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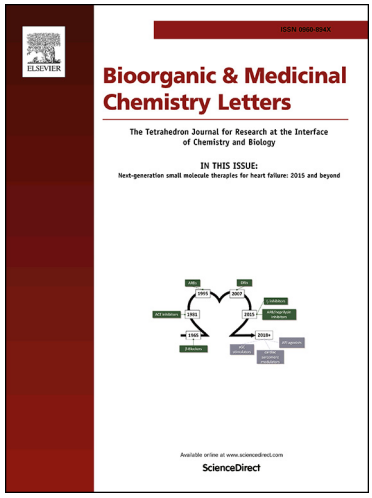

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PII: S0960-894X(18)30456-6
DOI: https://doi.org/10.1016/j.bmcl.2018.05.044
Reference: BMCL 25861

To appear in: *Bioorganic & Medicinal Chemistry Letters*

Received Date: 28 March 2018
Revised Date: 20 May 2018
Accepted Date: 23 May 2018

Please cite this article as: Dao, V.H., Ourliac-Garnier, I., Bazin, M-A., Jacquot, C., Baratte, B., Ruchaud, S., Bach, S., Grovel, O., Pape, P.L., Marchand, P., Benzofuro[3,2-*d*]pyrimidines inspired from cercosporamide *Ca*Pkc1 inhibitor: synthesis and evaluation of fluconazole susceptibility restoration, *Bioorganic & Medicinal Chemistry Letters* (2018), doi: https://doi.org/10.1016/j.bmcl.2018.05.044

Bioorganic & Medicinal Chemistry Letters
journal homepage: www.elsevier.com

Benzofuro[3,2-*d*]pyrimidines inspired from cercosporamide *Ca*Pkc1 inhibitor: synthesis and evaluation of fluconazole susceptibility restoration

Viet Hung Dao [a], Isabelle Ourliac-Garnier [b], Marc-Antoine Bazin [a], Catherine Jacquot [b], Blandine Baratte [c], Sandrine Ruchaud [c], Stéphane Bach [c], Olivier Grovel [d], Patrice Le Pape [b], Pascal Marchand [a,*]

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ARTICLE INFO

Article history:
Received
Revised
Accepted
Available online

Keywords:
Cercosporamide
Benzofuro[3,2-*d*]pyrimidine
Antifungal activity
Candida albicans Pkc1
Fluconazole susceptibility restoration

ABSTRACT

In a context of growing resistance to classical antifungal therapy, the design of new drugs targeting alternative pathways is highly expected. Benzofuro[3,2-*d*]pyrimidine derivatives, derived from (–)-cercosporamide, were synthesized and evaluated as potential *Candida albicans* PKC inhibitors in the aim of restoring susceptibility to azole treatment. Co-administration assay of benzofuropyrimidinedione **23** and fluconazole highlighted a synergistic effect on inhibition of cell growth of a *Candida albicans* resistant strain.

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Invasive fungal infections are a major cause of mortality worldwide, especially among vulnerable patients.[1] Among the 35876 invasive fungal disease cases identified in France on the 2001-2010 period, 43.4% are candidemia; this number increases among patients with hematologic malignancies and those with chronic renal failure.[2] *Candida albicans* remains the most prevalent species in invasive candidiasis in the United States[3] and Europe.[4] It is noteworthy that the proportion of infections caused by non-*albicans Candida* (NAC) species such as *C. glabrata*, *C. parapsilosis*, *C. tropicalis* and *C. krusei* has increased over the last two decades, species involvement depending on the infection site and the geography.[5] *Candida* pathogenicity is caused by a large number of mechanisms,[6] such as adherence to the host tissues and medical devices (biofilm), host recognition through binding to host cells and proteins and production of extracellular hydrolytic enzymes.[7] Its virulence depends on fungal but also on host factors in opportunistic situations.

In models of experimental infection, strains deleted for elements of MAPK-mediated signal transduction pathways exhibit a reduction or loss of virulence[8] and decrease in biofilm formation.[9] In *Candida albicans*, protein kinase C (called *Ca*Pkc1), one of the key proteins involved in MAPK pathways, is described as a regulator of cell wall integrity during growth, morphogenesis and response to cell wall stress.[10] In addition, La Fayette *et al.*[11] established a new role for PKC signaling in drug tolerance mechanisms. Given the limited number of antifungals used in clinic staments[12] and the emergence of drug resistance,[13] there is an urgent need to identify alternative targets in order to speed up the development of new generation of antifungals either more effective or able to restore susceptibility to classical antifungal drugs.[14] In this context, targeting PKC-mediated signal transduction pathway represents an new attractive strategy for antifungal therapy.[15]

(–)-Cercosporamide is a natural product isolated from the phytopathogen fungus *Cercosporidium henningsii*.[16] It was identified as a broad-spectrum antifungal agent displaying *in vitro* mean MIC value of 89 µg/mL[17] and of 10 µg/mL[18] against *C. albicans*. Interestingly, it appeared to act as a potent *Ca*Pkc1 ATP-competitive inhibitor with an IC_{50} of 44 nM.[18] Furthermore, cercosporamide inhibited human PKCα (IC_{50} = 1 µM) and PKCβ (IC_{50} = 0.3 µM)[18] and was later shown to inhibit other human kinases, including Mnk1/2, Jak3, GSK3β, ALK4 and Pim1, from nanomolar to low micromolar ranges.[19,20]

To the best of our knowledge, heterocyclic compounds displaying antifungal activity associated with *Ca*Pkc1 inhibition are not highlighted in the literature, except cercosporamide.[18] Consequently, in continuation of our successful attempts in the search of biologically active cercosporamide inspired derivatives,[21] we report here the synthesis and antifungal evaluation of benzofuro[3,2-*d*]pyrimidines targeting *Ca*Pkc1.

The strategy of mimicking natural product for the design of antifungal agents to combat fungal resistance was very recently validated by the discovery of xanthones derived from α-mangostin.[22] In addition, cercosporamide was found to recognize ATP-binding site of Mnk2 kinase through hydrogen bond network due to the 3-OH and the 4-$CONH_2$ of the phenyl portion, justifying the strategy of keeping dihydroxybenzofuran-carboxamide part of the natural product model for the design of new tricyclic compounds (Figure 1).[20]

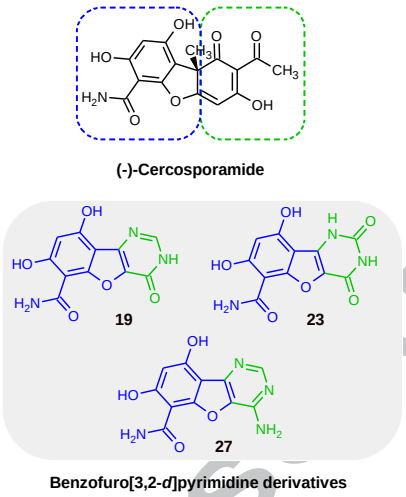
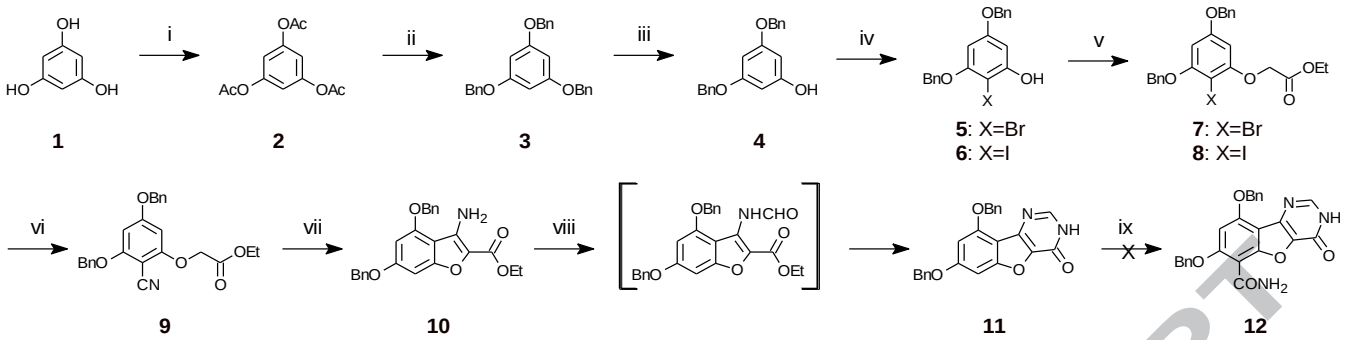


Fig. 1. Design of target compounds

In addition, our expertise in the synthesis of pyrimidine-fused heterocycles as biological agents targeting kinases was used for the design of the new compounds, constituting the third ring of the benzofuro[3,2-*d*]pyrimidine derivatives described in this study.[23]

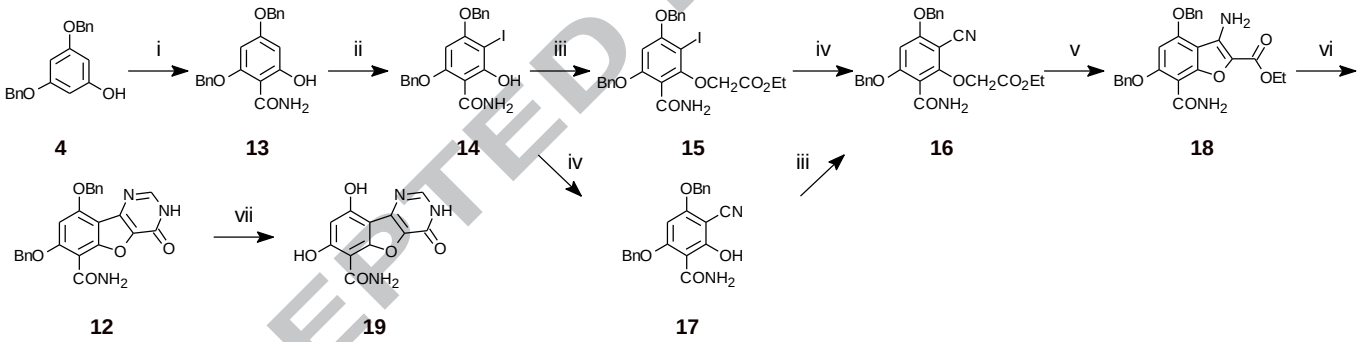
Benzofuran scaffold was first built from phloroglucinol **1** to achieve the suitable substitutions on the benzene ring and at the positions 2 and 3 for subsequent ring closure sequence (Scheme 1). 3,5-Dibenzyloxyphenol **4** was obtained in three steps by an initial tribenzylation of triacetoxyphloroglucinol **2** to avoid additive *C*-benzylation by direct *O*-benzylation of phloroglucinol **1**.[21,24] Mono-deprotection was carried out under transfer hydrogenation conditions using Pd/C and cyclohexene, in a mixture of ethyl acetate/ethanol at reflux, to afford compound **4** in a moderate yield (Scheme 1).[25] 2-Halo-3,5-dibenzyloxyphenols **5** and **6** were then synthesized by a mono-bromination or a mono-iodination procedure, respectively, very quickly at low temperature.[26] Afterwards, *O*-alkylation was realized using NaH as a base and ethyl bromoacetate at room temperature providing esters **7** and **8** in excellent yields.[27] In the next step, cyanation in the presence of copper cyanide in DMF gave ester **9** in a good yield from iodinated precursor **8** but the corresponding reaction, from the brominated counterpart **7**, remained less effective (19% of yield).[23b] Cyclization of the ethoxycarbonylmethylether **9** was performed in the presence of NaH to furnish benzofuran derivative **10** bearing amino group at position 3 and ester function at position 2.[28] Finally, benzofuro[3,2-*d*]pyrimidin-4-one derivative **11** was obtained through a ring closure reaction from benzofuran precursor **10** *via* a formamide intermediate reacting with ammonia.[27] Unfortunately, the carbamoyl group in position 6 could not be incorporated directly by electrophilic aromatic substitution using chlorosulfonyl isocyanate (CSI) followed by an acidic hydrolysis step.[21]



Scheme 1. (i) Ac_2O, pyridine, 120 °C, 5 h, 87%; (ii) BnCl, NaH, DMF, H_2O, 0 °C to rt, 10 h, 96%; (iii) C_6H_{10}, Pd-C 10%, AcOEt/EtOH (3/1), 110 °C, 2 h, 45%; (iv) NBS (1.0 eq.), CH_2Cl_2, -78 °C, 2 min, 95% for **5** or NIS (1.0 eq.), CH_2Cl_2, -78 °C, 2 min, 76% for **6**; (v) NaH, $BrCH_2CO_2Et$, DMF, rt, 12 h, 83% for **7** and 96% for **8**; (vi) CuCN, DMF, 160 °C, 1 h, 19% from **7** and 92% from **8**; (vii) NaH, DMF, 0 °C, 30 min, 66%; (viii) $HC(OEt)_3$, MW, 200 °C, 15 min then NH_3/MeOH 7N, MW, 140 °C, 15 min, 31%; (ix) CSI, CH_3CN, rt, 24h then HCl 1N, rt, 24 h, failure.

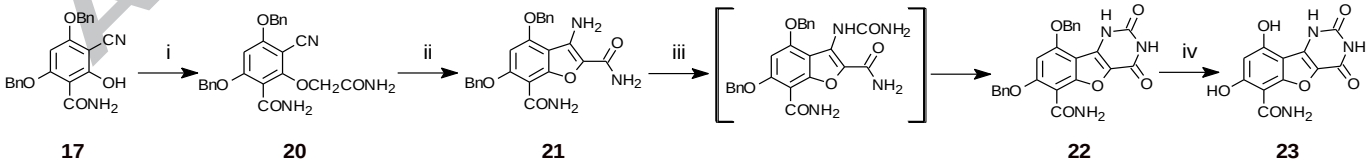
To circumvent this issue, we decided to introduce $CONH_2$ group at the beginning of the synthesis (Scheme 2). A direct aminocarbonylation of phenol derivative **4** in the presence of CSI afforded benzamide **13** after hydrolysis of the corresponding chlorosulfonyl intermediate. Afterwards, the same reaction sequence, as previously described in scheme 1, was applied to obtain benzofuro[3,2-*d*]pyrimidin-4-one derivative **12** bearing the carbamoyl appendage at C-6 position of the azaheterocycle. The only difference was the possibility to perform the mono-iodination at 0 °C *vs* -78 °C and according to the yields, it was better to carry out first the cyanation (compound **17**) and then *O*-alkylation (compound **16**). In the last step, benzyl cleavage was accomplished with concentrated sulfuric acid to provide the target compound **19**.

The synthetic route developed for the preparation of the 2,4-dioxo-1,2,3,4-tetrahydro[1]benzofuro[3,2-*d*]pyrimidine-6 carboxamide **23** is outlined in Scheme 3. To this end, *O*-alkylation of the benzamide derivative **17** was performed by the formation of the sodium salt in the presence of sodium hydride as base followed by its reaction with 2-iodoacetamide as described for the corresponding ester **16** (Scheme 2) but warming the medium at 60 °C.[29] Cyclization using KOH/EtOH at 60 °C furnished benzofuran-2,7-dicarboxamide **21** in acceptable yield. Indeed, we observed that these basic conditions were more efficient than NaH/DMF combination (50% *vs* 60% of yield).



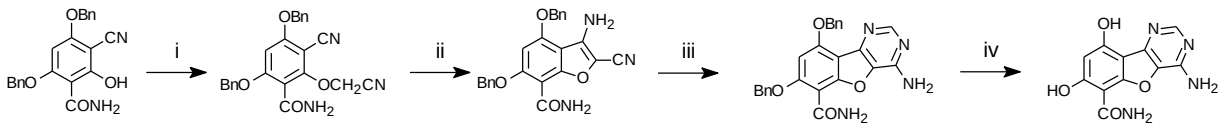
Scheme 2. (i) CSI, CH_3CN, 0 °C, 10 min then HCl 1N, rt, 10 h, 53%; (ii) NIS, CH_2Cl_2, 0 °C, 10 min, 80%; (iii) NaH, $BrCH_2CO_2Et$, DMF, rt, 24 h, 86% for **15** and 90% for **16** (iv) CuCN, DMF, 160 °C, 6 h, 20% for **16** and 73% for **17**; (v) NaH, DMF, 0 °C, 10 min, 67%; (vi) $HC(OEt)_3$, MW, 200 °C, 15 min then NH_3/MeOH 7N, MW, 140 °C, 15 min, 62%; (vii) H_2SO_4cc, rt, 15 min, 53 %.

Benzofuropyrimidinedione derivative **22** was then synthesized from benzofuran precursor **21** *via* an urea intermediate.[30] Finally, the dihydroxy analogue **23** was formed by treatment with concentrated sulfuric acid as procedure of debenzylation.



Scheme 3. (i) NaH, ICH_2CONH_2, DMF, 60 °C, 12 h, 69%; (ii) KOH, EtOH, 60 °C, 30 min, 60%; (iii) 1) KOCN, CH_3COOH, 100 °C, 1 h - 2) KOH, EtOH, 90 °C, 1 h, 56%; (iv) H_2SO_4cc, rt, 5 min, 48%.

Synthesis of the 4-aminobenzofuro[3,2-*d*]pyrimidine-6-carboxamide derivative **27** started by *O*-alkylation of 3-cyano-2-hydroxybenzamide **17** in the presence of K_2CO_3 and bromoacetonitrile in DMF at 80 °C.[22b] Heteroannulation of the corresponding cyanomethylether intermediate **24** using NaH furnished readily 3-amino-2-cyanobenzofuran-7-carboxamide **25** with suitable substitutions for the production of the fused 4-aminopyridine ring (compound **26**). Previous debenzylation conditions were then applied leading to the unprotected analogue **27**.



Scheme 4. (i) $BrCH_2CN$, K_2CO_3, DMF, 80 °C, 24 h, 72%; (ii) NaH, DMF, 0 °C, 5 min, 64%; (iii) $HC(OEt)_3$, MW, 200 °C, 15 min then NH_3/MeOH 7N, MW, 140 °C, 15 min, 45%; (iv) H_2SO_4cc, rt, 5 min, 66%.

Inhibition of PKC activity (*Ca*Pkc1) was initially investigated in order to check the involvement of this protein as the putative target of benzofuro[3,2-*d*]pyrimidine derivatives. Pkc activity of *C. albicans* (CAAL2) total protein extracts was measured using ELISA-based PKC Kinase Activity Assay (Enzo Life Sciences, Inc.) as previously described.[31] PKC inhibition activity was expressed as the ratio between the absorbance measured for protein extract treated with compounds **19**, **23** or **27** (100 µM) and untreated protein extract. The reference natural (–)-cercosporamide was provided by extraction and purification from a *Mycosphaerella henningsii* culture using a modified procedure of the literature (see Supplementary Material).[16,32]

As depicted in Figure 2A, when used at 100 µM cercosporamide displayed a moderate Pkc inhibitory activity of 39±3% in our conditions. Interestingly, compounds **19**, **23**, and **27** showed statistically ($p < 0.05$) significant higher inhibition with values of 88±2%, 71±5% and 81±3%, respectively, at 100 µM. The same experiments performed with active human PKC showed that all the compounds also displayed inhibition of the human enzyme counterpart (Figure 2B). All together these data supported a moderate activity on PKCs. Moreover the absence of selectivity for fungal *Ca*Pkc1 suggested potential additional fungal targets for these compounds as discussed below.

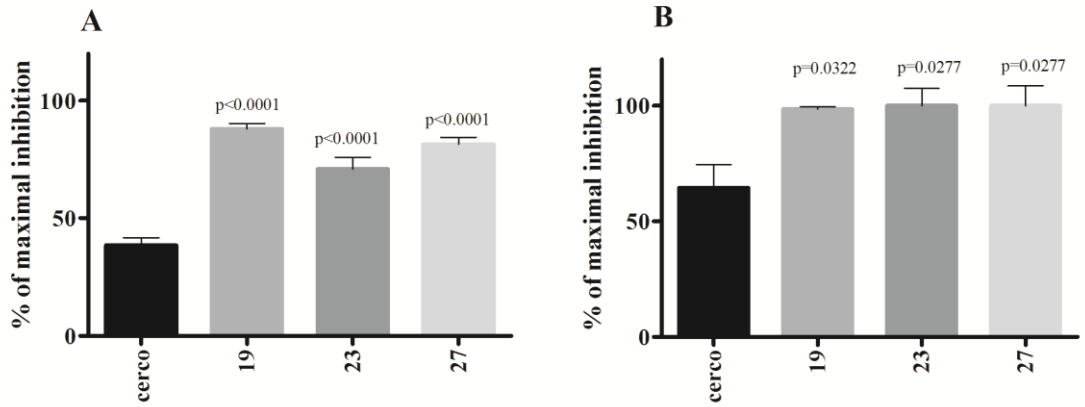
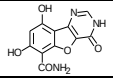
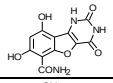
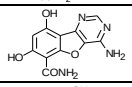
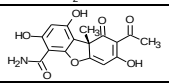


Fig 2. Percentage of inhibition of Pkc activity on protein extract of CAAL2 (A) and percentage of inhibition human PKCα activity (B). (**cerco**: cercosporamide, **19**: compound **19**, **23**: compound **23**, **27**: compound **27**). Mean percentage of inhibition of PKC activity were obtained for each compound (100 µM) and compared with mean results obtained for cercosporamide (100 µM) applying a one-way ANOVA followed by multiple comparisons with an uncorrected Fisher's LSD test. p values were expressed for uncorrected Fisher's LSD test.

Afterwards, compounds **19**, **23** and **27** were assessed for their *in vitro* antifungal properties alone or in combination with fluconazole against six *C. albicans* clinical isolates. Among the six strains chosen for this study, CAAL93 and CAAL97 were susceptible to fluconazole and CAAL2, CAAL28, CAAL111 and CAAL117 exhibited resistance through well characterized and distinct mechanisms, as we previously described.[33] Minimal inhibitory concentrations (MICs) were determined according to a slightly modified EUCAST reference method[34,35] and expressed as the compound concentration that produced 50% of growth inhibition compared to the drug-free growth control. In the experiments, cercosporamide exhibited MICs of about 100 µM (33 µg/mL) on CAAL93, CAAL97 and CAAL2 strains in agreement with previous findings.[17,18] Unfortunately, even at 300 µM (100 µg/mL), i. e. the highest tested concentration based on Lafayette *et al*. previous work,[11] no activity was measured for cercosporamide on resistant strains CAAL28, CAAL111 and CAAL117 (Table 1). Against all 6 strains studied, MICs for compounds **19**, **23**, and **27** were greater than 300 µM. Consequently, two hypotheses may arise from the intrinsic antifungal inactivity of the compounds: they might not reach the fungal cytosol or *C. albicans* Pkc1 would not be a main protein to target suggesting that *pkc* is not an essential gene.

Table 1. Activity of compounds **19**, **23** and **27**, cercosporamide and fluconazole on 6 clinical isolates of *C. albicans* and HeLa

Cpd	Structure	MIC values (µM)[a]						
		HeLa	CAAL93	CAAL97	CAAL 2	CAAL28	CAAL111	CAAL117

19		227.53 ±27.97	> 300	> 300	> 300	> 300	> 300	> 300
23		> 300	> 300	> 300	> 300	> 300	> 300	> 300
27		> 300	> 300	> 300	> 300	> 300	> 300	> 300
CERCO[b]		< 3	103.2 ±23.2	93.3 ±18.5	90.1 ±11.3	> 300	> 300	> 300
FLU[b]			0.14 ±0.07	0.19 ±0.11	> 100	> 100	> 100	> 100

[a] Values represent the mean ±SD of experiments performed in triplicate. MIC were determined as the compound concentration that produced 50% of growth inhibition relative to that of the drug-free growth control.
[b] CERCO: cercosporamide; FLU: fluconazole.

Initial hypothesis was that benzofuro[3,2-*d*]pyrimidine derivatives could restore susceptibility of fluconazole-resistant strains. Therefore, a checkerboard assay was performed with the same methodology as MIC determination (see Supplementary Material).[36] MICs of compounds **19**, **23**, and **27** alone and in combination with fluconazole were determined to calculate a fractional inhibitory concentration index (FICI) (Table 2).[37] FICI values were used to classify interactions between fluconazole and compounds **19**, **23**, or **27** as recommended by Odds:[38] "synergy" (FICI ≤ 0.5), "antagonism" (FICI > 4.0) and "no interaction" (FICI > 0.5–4.0). Combination of cercosporamide and fluconazole exhibited a synergistic effect only against CAAL2 (FICI = 0.50). Compound **23** and fluconazole showed a promising synergistic effect on inhibition of cell growth of CAAL111 strain (FICI = 0.35) contrary to combination of cercosporamide and fluconazole against that strain. Interestingly, this synergistic effect demonstrated interaction of the benzofuro[3,2-*d*]pyrimidine derivative **23** at cellular level. Compound **27** and fluconazole had no effect on cell growth when combined, whatever the strain assessed. Compound **19** combined with fluconazole decreased cell growth of CAAL2 and CAAL117 strains but calculation of FICI did not allow to conclude to any interaction.

Table 2. MIC results of checkerboard assays testing the combination of compounds **19**, **23** and **27** with fluconazole against 4 fluconazole-resistant *C. albicans* clinical isolates.

	MIC (µM) and FICI[a] determination											
	FLU	**19**	**FLU/19[b]**	**$FICI_{19}$**	**23**	**FLU/23[b]**	**$FICI_{23}$**	**27**	**FLU/27[b]**	**$FICI_{27}$**	**cerco**	**FLU/cerco[b]**	**$FICI_{cerco}$**
CAAL2	> 100	> 300	4/300	**1.06**	> 300	> 100/> 300	2.0	> 300	> 100/> 300	2.0	100	0.5/50	**0.50**
CAAL28	> 100	> 300	> 100/> 300	2.0	> 300	> 100/> 300	2.0	> 300	> 100/> 300	2.0	> 300	> 100/> 300	2.0
CAAL111	> 100	> 300	> 100/> 300	2.0	> 300	50/30	**0.35**	> 300	> 100/> 300	2.0	> 300	> 100/> 300	2.0
CAAL117	> 100	> 300	50/300	**1.25**	> 300	> 100/> 300	2.0	> 300	> 100/> 300	2.0	> 300	100/> 300	2.0

[a] FICI was the sum of the FICs of each of the drugs, which in turn is defined as the MIC of each drug when used in combination divided by the MIC of the drug when used alone. Highest FICI value could not be greater than 2.0 due to highest concentrations tested for each compound.
[b] MIC in combination expressed as [FLU]/[compound]

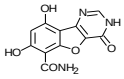
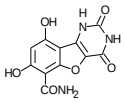
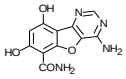
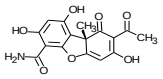
As MAP kinases were shown to be involved in biofilm formation,[9] an assay was performed to explore the potential antibiofilm effect of cercosporamide and compound **19**. CAAL121, CAAL124 and CAAL146 were selected for their ability to form biofilm. After 24h of incubation, mature biofilm was treated for 24h with cercosporamide or compound **19**. Minimal inhibitory concentrations (MICs) was determined as the antifungal concentration that produced 50 % of biofilm growth inhibition compared to the drug-free growth control. For cercosporamide, MICs of about 120 µM were measured on CAAL124 and CAAL146, whereas no effect was observed on CAAL121, even at 300 µM. In addition, no effect on biofilm growth was observed for compound **19** on any strain. Taking into account the results obtained for cercosporamide, inhibiting Pkc1 could be of interest in targeting biofilm growth. However, more experiments are necessary on all the benzofuro[3,2-*d*]pyrimidine series to develop more in depth the first biological data.

To investigate *in vitro* cytotoxic activity of benzofuro[3,2-*d*]pyrimidine derivatives, assay on Hela cells was performed. Compounds **19**, **23**, and **27** proved to be, at least 100 times, less cytotoxic than cercosporamide (Table 1).

Due to the common reports of tricyclic pyrimidine as kinase inhibitors[19,20] and the reported polypharmacology of the class, compounds **19**, **23**, and **27** and cercosporamide were evaluated by a kinase screen to investigate their selectivity profile.[23] Kinases available for this study were *Hs*CDK2/CyclinA, *Hs*CDK5/p25, *Hs*CDK9/CyclinT, *Hs*PIM1, *Hs*Haspin, *Mm*CLK1, *Rn*DYRK1A, *Ssc*GSK3α/β, *Ssc*CK1δ/ε and *Hs*Aurora B. As described in the literature,[19] cercosporamide showed inhibitory activity at sub-micromolar range against CDK2 (IC_{50}= 0.77 µM) and CDK9 (IC_{50}= 0.22 µM) (Table 3). In addition, lower inhibitory activity was measured against Aurora B (IC_{50}= 2.40 µM), CDK5 (IC_{50}= 5.60 µM) and PIM1 (IC_{50}= 8.10 µM), and no activity was detected against the other kinases. Tricyclic pyrimidinone **19** and its pyrimidinedione counterpart **23** remained inactive on the

panel of kinases suggesting that compound **23** restoring susceptibility of fluconazole-resistant strains (CAAL111), could be the result of PKC inhibition. Finally, 4-aminopyrimidine derivative **27** exhibited significant kinase inhibition towards PIM1 (IC_{50}= 1.29 µM), CLK1 (IC_{50}= 2.69 µM) and DYRK1A (IC_{50}= 8.23 µM) offering new potential targets for this compound. Indeed, after performing alignment of human kinase sequences with *C. albicans* genome, Fun31p, a serine/threonine kinase of this pathogen exhibits 51 % similarity with the kinase domain of human PIM1. This fungal kinase being implicated in cell wall damage regulation in *Candida albicans* could be a putative target for compound **27**.[39]

Table 3. Inhibitory activity of benzofuro[3,2-*d*]pyrimidines **19**, **23**, **27** and cercosporamide against a panel of 10 protein kinases.

Cpd	Structure	IC_{50} (µM)[a]									
		CDK2/ CyclinA	CDK5/ p25	CDK9/ CyclinT	PIM1	Haspin	CLK1	DYRK1A	GSK3α/β	CK1δ/ε	AurKB
19		>10	>10	>10	>10	>10	>10	>10	>10	>10	>10
23		>10	>10	>10	>10	>10	>10	>10	>10	>10	>10
27		>10	>10	>10	1.29	>10	2.69	8.23	>10	>10	>10
CERCO[b]		0.77	5.60	0.22	8.10	>10	>10	>10	>10	>10	2.40

[a] Values are the mean of at least two independent determinations and are within ± 15% SD.

[b] (–)-Cercosporamide is used as reference compound.

In summary, we have reported that cercosporamide is a valuable structural model for the design of promising tricyclic compounds restoring susceptibility of fluconazole-resistant strains through PKC inhibition. In addition, other kinases have appeared as interesting molecular targets for one of the benzofuro[3,2-*d*]pyrimidine derivatives. Furthermore, some pharmacomodulations are still requested to improve the effects observed in this study.

Acknowledgments

Authors are thankful to T. Robiou du Pont for performing fungal seedings and cultures, and M.-C. Boumard for helping in separation and purification of cercosporamide. They also acknowledge Dr. B. Misme-Aucouturier for helping in statistical analysis.

The authors also thank the Cancéropôle Grand-Ouest (axis: Nature sea products in cancer treatment), GIS IBiSA (Infrastructures en Biologie Santé et Agronomie, France), Biogenouest (Western France life science and environment core facility network) for supporting kinase screening facility (KISSf, Roscoff, France) and Consortium against Parasites and Fungi (CaPF).

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Supplementary Material

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Benzofuro[3,2-*d*]pyrimidines inspired from cercosporamide *Ca*Pkc1 inhibitor: synthesis and evaluation of fluconazole susceptibility restoration

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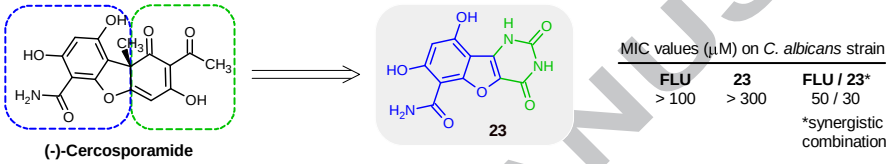
Viet Hung Dao, Isabelle Ourliac-Garnier, Marc-Antoine Bazin, Catherine Jacquot, Blandine Baratte, Sandrine Ruchaud, Stéphane Bach, Olivier Grovel, Patrice Le Pape, Pascal Marchand*



HIGHLIGHTS

Heterocyclic compounds inspired from cercosporamide were synthesized
Synergistic effect with fluconazole on inhibition of cell growth of a *Candida albicans* resistant strain is reported.
Kinase inhibitory activity was detected