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Triaryl methane derivatives as antiproliferative agents

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Abstract—Clotrimazole (CLT) **1**, a synthetic anti-fungal imidazole derivative, inhibits tumor cell proliferation and angiogenesis. In the current study, flow cytometric analysis demonstrated that the decrease in tumor cell growth by CLT **1** was associated with inhibition of cell cycle progression at the G_1–S phase transition, resulting in G_0–G_1 arrest. A series of CLT **1** analogues has been generated in order to develop CLT **1** derivatives that are devoid of the imidazole moiety which is responsible for the hepatoxicity associated with CLT **1** while retaining CLT **1** efficacy. The majority of these analogues demonstrate in vitro antiproliferative activity ranging from submicromolar to micromolar concentrations.

Cancer remains the second leading cause of death in the US and as a result there is a pressing need for novel and effective treatments. One characteristic of cancer cells, that distinguishes them from most normal cells, is their high proliferative index. As a result, targeting of proliferative pathways resulting in cell death via apoptosis or prevention of cell division via cell-cycle arrest, are considered effective strategies for fighting this disease. Calcium is one of the most versatile second messengers involved in cell growth, differentiation and signaling of apoptosis.[1] As a result, modulation of calcium signaling pathways in cells may play an important role in controlling cell growth and directing cell fate. Compounds designed to block Ca^{2+} influx have demonstrated inhibition of human prostate cancer cell proliferation in vitro and in a mouse model.[2]

Clotrimazole (CLT) **1** (Fig. 1) is a synthetic chlorinated imidazole derivative first synthesized by BAYER researchers.[3] CLT **1** is a broad-spectrum antimycotic agent used to treat pathogenic dermatophytes, yeast infections, and topical fungal infections caused by several species of Candida, Trichophyton, Microsporum, Epidermophyton and Malassezia.[4] The antifungal activity of CLT **1** is via the inhibition of sterol 14α-demethylase, a microsomal cytochrome P-450 enzyme.[5]

CLT **1**, like other antimycotic imidazole containing compounds, econazole **2**, metronidazole **3**, and miconazole **4** (Fig. 1), was found to inhibit voltage- and ligand-stimulated Ca^{2+} influx in nucleated cells.[6] Recent studies have demonstrated that CLT **1** has antiproliferative and antiangiogenetic activity.[7,8] This has led to the search for structural analogues of CLT **1** that retain biological activity after removal of the imidazole moiety which is responsible for hepatotoxicity that limits clinical application of CLT **1**. It has been demonstrated that the imidazole ring of CLT **1** is not required for inhibition of human erythrocyte Ca^{++} activated K^+ channels.[9] Furthermore, other imidazole containing antimytotic compounds like **2**, **3** and **4** have similar antimycotic activity as CLT **1**, but do not possess antiproliferative activity.[7] Taken together, these observations lead to the hypothesis that the antiproliferative activity of CLT **1** results from the non-imidazole part of its structure, while the imidazole moiety is responsible for antimycotic activity.

To test this hypothesis, the imidazole moiety of CLT **1** was removed via refluxing in 1 M HCl for 3 h (Scheme 1) to generate compound **5**. Compound **5** was subsequently transformed to compound **6** in the presence of NaI, chlorotrimethylsilane, MeCN, and CH_2Cl_2 at room temperature for 48 h. Both compounds **5** and **6** are considered to be the main metabolites of CLT **1** in humans.[9] A sulforhodamine B (SRB) assay,[10] using human colon adenocarcinoma cells (HT-29), was performed to compare the antiproliferative activity of CLT **1** with that of compounds **5** and **6**. The IC_{50} values were determined to be 5.1, 17.6 and 19.9 μM, respectively. To corroborate these results, a mitogen-induced cell proliferation assay,[11] using NIH 3T3 cells was performed

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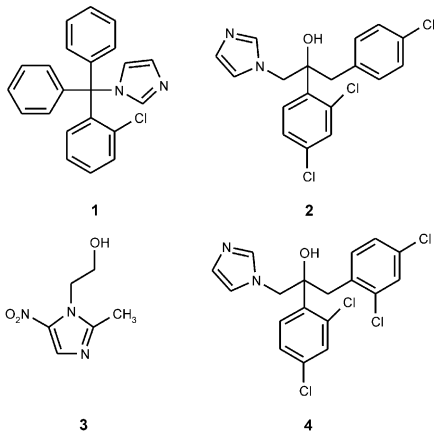
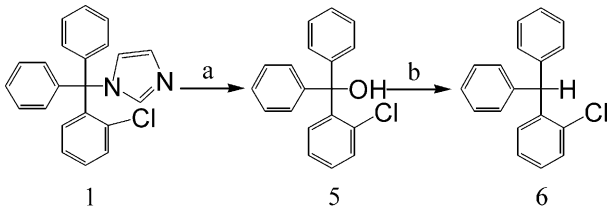


Figure 1. The structures of the antimitotic imidazole derivatives, Clotrimazole **1**, Econazole **2**, Metronidazole **3** and Miconazole **4**.



Scheme 1. (a) 1 M HCl, reflux, 3 h; (b) NaI, chlorotrimethylsilane, MeCN, CH_2Cl_2, rt, 48 h.

and the differences in IC_{50} values of these compounds were determined to be comparable to those obtained from SRB assays at 0.63, 2.1 and 3.9 μM, respectively. These results demonstrate that removal of the imidazole moiety does not completely inhibit the antiproliferative activity of CLT **1**, although the 3- to 6-fold increase in IC_{50} value illustrates the importance of the imidazole, either directly or as a result of contributions to the structural configuration of the molecule. Given the potential therapeutic benefit from removal of the imidazole moiety, these results warranted a more detailed investigation of the antiproliferative activity of non-imidazole structure.

Subsequently, the non-imidazole portion of CLT **1** was modified and a series of triaryl methane derivatives was synthesized using previously described synthetic procedures.[12] A mitogen-induced cell proliferation assay was used to evaluate the antiproliferative effect of these derivatives in NIH 3T3 cells.[11] Table 1 shown below summarizes the IC_{50} values and the percent inhibition at a concentration of 10 μM, for 23 compounds compared to CLT **1**.

The results clearly demonstrate the feasibility of generating therapeutically useful compounds through structural modification of the triaryl structure of CLT **1** while completely eliminating the imidazole moiety. Despite the modifications described in Table 1, all the compounds are predicted to have a similar conformation around the central methane atom. Several analogues were shown to possess similar or better antiproliferative activity than CLT **1** (see compounds **7**, **11**, **13**, **15**, **23**, **24**, **25** and **26**). The differences in potency may be the result of a general contribution of electron density to the molecule and/or from the introduction of specific functionalities on the aryl groups. These and other possibilities are currently under investigation. In addition, further modification and potency screening may result in a detailed understanding of the underlying mechanisms by which modification affects potency. The ultimate goal of these studies is the generation and characterization of potential therapeutic compounds that can be advanced to preclinical and clinical development.

To further investigate the effects of CLT **1** on cell cycle progression, DNA content analysis of the human non-small cell lung carcinoma cells (NCI-H460) was determined after treatment with CLT **1**.[13] Figure 2 shows the results upon treatment with different concentrations of CLT **1**. When cultures were synchronized by serum-starvation and subsequently released into complete medium and treated with vehicle alone (DMSO) for 16 h, cell cycle progression was evident with an increase in the number of cell progressing into S and G_2–M phases (A; 54.28 and 13.55%). However, cell cycle progression was dramatically blocked in G_0–G_1 when cells were treated with CLT **1**. In the presence of 10 μM CLT **1**, fewer cells exited out of G_0–G_1 phase (B; 43.99%) and consequently fewer cells progressed to S phase (B; 45.99%). Higher concentrations of CLT **1** (25 and 50 μM) resulted in a dose dependent increase in the number of cells remaining in G_0–G_1 phase at 68.53% (C) and 77.78% (D), respectively. There was a concomitant decrease in the number of cells in S phase. There was no indication that CLT **1** treatment-induces apoptosis as there was no increase in the sub-G_1 population of cells. Taken together, these results are consistent with previous findings that CLT **1** inhibits the expression of G_1 cyclins, such as cylclin D1 and cyclin E, through depletion of intracellular Ca^{2+} stores.[7] This depletion was shown to result in activation of RNA-dependent protein kinase (PKR), inducing phosphorylation of eIF2α at serine 51 and its concomitant inactivation. Inactivation of eIF2α inhibits formation of the ternary complex between Met-tRNA, eIF2α, and GTP, the rate-limiting step in translation initiation.[14,15]

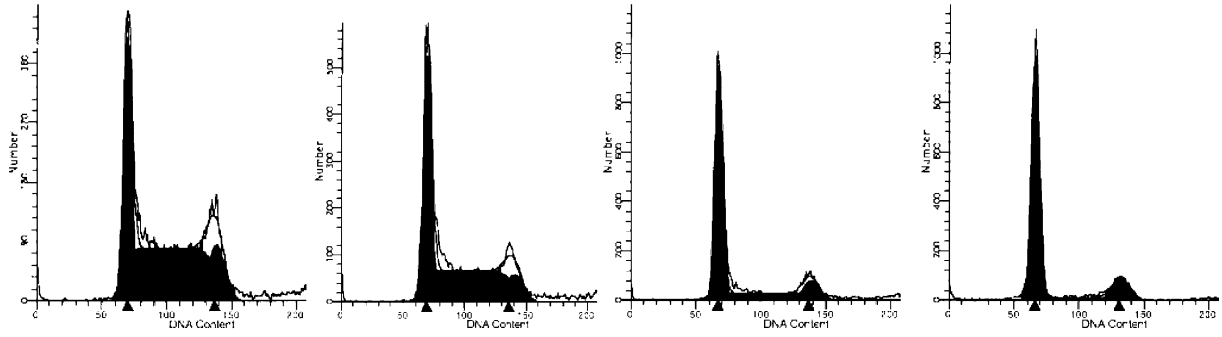
In order to assess whether the analogues decrease cell proliferation via a similar mechanism as CLT **1**, the flow cytometric analysis was repeated with compounds **26** and **27**. Table 2 summarizes the results and shows that both compounds produce a similar pattern of cell cycle progression as CLT **1**. Analysis of additional analogues is ongoing as well as in vitro studies aimed at understanding the detailed mechanism of action of the more promising compounds, including analysis of cyclin expression and induction of apoptosis.

In conclusion, the results of this study, together with previous findings, demonstrate that the antimycotic drug CLT **1** is an effective cell cycle inhibitor that specifically arrests the cell cycle in G_0–G_1 phase. Several analogues of CLT **1**, described in the current study, were also effective in vitro, with growth inhibition in the submicromolar to micromolar concentration range.

Table 1. Mitogen-induced cell proliferation assay[a] of the triaryl methane derivatives ($R_1R_2R_3CR_4$), CLT **1** analogues

Compd	R_1	R_2	R_3	R_4	IC_{50}, μM	% Inhibition at 10 μM
CLT					0.63	93.0
5	2-Cl–Ph	Ph	Ph	OH	2.1	31.0
6	2-Cl–Ph	Ph	Ph	H	3.9	91.8
7	Ph	Ph	Ph	OH	0.4–2.0	99.0
8	Ph	Ph	PhPh	OEt	2.2	99.0
9	2-OH–Ph	Ph	Ph	OH	3.3	99.0
10	2-Cl–Ph	2-OH–Ph	Ph	OH	1.0	99.0
11	Ph	2-OH–Ph	Ph	OH	0.8	99.0
12	Ph	4-CF_3–Ph	Ph	OH	3.1	98.0
13	2-Cl 4-NO_2 Ph	Ph	Ph	OH	0.3	94.0
14	4-Me–Ph	4-Me–Ph	4-Me–Ph	OH	3.0	98.0
15	4-Br–Ph	4-Br–Ph	Ph	OH	0.50	99.0
16	Ph	1-Naphthaline	Ph	OH	1.60	99.0
17	4-NO_2–Ph	Ph	Ph	CN	1.70	93.0
18	Ph	Cyclohexane	Ph	OH	9.50	94.0
19	Ph	Cyclohexane	Ph	CN	0.90	98.0
20	Ph	Piperidine	Ph	CO_2Et	2.20	96.0
21	2-Cl–Ph	Ph	Ph	CH_2CO_2H	1.20	92.0
22	2-Cl–Ph	Ph	Ph	CN	2.2	95.8
23	4-NH_2–Ph	4-NH_2–Ph	4-NH_2–Ph	OH	0.2	99.0
24	Ph	C_2Ph	Ph	OH	0.4	98.0
25	Ph	4-Cl–Ph	Ph	OH	0.8	99.0
26	Ph	Ph	C_2–Ph	OH	0.4	98.0
27	Ph	Ph	Ph	(*S*)-(+)-5-(Oxymethyl)-2-Pyrrolidinone	3.8	77.0

[a] The assay has been performed as in ref. 11.



Conc.	DMSO	10μM	25 μM	50 μM
G_0-G_1	32.17%	43.99%	68.53%	77.78%
S	54.28%	45.99%	19.72%	8.23%
G_2-M	13.55%	10.03%	11.75%	14.00%

Figure 2. DNA content analysis of NCI-H460 non-small cell lung cancer cells by flow cytometry. NCI-H460 cells were serum-starved for 56 h and then treated with various concentrations (10–50 μM) of CLT **1** for 16 h. Cells were stained with propidium iodide and analyzed by flow cytometry. Percentage of cells under different stages of cell cycles (G_0–G_1, S or G_2–M) is indicated below each graph.

Table 2. Effects of compounds **26** and **27** on cell cycle progression

Treatment[a]	G_0–G_1	S	G_2–M
DMSO	32.17	54.28	13.55
25 μM **26**	46.80	44.63	8.57
50 μM **26**	69.91	25.09	5.0
25 μM **27**	58.07	35.29	6.64
50 μM **27**	80.85	4.79	14.36

[a] DNA content analysis of NCI-H460 non-small cell lung cancer cells by flow cytometry. NCI-H460 cells were serum-starved for 56 h and then treated with various concentrations (25–50 μM) of CLT **1** analogues for 16 h. Cells were stained with propidium iodide and analyzed by flow cytometry. Percentage of cells under different stages of cell cycles (G_0–G_1, S or G_2–M) is shown.

Preliminary analysis of two analogues suggests they act in a similar manner to CLT **1**. These results are highly encouraging and have led to studies aimed at optimization of these analogues with a long-term goal of developing novel anti-cancer therapeutics based on the isolated triaryl structure of CLT **1**.

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11. The mitogen-induced cell proliferation assays were performed using NIH 3T3 mouse fibroblast cells (ATCC # CRL1658). The assay was performed essentially as described in ref 7 and the cited references therein. Briefly, the cells were exposed to a mitogenic stimulus (such as PDGF or bFGF) for 10 h, and then [^{3}H] thymidine added to the well. The cells were maintained in culture at 37 °C and 5% CO_2 for another 21 h, and then washed four times with DME medium. The acid-precipitable radioactivity was extracted with cold 10% TCA and after neutralization with 0.3 N NaOH, aliquots were counted in a Packard Tri-Carb Scintillation counter. For the cell proliferation assay, cells were seeded in culture plates and allowed to attach to the culture plates for 24 h. Different concentrations of the compounds or vehicle were subsequently added and the number of cells per surface area counted at regular intervals.
12. All compounds synthesized in this study gave satisfactory physical and spectroscopic analytical results. A general method of synthesis is represented by the synthesis of the triphenyl methanol. A mixture of substituted benzoyl chloride (1 equiv), substituted benzene (1 equiv) and aluminum chloride (1.1 equiv) were stirred at room temperature in methylene chloride. The resulting benzophenone product after purification was reacted with phenylmagnesium bromide and the resultant product was purified and used for the study. General references are: (a) Austin, P.; Johnson, J. *J. Am. Chem. Soc*. **1932**, 54, 647. (b) Rodriguez, G.; Bazen, G. *J. Am. Chem. Soc*. **1997**, 119, 343. (c) Houben, J. Chem. Ber. 1903, 36, 3087. (d) Morton, A., Stevens, J. *J. Am. Chem. Soc*. **1931**, 53, 4028.
13. Cell cycle analysis: H460 cells were synchronized in the medium without FCS for 56 h. Cells were then released into complete medium containing 0.1% DMSO, 10, 25 or 50 μM CLT 1. Cells were harvested 16 h following treatment, washed with cold PBS twice and fixed in 70% ethanol at 4 °C at least 4 h. The fixed cells were centrifuged at 1500 rpm for 4 min at 4 °C, washed twice with cold PBS containing 2% FBS, and treated with 3 mg/mL ribonuclease (Sigma Chemical Co.) and 50 μg/mL propidium iodide (PI) (Sigma Chemical Co.) for 30 min at 37 °C. Flow cytometry analyses were performed on the Becton Dickinson fluorescence-activated sorter FACScan by using the Becton Dickinson Cell Quest program. Data were evaluated using Modfit software (Verity software House, Topsham, ME, USA).
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