from 0.1 to 200 μM. Increase in caspase activation was calculated by dividing the percentage of apoptotic cells in each drug-treated sample (200 μM) by the percentage of apoptotic cells in untreated samples. Results are presented in Figure 4. Consistent with previous results, **6a** and **6c** compounds moderately increased apoptosis. However, **6b** and **6d** compounds seem to be promising anti-cancer agents as they strongly induced caspase activation (10-fold higher than in control cells) and cell death.

3. Discussion

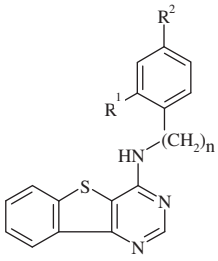
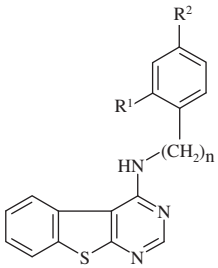
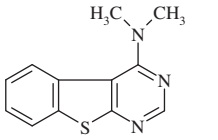
In the past ten years, quinazolines have been proposed as inhibitors of various tyrosine kinase receptors. Some have been commercialized such as erlotinib, gefitinib (anti-EGFR) and lapatinib (anti-EGFR and anti-ErbB-2 (erythroblastic leukemia viral oncogene homolog 2)), for treating non-small cell lung cancer and breast cancer respectively.

These compounds act as tyrosine kinase ATP-binding site competitors. In metastatic colorectal cancer, anti-EGFR targeted therapies are mainly based on the use of MoAbs, but TKI could be equally efficient. In order to develop novel compounds able to powerfully block the tyrosine kinase activity of RTKs (tyrosine kinase receptors), we designed a new series of thienopyrimidine compounds resembling a previously described series.[21] We synthesized molecules with a thienopyrimidine scaffold, as they are structurally close to the quinazoline skeleton and potentially able to inhibit RTKs. Moreover, authors have recently studied thienopyrimidine derivatives and evaluated their biological activities on different fields such as antimicrobial, antiviral, analgesic, anti-inflammatory and anticancer properties.[11–16]

We then evaluated the capacity of these compounds to induce apoptosis on KRAS or BRAF mutated colorectal cancer cell lines, as well as on Hela cells (not mutated for KRAS and BRAF). Since KRAS and BRAF are downstream effectors of the EGFR pathway, their mutations induce the continuous activation of the EGFR signaling pathway, which is responsible for the resistance to anti-EGFR MoAbs. The goal of this study was to evaluate the cytotoxicity of these new molecules according to the mutational status of KRAS and BRAF on the EGFR pathway.

We previously synthesized two series of 4-substituted [2,3-*d*] thienopyrimidine compounds: the **3a–e** series with a cyclohexyl cycle and the **6a–e** series possessing a benzenic cycle.[21] The C-4 substitution makes it possible to differentiate the compounds: an anilino group corresponds to the **a** series, an *ortho* methylanilino group to the **b** series, an *ortho* methoxyanilino group to the **c** series, a *para* methoxyanilino group to the **d** series and a dimethylamino substituent to the **e** series. In these compounds, the sulfur atom was positioned on the opposite side of the C-4 substituent. In the present work, we first synthesized two original series of 4-substitutedbenzo[4,5]thieno[3,2-*d*] pyrimidine in which the sulfur atom is located on the same

Table 1
Synthesized thienopyrimidine derivatives

	Compounds	*n*	R^1	R^2	Reference
	4a	1	H	H	30
	4b[a]	1	Methyl	H	–
	4c[a]	1	Methoxy	H	–
	4d[a]	1	H	Methoxy	–
	5a[a]	0	H	H	–
	5b[a]	0	Methyl	H	–
	6a	0	H	H	21
	6b	0	Methyl	H	21
	6c	0	Methoxy	H	21
	6d	0	H	Methoxy	21
	7a[a]	1	H	H	–
	7b[a]	1	Methyl	H	–
	6e	–	–	–	21

[a] The newly synthesized compounds were characterized by spectroscopic data.

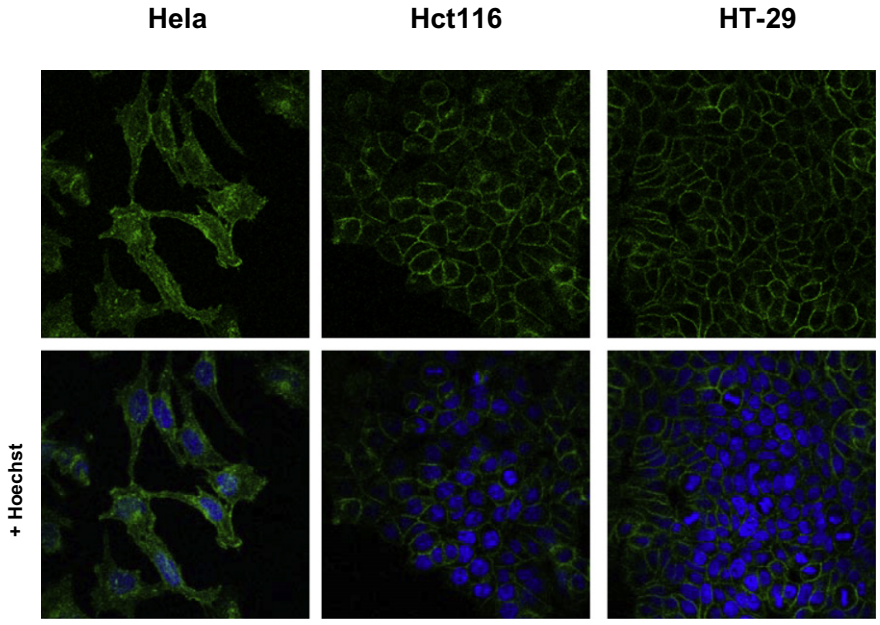


Figure 2. EGFR expression on Hela cell line and on colorectal cancer cell lines HCT116 and HT29 was measured by immunofluorescence as described in Experimental protocols. Green signal corresponds to EGFR and blue to nuclear staining. Images were taken using the LSM510 confocal microscope and subsequently analyzed with LSM Image browser software.

side of the C-4 substituent (series **4** and **5**). The first series is substituted by a benzyl amino group with different substitutions (series **4**) and the second by an anilino group (series **5**) (Table 1). The letter characterizes the same substitutions as in the **3** and **6** series. We additionally synthesized another original series (series **7**) where the sulfur atom is located on the opposite side of the C-4 substitution (like series **3** and **6**) but with an intercalated methylene group between the amino group and the phenyl cycle (like series **4**) (see Table 1).

Three of our synthesized compounds (**5b, 6b** and **6d**) exerted a cytotoxic activity comparable to gefitinib (selected as an anti-EGFR reference drug–see structure in Fig. 1) in the three cell lines studied. First, series **4** (see structure in Table 1), which possesses a methylene group intercalated between the amino group and the

Table 2
IC_{50} (μM) of the synthesized compounds on the three cell lines

	HELA	HCT116	HT29
4a	105.3 ± 17.4	NR	NR
4b	NR	NR	NR
4c	128.8 ± 14.1	NR	NR
4d	118.3 ± 20.7	NR	NR
5a	NR	NR	NR
5b	77.7 ± 7.4	121.0 ± 1.0	126.7 ± 5.4
6a	>130	NR	NR
6b	129.0 ± 0.8	100.7 ± 7.0	120.3 ± 12.0
6c	>130	NR	NR
6d	109.0 ± 9.9	92.0 ± 19.0	117.0 ± 9.9
6e	102.0 ± 22.9	NR	NR
7a	112.9 ± 3.1	>130	126.2 ± 3.2
7b	>130	NR	NR
gefitinib	108.3 ± 8.9	66.9 ± 16.7	110.3 ± 8.4

NR: not reached.

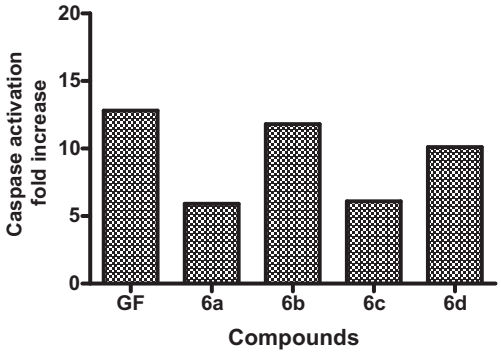


Figure 4. Caspase activation induced by different drugs on HT-29 cell line was evaluated by the differential anchorage probe technique, as described in Experimental protocols. Histograms represent the caspase activation fold change between control and cells treated with 200 μM of each drug.

phenyl cycle, showed weak activity against the Hela cell line and no activity against the mutated cell lines. Data from serie **7** support this result despite that these compounds seem slightly more active. We therefore conclude that the introduction of this methylene group conferring greater flexibility led to poorly active compounds. In fact, the methylene group interrupts the conjugation existing between the benzenic cycle and the rest of the molecule, possibly explaining the loss of activity of these compounds.

The location of the sulfur atom does not seem crucially important as compounds **5b** and **6b** showed interesting activity both against Hela and the mutated cell lines. In fact, these two compounds carry the same substitution on the aromatic ring (ortho methyl) and differ only with regard to the location of the sulfur atom (Table 1). On the contrary, compounds **5a** and **6a**, which differ only regarding the position of the sulfur atom (Table 1), exerted less or no activity on the three cell lines. Since neither of these two latter compounds possesses any substitutions on the aromatic ring, we think that these substitutions play a major role in the activity of the compounds. Moreover, the compounds inducing the greatest caspase activation on the HT-29 mutated cell line (**6b** and **6d**) possess substituted groups (methyl or methoxy) on the aromatic cycle (see structures in Table 1).

The general pharmacophore model for ATP-competitive binding inhibitors at the active site of RTK previously described[8,27,28] could explain the activity of some of our compounds.

This work demonstrates that three synthesized compounds (**5b, 6b**, **6d**) exert a comparable activity to the standard anti-EGFR gefitinib. The observed effect can be explained by the pharmacophore model for ATP-competitive binding inhibitors at the active site of EGFR. These newly synthesized thienopyrimidines have potential for treating colorectal cancer and could be studied in preclinical models.

4. Experimental

4.1. Chemistry

Melting points were determined on a Kofler apparatus (Wagner and Munz, Germany). IR spectra were taken on a IR 470 Spectrometer (Shimadzu, Japan). NMR spectra were recorded in DMSO-d_6 solution on a Bruker AMX 500 instrument (Germany) (^{1}H: 300 MHz, ^{13}C:125 MHz). The NH chemical shifts were confirmed by running D_2O exchange experiments. Thin layer chromatography (TLC) was performed using aluminum precoated plates (silica gel

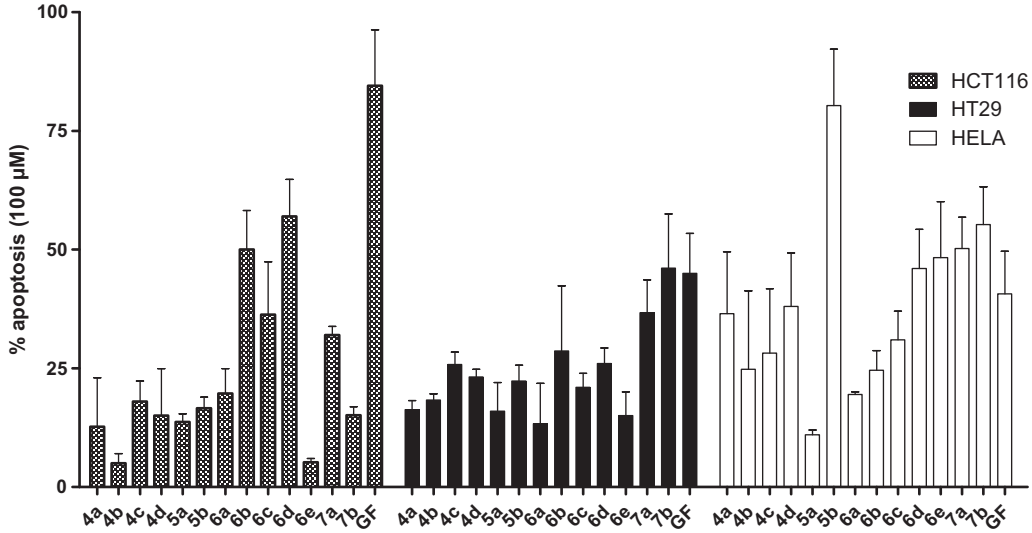


Figure 3. Apoptosis induced by 13 compounds and GF on Hela, HCT116 and HT29 cell lines. Apoptosis was measured by a flow cytometry technique with a fluorescent probe (TMRM) as described in Experimental protocols. Percentage of apoptotic cells induced by 100 μM of each drug was plotted. Experimentations were done in triplicate and presented as mean ± SD.

SDS 60F 254 Whatman, 0.2 mm thickness), eluted with a solution of methylene chloride/ethylacetate (75/25). Column chromatography was carried out on silica gel 60–200 μm (Merck).

4.1.1. General procedure for the preparation of 4-oxo-benzo [4,5]thienopyrimidines (Scheme 1: compound B and Scheme 2: compound B′)

The compounds were prepared according to previously described methods[22]

B: 4-oxo-benzo[4,5]thieno[3,2-d]pyrimidine[23,26]

B′: 4-oxobenzo[4,5]thieno[2,3-d]pyrimidine[25]

4.1.2. General procedure for the preparation of 4-chloro-benzo[4,5]thienopyrimidines (Scheme 1: compound C and Scheme 2: compound C')

The compounds were prepared according to previously described methods[25,29]

C: 4-chloro-benzo[4,5]thieno[3,2-d]pyrimidine

C′: 4-chloro-benzo[4,5]thieno[2,3-d]pyrimidine

4.1.3. General procedure for the preparation of 4-substituted-benzo[4,5]thieno[3,2-*d*] pyrimidines[29] (series 4 and 5, Scheme 1)

A mixture of 0.01 mole of 4-chlorothieno[3,2-d]pyrimidine (Scheme 1: compound C) and 0.02 mole of the appropriate amine in 50 mL of dimethylformamide was heated at reflux for 20 h. The DMF was evaporated under reduced pressure. The reaction mixture was treated with diethylether and the separated solid was filtered and recrystallized from ethanol.

4.1.3.1. *N*-benzyl-benzo[4,5]thieno[3,2-d]pyrimidin-4-amine[30] (4a). The appropriate amine used for the preparation of compound **4a** was the benzylamine.

4.1.3.2. N-(2-methylbenzyl)-benzo[4,5]thieno[3,2-d]pyrimidin-4-amine (4b). The appropriate amine used for the preparation of compound **4b** was the 2-methylbenzylamine

4.1.3.3. N-(2-methoxybenzyl)-benzo[4,5]thieno[3,2-d]pyrimidin-4-amine (4c). The appropriate amine used for the preparation of compound **4c** was the 2-methoxybenzylamine

4.1.3.4. N-(4-methoxybenzyl)-benzo[4,5]thieno[3,2-d]pyrimidin-4-amine (4d). The appropriate amine used for the preparation of compound **4d** was the 4-methoxybenzylamine

4.1.3.5. *N*-phenyl-benzo[4,5]thieno[3,2-d]pyrimidin-4-amine (5a). The appropriate amine used for the preparation of compound **5a** was the aniline

4.1.3.6. N-(2-methylphenyl)-benzo[4,5]thieno[3,2-d]pyrimidin-4-amine (5b). The appropriate amine used for the preparation of compound **5b** was the 2-methylaniline

4.1.4. General procedure for the preparation of 4-substituted-benzo[4,5]thieno[2,3-d] pyrimidines[29] (series 7, Scheme 2)

A mixture of 0.01 mole of 4-chlorothieno[2,3-d]pyrimidine (Scheme 2: compound C′) and 0.02 mole of the appropriate amine in 50 mL of dimethylformamide was heated at reflux for 20 hours. The DMF was evaporated under reduced pressure. The reaction mixture was treated with diethylether and the separated solid was filtered and recrystallized from ethanol.

4.1.4.1. *N*-benzyl-benzo[4,5]thieno[2,3-d]pyrimidin-4-amine (7a). The appropriate amine used for the preparation of compound **7a** was the benzylamine

4.1.4.2. N-(2-methylbenzyl)-benzo[4,5]thieno[2,3-d]pyrimidin-4-amine (7b). The appropriate amine used for the preparation of compound **7b** was the 2-methylbenzylamine

4.1.5. Physical and spectroscopic data

4.1.5.1. *N*-benzyl-benzo[4,5]thieno[3,2-d]pyrimidin-4-amine substituted derivatives. *4.1.5.1.1. N-(2-methylbenzyl)-benzo [4,5]thieno[3,2-d]pyrimidin-4-amine (**4b**).* Compound **4b** (R1 = CH_3, R2 = H): mp: 220–222 °C; yield 63%; IR KBr, cm^{-1}: 3438(ν_{NH}); ^1H NMR (300 MHz, DMSO-d_6): δ ppm 2.37 (s, CH_3), 4.77 (d, J = 5 Hz, CH_2), 7.21 (m, 4H), 7.62 (m, 2H, H6 and H7), 8.13 (d, J = 7.9 Hz, 1H, H8), 8.25 (s, NH), 8.32 (d, J = 7.8 Hz, 1H, H5), 8.59 (s, 1H, H2; ^{13}C NMR (75 MHz, DMSO-d_6): δ ppm 18.1 (CH_3), 41.1 (CH_2), 113.7 (C4a), 122.4 to 129.3 (C5 to C8 and C13 to C16), 133.5 (C8a), 135.0 (C12), 136.3 (C11), 138.8 (C4b), 154.2 (C2), 154.5 (C8b), 156.5 (C4).

*4.1.5.1.2. N-(2-methoxybenzyl)-benzo[4,5]thieno[3,2-d]pyrimidin-4-amine (**4c**).* Compound **4c** (R1 = OCH_3, R2 = H): mp: 211–213 °C; yield 60%; IR KBr, cm^{-1}: 3417 (ν_{NH}); ^1H NMR (300 MHz, DMSO-d_6): δ ppm 3.84 (s, OCH_3), 4.75 (d, J = 4.4 Hz CH_2), 7.04 (m, 4H), 7.63 (m, 2H, H6 and H7), 8.17 (d, J = 7.9 Hz, 1H, H8), 8.32(d, J = 7.7 Hz, 1H, H5), 8.33 (s, NH), 8.57 (s, 1H, H2; ^{13}C NMR (75 MHz, DMSO-d_6): δ ppm 38.6 (CH_2), 55.2 (OCH_3), 110.3 (C10), 114.2 (C4a), 120.0 to 129.9 (C5 to C8, C11 and C14 to C16), 131.9 (C8a), 139.3 (C4b), 154.8 (C2), 156.5 (C8b, C12), 157.1 (C4).

*4.1.5.1.3. N-(4-methoxybenzyl)-benzo[4,5]thieno[3,2-d]pyrimidin-4-amine (**4d**).* Compound **4d** (R1 = H, R2 = OCH_3): mp: 190–192 °C; yield 58%; IR KBr, cm^{-1}: 3458(ν_{NH}); ^1H NMR (300 MHz, DMSO-d_6): δ ppm 3.70 (s, OCH_3), 4.70 (s, 2H, CH_2), 6.87 (d, J = 7.8 Hz, 2H, H3′, H5′), 7.31 (d, J = 7.8 Hz, 2H, H2′, H6′), 7.61 (m, 2H, H6 and H7), 8.15 (d, J = 7.8 Hz, 1H, H8), 8.31 (d, J = 7.4 Hz, 1H, H5), 8.48 (s, NH), 8.61 (s, 1H, H2; ^{13}C NMR (75 MHz, DMSO-d_6): δ ppm 42.8 (CH_2), 54.9 (OCH_3), 113.6 (C13, C15), 114.2 (C4a), 122.9 to 125.2 (C5, C7, C8), 128.6 (C12, C16), 129.3 (C6), 131.3 (C8a), 133.9 (C11), 139.3 (C4b), 154.8 (C2), 156.8 (C8b), 158.1 (C4, C14).

4.1.5.2. *N*-phenyl-benzo[4,5]thieno[3,2-d]pyrimidin-4-amine and substituted derivatives. *4.1.5.2.1. **N**-phenyl-benzo[4,5]thieno[3,2-d]pyrimidin-4-amine (**5a**).* Compound **5a** (R = H): mp: 266–268 °C; yield 74%; IR KBr, cm^{-1}: 3460 (ν_{NH}); ^1H NMR (300 MHz, DMSO-d_6): δ ppm 6.39 (t, J = 6.7 Hz, 1H), 6.64 (t, J = 7.3 Hz, 2H), 6.84 (t, J = 7.1 Hz, 1H), 6.94 (t, J = 7.2 Hz, 1H), 7.02 (d, J = 7.6 Hz, 1H), 7.40 (d, J = 7.8 Hz, 1H, H8), 7.61 (d, J = 7.4 Hz, 1H, H5), 7.97 (s,1H, H2), 8.89 (s, 1H NH; ^{13}C NMR (75 MHz, DMSO-d_6): δ ppm 114.9 (C4a), 122.3 to 129.5 (C5 to C8 and C12 to C15), 133.4 (C8a), 138.6 (C10), 139.5 (C4b), 154.1 (C2), 155.3 (C8b), 156.0 (C4).

*4.1.5.2.2. N-(2-methylphenyl)-benzo[4,5]thieno[3,2-d]pyrimidin-4-amine (**5b**).* Compound **5b** (R = CH_3): mp: 162–164 °C; yield 55%; IR KBr, cm^{-1}: 3440(ν_{NH}); ^1H NMR (300 MHz, DMSO-d_6): δ ppm 2.19 (s, CH_3), 7.28 to 7.32 (m, 4H), 7.56 (t, J = 7.2 Hz, 1H), 7.65 (t, J = 7.3 Hz, 1H), 8.07 (d, J = 7.7 Hz, 1H, H8), 8.33 (d, J = 7.5 Hz, 1H, H5), 8.60 (s,1H, H2), 9.59 (s, 1H NH); ^{13}C NMR (75 MHz, DMSO-d_6): δ ppm 17.9 (CH_3), 113.5 (C4a), 123.0 to 130.4 (C5 to C8 and C12 to C15), 133.4 (C8a), 135.8 (C11), 136.1 (C10), 139.9 (C4b), 154.7 (C2), 156.3 (C8b), 156.8 (C4).

4.1.5.3. *N*-benzyl-benzo[4,5]thieno[2,3-d]pyrimidin-4-amine and substituted derivatives. *4.1.5.3.1. **N**-benzyl-benzo[4,5]thieno[2,3-d]pyrimidin-4-amine (**7a**).* Compound **7a** (R = H): mp: 168–170 °C; yield 86%; IR KBr, cm^{-1}: 3375(ν_{NH}); ^1H NMR (300 MHz, DMSO-d_6): δ ppm 4.90 (s, CH_2), 7.32 (m, 7H), 7.86 (s, 1H, H8), 7.98 (s, NH), 8.43 (s, 1H, H2), 8.51 (d, J = 7.4 Hz, 1H, H5; ^{13}C NMR (75 MHz, DMSO-d_6): δ ppm 43.5 (CH_2), 109.8 (C4a), 122.7 to 127.8 (C5 to C8 and C12 to C16), 131.0 (C4b), 134.0 (C8a), 139.5 (C11), 154.6 (C2), 156.6 (C4), 167.0 (C8b).

*4.1.5.3.2. N-(2-methylbenzyl)-benzo[4,5]thieno[2,3-d]pyrimidin-4-amine (**7b**).* Compound **7b** (R = CH_3): mp: 164–166 °C; yield 70%; IR KBr, cm^{-1}: 3417(ν_{NH}); ^1H NMR (300 MHz, DMSO-d_6): δ ppm 2.39 (s, CH_3), 4.83 (s, CH_2), 7.14 (m, 5H), 7.57 (t, J = 8.4 Hz, 1H, H6), 7.99 (s, NH), 8.09 (d, J = 7.2 Hz, 1H, H8), 8.44 (s, 1H, H2), 8.63 (d, J = 7.2 Hz, 1H, H5; ^{13}C NMR (75 MHz, DMSO-d_6): δ ppm 19.3 (CH_3), 34.4 (CH_2), 110.5 (C4a), 123.6 to 126.8 (C5 to C8 and C14 to C16), 130.2 (C4b), 131.8 (C13), 134.6 (C12), 135.5 (C8a), 137.7 (C11), 155.5 (C2), 157.3 (C4), 167.7 (C8b).

4.2. Biological assays

4.2.1. Cell culture

Colorectal carcinoma cell lines HT29 and HCT116, and Hela cell line were cultured in RPMI 1640 containing 10% heat-inactivated FCS supplemented with sodium pyruvate (1 mM) and glutamax 1X. Cells were cultured in 75 cm^2 flasks and maintained at 37 °C in a fully humidified 5% CO_2 atmosphere. Subcultures were assessed by using trypsin/EDTA solution (1:250) for 5 min at 37 °C.

4.2.2. Mitochondrial membrane potential measurement ($\Delta\Psi$)

Apoptosis was evaluated by mitochondrial potential measurement with TMRM, a fluorescent lipophilic cation, which does not accumulate in depolarized mitochondria.[31,32] Cells were seeded at a density of 10.10^3 per well in 96-well microtiter plates and cultured for 24 h. Cells were then treated for 24 h with various appropriate dilutions of tested compounds. Cells were incubated in Krebs Ringer buffered saline (NaCl 130 mM, KCl 3.6 mM, HEPES 10 mM, $NaHCO_3$ 2 mM, NaH_2PO_4 0.5 mM, $MgCl_2$ 0.5 mM, $CaCl_2$ 1.5 mM, glucose 4.5 g/L, pH 7.42) supplemented with 200 nM TMRM and maintained at 37 °C in 5% CO_2, 95% air atmosphere for 30 min. Verapamil (20 μM) was added during TMRM incubation to block multidrug-resistant (MDR) pumps. Cells were then incubated with trypsin at 37 °C, resuspended in Krebs Ringer buffered saline supplemented with 4% fetal calf serum, and maintained at 4 °C before analysis.

4.2.3. Caspase activation measurement

Differential anchorage probes (DAP) described in a previous work[33] are useful tools allowing the detection of molecular events inside living cells such as caspase activation. HT29 cells stably expressing C3/7 DAP were generated as previously described.[33] To evaluate caspase activation on these cells, the plates were first washed with PBS (100 μL per well). Then cells were detached with 1 mM EDTA diluted in PBS (30 μL per well) for 30 min at 37 °C. After detaching, permeabilization solution (intracellular saline solution (130 mM KCl, 10 mM NaCl, 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 1 mM $MgSO_4$, 5 mM succinate, pH 7.2) supplemented with 50 μM digitonin and 50 μM ethylene glycol tetraacetic acid (EGTA)) was added (100 μL per well). Plates were incubated 10 min at room temperature and analyzed by flow cytometry.

4.2.4. Flow cytometry

96-well microtiter plates were then analyzed on a FACSCalibur flow cytometer (Becton Dickinson, USA) equipped with a 488 nm argon ion laser. Fluorescence emission was measured in FL1 log mode (515–545 nm) for GFP and in FL3 log mode (>650 nm) for TMRM. Forward scatter (FSC) and side scatter (SSC) were used to gate out cellular fragments. Gates were determined to separate fluorescent and non-fluorescent cells on each mode. The number of cells was calculated using the Cell Quest software.

4.2.5. Immunofluorescence

Cells were seeded onto glass coverslips in 96-well plates and fixed 24 h later with 3.7% formaldehyde for 10 min at room temperature. After 3 washes in PBS, cells were permeabilized in 0.5% Triton X-100 for 5 min. Nonspecific binding was blocked by incubating cells in 0.2% gelatin/PBS for 30 min at RT, and staining was performed using a specific primary antibody anti-EGFR (clone 31G7, Zymed laboratories) with a dilution 1/100 overnight at 4 °C and a fluorescent secondary antibody conjugate (Alexa Fluor 488 (green), Molecular Probes) for 1 h at RT. Nuclear staining was subsequently done with a 1/5000 dilution of Hoechst 33258 (10 mg/mL) (Molecular Probes) for 10 min at RT. Coverslips were washed once in PBS and then mounted with Fluoromount G on glass slides. Image acquisition was performed using a Zeiss LSM 510 Meta microscope.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2012.09.034.

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