Rapid Discovery of Triazolobenzylidene-Thiazolopyrimidines (TBTP) as CDC25 Phosphatase Inhibitors by Parallel Click Chemistry and in Situ Screening

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The recent copper(I)-catalyzed version of the (3 + 2) cycloaddition between terminal alkynes and organic azides ("click" reaction), has emerged as a powerful tool for the chemoselective ligation of complementary reactants irrespective of their chemical types (small-molecule inhibitors, fluorophores, glycosides, proteins, oligonucleotides, etc.).[1] While providing with a modular approach toward small molecule conjugates of biological entities, that are invaluable for proteomic and cell biology studies, the "click" reaction has an extraordinary potential in drug discovery for the synthesis of ligands and inhibitors.[2] The key features of this reaction in this context are the following: (a) an almost total chemoselectivity, allowing the assembly of reactants decorated with diverse unprotected functionalities; (b) a complete regioselectivity (only 1,4-disubstituted 1,2,3-triazoles are formed); (c) excellent to quantitative yields.[3] Having this set of characteristics in mind, we devised an approach of parallel click synthesis of a novel type of CDC25 phosphatase inhibitors, associated with in situ biological screening. In this approach, nearly quantitative solution synthesis of cycloadducts would permitt their screening "as such", as long as the reaction medium remains homogeneous and the products fully soluble in water-miscible and test-compatible organic solvents. CDC25 phosphatases are key regulatory enzymes of the cell cycle[4−6] and constitute attractive targets in cancer therapy: out of the three occurring isoforms A, B, and C, the first two are found up-regulated in a number of metastatic diseases featured by their aggressiveness and resistance to chemotherapy.[7] Our laboratory recently identified benzylidene-thiazolopyrimidines (BTP) as novel heterocyclic inhibitors of CDC25 enzymes.[8,9] Analysis of the SARs in this series suggests that an extended conjugation through a substituted 2-thiocinnamide system is a critical feature for CDC25 inhibition (Figure 1).

Moreover, our recent modeling study suggested that the benzodioxolane and ester functions of BTP inhibitors **1** and **2** were specifically interacting with the catalytic site of CDC25 enzymes, whereas the phenol moiety was occupying some adjacent inhibitor binding pocket.[8] To allow an efficient probing of this inhibitor binding site with diverse chemical fragments, our plan toward novel triazole analogs (i.e., triazolobenzylidene-thiazolopyrimidines, TBTP) was based on the use of the phenyl ring as an (*o*,*m*,*p*)-orienting scaffold. Practically, our strategy was defined as the introduction of an (*o*,*m*,*p*)-azido function on the inhibitor skeleton (precursor), followed by parallel "click" reactions affording 1,4-disubstituted 1,2,3-triazole products (Scheme 1). This approach was chosen for two reasons. First, the azide function exerts electrowithdrawing effects on the pharmacophore and should have a negative influence on the bioactivity of precursors. On the other hand, the formed 1,4-triazoles constitute electrodonating nuclei that can be compared to the favored conjugated systems found in inhibitors **1** and **2**. In other words, the use of inactive starting materials leading to potentially bioactive products would eliminate fake-positive results due to traces of unreacted material. Second, our choice was to take advantage of the high and ever-expanding chemodiversity of commercial alkynes.[10] This strategy would allow the construction of a TBTP library containing three categories of analogous products, based on three (*o*,*m*,*p*)-azido precursors (acting as pharmacophore-scaffold elements), and diversity-oriented alkynes moieties (acting as pharmacomodulating elements). This is in marked contrast with the common use of click chemistry in drug discovery processes, where alkyne-bearing pharmacophores react with organic azides generated in situ.[11,12] This approach is hardly feasible in a context of library synthesis.

The three (*o*,*m*,*p*)-azido-BTP precursors, namely {*o*,*m*,*p*}-**4**, were obtained by condensing thiazolopyrimidine precursor **3**[9] with (*o*,*m*,*p*)-azidobenzaldehydes in Knoevenagel conditions (catalytic piperidine in warm ethanol). The (*o*,*p*)-azidobenzaldehydes were obtained directly from the corresponding nitrobenzaldehydes through aromatic nucleophilic substitution, using sodium azide in HMPT.[13] Interestingly, this useful reaction has not been previously described with the *p*-isomer. On the other hand, *m*-azidobenzaldehyde was obtained using a multistep literature procedure starting from *m*-nitrobenzaldehyde[14] (Scheme 2).

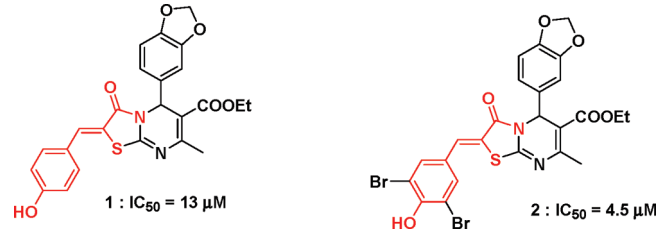

Figure 1. 2-Thiocinnamide pharmacophore of benzylidene-thiazolopyrimidine CDC25 inhibitors (BTP) recently described.[9]

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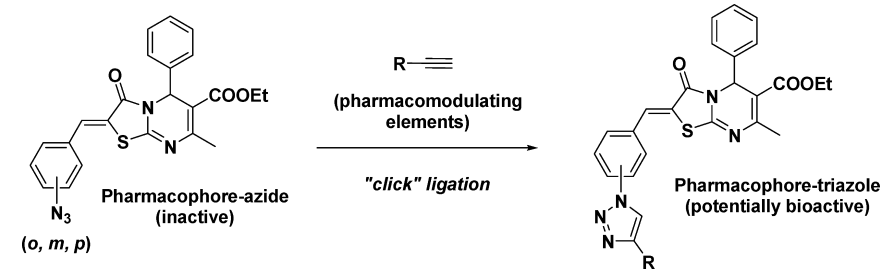
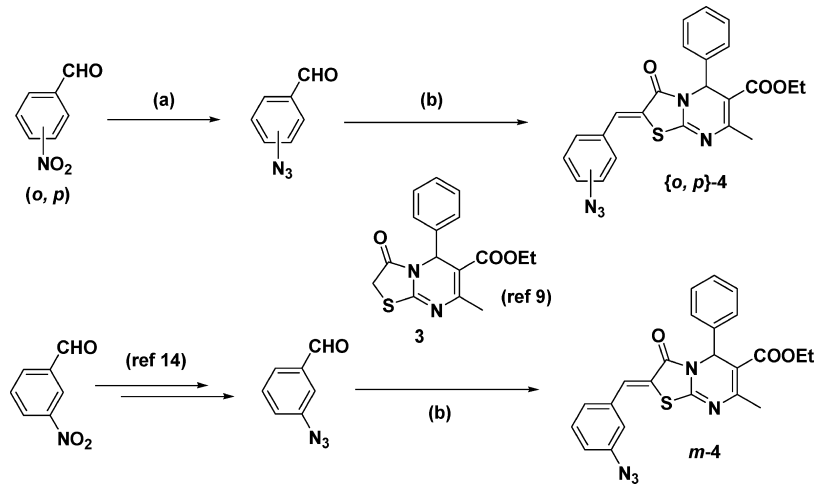
Scheme 1. Strategy for the Exploration of the CDC25 Inhibitor Binding Pocket Using Parallel Click Synthesis

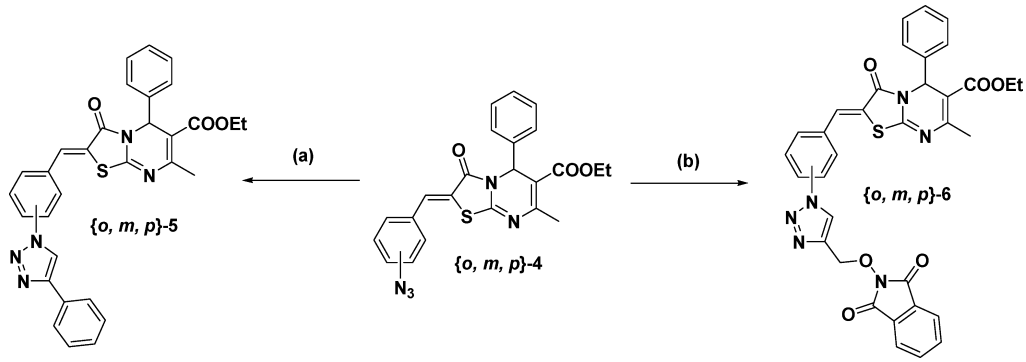


Scheme 2. Synthesis of the (*o*,*m*,*p*)-azido-BTP Precursors {*o*,*m*,*p*}-**4**[a]



[a] Reagents and conditions: (a) NaN_3, HMPT, rt (90% for *o*-isomer, 82% for *p*-isomer); (b) cat. piperidine, EtOH, reflux (56% *o*-**4**, 30% *m*-**4**, 60% *p*-**4**).

Scheme 3. Model Reactions of (*o*,*m*,*p*)-azido-BTP {*o*,*m*,*p*}-**4** with Representative Alkynes[a]



[a] Reagents and conditions: (a) phenylacetylene or (b) *N*-(propargyloxy)phthalimide, cat. sodium ascorbate, cat. $CuSO_4$, NMP, rt (90−98% {*o*,*m*,*p*}-**5** and {*o*,*m*,*p*}-**6**).

The possibility of performing click reactions with {*o*,*m*,*p*}-**4** substrates was investigated in standard conditions (i.e., 5% $CuSO_4$ and 10% sodium ascorbate in NMP), using phenylacetylene and *N*-(propargyloxy)phthalimide as representative alkyne moieties. NMP was chosen as a water-miscible solvent, which would allow direct dilution of reaction mixtures with the biological buffer prior to enzymatic assay with CDC25B. Those classical conditions led to complete consumption of the reactants, and formation of the expected TBTP cycloadducts {*o*,*m*,*p*}-**5** and {*o*,*m*,*p*}-**6** with nearly quantitative yields and crude purity >90% as judged by ^{1}H NMR (Scheme 3).

Importantly, the high substrate concentration (0.2 M) induced significant precipitation of the formed triazoles. This useful feature of click chemistry from a preparative standpoint corresponds to a major drawback in our solution-based, parallel synthesis strategy, where all products need to remain fully soluble for homogeneous sampling and biological evaluation. Therefore, we looked for a compromise between adequate reaction kinetic and sufficient dilution, when adapting the reaction conditions for parallel, 96-well plate synthesis. This was achieved by increasing the catalyst and reductant concentrations (i.e. 20% $CuSO_4$ and 100% sodium ascorbate), using substrate concentration of 14 mM for the (*o*,*m*)-azido-BTP {*o*,*m*}-**4**, and 85 mM for the *p*-azido-BTP *p*-**4**. Indeed, owing to different solubilities of azide precursors, parallel synthesis was performed in two distinct 96-wall plates for the (*o*,*m*) and *p*-series (Figure 2). As far as the alkyne reactants are concerned, we chose 29 terminal alkynes with high chemodiversity (aromatic alkynes, *O*-propargyl, and *N*-propargyl derivatives). Among these reactants, 25 were of commercial origin, and four had been

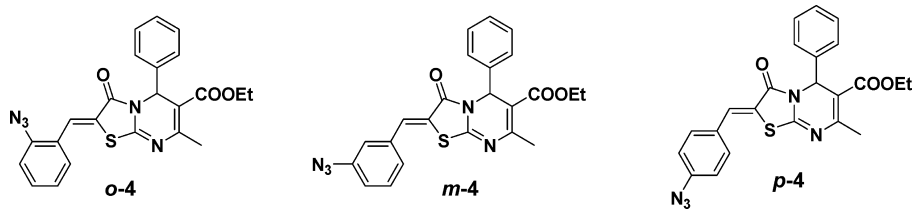


Figure 2. Azido-BTP precursors {*o*,*m*,*p*}-**4** used in this study.

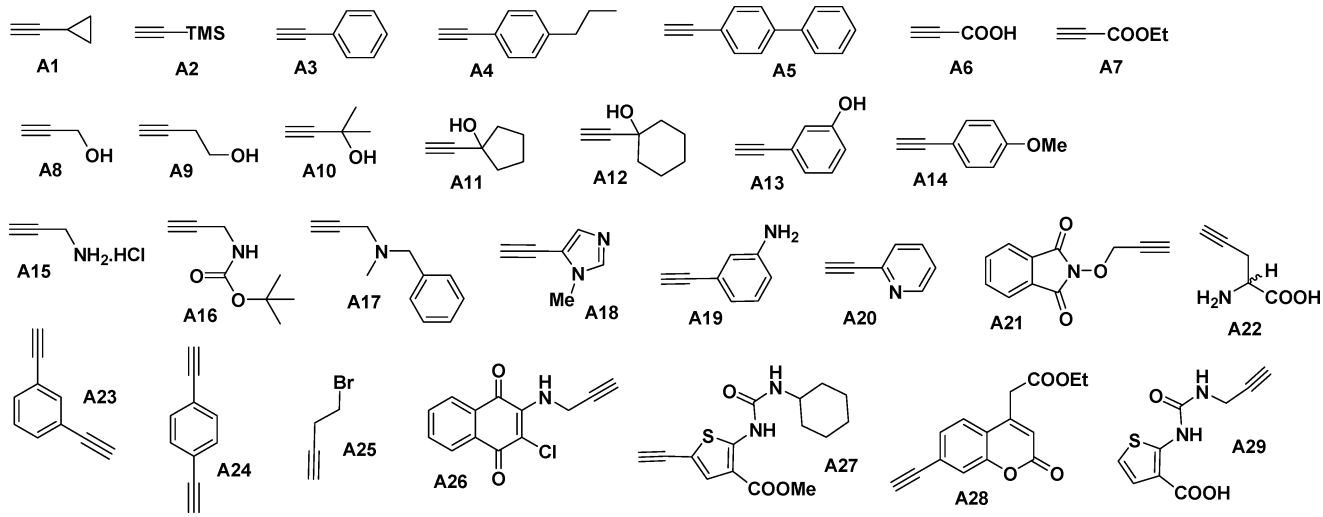


Figure 3. Commercial and synthetic alkynes used in this study.

previously synthesized in our lab (naphthoquinone derivative **A26**, thiophene derivatives **A27** and **A29**, and coumarin analog **A28**) (Figure 3).

Using the optimized parallel synthesis conditions, we found that 5 days of stirring on a microtitration plate at room temperature led to complete conversion of the (*o*,*m*,*p*)-azido-BTP substrates {*o*,*m*,*p*}-**4**. Formation of single, fully soluble products in virtually quantitative yields was observed in each well by TLC. Out of the 29 alkynes used, only (D,L)-propargylglycine failed to yield the desired triazoles. The identity of the formed products as TBTP cycloadducts was ascertained using ^{1}H NMR, showing complete disappearance of (*o*,*m*,*p*)-azido-BTP substrates as well as formation of the desired products with purity >90% (see the Supporting Information). Having secured the synthetic aspect of our strategy, we turned to the biological probing of the crude reaction mixtures ("as such" testing) in a parallel manner. An inhibitor concentration of 10 μM was chosen as a "cut-off" concentration that would permit the rapid identification of novel TBTP inhibitors of CDC25 phosphatases. The synthesis plates were thus sampled and directly diluted in the enzymatic buffer used for the assay. Importantly, neither any of the azido-BTP precursors {*o*,*m*,*p*}-**4**, nor the used alkynes {**A1, A29**}, NMP, or copper sulfate, showed significant inhibitory activity with IC_{50} > 50 μM [The solubility limits in biological buffer for azido-BTP precursors {*o*,*m*,*p*}-**4** is c.a. 50 μM. Therefore, the theoretical IC_{50} for those precursors can be expected to be much higher.]. As far as the cycloadducts are concerned, we found that none of the novel (*o*,*m*)-TBTP showed high CDC25B inhibition at 10 μM, with percentages of inhibition of 0−40% compared to the control (Table 1 in the Supporting Information). On the other hand, several *p*-TBTP derivatives were identified as potential inhibitors using the cut-off method, with percentages of inhibition around 50% (Table 1 in the Supporting Information). Following resynthesis and purification, a number of analogs were indeed found to be potent inhibitors, with low micromolar IC_{50} (Figure 4, Table 1).

Table 1. CDC25B Inhibitory Activities (IC_{50} μM) of the Hit Inhibitors

compound	percent inhibition of CDC25B at the cut-off concentration (10 μM crude compound)	IC_{50} (μM) for purified compounds
p-TBTP-A3	54	16.6 ± 6.3
p-TBTP-A14	50	ND[a]
p-TBTP-A19	53	6.6 ± 1.2
p-TBTP-A21	44	6.8 ± 0.2
p-TBTP-A27	46	9.5 ± 2.8
p-TBTP-A29	54	3.0 ± 0.1

[a] Not determined. The compound was not soluble at the tested concentrations.

From this work, key structure−activity relationships (SARs) could also be obtained: (i) *p*-TBTPs are the best CDC25 inhibitors in the TBTP series, over the (*o*,*m*)-TBTP analogs that are much weaker inhibitors. Notably, the electronic features of *p*-TBTP can be compared to the electro-enriched systems of 4-hydroxy-BTP **1** and 4-hydroxy-3,5-dibromo-BTP **2**, previously identified as hit CDC25 inhibitors in our laboratory;[8,9] (ii) in the *p*-TBTP series, good inhibitors are featured by aryltriazole moieties (click reaction with arylacetylene derivatives), over the ones possessing a triazolomethyl unit (click reaction with propargyl derivatives). Electro-enriched, lipophilic pendants seem thus to be favored, suggesting that an extended conjugated system (i.e. triazoloarylcinnamide) is a beneficial feature for CDC25 inhibition. The exception is polar triazolomethylurea derivative ***p*-TBTP-A29**, the activity of which can be accounted by specific H-bond networking with the inhibitor binding pocket.

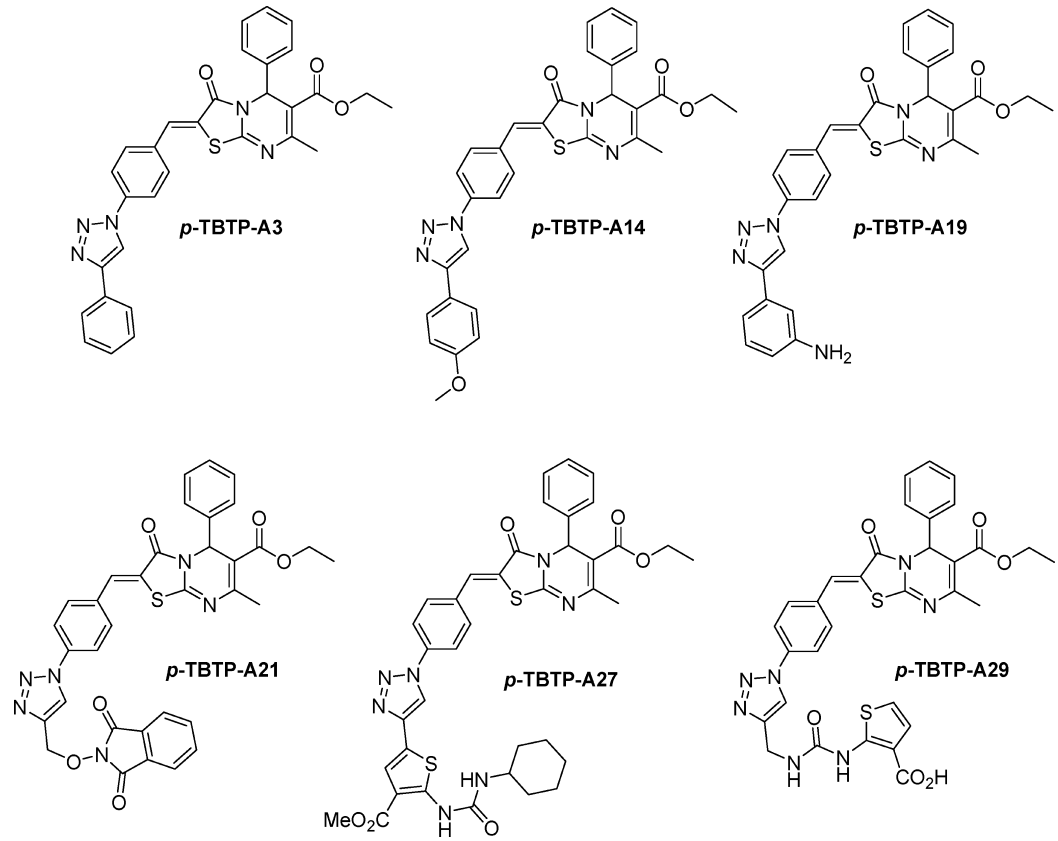


Figure 4. Hit TBTP inhibitors of CDC25B identified in this study.

Importantly, this derivative is the most potent inhibitor identified to date within the BTP[9]/TBTP series, with IC_{50} = 3.0 μM.

In conclusion, this paper highlights the relevance of click chemistry for the rapid discovery of novel CDC25 phosphatase inhibitors in a parallel manner.[2] Rational design of inactive azide precursors, and use of alkynes as pharmacomodulating elements, led to the generation of an 87-member triazole library with good to quantitative yields and high purities. This permitted the direct biological screening of reaction mixtures for bioactive cycloadducts, thus efficiently short-cutting the standard drug-discovery approach. From this strategy, novel micromolar inhibitors with an unprecedented triazolobenzylidene-thiazolopyrimidine (TBTP) skeleton were rapidly identified, along with key SARs in this series.

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Supporting Information Available. Synthetic procedures for standard and parallel synthesis, NMR data of crude and purified compounds, and preliminary inhibition results with the "cut-off" method. This information is available free of charge via the Internet at http://pubs.acs.org/.

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