Pergamon

Bioorganic & Medicinal Chemistry 9 (2001) 1365–1370

BIOORGANIC & MEDICINAL CHEMISTRY

# Investigation of the Biological Mode of Action of Clerocidin Using Whole Cell Assays

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Received 1 December 2000; accepted 20 December 2000

**Abstract**—Clerocidin, a diterpenoid natural product, has been shown in vitro to inhibit DNA religation following cleavage by topoisomerase II. Herein, we characterize the efficacy and specificity of clerocidin in HeLa cells. Our results suggest that clerocidin recognizes topoisomerase II as its main intracellular target and binds to this enzyme prior to formation of the 'cleavable complex' with DNA. These pharmacological features attest to the promising chemotherapeutic potential of this natural product. 

## Introduction

DNA topoisomerases are nuclear enzymes that alter DNA topology through a concerted breaking and rejoining of the phosphodiester backbone.[1–3] In eukaryotic cells, these enzymes are composed of three distinct subfamilies, among which topoisomerase II is unique in its ability to generate transient double-stranded breaks in DNA.[4,5] In an ATP-dependent process, the enzyme then allows the passage of another DNA strand through the break, before it reseals the broken backbone. This catalytic cycle results in both the removal of positive and negative supercoils and the unlinking of intertwined pairs of DNA duplexes.[6] By virtue of this activity, topoisomerase II plays a crucial role in many processes of DNA metabolism, including replication, transcription, recombination as well as chromosome segregation and condensation during mitosis.[7,8]

The above considerations defined topoisomerase II as a clinically important target and led to the development of several antitumor drugs, which currently include intercalating agents (such as acridines, ellipticines, and anthracyclines) or the nonintercalative epipodophyllotoxins.[9,10] These compounds, commonly referred to as 'topoisomerase II poisons', interfere with the enzyme during formation of its intermediary reversible complex with DNA, thereby preventing DNA religation and resulting ultimately in cellular death.[11,12] Recent data have also established a correlation between the ability of a particular agent to induce topoisomerase II-mediated DNA cleavage in vitro and its cytotoxicity in vivo.[13] Thus, identification of new compounds, which interfere with formation and dynamics of the topoisomerase–DNA cleavable complex in vitro has been viewed as a promising approach to discover novel anticancer agents.

Although formation of a ternary complex between enzyme–DNA-drug is essential for DNA cleavage, the pathway of its assembly is poorly understood and needs to be determined individually for each drug.[14] In principle, three different scenarios can account for formation of the ternary complex. In the first, the drug becomes part of the ternary complex primarily through interactions with DNA, which occur in the absence of topoisomerase II.[15,16] In the second, the drug binds initially to the resting state of topoisomerase II and becomes subsequently part of the ternary complex.[17] In the third case, the drug binds only to the topoisomerase–DNA complex and has minimal interactions with either the enzyme or the DNA independently.[18–20]

In the present study, we set out to investigate the topoisomerase II poisoning profile of clerocidin (Fig. 1)[21–24]

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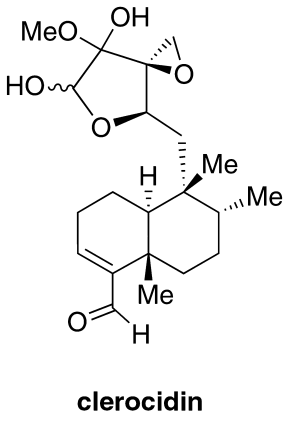
0968-0896/01/$ - see front matter 
PII: S0968-0896(01)00051-7

using whole cell assays. This objective is particularly salient since, in contrast to all other known topoisomerase II poisons, clerocidin was found to stimulate in vitro topoisomerase II-mediated DNA cleavage that is not reversible upon heat treatment or addition of salts.[25–28] Moreover, the chemical structure of clerocidin is unique, in that it lacks even a small planar moiety and consequently is not able to intercalate into the double helix of DNA.[26] Parallel to these studies, experiments in mice have shown that clerocidin displays a significant antitumor activity against murine tumor models (P388 lymphocytic leukemia screening model) at a single ip dose of 12.5 mg/kg and a low overall cytotoxicity ($LD_{50} = 250$ mg/kg).[21,24]

Our studies confirm that topoisomerase II is the intracellular target of clerocidin and suggest that this compound binds to the enzyme prior to formation of cleavable complex with DNA. This binding could interfere with the catalytic function of the enzyme on DNA, thereby preventing the completion of mitosis. Cells treated with clerocidin remained arrested at the G2/M phase and were forced to undergo apoptosis. In addition, we demonstrate that clerocidin has no effect in non-mitotic processes, which supports the notion of excellent target selectivity and attests to its high chemotherapeutic potential.




clerocidin

**Figure 1.** Chemical structure of clerocidin.

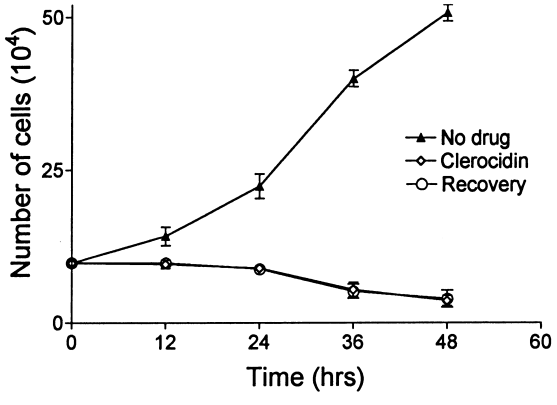



**Figure 2.** Growth arrest of HeLa cells induced by clerocidin. Cells were grown either in DMSO or 2 mg/mL clerocidin at 37 °C in complete media for the indicated times. Cells were harvested and the number of viable cells was determined using the trypan blue exclusion test. The indicated times for cells allowed to undergo recovery is equal to 1 h clerocidin treatment + time incubated in fresh media and allowed to recover in fresh media.

## Results

### Effect of clerocidin on growth of HeLa cells

The in vivo effect of clerocidin was examined using HeLa cells, an epithelial cell line derived from human cervical carcinoma. This was accomplished by measuring the growth characteristics of cells following treatment with the drug. As shown in Figure 2, HeLa cells grown in 2 μg/mL of clerocidin displayed an immediate cessation of propagation, while cells exposed to the vehicle solvent (DMSO) exhibited rapid exponential growth, which is characteristic of this cell line. Following 24 h of exposure to the drug, a 30% decrease in cell viability was observed and a substantial amount of cells were found floating in the culture media. These cells were determined to be dead on the basis on their inability to exclude trypan blue. Based on the documented 'irreversibility' of clerocidin in vitro,[26–28] the effect of a transient exposure of cells to this drug was next assessed. Interestingly, 1 h of exposure of HeLa

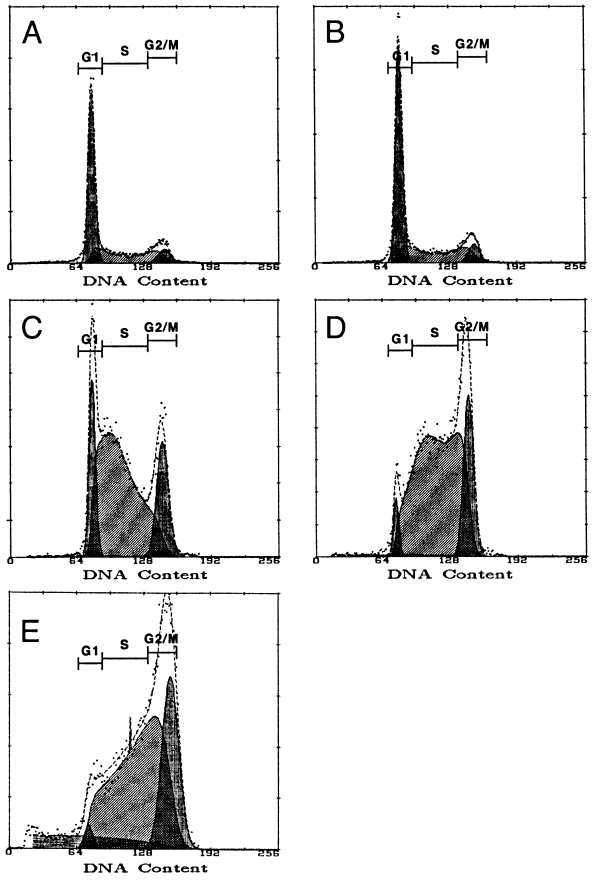



**Figure 3.** Cell cycle analysis in clerocidin-treated cells. (A) HeLa cells were treated for 1 h at 37 °C with DMSO and allowed to recover in fresh media for 17 h. Cells were treated with 2 μg/mL of the compound for 1 h (B) and allowed to recover in fresh media for (C) 5 h, (D) 11 h, or (E) 17 h. Cells were fixed and stained for DNA content with propidium iodide.

cells to 2 μg/mL of clerocidin, followed by removal of the drug and incubation of the cells in fresh media ('recovery') resulted in the block of cellular proliferation and produced results identical to the ones obtained with cells exposed continuously to this drug.

### Cell cycle analysis of clerocidin-treated cells

To test if the block in cellular proliferation was due to the conversion of topoisomerase II into a DNA-damaging agent, we probed whether clerocidin caused an arrest in the cell cycle. This was accomplished by treating HeLa cells with clerocidin for 1 h and subsequently replacing the drug with fresh media. After varying lengths of incubation, the cells were fixed and stained with propidium iodide to determine DNA content via flow cytometry.[29] As shown in Figure 3A, the majority of the control cells exposed to DMSO were in the G1 phase of cell cycle and only a small minority was detected in either the S or G2/M phase. The cell cycle profile of cells immediately after treatment with clerocidin was identical to those exposed to DMSO (Fig. 3B). However, 5 h after application of clerocidin, we observed an accumulation of cells in early S and G2/M phases (Fig. 3C). After 11 h post-clerocidin treatment, cells slowly progressed to the late S phase (Fig. 3D). At 17 h post-clerocidin treatment, cells were delayed at the late S phase, while the majority of cell population was arrested at the G2/M phase (Fig. 3E). Consistent with this cell cycle arrest only 2% of the cells were found at the G1 phase, which normally comprises 60% of control cells. Cells incubated for longer periods after clerocidin treatment found to be too damaged to process through the flow cytometer.

### Induction of apoptosis in HeLa cells by clerocidin

It is well established that prolonged cell cycle arrest leads to programmed cell death (apoptosis).[12,30] We, therefore, reasoned that irreparable DNA damage induced by clerocidin should result in an increased number of apoptotic cells. To test this hypothesis, we assayed for hallmark changes in nuclear morphology characteristic of apoptosis. HeLa cells grown on coverslips were treated for 1 h with 2 μg/ml of clerocidin and subsequently washed and incubated in fresh media. After varying times, the cells were fixed and stained with Hoechst dye to visualize nuclear structure. Cells treated with DMSO displayed a normal oval shaped nucleus, as shown in Figure 4A. However, 17 h after exposure to clerocidin, about 40% of the cells exhibited nuclear blebbing, which is early and unequivocal morphological evidence for the onset of apoptosis (Fig. 4B). After 23 h, almost 75% of cells displayed convolutions in their nuclear structure (Fig. 4C). The large size of the cell after clerocidin treatment is likewise consistent with the delay in progression through S phase and the G2/M arrest observed in our FACS analysis (Fig. 3). Moreover, the ability of clerocidin to induce apoptosis in HeLa cells was verified by its ability to cause DNA fragmentation. Cells incubated in fresh media for at least 18 h, following exposure to clerocidin for 1 h yielded the characteristic DNA laddering on agarose gels (data not shown).

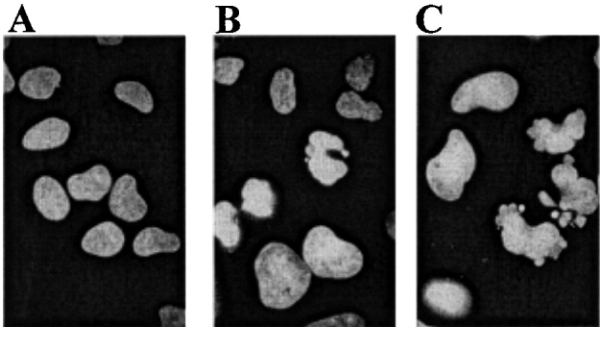



**Figure 4.** Morphological analysis of clerocidin-induced apoptosis. (A) HeLa cells were treated with DMSO for 1 h and allowed to recover for 23 h. In clerocidin treatment, cells were incubated with 2 μg/mL clerocidin for 1 h and allowed to recover in fresh media for (B) 17 h and (C) 23 h. Nuclear morphology was determined by fluorescence microscopy via staining DNA with Hoechst dye.

### Effect of clerocidin on VSV-G transcription, translation and transport

To probe if clerocidin has an effect in non-mitotic processes, we examined the expression and transport of the Vesicular Stomatitis Virus Glycoprotein (VSV-G) on the cell surface.[31] These experiments were performed with HeLa cells, which were treated for 1 h with clerocidin and subsequently washed and incubated in fresh media. At the indicated times, the cells were infected with VSV-G and the progression of glycoprotein from the ER to the plasma membrane was monitored by immunofluorescence with an antibody against VSV-G protein. As shown in Figure 5A, control cells (treated with DMSO) exhibited transport of VSV-G to the Golgi and the cell surface as evidenced by the spikes emanating from the plasma membrane. Interestingly, 1 h (Fig. 5B) and 18 h (Fig. 5C) after clerocidin treatment, cells retained their ability to express and transport the viral protein. This observation suggests that even though clerocidin inhibits proliferation, the cell is nonetheless

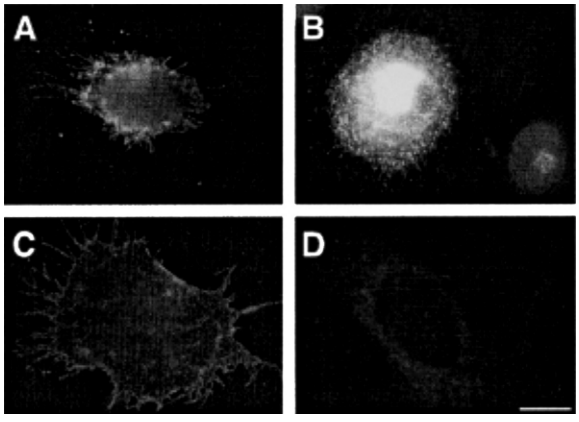



**Figure 5.** Effect of clerocidin on VSV-G transport. After treatment with clerocidin cells were infected with VSV and the progression of the G-protein was monitored by immunofluorescence. Treatments were as follows: A) DMSO for 1 h followed by 23 h incubation in fresh media; (B) 1 h with 2 μg/mL clerocidin; (C) 1 h clerocidin followed by 17 h incubation in fresh media; and (D) 1 h clerocidin followed by 23 h incubation in fresh media. Bar = 20 μm.

still capable of carrying out many physiological processes, including transcription, translation and secretion. The only noticeable effect that clerocidin had at this stage is a doubling in size of the cell, which in turn is consistent with the FACS data showing a delay in the progression through the S-phase. However, 23 h post clerocidin treatment (Fig. 5D), we observed no expression of VSV-G, suggesting, in agreement with the observed apoptosis (Fig. 4C), that these cells are unable to perform some, if not all, of the above functions.

## Discussion

Clerocidin is a natural product that displays potent antitumor activities which, based on in vitro studies, have been postulated to derive from topoisomerase II poisoning.[26–28] However, in sharp contrast to all other known topoisomerase II poisons, clerocidin was shown to induce topoisomerase II-mediated DNA cleavage that is not reversible upon heat treatment of addition of salts.[25,26] To examine if the topoisomerase II poisoning activity that clerocidin displays in vitro correlates well with its in vivo profile, we first examined its effect on the growth of HeLa cells. As anticipated, clerocidin induced cell growth arrest in a time-dependent manner (Fig. 2). Of remarkable interest was the observation that even a limited exposure to clerocidin inhibits the growth of an asynchronous population of cells, since it implies that this compound does not require topoisomerase II to be complexed with DNA in order to bind. Rather, if topoisomerase II is indeed the biochemical target of clerocidin, then this drug may bind to the enzyme in its resting state independently of the formation of the cleavable complex.

It is well documented that topoisomerase II is involved in DNA replication and transcription as well as in the final stages of chromosome segregation and condensation.[7] Based on this evidence, we hypothesized that if topoisomerase II is the cellular target of clerocidin, then cells treated with this drug should exhibit a change in kinetics of the synthesis (S) and G2/mitosis (M) phases of cell cycle. The data shown in Figure 3 demonstrates that clerocidin induces a substantial delay in the cellular progression through the S phase and a final accrual of cells at the G2/M phase. Ultrastructural data presented in Figures 4 and 5 also show an increase in cell size consistent with the protracted time that the cell remains in S phase. These observations are in full agreement with a drug that induces cell cycle arrest by interfering with topoisomerase II.[7,32] Notably, it has been shown that topoisomerase I, an enzyme that induces single-strand breaks in DNA, can functionally substitute for topoisomerase II in relaxing the supercoiled DNA during early S phase.[32] Thus, although topoisomerase II is dispensable during DNA transcription and replication, it is nonetheless essential during the M phase for the removal of interlocks between sister chromatids generated during replication.[33,34] Mutations or drugs that block the catalytic function of topoisomerase II are known to prevent the completion of mitosis, apparently because catenation between sister chromatids physically impedes their segregation.[34] The FACS data obtained with clerocidin corroborates these findings: progression through S phase was slowed but not blocked due to the functional redundancy of topoisomerases I and II; the irreversible block in M phase occurred due to the inability to segregate sister chromatids, which is a unique activity of topoisomerase II. These findings further reinforce the notion that topoisomerase II is the intracellular target of this compound.

The cell cycle arrest at the G2/M phase, induced by clerocidin, should trigger an M phase checkpoint. Failure to resolve the problem that causes cellular arrest at this control point should theoretically initiate programmed cell death.[30,35–37] In accordance with this postulate, our FACS data shows that clerocidin causes an increase in the number of cells with less than a 2 N DNA content and a broadening of peaks denoting the phases of cell cycle. (Fig. 3). These findings strongly suggest that DNA fragmentation, which in turn is a biochemical landmark for apoptosis, has occured. Evidence for apoptotic death was obtained by morphological analysis of clerocidin-treated HeLa cells (Fig. 4) and by DNA fragmentation (data not shown).

In addition to topoisomerase II, clerocidin could, in principle, bind to a secondary intracellular target, which in turn may be responsible for the observed cell growth arrest, or interfere simultaneously with other non-mitotic processes. This issue of target selectivity is particularly conspicuous, since several topoisomerase II poisons are known to also affect other cellular functions. For example, the topoisomerase II poisons teniposide and VP-16 are known to also affect nucleoside uptake into HeLa cells and exhibit radical-mediated cytotoxicity.[38,39] To assess the issue of target selectivity, we examined the effect of clerocidin in VSV-G transport. Upon infection of the cells with the virus, the viral DNA is integrated into the host genome where it is transcribed, translated and transported through the secretory pathway to the cell surface. As such, this assay provides a comprehensive test of the integrity of various organelles and normal function of physiological processes that are not related to cellular division. Our data shown in Figure 5 demonstrate that clerocidin does not interfere with the above cellular processes and further indicate that the in vivo effects of this drug rest upon its binding to topoisomerase II.

## Conclusion

In conclusion, our findings suggest that the sole intracellular target of clerocidin is indeed topoisomerase II. Analysis of the intracellular effect of this compound indicates that clerocidin binds to topoisomerase II prior to formation of the cleavable complex with DNA. The enzyme/drug complex then exhibits its lethal effect in cells during religation of the cleaved DNA, thereby leading to cell cycle arrest at the G2/M phase and ultimately apoptosis. Of particular interest is the observation that transient (1 h) exposure to clerocidin causes an inhibition of cellular proliferation, with the same

efficacy as cells grown in continuous presence of this drug. This characteristic could result from the 'irreversible' nature of the topoisomerase II-mediated DNA cleavage that is observed using clerocidin in in vitro studies. This property provides an obvious advantage over other topoisomerase II poisons whose effects are reversible and may therefore require repeated or continuous administration. We have also shown that clerocidin has little or no effect in non-mitotic processes and appears to be target specific for topoisomerase II. Combination of the above properties suggests that clerocidin may have minimal clinical side effects and a high chemotherapeutic potential.[40] Moreover, the peculiar chemical structure of clerocidin,[21–24] together with its intriguing biological activity[26–28] and its recently accomplished total synthesis[41,42] may serve as leads for the development of novel antitumor agents.

## Experimental

### General

Clerocidin was obtained from Leo Pharaceutical Products (Denmark). The clerocidin stock solution was prepared at 5 mg/ml in dimethylsulfoxide (DMSO) and stored at −20 °C. HeLa cells were grown in DMEM (GIBCO/BRL) supplemented with 10% fetal bovine serum, 1 mM glutamine, and 1% penicillin/streptomycin. Cells were grown in a 37 °C incubator with 5% $CO_2$. Cells used for fluorescence microscopy analysis were grown on 12 mm glass coverslips (Fisher Scientific) coated with Pronectin F (Protein Polymer Tech).

### Cell growth assays

Cell number and viability experiments were performed one day after plating cells on a 6 cm tissue culture dish at which time the HeLa cells achieved a density of 100,000 cells/dish. Cells were treated with either DMSO or clerocidin in complete medium at 37 °C for the indicated time. Cells transiently exposed to 2 μg/mL clerocidin were incubated with drug for 60 min at 37 °C, then washed twice with PBS and incubated with fresh media for the indicated amount of time. Cells were then trypsinized and resuspended in fresh culture medium and trypan blue to measure cell viability. Total number of viable cells was counted on a hemocytometer (VWR Scientific). Experiments were performed in triplicate and repeated at least three times.

### Flow cytometric analysis of cell cycle dynamics

$1\times10^6$ HeLa cells were plated on a 6 cm tissue culture dish and cells were allowed to recover 24 h before any treatment. Cells were treated with either DMSO or 2 μg/mL clerocidin for 1 h in complete medium at 37 °C, washed twice with PBS and incubated in fresh media for the indicated time. Cells were harvested with trypsin and washed twice with PBS. Samples were fixed in 70% ethanol and stored at 4 °C for at least 24 h. Cells were then washed once with McIlvaine's buffer (0.2 M $Na_2HPO_4$, 0.1 M citric acid, pH 7.5) and once with PBS. Samples were stained with propidium iodide staining solution (PBS containing 100 μg/mL RNase A and 10 μg/mL propidium iodide [Sigma]). The samples were processed on a Coulter Elite flow cytometer and the data analyzed with the Multiplus AV program. The data is representative of two independent experiments.

### Nuclear morphology of apoptotic cells

HeLa cells grown on coverslips were treated with clerocidin as described above and fixed with 4% formaldehyde (in PBS) for 10 min at room temperature. Samples were washed in PBS and stained with 10 μg/mL Hoechst dye (Sigma) for 5 min and washed with PBS. Coverslips were mounted and viewed under a fluorescent microscope.

### VSV-G Transport

HeLa cells were plated on glass coverslips at least 18 h prior to use in the experiment. Cells were incubated with either DMSO or 2 μg/mL clerocidin for 1 h at 37 °C, washed extensively with PBS and incubated with fresh media under normal growth conditions. At the indicated times cells were infected with VSV tsO45 as described elsewhere.[29] Cells were fixed with 4% formaldehyde and stained with P5D4 (an antibody recognizing the VSV-G protein) and fluorescein conjugated goat anti-mouse antibody (Boehringer Mannheim Biochemicals, IN, USA).

## Acknowledgements

Financial support from the Cancer Research Coordinating Committee, the American Cancer Society (IRG, RPG CDD-9922901) and the Hellman Foundation (Faculty Research Fellowship to EAT) is gratefully acknowledged. We also thank the UCSD Cancer Center and Dr. P. R. Rasmussen (Leo Pharmaceutical Products, Denmark) for a generous gift of clerocidin.

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