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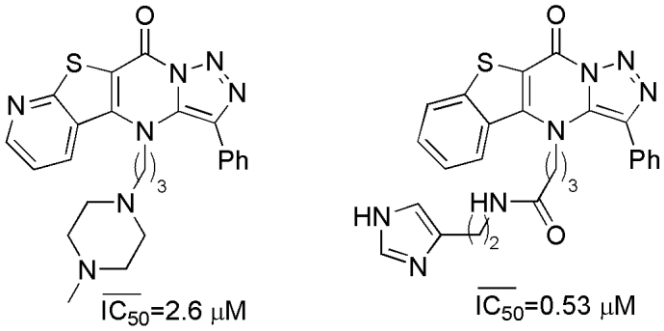

Dimroth rearrangement occurred in the synthesis of annelated thieno[2,3-*e*][1,2,3]triazolo[1,5-*a*]pyrimidine core, giving the linear isomer thieno[3,2-*d*][1,2,3]triazolo[1,5-*a*]pyrimidine. Derivatives of this skeleton possess potent antitumor activity.

An Unexpected Dimroth Rearrangement Leading to Annelated Thieno[3,2-d][1,2,3]triazolo[1,5-a]pyrimidines with Potent Antitumor Activity

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Research Highlights

- An unusual Dimroth rearrangement led to new thienotriazolopyrimidine derivatives.
- The biological screenings showed their strong antiproliferative activity.
- The most active compound showed low toxicity and high potency *in vivo*.

An Unexpected Dimroth Rearrangement Leading to Annelated Thieno[3,2-*d*][1,2,3]triazolo[1,5-*a*]pyrimidines with Potent Antitumor Activity

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ABSTRACT

An unusual Dimroth rearrangement occurring in the reaction leading to annelated thieno[2,3-*e*][1,2,3]triazolo[1,5-*a*]pyrimidine core allowed the isolation of the linear isomer thieno[3,2-*d*][1,2,3]triazolo[1,5-*a*]pyrimidine. By decorating the linear isomer with the same chains that improved the biological activity of the angular isomers, new annelated thieno[3,2-*d*][1,2,3]triazolo[1,5-*a*]pyrimidines were designed and synthesized. They were selected by the Development Therapeutical Program (DTP) of the National Cancer Institute (NCI) for the anticancer screening against a panel of 60 human tumor cell lines. The biological results showed that the new derivatives exhibited strong antiproliferative activity up to nanomolar concentration. *In vivo* screenings of the most active compound, the *N*-[2-(1*H*-imidazol-4-yl)ethyl]-4-(3-phenyl-10-oxo-4,10-dihydrobenzothieno[3,2-*d*][1,2,3]triazolo[1,5-*a*]pyrimidin-4-yl)butanamide, showed its low toxicity and high potency.

Keywords: Annelated thienotriazolopyrimidines, Domino reactions, Dimroth rearrangement, Developmental Therapeutics Program (DTP), Anticancer agents.

Highlights:

- An unusual Dimroth rearrangement led to new thienotriazolopyrimidine derivatives.
- The biological screenings showed their strong antiproliferative activity.
- The most active compound showed low toxicity and high potency *in vivo*.

1. INTRODUCTION

Heterocyclic compounds represent the most numerous class that is present among the known drugs [1]. Typically, they are scaffolds that need to be decorated with selected substituents to exert the biological effect. Since many years we have been interested in the design and synthesis of compounds with antiproliferative activity. *In silico* design methodology (molecular docking, chemometric protocols, etc) allowed to identify annelated pyrrolo[3,2-*e*]pyrimidine derivatives of type **1** [2] and indolo[2,3-*e*][1,2,3]triazolo[1,5-*a*]pyrimidines of type **2** [3] with a wide spectrum of antitumour activities (Figure 1).

Fig. 1.

The synthesis of annelated pyrrolo-pyrimidine core of type **1** was performed through the domino reaction involving 2-amino-3-cyanopyrroles and N-[bis(methylthio)methylene]amino moiety (BMMA) [4]. While in the synthesis of indolo[2,3-*e*][1,2,3]triazolo[1,5-*a*]pyrimidine core **2** was useful employed the 1,3-cycloaddition reaction (13DCR) of ortho-azido derivatives and acetonitriles in domino reaction [3]. The 13DCRs have been widely applied for many years in the synthesis of polyheterocycles as scaffolds in the search of biologically active compounds [5-9]. Moreover, the 13DCRs were recently applied in the synthesis of annelated thieno[2,3-*e*][1,2,3]triazolo[1,5-*a*]pyrimidine cores of type **3** (Scheme 1) [10], designed through the Virtual Lock And Key (VLAK) protocol [11].

Scheme 1.

They were synthesized starting from the ethyl 3-azido-benzo[*b*]thiophene-2-carboxylate (**4a**) or the aza analog ethyl 3-azidothieno[2,3-*b*]pyridine-2-carboxylate (**4b**) and acetonitriles **5** (Scheme 1) to give the intermediates **6**. These last were decorated through optimized protocols to give **3**.

Three of these derivatives **3aA**, **3bA**, and **3bB** (Figure 2) showed excellent antiproliferative activity up to sub-nanomolar concentration against a large spectrum of human tumor cell lines [10].

Fig. 2.

2. RESULTS AND DISCUSSION

2.1 Chemistry

The first step in the synthesis of **3aA** starts from the reaction of the core **7a** with ethyl 4-bromobutyrate in-N,N-DiMethylFormamide (DMF) and K_2CO_3 (Scheme 2) [10]. In addition to the expected product **8**, the linear isomer **9** was isolated, in low yield.

Scheme 2.

This experimental evidence is not entirely surprising. In fact it is known as the pyrrolo/indolo -[1,2,3]triazolo[1,5-*a*]pyrimidines **10**, in basic conditions, are converted to linear isomers **11** through a Dimroth rearrangement (Scheme 3) [12,13].

Scheme 3.

Therefore, in basic conditions, the presence of the pyrimidine ring induced a partial rearrangement to linear isomer **9**, before the reaction could reach total conversion to final angular isomer **8**.

The serendipitous experimental evidence promoted us to synthesize new derivatives of the linear isomer with the same chains that had increased the antiproliferative activity of the angular isomer [10].

Different attempts were carried out to optimize the reaction conditions in order to address the isolation of the linear isomer in good yields, as reported in experimental section.

Thus, the linear isomer **9** was hydrolyzed with NaOH in EtOH/H_2O mixture to give the corresponding linear intermediate **12**. Finally, the derivative **13** was obtained by the reaction of the carboxylic acid derivative **12** with histamine and EDCI/DMAP (EDCI, 1-Ethyl-3-(3-Dimethylaminopropyl)CarbodiImide; DMAP, 4-DiMethylAminoPyridine) (Scheme 4).

Scheme 4.

Again a partial rearrangement was observed in the reaction leading to the chloropropyl derivative **14**. Thus, this reaction gave also the linear isomer **15**. Upon refluxing isomer **15** in the respective reagent under *solvent-free* conditions, derivative **16** was obtained (Scheme 4).

2.2 Biological screening

The new derivatives **13**, **16** and the intermediates **9**, **12**, and **15** were subjected to the NCI disease-oriented human cell lines screening assay to be evaluated for their *in vitro* antitumor activity.

Derivatives **12**, **13**, and **16** passed the selection criteria adopted by DTP NCI screening and consequently were *in vitro* analyzed.

A single dose (10 μM) of the accepted compounds was tested against a panel of approximately 60 human tumor cell lines grouped in nine disease subpanels; namely, breast, central nervous system, colon, leukemia, melanoma, non small-cell lung, ovarian, renal, and prostate tumors cell lines. In table 1 the overview of one dose screening for the new derivatives **12**, **13** and **16** is reported (Supplementary material) [14-16].

Table 1.

With the aim to compare the antiproliferative activity, the biological data of the previously angular isomers **3** were also reported [10].

In the biological protocol, each cell line is inoculated and preincubated on a microtiter plate. Test compounds were then added at a single concentration and the culture is incubated for 48 h. End-point determinations were made with alamar blue [17]. Results for each tested compound are reported as the percent of growth (G%) of the treated cells when compared to the untreated control cells. Compounds which reduced the growth of any one of the cell lines to approximately 32% or less, are passed on for evaluation in the full panel of 60 cell lines over a 5-log dose range. The new annelated thieno[3,2-*d*][1,2,3]triazolo[1,5-*a*]pyrimidines generally exhibited excellent antiproliferative activity. In particular, derivatives **13** and **16** showed G% mean values of -39 and 13, respectively (Table 1).

These two derivatives exhibited G% values significantly under the level of 32% for at least one tumor cell line. Thus, according with the selection criteria of DTP NCI protocol, they were passed to five dose concentrations screening.

In the five dose screening, the antitumor activity is given by three parameters for each cell line: pGI_{50} value (GI_{50} is the molar concentration of the compound that inhibits 50% of cell growth), pTGI value (TGI is the molar concentration of the compound leading to total inhibition of cell growth), and pLC_{50} value (LC_{50} is the molar concentration of the compound that induces 50% cell death). Moreover, a Mean Graph MIDpoint (MG_MID) is calculated for each of the mentioned parameters, giving an average activity parameter over all cell lines. For the calculation of the MG_MID, insensitive cell lines are included with the highest concentration tested. The discovery of compounds with new selectivity patterns is one of the targets of the Developmental Therapeutical Screening Program (DTP) screening program. Selectivity of a compound towards certain cell line is characterized by a high deviation of the particular cell line parameter compared to the Mean Graph MIDpoint (MG_MID) value. The overview of pGI_{50},

pTGI, and pLC_{50} values are reported in table 2, together with the number of human tumor cell lines investigated. Also, the biological data of the previously reported angular isomers **3** [10] are presented with the aim to compare the results.

Table 2.

Evaluation of the detailed biological data (Table 3) revealed that the derivatives selected for the five dose screening, showed a significant antiproliferative activity against all human tumor cell lines investigated, generally in the sub-micromolar concentration.

Table 3.

The analysis of the biological data, as MG_MID values of angular isomers **3aA**, **3bA**, and **3bB** [10] and the new linear isomers **13** and **16** revealed that isomer **13** is the most active compound with pGI_{50} and pTGI values of 6.28 and 5.51, respectively, followed by angular isomer **3aA**, and **16** (Table 3).

With respect to the tumor subpanels (Table 3), compound **13** was particularly effective against colon cancer and leukemia cell lines. In fact, the calculated pGI_{50} MG_MID values for these sub-panels (6.86 and 7.31, respectively) were always much higher than the overall MG_MID value (6.28).

Compound **13** showed excellent activity against HCT-116 ($pGI_{50}>8.00$), SF-539, ($pGI_{50}=7.69$), CCRF-CEM ($pGI_{50}=7.81$), and SN12C ($pGI_{50}=7.96$).

Moreover, it has to be underlined that the anticancer activity of **13** against the OVCAR-8 cell line with $pGI_{50}>8$ and $pLC_{50}<4$, demonstrating the highest potency and least toxicity. A similar behavior was observed in case of the angular isomer **3aA** against OVCAR-8 ($pGI_{50}=6.49$, $pLC_{50}<4$) and HCT-116 ($pGI_{50}=6.47$, $pLC_{50}<4$) cell lines [10].

Particular attention should also be paid when comparing the antiproliferative activity of angular isomers **3aA** and **3bB** and the corresponding linear isomers **13** and **16**. Although having the same side chain, the different structural conformation (angular or linear) of the tetracyclic system deeply influences the biological activity.

Compound **13** with the highest pGI_{50} value, was selected by DTP NCI protocol for the acute toxicity test. This consists of a rapid procedure used to measure the concentration that will make the tested organisms sick.

Thus, three different mice received doses of 400, 200 and 100 mg/Kg of compound **13**. The mice are observed for a period of two weeks. They are sacrificed if they lose more than 20% of their body weight or if there are other signs of significant toxicity. If all three mice sacrificed, then the next three dose levels (50, 25, 12.5 mg/kg) should be tested in a similar way. This process is repeated until a tolerated dose is found.

The obtained data showed that compound **13** was not toxic at all tested concentrations; no negative effects were observed in the screened mice for the two weeks of tests.

Therefore, compound **13** is considered to be a good candidate as anticancer agent.

Further *in vivo* analyses and studies are in progress by DTP NCI, to elucidate the mechanism of action, and will be reported elsewhere.

The biological data provided by DTP NCI screening were useful to analyze the different biological activity spectrum of the angular and linear isomers. Since many years, the analysis COMPARE [18] is available from DTP NCI. This facility is able to match the biological data of a screened compound with specific databases, with the aim of finding the drug that best fits the input compound.

The most active compound **13** tested as seed against the NCI "Standard Agents" database, showed a Pearson correlation coefficient (PCC) of 0.551 for Aclacinomycin A and of 0.470 for *N*,*N*-dibenzyldaunomycin, known inhibitors of topoisomerase II.

Recently, CellMiner, an evolution of COMPARE analysis, was developed [19]. This is a web-based suite of Genomic and Pharmacologic Tools to explore transcript and drug patterns in the NCI-60 cell line set. Thus, by submitting the NCI DTP results of the most active compound **13** and its angular isomer **3aA**, it emerged that they have different biological profiles. In fact, the PCC of **13** on **3aA** is 0.716 and the significant gene correlations and the microRNA correlations showed a quite different patterns (supplementary material).

3. CONCLUSIONS

An unusual Dimroth rearrangement in the reaction leading to annelated thieno[2,3-*e*][1,2,3]triazolo[1,5-*a*]pyrimidine core allowed to identify annelated thienotriazolopyrimidines with antitumor activity up to nanomolar concentration. The derivative *N*-[2-(1*H*-Imidazol-4-yl)ethyl]-4-(3-phenyl-10-oxo-4,10-dihydrobenzothieno[3,2-*d*][1,2,3]triazolo[1,5-*a*]pyrimidin-4-yl)butanamide **13** revealed to be an excellent candidate as antitumor agent. In fact, it showed antiproliferative activity against all tested tumor cell lines, up to nanomolar concentration. Moreover, preliminary *in vivo* tests showed that it has high potency and low cytotoxicity. Two factors contributed to the identification of this compound: the computational approach VLAK applied taking the advantage of old biological results on indolo[2,3-*e*][1,2,3]triazolo[1,5-*a*]pyrimidines, and the serendipitous synthesis that drove to build the linear core of type **13**. Finally, this scaffold represents a new ring system that can be used as lead in further optimization. The statistical analysis of the biological data of the designed compounds,

performed through COMPARE and CellMiner Tools, allowed to underline the difference in biological property between the angular and linear isomers.

4. EXPERIMENTAL

4.1. Chemistry

Unless otherwise indicated, all reagents and solvents were purchased from commercial sources and used without further purification. All melting points (°C) were determined on a Büchi–Tottoli capillary apparatus and are uncorrected; IR spectra were determined in bromoform with a Jasco FT/IR 5300 spectrophotometer. ^{1}H NMR, ^{13}C NMR spectra were recorded in DMSO-d_6 solution, unless otherwise specified, at 200 and 50.3 MHz, respectively, using a Bruker AC-E series 200 MHz spectrometer. Chemical shift values are given in ppm and referred as the internal standard to TMS (tetramethylsilane). The purity of all compounds screened in biological assays was determined to be >95% by HPLC/MS analysis. Mass spectroscopy was performed using a GC-MS Shimadzu QP5050 with EI (75ev). Microanalyses were in agreement with theoretical values ±0.4%. Thin layer chromatography was performed on precoated (0.25 mm) silica gel GF_{254} plates, compounds were detected with 254nm UV lamp. Column chromatography was performed with Merck silica gel ASTM (230–400 mesh), or Merck aluminium oxide 90 (70-230 mesh), and with a Biotage FLASH40i chromatography module (prepacked cartridge system).

4.1.1. General procedure for preparation of ethyl 4-(5-oxo-3-phenylbenzo[4,5]thieno[2,3-*e*][1,2,3]triazolo[1,5-*a*]pyrimidin-4(5*H*)-yl)butanoate (**8**) and ethyl 4-(10-oxo-3-phenylbenzo[4,5]thieno[3,2-*d*][1,2,3]triazolo[1,5-*a*]pyrimidin-4(10*H*)-yl)butanoate (**9**).

To a stirred suspension of **7a** (1.0 mmol) in *N*,*N*-dimethylformamide (5 mL), potassium carbonate (3.0 mmol) and ethyl 4-bromobutyrate (5.0 mmol) were added. The mixture was

refluxed for 4 h, cooled to room temperature, and then slowly poured onto ice-water. The crude solid was filtered and purified by column chromatography using dichloromethane/ethyl acetate 98:2 as eluent to afford both **8** (0.11 g, yield 26%), and **9** (0.30 g. yield 70%).

4.1.1.1. Ethyl 4-(5-oxo-3-phenylbenzo[4,5]thieno[2,3-*e*][1,2,3]triazolo[1,5-*a*]pyrimidin-4(5*H*)-*yl*)butanoate (**8**): Mp 123.6-124.0°C. IR: 1725, 1677 (2CO) cm^{-1}. ^{1}H NMR ($CDCl_3$) δ: 1.22 (t, J= 7.4 Hz, 3H, $OCH_2\underline{CH_3}$), 2.07-2.15 (m, 2H, $CH_2\underline{CH_2}CH_2$), 2.41 (t, J= 7.2 Hz, 2H, CH_2CO), 4.04-4.12 (q, J= 7.4 Hz, 2H, $O\underline{CH_2}CH_3$), 5.04 (t, J= 7.2 Hz, 2H, NCH_2), 7.49-7.62 (m, 5H, C8-H, C9-H, C3'-H, C4'-H, C5'-H), 7.90 (d, J= 7.4 Hz, 1H, C7-H), 8.48 (d, J= 7.4 Hz, 1H, C10-H), 8.60 (d, J= 8.0 Hz, 2H, C2'-H, C6'-H). ^{13}C NMR ($CDCl_3$) δ: 14.1 (q), 23.6 (t), 31.1 (t), 55.3 (t), 60.7 (t), 115.7 (s), 123.4 (d), 124.3 (d), 125.0 (d), 127.0 (dx2), 128.6 (s), 128.8 (dx2), 129.5 (d), 129.9 (d), 134.1 (s), 139.2 (s), 142.1 (s), 145.9 (s), 152.0 (s), 155.5 (s), 172.2 (s). Elem. Anal. calcd. for $C_{23}H_{20}N_4O_3S$: C, 63.87; H, 4.66; N, 12.95; found: C, 63.61; H, 4.83; N, 12.54.

4.1.1.2. Ethyl 4-(10-oxo-3-phenylbenzo[4,5]thieno[3,2-*d*][1,2,3]triazolo[1,5-*a*]pyrimidin-4(10*H*)-yl)butanoate (**9**): Mp 217.2-217.7°C. IR: 1730, 1690 (2CO) cm^{-1}. ^{1}H NMR ($CDCl_3$) δ: 1.26 (t, J= 7.4 Hz, 3H, $OCH_2\underline{CH_3}$), 2.21-2.34 (m, 2H, $CH_2\underline{CH_2}CH_2$), 2.59 (t, J= 7.3 Hz, 2H, CH_2CO), 4.12-4.20 (q, J= 7.4 Hz, 2H, $O\underline{CH_2}CH_3$), 4.67 (t, J= 7.3 Hz, 2H, NCH_2), 7.24-7.63 (m, 5H, C6-H, C7-H, C3'-H, C4'-H, C5'-H), 7.90 (d, J= 7.0 Hz, 1H, C8-H), 8.35 (d, J= 8.1 Hz, 2H, C2'-H, C6'-H), 9.04 (d, J= 7.0 Hz, 1H, C5-H). ^{13}C NMR ($CDCl_3$) δ: 14.2 (q), 24.0 (t), 30.9 (t), 60.7 (t), 67.2 (t), 123.1 (d), 125.6 (d), 126.2 (d), 126.5 (dx2), 127.4 (d), 128.0 (s), 128.5 (s), 128.6 (dx2), 129.5 (d), 131.1 (s), 133.2 (s), 135.2 (s), 137.9 (s), 140.4 (s), 157.6 (s), 172.9 (s). Elem. Anal. calcd. for $C_{23}H_{20}N_4O_3S$: C, 63.87; H, 4.66; N, 12.95; found: C, 63.93; H, 4.74; N, 12.87.

4.1.2. 4-(10-Oxo-3-phenylbenzo[4,5]thieno[3,2-*d*][1,2,3]triazolo[1,5-*a*]pyrimidin-4(10*H*)-yl)butanoic acid (**12**).

To a stirred suspension of the ethoxycarbonyl derivative **9** (0.5 mmol) in absolute ethanol (5 mL), a solution of potassium hydroxide (0.13 g, 2.5 mmol) in water (2 mL) was added dropwise. The mixture was stirred at room temperature for 1-8 h. The solvent was evaporated under reduced pressure. The residue was dissolved in water (10 mL) and then carefully adjusted to pH=1 with 6N hydrochloric acid. The precipitated solid was filtered, dried and recrystallized from ethanol to afford the corresponding acid **12** as white solid, 0.40 g. Yield 100%. Mp 219.3-220.0°C. IR: 3519-2900 (OH), 1720, 1681 (2CO) cm^{-1}. ^{1}H NMR δ: 1.94-2.05 (m, 2H, $CH_2\underline{CH_2}CH_2$), 2.33 (t, J= 7.4 Hz, 2H, CH_2CO), 4.96 (t, J= 7.4 Hz, 2H, NCH_2), 7.52-7.69 (m, 5H, C6-H, C7-H, C3'-H, C4'-H, C5'-H), 8.12 (d, J= 7.3 Hz, 1H, C8-H), 8.38 (d, J= 7.3 Hz, 1H, C5-H), 8.52 (d, J= 8.0 Hz, 2H, C2'-H, C6'-H), 10.62 (s, 1H, COOH). ^{13}C NMR δ: 22.8 (t), 29.8 (t), 54.5 (t), 123.0 (d), 123.3 (d), 124.0 (s), 124.7 (d), 125.8 (dx2), 128.0 (s), 128.1 (d), 128.2 (dx2), 129.1 (d), 131.2 (s), 133.4 (s), 135.4 (s), 137.9 (s), 140.7 (s), 157.5 (s), 173.9 (s). Elem. Anal. calcd. for $C_{21}H_{16}N_4O_3S$: C, 62.36; H, 3.99; N, 13.85; found: C, 62.43; H, 3.87; N, 13.89.

4.1.3. *N*-(2-(1*H*-Imidazol-4-yl)ethyl)-4-(10-oxo-3-phenylbenzo[4,5]thieno[3,2-*d*][1,2,3]triazolo[1,5-*a*]pyrimidin-4(10*H*)-yl)butanamide (**13**).

To a stirred suspension of the acid **12** (0.44 mmol) in dioxane (5 mL), 4-dimethylaminopyridine (DMAP), 0.05 g, 0.45 mmol) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDCI) (0.17g, 9.2 mmol) were added under nitrogen atmosphere, at 0°C. After 2 h of stirring at room temperature, histamine (0.102 g, 9.2 mmol) was carefully added and the reaction mixture was incubated at 50°C for 12h. The reaction mixture was evaporated under reduced pressure and the crude product was purified by column chromatography using dichloromethane/methanol 9:1 as

eluent. Pale yellow solid, 0.15 g. Yield 70%. Mp 140.7-141.2°C. IR: 3360 (NH), 1722, 1650 (2CO) cm^{-1}. ^1H NMR δ: 1.96-2.05 (m, 2H, $CH_2\underline{CH_2}CH_2$), 2.17 (t, J= 7.4 Hz, 2H, $COCH_2$), 2.53 (t, J= 7.3 Hz, 2H, $\underline{CH_2}CH_2NH$), 3.17 (t, J= 7.3 Hz, 2H, $CH_2\underline{CH_2}NH$), 4.97 (t, J= 7.4 Hz, 2H, NCH_2), 6.72 (s, 1H, C5"-H), 7.41-7.99 (m, 8H, C6-H, C7-H, C3'-H, C4'-H, C5'-H, C2"-H, 2NH), 8.16 (d, J= 7.4 Hz, 1H, C8-H), 8.44 (d, J= 7.4 Hz, 1H, C5-H), 8.56 (d, J= 8.1 Hz, 2H, C2'-H, C6'-H). ^{13}C NMR δ: 24.1 (t), 26.9 (t), 32.0 (t), 40.7 (t), 55.5 (t), 122.2 (d), 123.7 (s), 124.1 (d), 124.9 (d), 125.5 (d), 126.5 (dx2), 128.4 (d), 128.9 (dx2), 129.7 (d), 131.1 (s), 133.7 (d), 134.6 (s), 135.5 (s), 137.3 (s), 141.1 (s), 142.3 (s), 151.4 (s), 154.5 (s), 170.7 (s). Elem. Anal. calcd. for $C_{26}H_{23}N_7O_2S$: C, 62.76; H, 4.66; N, 19.71; found: C, 62.83; H, 4.72; N, 19.67.

4.1.4. General procedure for preparation of 4-(3-chloropropyl)-3-phenylpyrido[3',2':4,5]thieno[3,2-*d*][1,2,3]triazolo[1,5-*a*]pyrimidin-10(4*H*)-one (**15**).

To a stirred suspension of **7b** (0.62 g, 1.93 mmol) in *N*,*N*-dimethylformamide (12 mL), potassium carbonate (0.8 g, 5.81 mmol) and 1-bromo-3-chloropropane (1.52 g, 5,79 mmol) were added. The mixture was refluxed for 5 h, cooled to room temperature, and then slowly poured onto ice-water. The precipitate solid was filtered and purified by column chromatography using dichloromethane/ethyl acetate 98:2 as eluent to afford both **14** (0.12 g, yield 15%), and **15** (0.51 g. yield 67%).

4.1.4.1. 4-(3-Chloropropyl)-3-phenylpyrido[3',2':4,5]thieno[2,3-*e*][1,2,3]triazolo[1,5-*a*]pyrimidin-5(4*H*)-one (**14**). Pale yellow solid. Mp 200.8-201.7°C. IR: 1645 (CO) cm^{-1}. ^1H NMR ($CDCl_3$) δ: 2.28-2.43 (m, 2H, $CH_2\underline{CH_2}CH_2$), 3.63 (t, J= 6.3 Hz, 2H, CH_2Cl), 5.13 (t, J= 6.3 Hz, 2H, CH_2N), 7.47-7.57 (m, 4H, C9-H, C3'-H, C4'-H, C5'-H), 8.56 (d, J= 8.2 Hz, 2H, C2'-H, C6'-H), 8.70 (dd, J=1.7, 6.6 Hz, 1H, C10-H), 8.79 (dd, J=1.7, 4.6 Hz, 1H, C8-H). ^{13}C NMR δ: 31.2 (t), 41.3 (t), 53.6 (t), 100.0 (s), 115.0 (s), 120.2 (d), 127.0 (dx2), 128.2 (s), 128.8 (dx2),

130.2 (d), 132.2 (d), 139.2 (s), 145.7 (s), 151.5 (d), 151.9 (s), 152.9 (s), 163.4 (s). Elem. Anal. calcd. for $C_{19}H_{14}ClN_5OS$: C, 57.65; H, 3.56; N, 17.69; found: C, 57.76; H, 3.39; N, 17.43.

4.1.4.2. 4-(3-Chloropropyl)-3-phenylpyrido[3',2':4,5]thieno[3,2-*d*][1,2,3]triazolo[1,5-*a*]pyrimidin-10(4*H*)-one (**15**). White solid. Mp 195.4-196.6°C. IR: 1630 (CO) cm^{-1}. ^1H NMR ($CDCl_3$) δ: 2.45-2.59 (m, 2H, $CH_2\underline{CH_2}CH_2$), 3.84 (t, J= 6.2 Hz, 2H, CH_2Cl), 4.93 (t, J= 6.2 Hz, 2H, CH_2N), 7.33-7.41 (m, 3H, C3'-H, C4'-H, C5'-H), 7.54 (dd, J= 4.6, 7.2 Hz, 1H, C6-H), 7.70 (d, J= 8.2 Hz, 2H, C2'-H, C6'-H), 8.92 (dd, J= 1.7, 4.6 Hz, 1H, C5-H), 9.40 (dd, J= 1.7, 7.2 Hz, 1H, C7-H). ^{13}C NMR ($CDCl_3$) δ: 29.7 (t), 41.1 (t), 65.1 (t), 121.3 (d), 122.5 (d), 125.8 (dx2), 127.7 (d), 128.7 (dx2), 130.7 (s), 133.1 (s), 134.0 (s), 134.2 (s), 134.4 (d), 137.7 (s), 151.6 (s), 157.4 (s), 161.7 (s). Elem. Anal. calcd. for $C_{19}H_{14}ClN_5OS$: C, 57.65; H, 3.56; N, 17.69; found: C, 57.59; H, 3.64; N, 17.63.

4.1.5. General procedure for preparation of 4-(3-(4-methylpiperazin-1-yl)propyl)-3-phenylpyrido[3',2':4,5]thieno[3,2-*d*][1,2,3]triazolo[1,5-*a*]pyrimidin-10(4*H*)-one (**16**).

A stirred suspension of **15** (0.10 g, 0.25 mmol) in 1-methylpiperazine (3 mL) was heated under reflux for 2 h. After cooling the mixture was poured onto ice-water and the precipitate solid was filtered. Purified by alumina column chromatography using dichloromethane as eluent, to afford **16** as a yellow solid, 0.05 g. Yield 40%. Mp 149.6-150.8°C. IR: 1635 (CO) cm^{-1}. ^1H NMR ($CDCl_3$) δ: 2.14-2.24 (m, 2H, $CH_2\underline{CH_2}CH_2$), 2.29 (s, 3H, CH_3), 2.49-2.67 (m, 10H, $CH_2N(CH_2CH_2)_2N$), 4.77 (t, J= 6.6 Hz, 2H, CH_2N), 7.28-7.51 (m, 3H, C3'-H, C4'-H, C5'-H), 7.65 (dd, J= 4.6, 7.4 Hz, 1H, C6-H), 8.39 (d, J= 8.0 Hz, 2H, C2'-H, C6'-H), 8.88 (dd, J= 1.7, 4.6 Hz, 1H, C5-H), 9.31 (dd, J= 1.7, 7.4 Hz, 1H, C7-H). ^{13}C NMR ($CDCl_3$) δ: 26.1 (t), 46.0 (q), 53.3 (t), 55.0 (tx2), 55.1 (tx2), 67.0 (t); 112.9 (s), 121.2 (d), 122.4 (s), 125.7 (dx2), 127.6 (d), 128.6

(dx2), 130.8 (s), 132.8 (s), 133.6 (s), 134.3 (d), 137.7 (s), 151.4 (d), 157.6 (s), 161.6 (s). Elem. Anal. calcd. for $C_{24}H_{25}N_7OS$: C, 62.72; H, 5.48; N, 21.33; found: C, 62.82; H, 5.44; N, 21.37.

4.2. Biology

The human tumor cell lines of the cancer screening panel are grown in RPMI 1640 medium containing 5% fetal bovine serum and 2 mM L-glutamine. For a typical screening experiment, cells are inoculated into 96 multiwell microtiter plates in 100 µl at plating densities ranging from 5.000 to 40.000 cells/well depending on the doubling time of individual cell lines. After cell inoculation, the microtiter plates are incubated at 37°C, 5 % CO_2, 95 % air and 100 % relative humidity for 24 h prior to addition of tested compounds.

After 24 h, two plates of each cell line are fixed *in situ* with trichloro acetic acid (TCA), to represent a measurement of the cell population for each cell line at the time of drug addition (time zero, Tz). Tested compounds are solubilized in dimethyl sulfoxide at 400-fold the desired final maximum test concentration and stored frozen prior to use. At the time of compound addition, an aliquot of frozen concentrate is thawed and diluted to twice the desired final maximum test concentration with complete medium containing 50 µg/mL gentamicin. Additional four, ten-fold or ½ log serial dilutions are made to provide five concentrations of the test compounds plus control. Aliquots of 100 µl of the different drug dilutions are added to the appropriate microtiter wells already containing 100 µl of medium, resulting in the required final concentrations.

Following drug addition, the plates are incubated for an additional 48 h at 37°C, 5 % CO_2, 95 % air, and 100 % relative humidity. For adherent cells, the assay is terminated by the addition of cold TCA. Cells are fixed *in situ* by the gentle addition of 50 µl of cold 50 % (w/v) TCA (final concentration, 10 % TCA) and incubated for 60 min at 4°C. The supernatant is discarded, and

the plates are washed five times with tap water and air dried. Sulforhodamine B (SRB) solution (100 µl) at 0.4 % (w/v) in 1 % acetic acid is added to each well, and plates are incubated for 10 min at room temperature. After staining, unbound dye is removed by washing five times with 1 % acetic acid and the plates are air-dried. Bound stain is subsequently solubilized with 10 mM trizma base, and the absorbance is read on an automated plate reader at a wavelength of 515 nm. For suspension cells, the methodology is the same except that the assay is terminated by fixing settled cells at the bottom of the wells by gently adding 50 µl of 80 % TCA (final concentration, 16 % TCA). Using the seven absorbance measurements [time zero, (Tz), control growth, (C), and test growth in the presence of drug at the five concentration levels (Ti)], the percentage growth is calculated at each of the drug concentrations levels. Percentage growth inhibition is calculated as:

$$[(Ti-Tz)/(C-Tz)] \times 100 \text{ for concentrations for which } Ti \geq Tz$$

$$[(Ti-Tz)/Tz] \times 100 \text{ for concentrations for which } Ti < Tz.$$

Three dose response parameters are calculated for each tested compounds. Growth inhibition of 50 % (GI_{50}) is calculated from $[(Ti-Tz)/(C-Tz)] \times 100 = 50$, which is the drug concentration resulting in a 50% reduction in the net protein increase (as measured by SRB staining) in control cells during the drug incubation. The drug concentration resulting in total growth inhibition (TGI) is calculated from Ti = Tz. The LC_{50} (concentration of drug resulting in a 50% reduction in the measured protein at the end of the drug treatment as compared to that at the beginning) indicating a net loss of cells following treatment is calculated from $[(Ti-Tz)/Tz] \times 100 = -50$. Values are calculated for each of these three parameters if the level of activity is reached; however, if the effect is not reached or is exceeded, the value for that parameter is expressed as greater or less than the maximum or minimum concentration tested.

5. SUPPLEMENTARY MATERIAL

SM1: ^{1}H and ^{13}C NMR spectra for derivatives **13** and **16**; DTP NCI biological data for all compounds: one dose screening (derivatives **12**, **13**, **16**), five dose screening (derivatives **13**, **16**), and *in vivo* toxicity report (derivative **13**). **SM2**: Compare and CellMiner analyses.

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LIST OF CAPTIONS:

Figure 1. Annelated pyrrolo[3,2-*e*]pyrimidine derivatives **1** and indolo[2,3-*e*][1,2,3]triazolo[1,5-*a*]pyrimidine derivatives **2** with antitumor activities.

Figure 2. Annelated thieno[2,3-*e*][1,2,3]triazolo[1,5-*a*]pyrimidines with good antitumor activity (**3aA**, pGI50 = 4.73 - 6.74; **3bA**, pGI50 = 4.47 - 7.02; **3bB**, pGI50 = 5.03 - 6.80).

Scheme 1. Synthetic route to annelated thieno[2,3-*e*][1,2,3]triazolo[1,5-*a*]pyrimidine derivatives.

Scheme 2. Synthesis of ethyl 4-(5-oxo-3-phenylbenzo[4,5]thieno[2,3-*e*][1,2,3]triazolo[1,5-*a*]pyrimidin-4(5*H*)-yl)butanoate (**8**) and its linear isomer **9**.

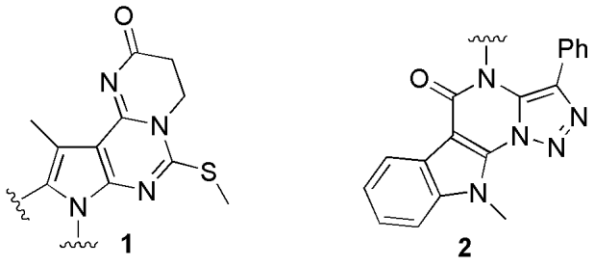
Scheme 3. Dimroth rearrangement of annelated [1,2,3]triazolo[1,5-*a*]pyrimidines.

Scheme 4. Synthesis of linear isomers of the targeted compounds.

Table 1. Overview of one dose screening for compounds **3aA**, **3bA**, **3bB**, **12**, **13** and **16** against all sub-panels (G%).

Table 2. Overview of five dose screening tests results for compounds **13**, **16** and the angular isomers **3**.

Table 3. DTP NCI five dose screening for compounds **13**, **16** and the angular isomers **3**.



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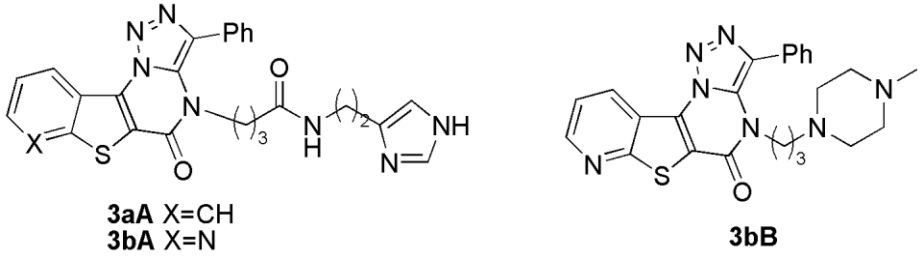

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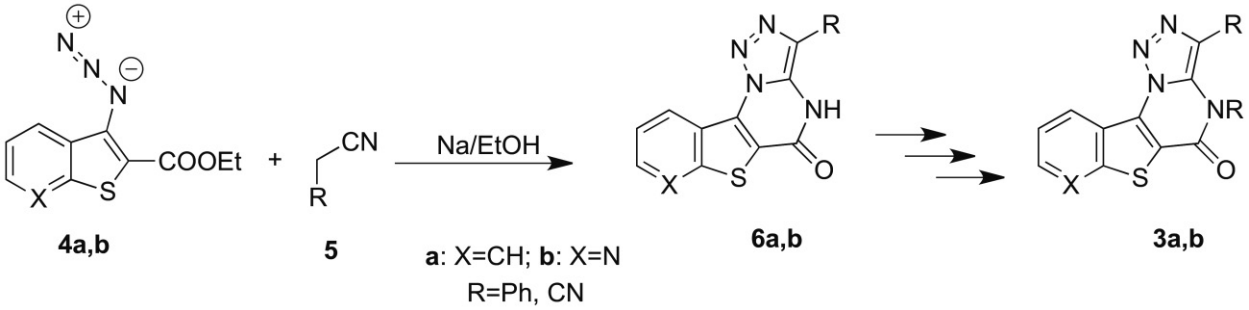

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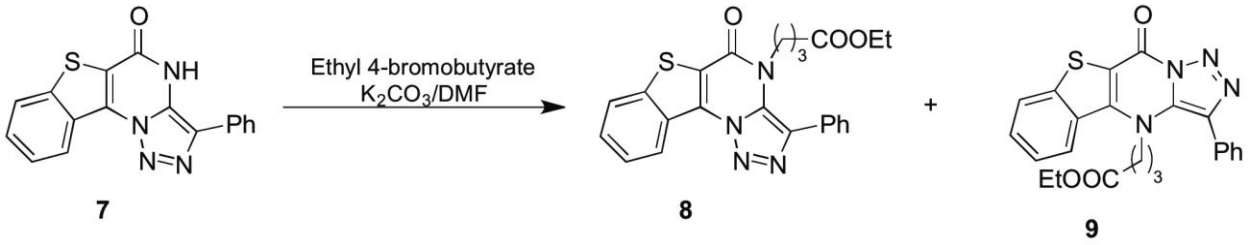
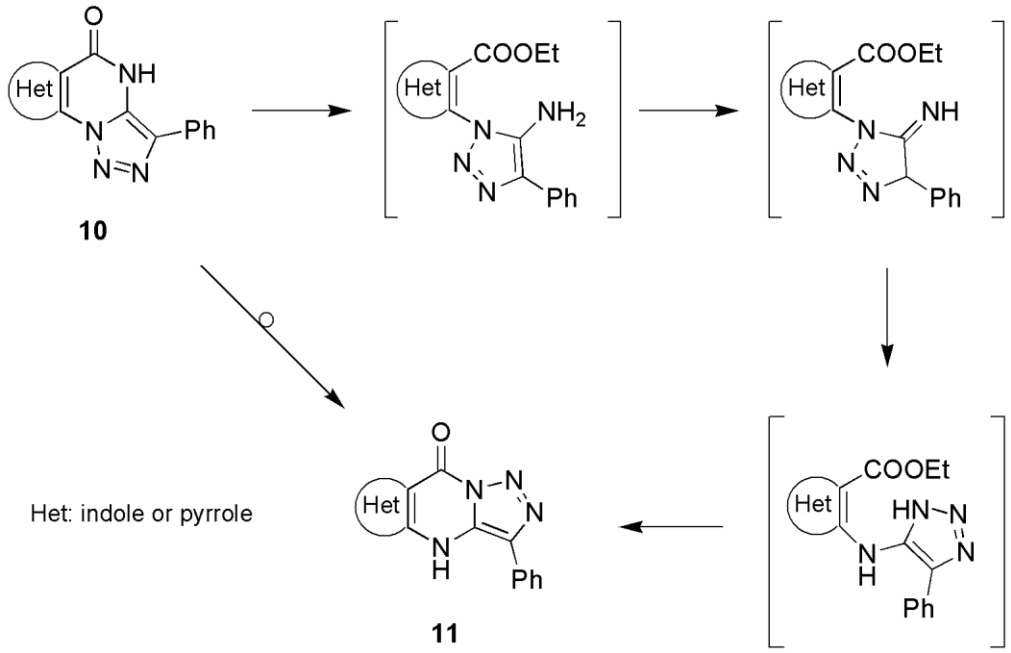
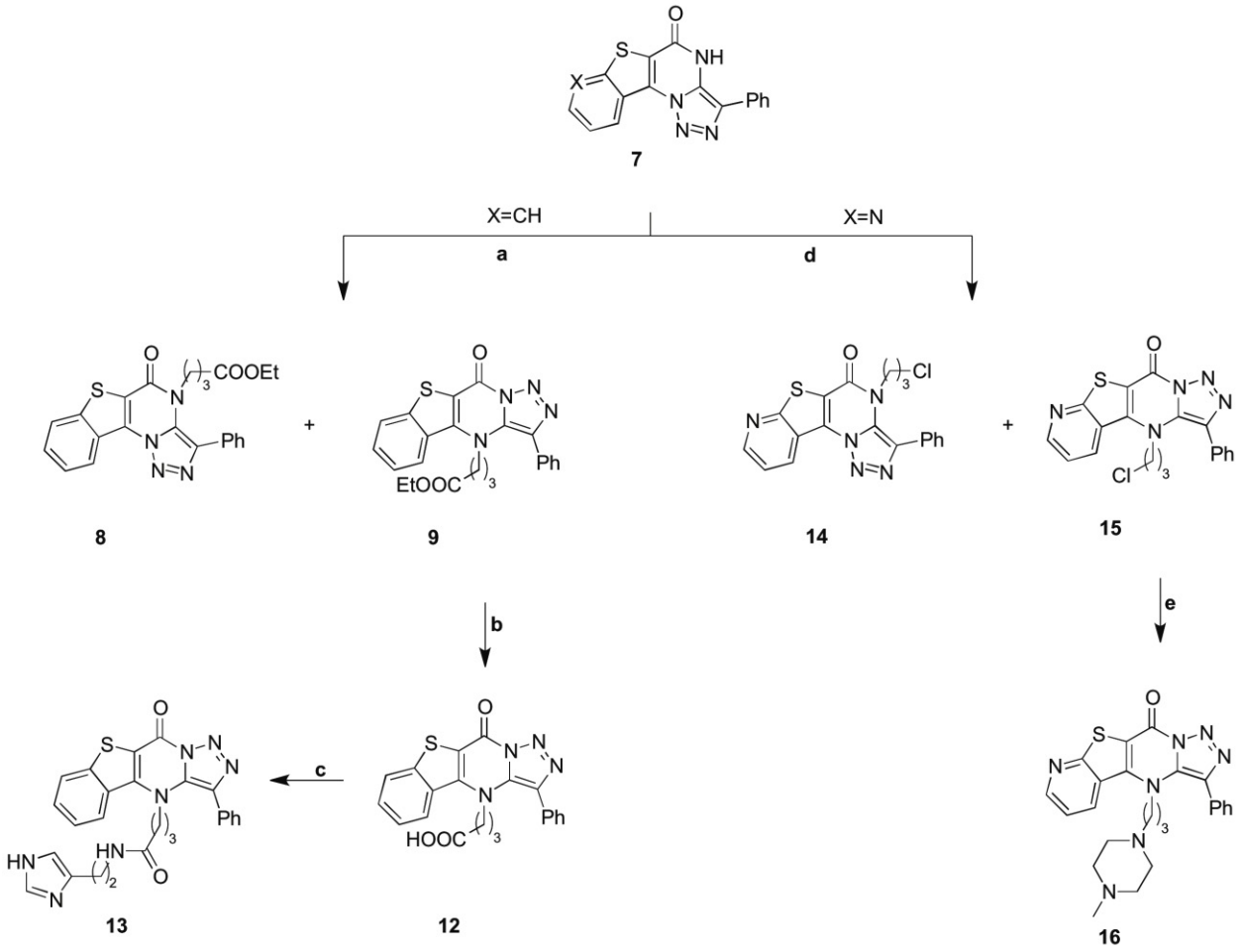


Scheme 2. Synthesis of ethyl 4-(5-oxo-3-phenylbenzo[4,5]thieno[2,3-*e*][1,2,3]triazolo[1,5-*a*]pyrimidin-4(5*H*)-yl)butanoate (**8**) and its linear isomer **9**.



Scheme 3. Dimroth rearrangement of annelated [1,2,3]triazolo[1,5-*a*]pyrimidines.



Scheme 4. Synthesis of linear isomers of the targeted compounds.

Table 1. Overview of one dose screening for compounds **3aA**, **3bA**, **3bB**, **12**, **13** and **16** against all sub-panels (G%).

	Derivative ([#]G%)					
PANEL	**3aA**	**3bA**	**3bB**	**12**	**13**	**16**
BreastCancer	-5	79	-23	79	-28	26
CNS Cancer	-18	60	-43	88	-50	15
Colon Cancer	-49	49	-37	90	-55	-13
Leukemia	71	61	-22	102	55	-1
Melanoma	-57	71	-78	95	-70	48
Non-Small Cell Lung Cancer	-26	88	14	85	-32	-6
OvarianCancer	-16	59	7	105	-41	-20
Prostate Cancer	-41	46	39	92	-92	-11
RenalCancer	-34	79	-14	75	-51	47
[*]Overall average	**-21**	**69**	**-23**	90	**-39**	**13**

[#]Subpanel cellular growth percentage average. [*]In bold the G% mean of compounds passed on for evaluation in the full panel of 60 cell lines over a five-log dose range.

Table 2. Overview of five dose screening tests results for compounds **13**, **16** and the angular isomers **3**.

Compd	pGI_{50}			pTGI			pLC_{50}		
	#	range	MG_MID	#	range	MG_MID	#	range	MG_MID
3aA	54	4.73-6.74	5.81	52	<4.00-5.78	5.29	44	<4.00-5.35	4.62
3bA	59	4.47-7.02	5.29	59	<4.00-5.35	4.58	59	<4.00-4.41	4.15
3bB	59	5.03-6.80	5.30	59	4.75-5.76	4.94	59	4.39-5.42	4.60
13	56	5.29->8.00	6.28	54	<4.00-6.91	5.51	49	<4.00-5.50	4.89
16	57	4.70-5.99	5.59	57	4.38-5.61	5.26	49	<4.00-5.24	4.78

Number of cell lines investigated

Table 3. DTP NCI five dose screening for compounds **13**, **16** and the angular isomers **3**.

		Derivative														
		3aA			**3bA**			**3bB**			**13**			**16**		
PANEL	[#]CELL LINE	**pGI_{50}**	**pTGI**	**pLC_{50}**	**pGI_{50}**	**pTGI**	**pLC_{50}**	**pGI_{50}**	**pTGI**	**pLC_{50}**	**pGI_{50}**	**pTGI**	**pLC_{50}**	**pGI_{50}**	**pTGI**	**pLC_{50}**
Breast Cancer	BT-549	4.76	4.38	<4.00	NT	NT	NT	NT	NT	NT	5.48	4.94	4.29	4.83	4.51	4.19
	HS 578T	5.76	5.39	NT	4.95	4.50	4.06	5.19	4.79	4.40	5.83	5.42	NT	4.83	4.38	4.00
	MCF7	5.84	5.51	5.19	4.75	4.00	4.00	5.11	4.82	4.53	5.94	5.58	5.21	5.81	5.52	5.24
	MDA-MB-231/ATCC	5.72	5.44	5.17	4.75	4.46	4.17	5.16	4.87	4.57	5.73	5.46	5.18	5.77	5.45	NT
	MDA-MB-468	5.77	5.43	5.10	5.09	4.56	4.10	5.16	4.86	4.55	6.47	5.92	5.42	5.74	5.48	5.22
	T-47D	5.85	5.35	<4.00	5.73	4.67	4.19	5.10	4.80	4.50	5.90	5.20	4.51	4.70	4.42	4.15
	panel average	5.62	5.25	4.69	5.05	4.44	4.10	5.14	4.83	4.51	5.89	5.42	4.92	5.28	4.96	4.56
CNS Cancer	SF-268	5.75	5.43	5.11	4.93	4.54	4.14	5.19	4.88	4.57	5.81	5.49	5.17	5.79	5.48	5.17
	SF-295	5.77	5.44	NT	5.76	4.79	4.27	5.25	4.91	4.57	6.34	5.68	5.27	5.71	5.43	5.16
	SF-539	6.18	5.66	5.24	6.50	4.83	4.27	5.24	4.87	4.51	7.69	6.91	5.50	5.77	5.48	5.18
	SNB-19	5.77	5.30	<4.00	4.97	4.47	4.00	5.08	4.80	4.53	5.29	4.66	4.27	5.60	5.25	4.00
	SNB-75	5.73	5.39	5.06	4.82	4.54	4.26	5.17	4.88	4.59	5.71	5.45	5.20	5.11	4.66	4.31
	U251	5.98	5.65	5.33	5.89	4.83	4.34	5.28	4.95	4.62	6.78	5.73	5.37	5.74	5.46	NT
	panel average	5.86	5.48	4.95	5.48	4.67	4.21	5.20	4.88	4.57	6.27	5.65	5.13	5.62	5.29	4.76
Colon Cancer	COLO 205	5.96	5.32	<4.00	5.20	4.63	4.23	5.13	4.84	4.54	6.48	5.65	5.23	5.79	5.49	5.19
	HCC-2998	5.75	5.38	5.01	5.69	4.52	4.00	5.17	4.87	4.58	NT	NT	NT	5.73	5.46	5.19
	HCT-116	6.47	5.78	<4.00	6.42	5.00	4.41	5.28	4.93	4.58	8.00	NT	NT	5.77	5.46	5.16
	HCT-15	5.90	5.60	5.30	5.24	4.00	4.00	5.21	4.88	4.55	6.43	5.72	5.24	5.74	5.41	5.08
	HT29	5.87	5.50	5.13	5.41	4.26	4.00	5.54	5.02	4.63	6.17	5.70	5.34	NT	NT	NT
	KM12	5.94	5.55	NT	5.72	4.62	4.08	5.37	4.97	4.61	6.47	5.70	5.31	5.76	5.47	5.17
	SW-620	6.39	NT	NT	6.48	4.84	4.21	5.27	4.94	4.61	7.62	6.00	5.38	5.77	5.43	5.09
	panel average	6.04	5.52	4.69	5.74	4.55	4.13	5.28	4.92	4.59	6.86	5.75	5.30	5.76	5.45	5.15
Leukemia	CCRF-CEM	NT	4.00	<4.00	6.65	5.35	4.00	5.81	5.28	4.61	7.81	6.54	5.28	5.78	5.37	4.00
	HL-60(TB)	5.85	5.20	<4.00	5.85	5.32	4.30	6.10	5.76	5.42	7.14	5.49	4.64	5.73	5.39	5.04
	K-562	6.36	NT	NT	6.51	4.81	4.00	6.06	5.69	5.32	7.64	5.99	5.29	5.72	5.32	4.00
	MOLT-4	5.93	NT	NT	5.65	4.62	4.00	5.81	5.31	4.67	NT	NT	NT	5.70	5.35	4.94
	RPMI-8226	NT	NT	<4.00	5.29	4.45	4.00	5.21	4.80	4.39	NT	NT	NT	5.74	5.36	4.00
	SR	NT	NT	NT	5.60	5.21	4.00	5.96	5.59	4.92	6.66	6.18	5.30	5.65	5.32	NT
	panel average	6.05	4.60	<4.00	5.93	4.96	4.05	5.83	5.41	4.89	7.31	6.05	5.13	5.72	5.35	4.40
Melanoma	LOX IMVI	5.80	5.53	5.27	4.91	4.58	4.24	5.27	4.94	4.60	5.82	5.54	5.26	5.78	5.47	NT
	M14	5.77	5.42	NT	5.46	4.71	4.30	5.67	5.13	4.68	6.49	5.69	NT	5.73	5.44	5.14
	MALME-3M	5.60	5.26	<4.00	4.74	4.43	4.12	5.03	4.75	4.47	5.80	5.48	5.17	5.71	5.44	5.17
	MDA-MB-435	5.83	5.51	5.20	6.31	4.69	4.00	5.93	5.52	4.88	6.46	5.73	5.33	5.73	5.45	5.17
	SK-MEL-2	NT	NT	NT	4.75	4.41	4.07	5.06	4.78	4.50	NT	NT	NT	NT	NT	NT
	SK-MEL-28	5.76	5.49	5.23	4.77	4.46	4.14	5.06	4.80	4.55	5.77	5.50	5.23	4.80	4.53	4.26
	SK-MEL-5	5.72	5.39	5.05	4.79	4.00	4.00	5.14	4.86	4.58	5.69	4.96	4.48	5.77	5.50	5.24
	UACC-257	5.69	5.34	<4.00	4.74	4.45	4.16	5.64	5.12	4.69	5.67	5.39	5.11	5.74	5.47	5.20
	UACC-62	5.72	5.45	5.18	4.80	4.52	4.24	5.05	4.79	4.53	5.74	5.45	5.15	4.78	4.46	4.15
	panel average	5.74	5.42	4.85	5.03	4.47	4.14	5.32	4.97	4.61	5.93	5.47	5.10	5.51	5.22	4.90
Non-Small Cell Lung Cancer	A549/ATCC	5.68	5.34	NT	5.06	4.46	4.00	5.17	4.86	4.55	5.89	5.49	NT	5.75	5.47	NT
	EKVX	5.57	5.06	<4.00	4.82	4.35	4.00	5.19	4.88	4.56	5.60	4.80	4.00	5.67	5.31	4.08
	HOP-62	5.64	5.28	<4.00	4.78	4.50	4.21	5.09	4.81	4.54	5.88	5.54	5.20	5.75	5.48	5.21
	HOP-92	5.73	NT	<4.00	4.77	4.40	4.04	5.24	4.89	4.54	5.39	4.69	4.00	5.20	4.61	4.15
	NCI-H226	4.73	4.42	4.12	4.93	4.53	4.13	5.13	4.82	4.50	5.56	5.13	4.39	4.86	4.53	4.19
	NCI-H23	5.53	4.93	<4.00	4.84	4.49	4.15	5.09	4.81	4.54	5.87	5.45	NT	5.67	5.29	4.47
	NCI-H322M	5.65	5.26	4.67	4.83	4.44	4.05	5.21	4.89	4.57	5.56	5.01	4.41	5.76	5.46	5.16
	NCI-H460	5.73	5.42	5.11	5.44	4.88	4.37	5.16	4.86	4.57	5.80	5.40	NT	5.72	5.40	5.09
	NCI-H522	5.98	4.93	4.09	5.53	4.64	4.22	5.14	4.84	4.54	7.36	5.51	4.00	5.72	5.43	NT
	panel	5.58	5.08	4.25	5.00	4.52	4.13	5.16	4.85	4.55	5.88	5.22	4.33	5.57	5.22	4.72

	average															
Ovarian Cancer	IGROV1	NT	NT	NT	5.02	4.56	4.12	5.23	4.86	4.49	7.00	5.68	5.30	5.70	5.35	5.01
	NCI/ADR-RES	5.49	4.00	<4.00	4.47	4.00	4.00	5.14	4.85	4.55	5.97	4.00	4.00	5.65	5.24	4.36
	OVCAR-3	5.82	5.50	5.18	4.96	4.63	4.30	5.64	5.12	4.69	5.78	5.51	5.25	5.74	5.46	5.18
	OVCAR-4	5.76	5.42	NT	4.81	4.49	4.17	5.14	4.85	4.57	5.74	5.42	5.10	5.73	5.41	5.09
	OVCAR-5	5.92	5.56	NT	4.94	4.49	4.03	5.05	4.77	4.49	7.25	5.87	5.39	5.57	5.13	4.00
	OVCAR-8	6.49	5.55	<4.00	7.02	4.76	4.17	5.11	4.81	4.52	8.00	5.52	4.00	5.71	5.35	4.00
	SK-OV-3	5.46	4.81	4.18	4.84	4.56	4.27	5.05	4.80	4.55	5.62	5.07	4.53	4.73	4.48	4.22
	panel average	5.82	5.14	4.34	5.15	4.50	4.15	5.19	4.87	4.55	6.48	5.30	4.80	5.55	5.20	4.55
Prostate Cancer	DU-145	5.81	5.53	5.24	5.56	4.71	4.21	5.23	4.91	4.60	5.97	5.65	5.32	5.73	5.44	5.14
	PC-3	NT	4.00	<4.00	4.96	4.00	4.00	5.20	4.87	4.54	7.22	5.66	5.14	NT	NT	NT
	panel average	5.81	4.77	4.62	5.26	4.36	4.11	5.22	4.89	4.57	6.60	5.66	5.23	5.73	5.44	5.14
Renal Cancer	786-0	5.80	5.45	NT	4.90	4.59	4.28	5.13	4.84	4.54	5.98	5.63	5.27	5.73	5.46	5.18
	A498	5.81	5.52	5.24	5.17	4.65	4.27	6.80	5.03	4.66	5.96	5.61	5.25	4.95	4.62	4.28
	ACHN	5.78	5.52	5.26	4.81	4.51	4.20	5.09	4.82	4.56	5.80	5.50	5.20	5.79	5.48	5.17
	CAKI-1	6.74	5.77	5.35	5.54	4.84	4.14	5.21	4.89	4.56	NT	NT	NT	5.79	5.48	5.18
	RXF 393	5.60	5.30	<4.00	4.83	4.47	4.11	5.20	4.87	4.55	5.66	5.38	5.11	5.77	5.50	5.23
	SN12C	6.57	5.73	5.29	6.48	4.87	4.38	5.14	4.85	4.55	7.96	5.69	5.26	5.74	5.39	NT
	TK-10	5.67	5.40	NT	4.87	4.55	4.24	5.16	4.87	4.57	5.66	5.43	5.19	5.67	5.43	NT
	UO-31	5.72	5.43	NT	4.93	4.57	4.22	5.29	4.92	4.55	5.89	5.58	5.28	5.99	5.61	5.23
	panel average	5.96	5.52	5.03	5.19	4.63	4.23	5.38	4.89	4.57	6.13	5.55	5.22	5.68	5.37	5.05
	MG_MID	5.81	5.29	4.62	5.29	4.58	4.15	5.30	4.94	4.6	6.28	5.51	4.89	5.59	5.26	4.78

[#]NT: not tested