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Original article

Synthesis and study of antiproliferative activity of novel thienopyrimidines on glioblastoma cells

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ABSTRACT

The receptor tyrosine kinases (for example EGFR, PDGFR, VEGFR) are a transmembrane protein family which plays a crucial role in tumor growth, survival, metastasis dissemination and angiogenesis. During the past 10 years, many tyrosine kinase inhibitors (TKIs) have been approved for cancer treatment (imatinib, gefitinib, erlotinib, sunitinib, sorafenib). These compounds generally possess a pyrrolo- or pyrimido- pyrimidine scaffold or approaching molecular structure. We synthesized 10 thienopyrimidine compounds (including 5 newly synthesized) whose scaffold is very similar to the agents cited above. The cytotoxicity of these agents was evaluated using a MTT assay and a flow cytometry technique on glioblastoma cell lines. Two compounds showed a similar cytotoxicity to the standard anti-EGFR gefitinib (IC50: gefitinib = 51.9 μM, **6b** = 61.8 μM, **6c** = 41.2 μM), suggesting a blockade of the EGFR pathway by binding to the TK receptor.

1. Introduction

The receptor tyrosine kinases (RTK) are a transmembrane protein family which plays a crucial role in tumor growth, survival, metastasis dissemination and angiogenesis. They are overexpressed in several types of tumors (in particular EGFR (epidermal growth factor receptor), PDGFR (platelet-derived growth factor receptor), FGFR (fibroblast growth factor receptor) and VEGFR (vascular endothelial growth factor receptor)) and they mediate the cellular transduction pathway consecutively to the binding of growth factors to their extracellular domain. Proliferation signaling is mediated after this binding by phosphorylation of the TK domain, which leads to a cascade of downstream signaling pathways. Abnormal signaling via these TK is linked to cancerous pathologies and to pathologies other than cancer, for example, cardiovascular and immunoinflammatory pathologies [1,2].

The ATP binding site of tyrosine kinase (TK) receptors could constitute a viable target for drug design [3,4] in these pathologies. Novel targeted therapies against these receptors have been synthesized and are now commonly used in clinical practice in various cancerous pathologies. Among these new agents, the TKIs (TK inhibitors) are small molecules that bind to the intracellular domain of growth factor receptors, inhibit autophosphorylation and consequently block the proliferation signaling pathway. These agents then prevent the proliferation of cells which have amplification or dysregulation of growth factor pathways. Compounds based on quinazoline, indolin or pyridopyrimidine scaffolds [5–11] have been synthesized and allow ATP-competitive binding on the TK domain of the growth factor receptors.

During the past 10 years, many TKIs have been approved for cancer treatment. Imatinib (anti Bcr/Abl kinase, c-kit, PDGFR) was the first TKI commercialized and became the standard therapy for chronic myelogenous leukemia (CML) with very impressive results, and for gastrointestinal stromal tumors (GIST). Erlotinib and gefitinib (anti-EGFR) are currently used in non-small cell lung cancer (NSCLC), sunitinib (anti VEGFR-2, PDGFR-β, c-kit and FLT-3 (FMS-like tyrosine kinase 3)) in renal cancer and GIST, sorafenib (anti Raf-kinase, VEGFR-2, PDGFR-β and c-kit) in renal cancer and

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hepatocellular carcinoma, and lapatinib (anti-EGFR and ErbB-2 (erythroblastic leukemia viral oncogene homolog 2)) in breast cancer. These compounds generally possess a pyrrolo- or pyrimidopyrimidine scaffold or approaching molecular structure.

In a previous work we synthesized a series of 3-substituted 1,2,3,4-tetrahydroquinazoline and 3-substituted thieno[2,3-d]pyrimidin-4-one compounds which presented a noticeable platelet antiaggregating power [12,13]. The most potent activity was exhibited by the thienopyrimidinone derivatives. These thienopyrimidine compounds present a similar scaffold to the agents cited above. Several studies have shown that the presence of a quinazoline skeleton substituted in position 4 by various substituted anilino groups potentially increased the EGFR inhibitory effect [14,15]. According to the results observed with the former thienopyrimidinone derivatives as platelet antiaggregating agents, the substitution of these compounds at the 4 position could lead to new PDGFR or EGFR pathway inhibitors. Moreover, authors recently show that compounds based on this thienopyrimidine scaffold could present an interest in the inhibition of VEGFR and PDGFR kinase domains [16]. This study describes the chemical synthesis of 10 thienopyrimidine derivatives and evaluates their activity on the epidermal growth factor receptor pathway.

2. Chemistry

The derivatives of thieno[2,3-d]pyrimidin-4-one were prepared according to a method described by Gewald [17,18], which involves the cyclization of the appropriate derivative of ethyl-2-aminothiophene-3-carboxylate with formamide. The use of phosphorus oxychloride affords the corresponding chlorosubstituted compounds which, by refluxing in dimethylformamide with the appropriate aniline, lead to the final derivatives of 4-anilinothieno [2,3-d]pyrimidine according to Scheme 1. Compounds synthesized are summarized in Table 1 with the different substitution groups.

3. Results

3.1. Cell characterization

Expression of EGF receptor was assessed on DBTRG.05-MG and U87-MG glioblastoma cell lines by immunocytochemistry and immunoblotting. Both techniques gave very similar results. The immunoblots in Fig. 1 show a 170 kDa band corresponding to EGFR for the DBTRG.05-MG cell line, demonstrating that DBTRG.05-MG displays high constitutive EGFR expression. This band was not present for the U87-MG cell line, representing a negative control of EGFR expression.

Table 1
Synthesized thieno[2,3-d]pyrimidine derivatives.

Compound	X	R^1	R^2	Reference
3a	Anilino	H	H	[25]
3b	Anilino	Methyl	H	[22]
3c	Anilino	Methoxy	H	[26]
3d	Anilino	H	Methoxy	[25]
3e	Dimethylamino	–	–	[27]
6a[a]	Anilino	H	H	–
6b[a]	Anilino	Methyl	H	–
6c[a]	Anilino	Methoxy	H	–
6d[a]	Anilino	H	Methoxy	–
6e[a]	Dimethylamino	–	–	–

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

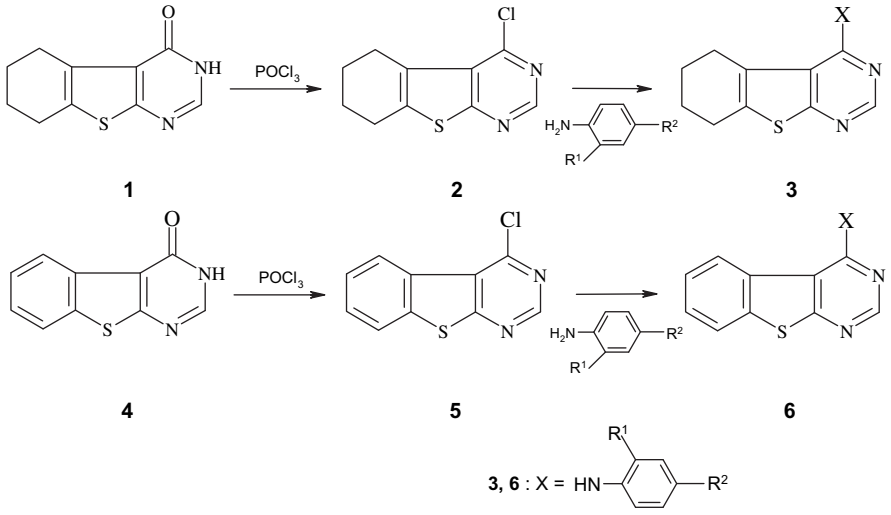
3.2. Cytotoxicity of the synthesized compounds

We assessed the cytotoxicity of DMSO, which was used as a dilution vehicle for the agents tested. DMSO showed very weak cytotoxicity (<10%) for concentrations ≤1% of DMSO. These DMSO concentrations corresponded to tested agent concentrations ≤200 μM. Consequently, concentrations ≤200 μM can be considered specific of the cytotoxicity of the tested agent itself and can be taken into account.

Gefitinib, which was used as a reference, was very cytotoxic on DBTRG.05-MG cells in a dose-dependent manner. IC_{50} was 51.9 ± 3.7 μM. Fig. 2A shows that two synthesized compounds (**6b** and **6c**) exerted a comparable cytotoxicity to gefitinib with IC_{50} of about 50 μM (IC_{50} **6b** = 61.8 ± 0.9 μM, IC_{50} **6c** = 41.2 ± 1.2 μM). Another group of synthesized agents (**6a**, **6d**, **6e**, **3d** and **3e**) was less cytotoxic (Fig. 2A and B) with IC_{50} around 100–150 μM (IC_{50} **6a** = 109.1 ± 9.3 μM, IC_{50} **6d** = 117.2 ± 8.3 μM, IC_{50} **6e** = 138.5 ± 1.9 μM, IC_{50} **3d** = 151.7 ± 11.7 μM, IC_{50} **3e** = 149.4 ± 13.9 μM). Finally, three compounds (**3a**, **3b** and **3c**) had very low cytotoxicity (IC_{50} **3a** = 584.7 ± 179.9 μM, IC_{50} **3b** = 474.1 ± 8.7 μM, IC_{50} **3c** = 571.0 ± 61.1 μM).

3.3. Apoptosis induced on two glioblastoma cell lines

Cell lines of glioblastoma (U87-MG and DBTRG.05-MG) were treated with increasing concentrations of **6b** (which shows



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

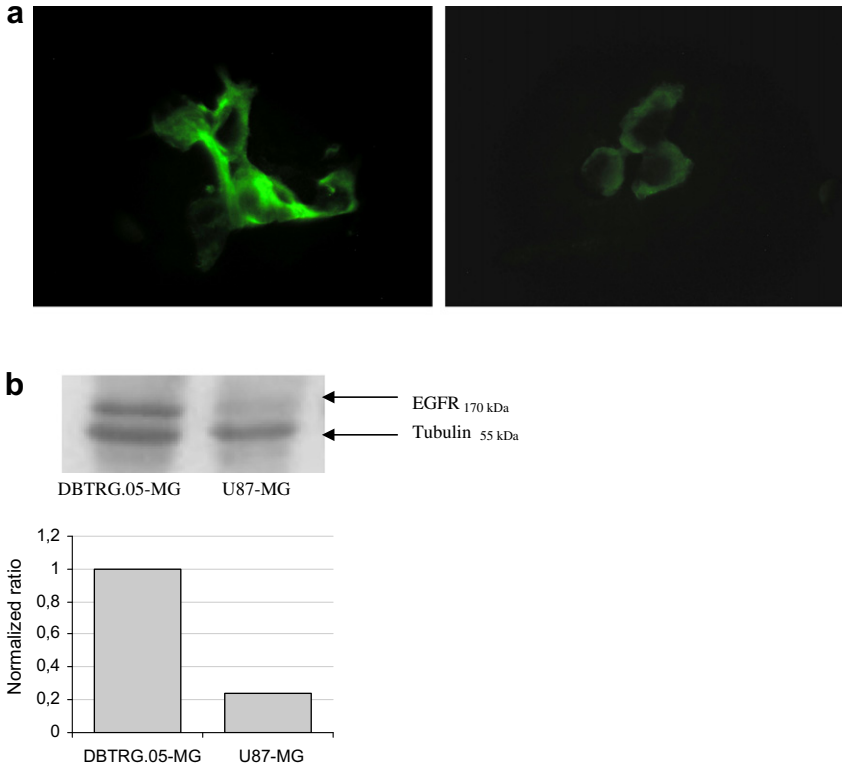


Fig. 1. EGFR expression on DBTRG.05-MG and U87-MG cell lines measured by immunocytochemistry (A) and western blotting analysis (B) as described in Experimental protocols. A: EGFR expression on DBTRG.05-MG left panel, on U87-MG right panel. B: Tubulin constitutes a control of the loaded quantity of protein. Histograms represent the ratio of EGFR band normalized with the tubulin band quantified using Image J software.

important cytotoxicity on DBTRG.05-MG) and **3c** (which induces no cytotoxicity on DBTRG.05-MG). The drug-induced depolarization of the mitochondrial membrane was used as a marker of apoptotic cells and was analyzed by flow cytometry, as described in Experimental protocols.

Fig. 3 shows the percentage of apoptotic cells achieved with **6b** (Fig. 3A) and **3c** (Fig. 3B). **6c** induces apoptosis on DBTRG cell line expressing EGFR (47% of apoptotic cells at 100 μM) meanwhile no induction of apoptosis is seen on U87 cell line lacking this receptor (4% of apoptotic cells at 100 μM). On the other hand **3c** induces no apoptosis on DGTRG neither than on U87 (Fig. 3B).

4. Discussion

In this work we evaluated the cytotoxicity of synthesized substituted thienopyrimidines on glioblastoma cell lines in order to assess the EGFR pathway blockade power of these agents. Cytotoxicity assays were performed using an MTT colorimetric assay evaluating the number of surviving cells after incubation for 48 h with the tested compounds.

In a previous study [19], gefitinib, which is used in NSCLC, was cytotoxic on the DBTRG.05-MG cell line expressing EGF receptor, so it can be used as a standard for assessing anti-EGFR agents. In our hands, gefitinib was very cytotoxic.

Two series of thienopyrimidine compounds were synthesized: the **3a**–**e** series with a cyclohexyl cycle and the **6a**–**e** series possessing a benzenic cycle. C-4 substitution permits to differentiate the synthesized agents: by an anilino group (**6a** and **3a**), by an ortho methylanilino group (**6b** and **3b**), by an ortho methoxyanilino group (**6c** and **3c**), by a para methoxyanilino group (**6d** and **3d**) and by a dimethylamino substituent (**6e** and **3e**).

Two compounds (**6b** and **6c**) showed a comparable cytotoxicity to gefitinib. In their structure they contain an aromatic cycle. On the other hand, the corresponding molecules of the **3** series (**3b** and **3c**) with the same C-4 substitution but without the aromaticity were weakly cytotoxic (4 times less cytotoxic). It seems therefore that the presence of this aromatic cycle near the thienopyrimidine scaffold is essential for anti-EGFR activity. Given this result, we replaced the aromatic substituent with an aliphatic group such as a dimethylamino moiety. **6e** was the least cytotoxic. In fact, replacement of the anilino group by a dimethylamino group led to a smaller conjugation. Since conjugation seems to participate in RTK binding, our finding confirmed the hypothesis about the importance of having an aromatic group linked to the C-4.

Substitution on this phenyl ring and the position of substitution could be of great importance. In fact, **6a** compound with no substitution and **6d** compound with a substitution in the para position were less cytotoxic than the substituted molecules in the ortho position (**6b** substituted by a methyl group and **6c** by a methoxy group). **6b** and **6c** showed comparable cytotoxicity, so the nature of the substitution seems to carry less importance than the position itself. This ortho substitution might therefore represent a privileged conformational position for RTK binding and for greater activity.

To assess whether the cytotoxic potency of these compounds is linked to EGFR binding, induction of apoptosis has been evaluated on glioblastoma cell lines expressing EGFR or not. We found that one of the most potent synthesized compound (**6b**) exerted a strong induction of apoptosis on the EGFR expressing DBTRG.05-MG cell line while it did not induce any apoptosis on U87-MG cell line which do not possess the EGF receptor. These data indicate that the effect observed seems to be linked to EGFR pathway blockade.

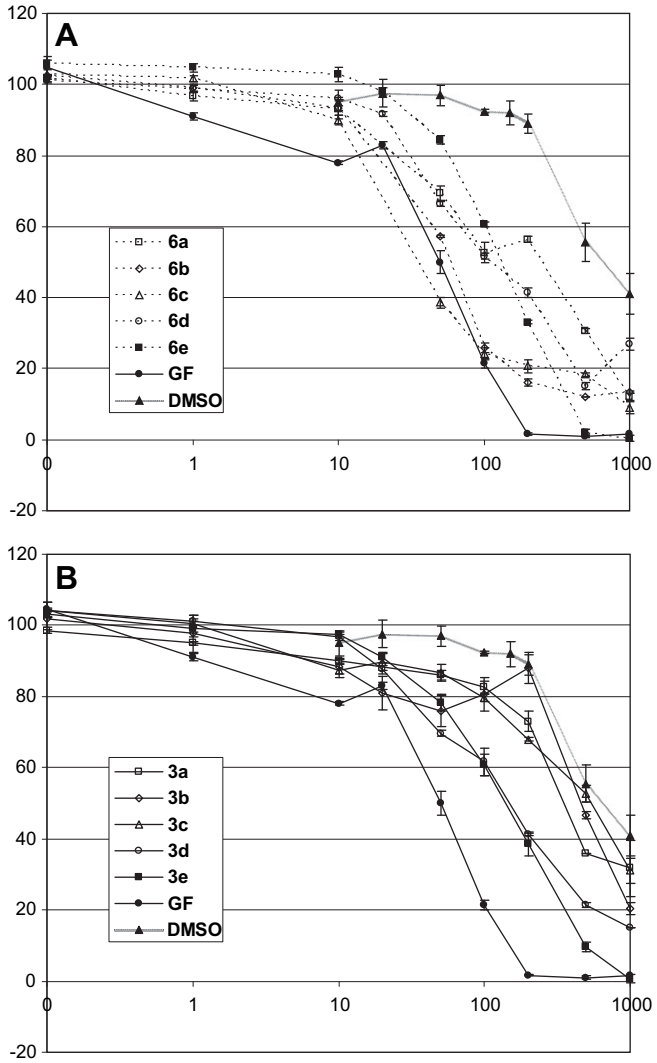


Fig. 2. Cytotoxicity of different thienopyrimidine compounds on DBTRG.05-MG cells. Cytotoxicity of anti-EGFR gefitinib and vehicle DMSO were also plotted. X-axis in μM. A: compounds with a benzenic cycle. B: compounds with a cyclohexyl cycle. Cell viability was determined using MTT assay as described in Experimental protocols. Each drug concentration was tested in triplicate. Data are presented as mean ± SD.

A general pharmacophore model for ATP and ATP-competitive inhibitors in the active site of RTK, notably EGFR TK, has been proposed [4,20,21]. It consists of a hydrophobic binding site for the adenine ring of ATP as well as for the heterocyclic scaffold of inhibitors such as quinazoline, pyridopyrimidine or eventually thienopyrimidine [4]. Three binding modes have been described differing in their inhibitor skeleton position. Most compounds with the pyrimidine scaffold have two H-bonding regions but a few have an additional 2-NH_2 group that could increase their binding potency. Our synthesized compounds do not possess this 2-NH_2 moiety but it can be hypothesized that they can achieve the two other hydrogen bonds, on one hand, with the 3-nitrogen atom and on the other, with the 4-anilino group. Moreover, one of the most potent compound synthesized (**6c**) could produce a third H-bonding with the oxygen atom of the methoxy group.

This work demonstrates that on glioblastoma cell lines harbouring EGF receptor, two of the new synthesized thienopyrimidine compounds exert strong cytotoxic activity comparable to that of the standard anti-EGFR gefitinib. The effect seems to be linked to the blockade of the EGFR pathway by binding to the TK domain. These agents could therefore have potential in treating this pathology and could be tested on other cell lines from other cancerous pathologies.

5. Experimental protocols

5.1. Chemistry

Melting points were determined on a Kofler apparatus. IR spectra were taken on a Shimadzu IR 470 Spectrometer. NMR spectra were recorded in DMSO-d_6 solution on a Bruker AMX 500 instrument (^{1}H: 300 MHz, ^{13}C: 75 MHz).

Thin layer chromatography (TLC) was performed using aluminium precoated plates (silica gel SDS 60F 254 Whatman, 0.2 mm thickness). Column chromatography was carried out on silica gel 60–200 μm (Merck).

5.1.1. General procedure for the preparation of thieno[2,3-d] pyrimidin-4-one derivatives (Scheme 1: compounds **1** and **4**)

The compounds were prepared according to previously described methods [17,18].

5,6,7,8-Tetrahydrobenzo[b]thieno[2,3-d]pyrimidin-4(3H)-one [18]
Benzo[b]thieno[2,3-d]pyrimidin-4(3H)-one[17]

5.1.2. General procedure for the preparation of 4-chlorothieno[2,3-d]pyrimidine derivatives (Scheme 1: compounds **2** and **5**)

The compounds were prepared according to previously described methods [22,23].

4-Chloro-5,6,7,8-tetrahydrobenzo[b]thieno[2,3-d]pyrimidine [22]
4-Chlorobenzo[b]thieno[2,3-d]pyrimidine [23]

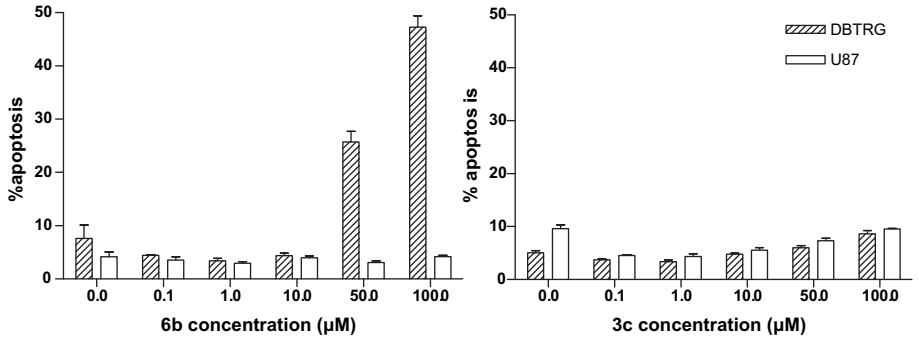


Fig. 3. Induction of apoptosis by **6b** (left panel) and **3c** (right panel) on DBTRG.05-MG and U87-MG cell lines measured by assessment of mitochondrial potential by flow cytometry as described in Experimental protocols. Each concentration was tested in triplicate. Data are presented as mean ± SD.

*5.1.3. General procedure for the preparation of 4-anilinothieno[2,3-d]pyrimidine and substituted derivatives [24] (Scheme 1: compounds **3** and **6**)*

A mixture of 0.01 mol of 4-chlorothieno[2,3-d]pyrimidine (Scheme 1: compounds **2** and **5**) and 0.02 mol 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.

1. *4-Anilino-5,6,7,8-tetrahydrobenzo[b]thieno[2,3-d]pyrimidine* [25] (**3a**)
2. *4-(2-Methylanilino)-5,6,7,8-tetrahydrobenzo[b]thieno[2,3-d] pyrimidine* [22] (**3b**)
3. *4-(2-Methoxyanilino)-5,6,7,8-tetrahydrobenzo[b]thieno[2,3-d] pyrimidine* [26] (**3c**)
4. *4-(4-Methoxyanilino)-5,6,7,8-tetrahydrobenzo[b]thieno[2,3-d] pyrimidine* [25] (**3d**)
5. *Procedure for the preparation of 4-dimethylaminobenzo[b]thieno [2,3-d]pyrimidine derivatives*
6. *4-Dimethylamino-5,6,7,8-tetrahydrobenzo[b]thieno[2,3-d] pyrimidine* [27] (**3e**)

*5.1.4. 4-Dimethylaminobenzo[b]thieno[2,3-d]pyrimidine (**6e**)*

0.01 mol of 4-chlorothieno[2,3-d]pyrimidine (Scheme 1: compounds **2** and **5**) 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.

5.1.5. Physical and spectroscopic data

5.1.5.1. 4-Anilinobenzo[b]thieno[2,3-d]pyrimidine and substituted derivatives. The general structure of the synthesized compounds is described in Fig. 4.

*5.1.5.1.1. 4-Anilinobenzo[b]thieno[2,3-d]pyrimidine (**6a**).* Compound **6a** (R^1=R^2=H): melting point: 166 °C; yield 60%; IR KBr, cm^{-1}: 3416 (ν_{NH}); ^{1}H NMR (300 MHz, DMSO-d_6): δ ppm 7.16 (t, J = 7.0 Hz, 1H), 7.39 (t, J = 7.0 Hz, 1H), 7.62 (m,4H), 8.14 (d, J = 7.0 Hz, 1H, H8), 8.56 (s, 1H, H2), 8.58 (d, J = 7.0 Hz, 1H, H5), 9.16 (s, NH), ^{13}C NMR (75 MHz, DMSO-d_6): δ ppm 111.6 (C4a), 123.1 à 128.3 (C5 à C8 et C12 à C16), 130.8 (C4b), 134.4 (C8a), 139.0 (C11), 154.5 (C2), 155.7 (C4), 168.2 (C8b).

*5.1.5.1.2. 4-(2-Methylanilino)benzo[b]thieno[2,3-d]pyrimidine (**6b**).* Compound **6b** (R^1=CH_3, R^2=H): melting point: 142 °C; yield 40%; IR KBr, cm^{-1}: 3375 (ν_{NH}); ^{1}H NMR (300 MHz, DMSO-d_6): δ ppm 2.18 (s, CH_3), 7.30 (m, 4H), 7.59 (m, 2H, H6 and H7), 8.14 (d, J = 7.5 Hz, 1H, H8), 8.42 (s, 1H, H2), 8.67 (d, J = 7.5 Hz, 1H, H5), 8.91 (s, NH), ^{13}C NMR (75 MHz, DMSO-d_6): δ ppm 17.9 (CH_3), 110.5 (C4a), 123.0 à 134.2 (C4b, C5 à C8 et C12 à C16), 134.9 (C8a), 137.3 (C11), 154.6 (C2), 156.2 (C4), 167.8 (C8b).

*5.1.5.1.3. 4-(2-Methoxyanilino)benzo[b]thieno[2,3-d]pyrimidine (**6c**).* Compound **6c** (R^1=OCH_3, R^2=H): melting point: 172 °C; yield 45%; IR KBr, cm^{-1}: 3442 (ν_{NH}); ^{1}H NMR (300 MHz, DMSO-d_6): δ ppm 3.88 (s, 3H, OCH_3), 7.04 (t, J = 7.0 Hz, 1H), 7.21 (m, 2H), 7.64 (m, 2H), 7.99 (d, J = 8 Hz, 1H), 8.18 (d, J = 7 Hz, 1H, H8), 8.47 (d, J = 7 Hz, 1H, H5), 8.56 (s, 1H, H2), 8.63 (s, NH), ^{13}C NMR (75 MHz, DMSO-d_6): δ ppm 55.8 (OCH_3), 110.9 (C4a), 111.2 à 126.3 (C5 à C8 et C13 à C16), 127.5 (C4b), 130.5 (C8a), 134.4 (C11), 151.0 (C12), 154.4 (C2), 155.3 (C4), 167.5 (C8b).

*5.1.5.1.4. 4-(4-Methoxyanilino)benzo[b]thieno[2,3-d]pyrimidine (**6d**).* Compound **6d** (R^1=H, R^2=OCH_3): melting point: 150 °C; yield 65%; IR KBr, cm^{-1}: 3434 (ν_{NH}); ^{1}H NMR (300 MHz, DMSO-d_6): δ ppm 3.78 (s, OCH_3), 6.98 (d, J = 8.0 Hz, 2H), 7.51 (d, J = 8.0 Hz, 2H), 7.59 (m, 2H, H6 and H7), 8.12 (d, J = 7.0 Hz, 1H, H8), 8.49 (s, 1H, H2), 8.61 (d, J = 7.0 Hz, 1H, H5), 8.97 (s, NH), ^{13}C NMR (75 MHz, DMSO-d_6): δ ppm 55.1 (OCH_3), 110.8 (C4a), 113.5 (C13, C15), 125.8 (C12, C16), 123.0 à 126.4 (C5 à C8), 130.9 (C4b), 131.5 (C8a), 134.3 (C11), 154.5 (C14), 156.0 (C2), 156.2 (C4), 167.8 (C8b).

*5.1.5.2. 4-Dimethylaminobenzo[b]thieno[2,3-d]pyrimidine (Fig. 5) (**6e**).* Compound **6e**: melting point: 96 °C; yield 55%; ^{1}H NMR (300 MHz, DMSO-d_6): δ ppm 3.11 (s, 6H, CH_3), 8.03 (d, J = 3.0 Hz, 1H, H8), 8.11 (d, J = 3.0 Hz, 1H, H5), 8.62 (s, 1H, H2), ^{13}C NMR (75 MHz, DMSO-d_6): δ ppm 40.5 (CH_3), 112.6 (C4a), 123.0 à 126.2 (C5 à C8), 131.1 (C4b), 134.2 (C8a), 153.6 (C2), 161.4 (C4), 168.9 (C8b).

5.2. Biological assays

5.2.1. Cell culture

Human glioblastoma cell line U87-MG (ECACC No: 89081402) and DBTRG.05-MG (ECACC No: 93061119) was purchased from the European Collection of Cell Cultures (ECACC, Salisbury, United Kingdom). DBTRG.05-MG cells were cultured in RPMI 1640 containing 10% heat-inactivated FCS supplemented with L-glutamine (2 mM), penicillin–streptomycin (100 units/mL–100 µg/mL), sodium pyruvate (1 mM) and hypoxanthine–thymidine (1%). U87-MG cells were cultured in EMEM containing 10% decomplemented FCS and supplemented with L-glutamine (2 mM), penicillin–streptomycin (100 units/mL–100 µg/mL), non-essential amino acids (1%), sodium pyruvate (1 mM). Cells were cultured in 75 cm^2 flasks (Greiner Bio-One GmbH, Frickenhausen, Germany) 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.

5.2.2. Immunocytochemistry

Detection of EGFR expression was carried out by immunocytochemistry [28,29] on DBTRG.05-MG cell line and on U87-MG cell line. Cells were seeded onto a glass coverslip. At confluence, the cells were washed twice with PBS. For EGFR determination, cells were fixed for 20 min at −20 °C with a methyl alcohol/acetone mixture (50/50, v/v). The cells were then incubated in the dark for 2 or 6 h with a primary mouse monoclonal anti-EGFR antibody diluted (1:100) with 1% BSA–PBS in order to determine EGFR. The

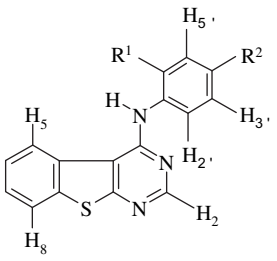


Fig. 4. General structure of the 4-substituted benzo[b]thieno[2,3-d]pyrimidine derivatives.

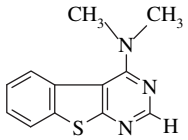


Fig. 5. Structure of 4-dimethylaminobenzo[b]thieno[2,3-d]pyrimidine.

coverslip was rinsed twice with 1% BSA–PBS for 15 min followed by an additional washing step with PBS. The glass coverslip was dried and cells were incubated for 1 h with the secondary FITC-conjugated goat anti-mouse antibody diluted (1:50) with 1% BSA–PBS. The coverslip was rinsed, dried and mounted in mineral oil on a microscope slide. Fluorescent images of immunolabeled cells were observed using an Olympus BH-2 fluorescent microscope and captured with an Olympus C-5060 digital camera.

5.2.3. Immunoblot analysis of EGFR

Cells were cultured in 60 mm Petri dishes and incubated with various concentrations of temozolomide, carboplatin, gefitinib or bortezomib for 48 h. Cells were lysed at +4 °C with lysis buffer (protease inhibitor cocktail (Sigma–Aldrich) supplemented with 0.3% SDS and 0.75% β-mercaptoethanol), sonicated and cleared by centrifugation. Supernatant was collected and protein concentration determined using the Bio-Rad Protein Assay kit (Bio-Rad Laboratories GmbH, München, Deutschland). An equal volume of Laemmli solution supplemented with β-mercaptoethanol (5%) was added to a supernatant aliquot and the mixture was heated for 10 min at 80 °C. Samples (15 μg of protein) were loaded on each lane and proteins were separated by 10% SDS polyacrylamide gel electrophoresis. Proteins were then transferred onto a PVDF (polyvinylidene fluoride) membrane (NEN Life Science Product, Boston, MA). Membrane was blocked for 2 h in TBST (Tris buffered saline Tween 20) containing 10% low fat dry milk and probed with primary antibody diluted in TBST with 5% low fat dry milk overnight at 4 °C. Primary antibody dilutions were as follows: 1/4000 for mouse anti-α-tubulin antibody, 1/1000 for mouse anti-EGFR antibody. The membrane was then washed 4 times with TBST, incubated with horseradish peroxidase (HRP)-conjugated anti-mouse secondary antibody (1/2000 in TBST with 5% low fat dry milk) for 2 h at room temperature. After washing 4 times for 10 min with TBST, protein bands were visualized by using the Western Lightning enhanced chemiluminescence kit (PerkinElmer Life Sciences, Boston, MA).

5.2.4. Cytotoxicity assay

The effect of the tested compounds on cell viability was assessed using the MTT assay as described [30]. The MTT assay is based on the capacity of mitochondrial dehydrogenase enzymes in living cells to convert the yellow water-soluble substrate MTT into a dark blue formazan product. The amount of formazan is directly proportional to the viable cell number. Briefly, glial cells were seeded at a density of 6×10^3 per well in 96-well microtiter plates and cultured for 72 h. Cells were treated for 72 h with various appropriate dilutions of tested compounds as described above. Thereafter, cells were stained with 200 μL MTT solution (0.5 mg/mL) at 37 °C. DMSO was added to each well to solubilize the intracellular formazan crystals. After staining homogenization, absorbances were measured using a multi-well scanning spectrophotometer (Titertek Multiskan Plus, Labsystems, Finland) at a wavelength of 570 nm using a reference of 630 nm. Data are expressed as the percentage of viability compared to untreated control wells performed in the same experiment. The cell viability curves were plotted and the IC_{50} (concentration required to inhibit 50% of cell growth) values determined.

5.2.5. Apoptosis determined by flow cytometry

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 6×10^3 per well in 96-well microtiter plates and cultured for 48 h. Cells were then treated for 48 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 HBSS supplemented with 4% fetal calf serum, and maintained at 4 °C. 96-well microtiter plates were then analyzed on a FACSCalibur flow cytometer (Becton Dickinson) equipped with a 488-nm argon ion laser and fluorescence emission was measured in FL3 log mode. Forward scatter (FSC) and side scatter (SSC) were used to gate out cellular fragments. The number of apoptotic cells was calculated using the Cell Quest software.

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