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Synthesis and biological evaluation of combretastatin analogs as cell cycle inhibitors of the G1 to S transition in *Saccharomyces cerevisiae*

Paola Coccetti [a,*], Giuseppe Montano [b], Alessandro Lombardo [b], Farida Tripodi [a], Fulvia Orsini [b], Roberto Pagliarin [b]

[a] Dipartimento di Biotecnologie e Bioscienze, Università Milano-Bicocca, P.zza della Scienza, 2, 20126 Milano, Italy
[b] Dipartimento di Chimica Organica e Industriale, Università di Milano, Milano, Italy

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ABSTRACT

A series of *Z* and *E* combretastatin A-4 analogs bearing different substituents (OH, F, NO_2, NH_2, $B(OH)_2$) in the 3′ position were synthesized. These derivatives and Z and E combretastatin A-1 were analysed by monitoring their ability to inhibit cell growth in *Saccharomyces cerevisiae*. Combretastatin A-1 (**2a**), A-4 (**2b**) and compound **2c** were found to inhibit yeast growth. Moreover, combretatstatin A-4 (**2b**) and compound **2c** induced a G1 arrest by affecting the synthesis of Clb5 protein, the principal S-phase cyclin. The G1 arrest is coincident with the activation of the stress activated kinase Snf1.

The combretastatins were originally isolated from the South African tree *Combretum caffrum*.[1] Combretastatin A-4 (CA4) is a natural *cis*-stilbene used in traditional medicine for the treatment of hepatitis and malaria.[2,3] CA4 is a microtubule-destabilizing agent that inhibits microtubule assembly by binding to tubulin, similar to the well-known microtubule-targeted agent colchicine.[4] CA4P, the water soluble prodrug, induces microtubule disassembly and leads to a dramatic cytotoxicity in proliferating vascular endothelial cells which show a strong inhibition of malignant hemangioendothelioma cell proliferation.[5,6] In experimental tumors, anti-vascular effects are observed within minutes of CA4P administration which rapidly leads to extensive ischemic necrosis in areas that are often resistant to conventional anti-cancer treatments.[5,7–10] In keeping with their tumor vascular disrupting activity at well tolerated doses,[11,12] combretastatin A-4-P and combretastatin A-1-P (the prodrug of the natural parent CA1) are now collectively classified as vascular disrupting agents (VDAs), since they cause rapid shut down of the established tumor vasculature.[13] CA4P, which is the most widely studied VDA of the microtubule depolymerising family, is being evaluated in Phase III clinical trials for cancer treatment.[13] CA1P, which is an even more potent VDA than CA4P,[14] is also now being clinically tested.[13]

CA4P addition to proliferating endothelial cells damages mitotic spindles, arrests cells at metaphase with a post-synthetic DNA content and leads to the death of mitotic cells. The mitotic arrest is associated with elevated levels of cyclin B1 and of $p34^{cdc2}$ activity.[5]

Although most previous studies have focused on the anti-vascular effect of CA4P and used the endothelial cells as major target cells, it has also been suggested that the anti-tumor effect of CA4P may be an integrated consequence of the destruction of tumor vascular network and direct killing of tumor cells. In fact, it was shown that CA4P has primary anti-neoplastic activity against human anaplastic thyroid carcinoma cell lines and xenograft tumors.[15]

Recently, it has been demonstrated that CA4 stimulates both extracellular signal-regulated kinases (ERK1/2) and p38 MAPK in human hepatocellular carcinoma cell lines and that only the inhibition of p38 MAPK synergistically enhances the cytotoxicity of CA4,[16] providing novel evidence that the combination of p38 MAPK inhibitors with CA4 may represent a promising strategy for cancer therapy. In addition, it was also shown that CA4 inhibits growth in several human gastric cancer cell lines and, more interestingly, the inhibition of the oncogenic pathway of AKT by CA4 results in reduced metastasis.[17]

Further investigation identifies CA4 as an activator of AMP-activated protein kinase (AMPK),[18] which is a key regulator of energy balance involved in response to cellular stress in mammalian cells.[19] It is known that activation of AMPK, through the up-regulation of p53, increases the level of the Cdk1 inhibitor $p21^{CIP}$, which in turn induces a G1 cell cycle arrest.[20]

Although the anti-fungal activity of combretastatins has been previously reported,[21,22] the effects on yeast cell cycle progression

* Corresponding author. Tel.: +39 02 64483521; fax: +39 02 64483519.
E-mail address: paola.coccetti@unimib.it (P. Coccetti).

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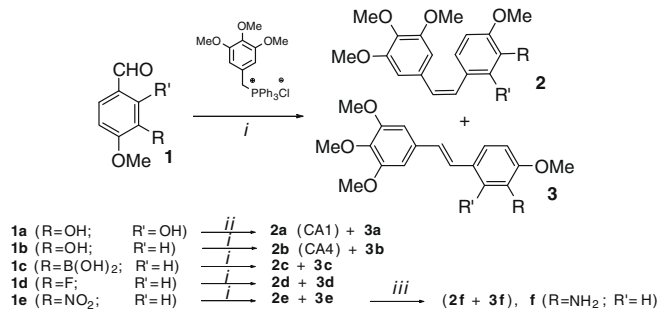
have not yet been evaluated. One of the best analysed organisms for cell cycle studies is the budding yeast *Saccharomyces cerevisiae*, which can grow on synthetic media at widely different growth rates. It is also well known at both the genetic and biochemical level, being the first eukaryote for which the complete genome sequence was obtained.[23] Genome-wide functional analyses, including the analysis of transcriptional responses to a range of stimuli, and comprehensive maps of protein–protein interactions further facilitate the study of the yeast cell cycle.[24,25] Finally, the high degree of conservation with mammalian cells in terms of gene sequences, proteins and biochemical functions makes *S. cerevisiae* very appealing for anti-cancer drug discovery.[26] In budding yeast, the cell cycle begins with the synthesis of the initiator cyclin Cln3, which binds to and activates the principal cyclin-dependent kinase Cdk1.[27] Substrate of the Cln3/Cdk1 complex is the transcriptional inhibitor Whi5 which, upon phosphorylation, is translocated out of the nucleus releasing the SBF and MBF transcription factors.[28,29] SBF and MBF transcription factors promote the expression of the other G1 cyclins, Cln1 and Cln2, responsible for bud formation and spindle pole body duplication,[27,30] and of the S-phase cyclins Clb5 and Clb6.[31] In G1 phase, Clb5,6/Cdk1 complexes are inhibited by the Cdk inhibitor Sic1 which prevents premature DNA replication.[32] This inhibition is removed following phosphorylation of Sic1 by Cln1,2/Cdk1 complexes, promoting its degradation via an ubiquitin-mediated mechanism, allowing Clb5,6/Cdk1-dependent onset of DNA replication.[33,34]

With the goal of elucidating the effects of combretastatins on cell cycle progression, the in vivo activity of compounds **2**(**a**–**f**) and **3**(**a**–**f**) was analysed by monitoring their ability to inhibit yeast cell growth. We synthesized the CA1 (**2a**, **3a**), the CA4 (**2b**, **3b**), and the eight CA4 derivatives **2**(**c–f**) and **3**(**c–f**), where the hydroxy group of the natural compound was replaced by fluoro, nitro, amino, and boronic acid moieties, either as *Z* or *E* isomer. The strategy used to prepare the cis and trans analogs is outlined in Scheme 1. CA1 (**2a**, **3a**) was obtained according to a methodology described in the literature,[35] whereas the eight derivatives **2**(**b–e**) and **3**(**b–e**) were prepared following a protocol alternative to those reported in the literature. All compounds are already known in the literature[36–39] but we developed a clean and more convenient methodology for their synthesis, which allowed us to skip the use of moisture-sensitive reagents and critical reaction conditions. We thus synthesized compounds **2**(**b–e**) and **3**(**b–e**) by a transfer-phase Wittig coupling of *p*-methoxybenzaldehyde bearing in position 3′ a boronic moiety, a fluoro atom or a nitro group, respectively, and triphenyl(3,4,5-trimethoxybenzyl)phosphonium chloride.[40] The most versatile reaction conditions were dichloromethane and a water solution saturated with potassium hydrogen carbonate. The cis/trans mixtures were purified and isomers were separated by flash chromatography in a gradient of acetone/petroleum ether. The compounds **2e** and **3e** were further submitted to a reduction reaction,[41] using sodium dithionite as reducing agent; the amino derivatives **2f** and **3f** were obtained with 60% yield after column purification. The boronic acid bioisostere **2c** was presented by Kong et al.[37] as a final product of two reactions performed in anhydrous conditions and the fluoro derivative **2d** was synthesized by Lawrence et al.[38] with a Wittig coupling at −78 °C. All compounds **2**(**b–e**) and **3**(**b–e**) were prepared in a single step from the corresponding aldehyde and phosphonium salt without dry solvents or low-temperature conditions.



Scheme 1. Reagents and conditions: (i) $KHCO_3/H_2O/CH_2Cl_2$, rt; (ii) (1) TBDMSCl/DIPEA/DMF; (2) BuLi/THF, from −20 °C to rt; (3) TBAF, THF; (iii) $Na_2S_2O_4$, acetone/water 2:1, 50 °C.

Exponentially growing cells of BY4741 *S. cerevisiae* strain (Supplementary data Table 2) were then grown in complete medium containing 2% glucose and at time 0 compounds shown in Scheme 1 (**2a–f** and **3a–f**) were added at a final concentration of 150, 300, and 450 μM. Samples were taken at the indicated time points to determine cell growth, budding index and DNA content by FACS analysis (Fig. 1A and B). Compounds **2d–f** and **3a–f** and compounds **3a**, **3b** and **3c** had no effect on yeast growth at all tested concentrations (data not shown). On the contrary, when log-phase cultures were grown and treated with molecules **2a**, **2b**, and **2c**, cell growth arrested in a dose-dependent manner (Fig. 1A). Remarkably, some differences were observed among the behaviour of the various compounds: molecule **2a** caused a small increase of budding index in comparison to the control culture, the majority of the population being arrested with a post-synthetic DNA content (Fig. 1A and B) and with short spindles typical of cells blocked in metaphase (data not shown). On the other hand, compounds **2b** and **2c** induced a strong reduction of budding index (42% with 450 μM, compare with 70% of the untreated culture, Fig. 1A) and about 50% of the population was completely arrested in G1 phase after 3 h treatment (Fig. 1B). In order to understand the molecular mechanism that accounts for G1 arrest mediated by compounds **2b** and **2c**, we analysed the pattern of expression of the main regulators of G1 phase, the cyclins Cln1, Cln2 and Clb5 and the inhibitor Sic1, following treatment with the drugs. To this end, strains bearing a TAP-tagged version of Cln1, Cln2, Clb5, and Sic1 were grown until exponential phase and treated with 450 μM of compounds **2b** and **2c** as reported above. Western blot analysis of samples taken after 3 h treatment showed that combretastatins **2b** and **2c** downregulated the level of Clb5 cyclin, which was required for the onset of DNA replication (Fig 1C). Cln1, Cln2, and Sic1 were still present in G1 arrested cells (Fig. 1C).

To further investigate whether the down-regulation of Clb5 expression was dependent on the presence of compounds **2b** and **2c**, exponentially growing Clb5-TAP strain was synchronized in G1 phase by α-factor treatment and released in fresh medium in the presence or absence of combretastatin **2b** or **2c**. As expected, control cells started to enter S-phase 20–30 min after the release, in keeping with the increase of Clb5 level (Fig. 2A and B). In contrast, Clb5 was not detectable in cells treated with both compounds **2b** and **2c** and, consistently, DNA replication did not begin (Fig. 2A and B).

Taken together these data suggest that combretastatins **2b** and **2c** determine a G1 arrest due to a decreased level of Clb5, which is not sufficient to start DNA replication. This shows a new block of yeast cell cycle which has not previously been identified in mammalian cells.

Since CA4 is known to induce AMPK activation in several mammalian cell lines,[18] we analysed the effect of compounds **2b** and **2c** on the activation of Snf1, the yeast orthologue of AMPK.[42,43] Snf1 plays an important role in regulating a wide range of responses to stress caused by nutrient limitation and salt stress[43,44] and phosphorylation on threonine 210 (T210), by one of the redundant upstream kinases Sak1, Tos3 or Elm1, is required for its activation.[45,46] We then tested whether Snf1 became phosphorylated at T210 following **2b** and **2c** treatment. Exponentially growing cells

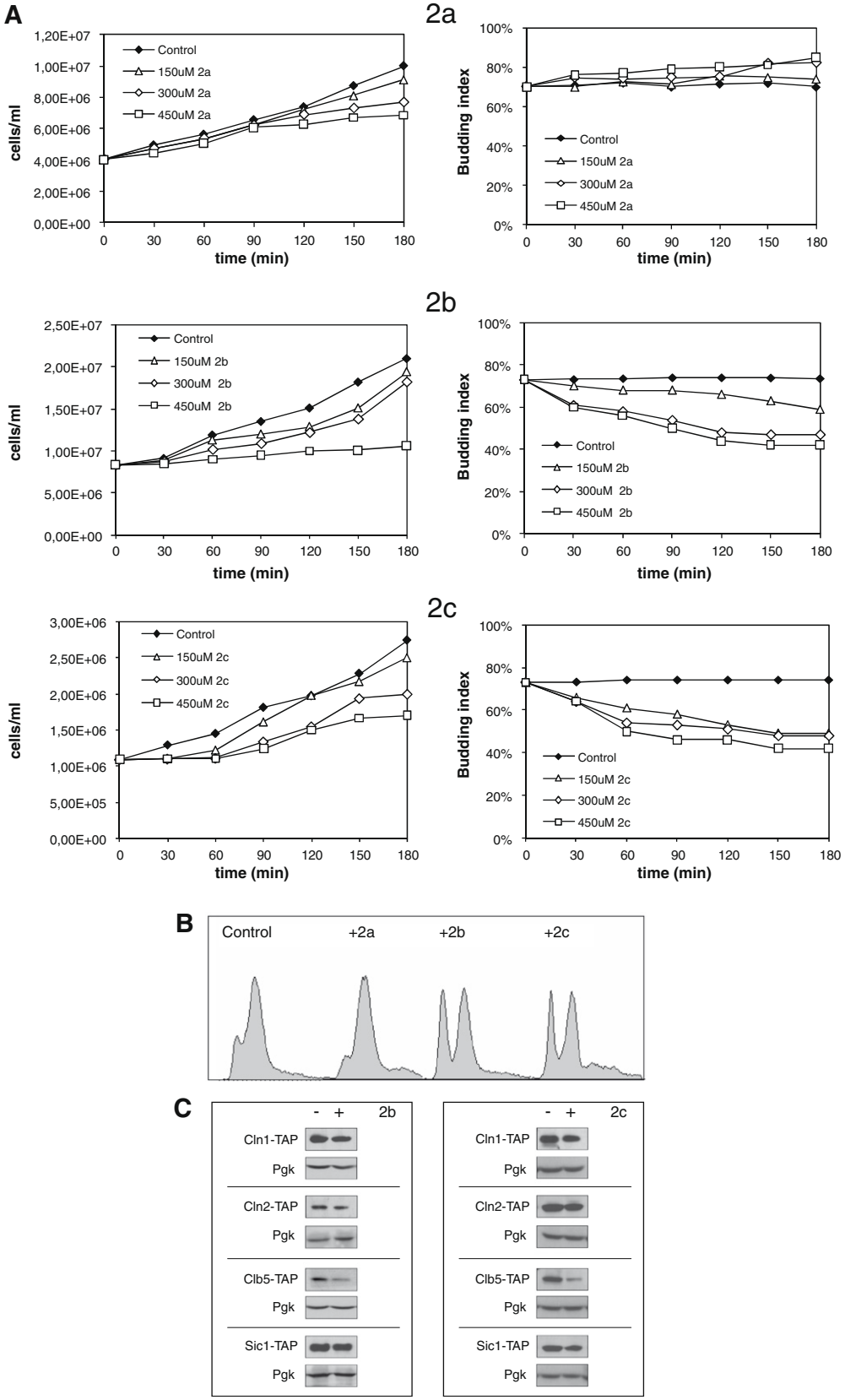


Figure 1. Combretatstatin **2a**, **2b** and **2c** inhibit the growth of budding yeast. Exponentially growing cells of BY4741 strain in YP medium, supplemented with 2% glucose, were treated with combretastatin **2a**, **2b** and **2c** (150 μM, 300 μM and 450 μM) at 25 °C. Samples were taken at the indicated time points to assay (A) cell density and budding index; (B) DNA content by FACS analysis. (C) Protein levels by western blot analysis of TAP-tagged proteins from strains treated for 3 h with 450 μM of compounds **2b** and **2c**. Anti-TAP antibody was used and Pgk1 was used as a loading control.

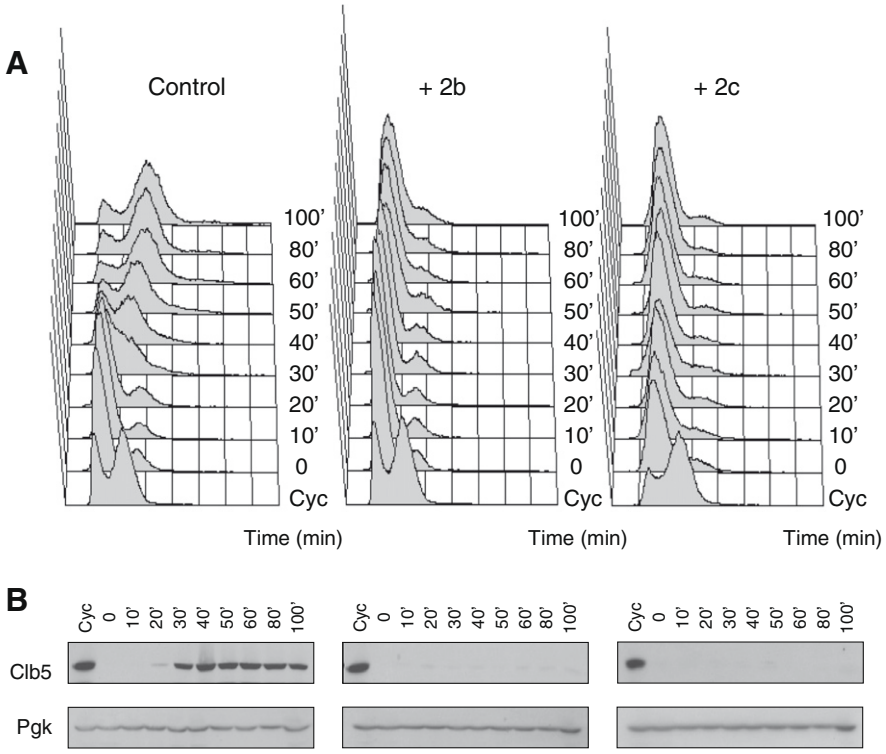


Figure 2. G1 arrested cells treated with combretastatin **2b** and **2c** are unable to traverse the G1 phase of the cell cycle. Clb5-TAP strain was synchronized in G1 phase by addition of α-factor treatment, then washed and released in fresh medium containing compounds **2b** and **2c** (450 μM); in control culture only DMSO was added. Samples were taken at the indicated time points to assay (A) DNA content by FACS analysis; (B) Clb5 level by western blot analysis using anti-TAP antibody. Pgk1 was used as a loading control.

of a yeast strain expressing Snf1-9myc were treated with compounds **2b** and **2c** for 3 h. Western blot analysis with specific anti-P-Thr-210 antibody revealed a very low level of Snf1 phosphorylation in the presence of combretastatin **2c**, while compound **2b** caused a consistent T210 phosphorylation on Snf1, comparable to that observed when cells were grown in low glucose concentration (0.05%), a known Snf1-activating condition (Fig. 3).[47]

Results reported in this Letter newly indicate an involvement of combretastatin **2b** and **2c** in arresting yeast growth inducing a G1 block not previously identified in mammalian cells, since they prevent DNA replication by affecting the synthesis of Clb5 protein, the principal S-phase cyclin. Furthermore, the G1 arrest is coincident with the activation of the stress activated kinase Snf1 in the case of compound **2b** and to a lesser extent with combretastatin **2c**. It has previously been reported that the homologue of Snf1 in mammalian cells—AMPK kinase—regulates cell proliferation by performing an energy-checkpoint at G1/S phase by the accumulation of p53 and of the cyclin-dependent kinase inhibitors, p21 and p27.[48] Another Snf1 homologue—DmAMPK in *Drosophila melanogaster*—was also found to be involved in cell cycle arrest, at the G1/S boundary, resulting in reduced levels of the rate-limiting protein Cyclin E.[49]

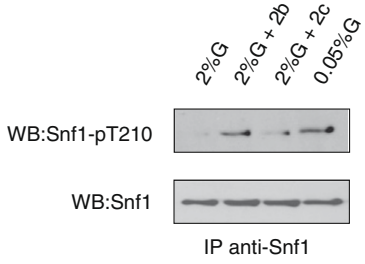


Figure 3. Snf1 is phosphorylated on Thr210 in cells treated with combretatstatin **2b**. Exponentially growing cells of Snf1-9myc strain were treated with 450 μM of combretastatin A4 **2b** and **2c** at 25 °C. In the control culture only DMSO was added (lane 1, cells grown in 2% glucose). After 3 h samples were taken and Snf1-9myc was immunopurified from 500 μg of total protein extract with anti-myc antibody. Western blot analysis to detect Snf1 phosphorylation on Thr210 was made with anti-Thr210P antibody and with anti-myc antibody to detect Snf1 total level.

Considering that CA4 has been identified as an activator of AMPK in several mammalian cell lines[18] and that data presented in this Letter show a similar role for Snf1, a full understanding of Snf1/AMPK signalling pathways could be useful for the development of optimal drugs for various diseases, since the AMPK pathway is deregulated in several cancer types.

Furthermore, the novel results presented here underline once more the importance of budding yeast as a model organism for understanding how intracellular and extracellular signals are transmitted to cell cycle machinery. These studies are of great importance considering that proliferative disorders are now such a major challenge for human health.

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Supplementary data

Supplementary data (synthesis, spectral data description, Table 2 of yeast strains used and yeast experiments performed) associated

with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.03.066.

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