Research Article

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European Journal of Experimental Biology
ISSN 2248-9215

2020
Vol.10 No.2:4

DOI: 10.36648/2248-9215.10.2.101

An *In Vitro* Evaluation of the Anti-Cancer Potential of Novel 1, 2, 4, 5-Tetraoxanes for Chronic Lymphocytic Leukemia at Physiologically Relevant Oxygen Tension

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Received Date: March 11, 2020; **Accepted Date:** March 18, 2020; **Published Date:** March 27, 2020

Citation: Reilly AO, Rawe S, Redmond R, Meade E, Murphy J, Garvey M (2020) An *In Vitro* Evaluation of the Anti-Cancer Potential of Novel 1, 2, 4, 5-Tetraoxanes for Chronic Lymphocytic Leukemia at Physiologically Relevant Oxygen Tension. Eur Exp Biol Vol.10 No.2:4.

Abstract

Chronic Lymphocytic Leukemia (CLL) is the most prevalent leukemia amongst adults in Europe and North America with a median age of diagnosis of 72 years. Risk factors for this disease include sex, ethnicity, age, hereditary influences and xenobiotic exposure. CLL is characterized by clonal proliferation with progressive accretion of B-cell lymphocytes expressing CD19+, CD5+ and CD23+ typically infiltrating the lymph system, spleen and bone marrow of susceptible persons. Treatment of CLL is via monotherapy with alkylating agents such as chlorambucil for elderly patients. As a relatively cheap chemotherapeutic with low toxicity chlorambucil has the disadvantage of having a low response and risk of side effects including myelodysplasia. Monoclonal antibodies such as Alemtuzumab have emerged as an alternative/combination therapy however; such therapy is typically more expensive and less convenient as an IV injection. This study reports on an investigation into novel drug therapy options for treatment of CLL and provides a comparative toxicity profile against non-tumour cells to determine selectivity. Five synthetic 1, 2, 4, 5-tetraoxanes, the semi-synthetic artemisinin derivative dihydroartemisinin (DHA) and clinically used chlorambucil were evaluated for their antiproliferative activity in two leukemic cell lines at physiologically relevant oxygen tension (5% v/v). All five tetraoxanes and DHA performed significantly better that chlorambucil against HL-60 cells with single digit micromolar antiproliferative IC50 values. Three tetraoxanes and DHA also displayed higher selectivity for tumour over non-tumour cells. Against the hard-to-treat MEC-1 cell line, all compounds showed less antiproliferative activity than against the HL-60 cell line. Only one of these compounds, a synthetic tetraoxane, had a selectivity index greater than 1, outperforming both chlorambucil and DHA and demonstrating that 1, 2, 4, 5-tetraoxanes have potential as a new class of anti-leukemia therapeutics.

Keywords: Normoxia; Artemisinins; Dihydroartemisinin; Endoperoxides; 1, 2, 4, 5-Tetraoxanes; Leukemia; Oxygen tension; Chlorambucil

Introduction

In the western world, Chronic Lymphocytic Leukemia (CLL) is the most common type of leukemia in adults, accounting for 37% overall [1]. Additionally, 54% of CLL cases are typically diagnosed in people over the age of 70, with a median age of 71 years at diagnosis [2,3]. CLL is characterized by the accumulation of monoclonal, functionally immature CD5+ B-lymphocytes in the bone marrow, blood and lymph nodes [4]. Although once thought to be an indolent, slowly progressing disease, it is now evident that CLL is a heterogeneous disease, which in the last number of years has been biologically characterized for predicting progression rate, response to therapy and probability of relapse [5]. The overall survival rate at five years is 87% for patients with good prognosis and 29% for poor prognosis CLL, demonstrating the significant need for more effective treatment regimens for patients with aggressive, hard-to-treat forms of the disease [6]. Also, the development of resistance and intolerance to currently used therapeutic agents ' remains a significant concern. Consequently, there is a constant need to develop new treatments, including new chemotherapies, to conquer treatment resistance and increase overall survival rates for all CLL patient subgroups.

Over the last 50 years, organic endoperoxides have emerged as effective antimalarial agents. Following the isolation of the natural product artemisinin from the sweet wormwood plant (*Artemisia annua*) in 1972, its semi-synthetic derivatives-artemether, artesunate and dihydroartemisinin (DHA) have become established treatments for malaria **(Figure 1)**. While their mechanism of action is still not fully understood, the endoperoxide bond, contained within a 1, 2, 4-trioxane ring in the artemisinins, is essential for activity [7]. Other synthetic endoperoxides-containing heterocyclic compounds, most notably 1, 2, 4-trioxolanes and 1, 2, 4, 5-tetraoxanes, have also

emerged as new antimalarial and promising drug candidates [8-10]. For example, RKA182 reached pre-clinical development and was discarded only as it did not represent a single dose treatment [10]. 1, 2, 4, 5-Tetraoxanes contain two endoperoxides bridges within the six-membered heterocyclic structure and be more stable and often more potent than the artemisinins in terms of their antimalarial activity [10,11]. More recently, researchers have focused on the promising anti-tumour activity of these compounds. They have been found to possess anti-proliferative activity in sub-micromolar levels in several cancer cell lines, including leukemia [11,12]. These promising compounds remain relatively under-investigated as a new class of anticancer agents, although recent research demonstrates their potential [13,14].

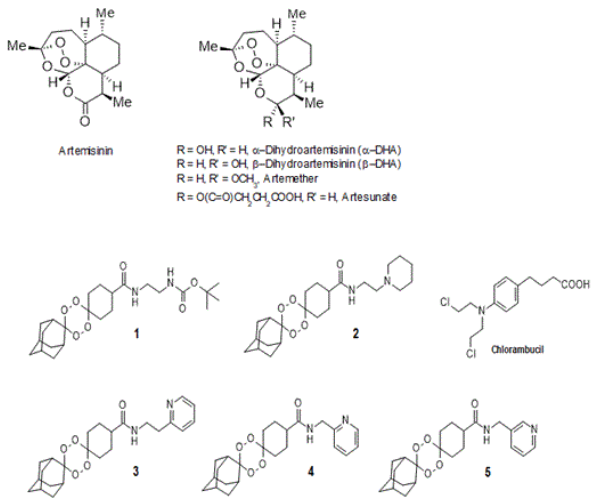

Figure 1: Artemisinin and clinically used antimalarial semi-synthetic artemisinin derivatives including Dihydroartemisinin (DHA) and 1, 2, 4, 5-Tetraoxanes 1 to 5 and the Chlorambucil (CLB).

Biocompatibility testing is typically conducted at oxygen levels of 21% v/v (atmospheric O_2 levels). However, the physiological oxygen levels to which cells are exposed *in vivo* is much lower, ranging from 13% to 3% depending on the location of the cells [15]. An O_2 level of 5% (40 mmHg) has been previously established as the physiological O_2 levels available to blood cells *in vivo* [16,17]. Numerous studies have been conducted to investigate the effect of various O_2 levels, such as atmospheric O_2 (21% or 160 mmHg) and *in vivo* O_2 levels on both commercial and primary cell line cultures. These studies have shown the importance of using physiological O_2 levels that mimic that of *in vivo* conditions [16,18-20]. Furthermore, this research group reported a significant increase in efficacy of artesunate when evaluated at physiological oxygen levels in comparison to atmospheric oxygen levels [18,20-22]. Therefore, the current study aimed to investigate the anti-tumour activity of five novel 1,2,4,5-tetraoxanes 1-5 **(Figure 1)** compounds on two leukemia cell lines and one non-cancer B-lymphoblast cell line at physiologically relevant oxygen tension (5%, defined here as normoxia), and following the International Organization for Standardization (ISO). The IC_{50} value of each compound on all three cell lines was determined to demonstrate the Selectivity Index (SI) of each compound for all cell types and to identify the potential for a therapeutic window for treatment. These values were compared to those obtained for dihydroartemisinin and chlorambucil (CLB) **(Figure 1)**, which is used clinically to treat CLL. The mode of cell death induced by the compounds was also investigated using flow cytometric analysis and colorimetric assays.

Materials and Methods

Cell culture parameters

All cell culture conducted throughout this study was maintained at the physiologically relevant O_2 level of 5% (v/v), the accepted physiological oxygen levels of human venous blood [17]. This was achieved using oxygen controlled Thermo 3110 CO_2 Incubator (Thermo Fisher Scientific, UK). HL-60 and MEC-1 cells were cultured RPMI 1640 medium (Sigma-Aldrich, cat. No. R8758, Ireland) supplemented with 10% Fetal Bovine Serum (FBS; Sigma-Aldrich, cat. no. F7524, Ireland) and 1% Penicillin-Streptomycin (Sigma-Aldrich, cat. No. P4333, Ireland). BL-2052 cells were maintained in RPMI 1640 medium supplemented with 10% FBS, 1% Penicillin-Streptomycin and 1 mM sodium pyruvate (Sigma-Aldrich, cat. No. S8636, Ireland). All cell lines were maintained at 37°C, 5% O_2, and 5% CO_2 in a fully humidified atmosphere in a Thermo 3110 CO_2 Incubator.

Compound preparation and addition

The novel compounds were synthesized by Robert Redmond and Dr. Sarah Rawe at the School of Chemistry and Pharmaceutical Science, Technological University Dublin, Ireland. Full details of the syntheses and characterization of these compounds along with the 1H and 13C NMR spectra are provided in the supporting information. The novel compounds, along with the two reference compounds, DHA) and CLB has dissolved in 100% DMSO to the following concentrations: 50 mM (novel tetraoxanes 1-5); 100 mM (DHA and CLB). All stocks were divided into 20 µL aliquots and stored at -80°C. On the day of each experiment, an aliquot of each compound was thawed and further dissolved in RPMI medium to a concentration of 2 times the final desired concentration in 0.2% (v/v) DMSO before addition to cells. After addition, cells were exposed to a range of drug concentrations for a set period. DMSO concentration remained constant at 0.1% at all drug concentrations employed. Cells were counted using the Z2 Particle analyzer (Beckman Coulter, FL, USA) and seeded into 96-well plates (Sarstedt, Germany) in 100 µL fresh medium at the following seeding densities: 20,000 cells/well (HL60), 50,000 cells per well (MEC-1). After 24 hours, 100 µL of either fresh medium, 0.2% (v/v) DMSO in fresh medium (vehicle control) or 2 times final concentration of the compound in 0.2 % (v/v) DMSO and fresh medium was added to the appropriate triplicate wells.

IC_{50} value determination

The ISO recognized MTT assay was used to analyze the IC_{50} value for each compound at a 24 hour time point. Briefly, after the 24-hour incubation with the test compound, 20 µL MTT (5 mg/mL) was added to each well and incubated for 2 hours (final MTT conc. on cells 0.5 mg/mL). The 96-well plate was then centrifuged for 5 minutes at 157 g at 4°C in a Jouan MR23i centrifuge (ThermoFisher, MA, USA) and the supernatant removed. 200 µL DMSO was added to each well and incubated at room temp in the dark for 20 minutes. Absorbance was read at 584 nm on an Optima plate reader. After a preliminary screen of the compounds, the following concentration ranges were chosen for the determination of IC_{50} values for HL-60 cell line: 0-25 µM (tetraoxanes 1-5), 0 µM-5 µM (DHA), and 0 µM-100 µM (CLB). For MEC-1 BL-2052 cell lines higher concentrations were required: 0-50 µM (tetraoxanes 1-5), and 0-100 µM (DHA and CLB).

Cell lysis evaluation

Lactate Dehydrogenase (LDH) is a cytoplasmic enzyme that is present in all cells and can be used as a marker for the determination of necrosis [21,22]. The Pierce LDH cytotoxicity assay kit (Bioscience, Ireland), a colorimetric method for determination of cell plasma membrane damage and leakage of LDH into the cell culture media was used to evaluate cell lysis induced by compounds after 24 hours of exposure [23]. Cells were exposed to concentration ranges (as stated above for MTT assay) of each compound, 0.1% DMSO (vehicle control/spontaneous LDH release control) or maximum LDH release the control (lysis buffer) at normoxia O_2 levels of 5%, in triplicate. After a 24 hour incubation period, the 96-well plate was centrifuged using the Jouan MR23i centrifuge at 157 g for 5 minutes at 4°C. 50 µL of cell culture medium was removed and added to a new 96 well plate. 50 µL of the reaction mixture on the 96 well plate reader at 485 nm. Percentage cytotoxicity was determined by subtracting the absorbance of the spontaneous LDH release control (vehicle control, 0.1% DMSO) from the absorbance of each sample, and expressing this value as a percentage of total LDH activity as per kit instructions was added to each plate mixed and then incubated at room temperature in the dark for 30 minutes. 50 µL of stop solution was added, and absorbance was read

Apoptosis detection

The FITC Annexin V Apoptosis Detection Kit I (BD Bioscience, cat. no. 556547, Ireland), was used as per protocol. Briefly, after exposure to compounds (IC_{50} concentration as determined by MTT) flow cytometric analysis was performed at three-time points; 12 hours, 24 hours and 48 hours. Cells were transferred to 0.5 mL Eppendorf tubes and centrifuged for 5 minutes at 120 g at 4°C using the MILRO 200R Centrifuge. The supernatant was removed, and a 100 µL 1X binding buffer added. 5 µL FITC and 5 µL PI dye were added to appropriate tubes and incubated at room temperature, in the dark, for 15 minutes. 100 µL of 1X binding buffer was added, and samples were run on the BD Accuri C6 Flow Cytometer. For analysis, viable cells were identified as both Annexin V-FITC and PI negative (FITC-/PI-), whereas cells in early-stage apoptosis were Annexin V-FITC positive but PI negative (FITC+/PI-). Cells that are in late-stage apoptosis or dead are positive for both Annexin V-FITC and PI (FITC+/PI+) and necrotic cells were detected as PI-positive, Annexin V negative (FITC-/PI+) [24].

Compound stability evaluation

Stability testing is a critical part of the compound analysis and is essential for drug development. For that reason, preliminary stability testing was conducted in the form of an MTT assay on the 6 novel compounds under study. Specifically, after nine months' storage at -80°C in 100% DMSO, an MTT assay was used to re-evaluate the IC_{50} concentrations of all six novel compounds, using the most sensitive of the three cell lines analyzed, HL-60s as previously described.

Statistical analysis

MTT and LDH graphs and analysis were generated by nonlinear regression using GraphPad Prism version 5.03; standard curves were generated from the mean of triplicate experiments ± the Standard Error of the Mean (SEM). Flow cytometric data were processed using Microsoft Office Excel; results are expressed as mean ± Standard Deviation (SD). Two-tailed unpaired t-test was used to identify statistically significant differences between non-cancer cell line (BL-2052) IC_{50} values and HL-60 IC_{50} values, with a p-value of less than 0.05 indicating statistical significance. One-tailed paired t-test was used to identify statistically significant differences between compound activities after nine months of storage, a p-value of less than 0.05 indicating statistical significance.

Result

Cell viability- IC_{50} determination

Determination of IC_{50} values for each compound at 5% O_2 tension indicates the potent anti-cancer properties of the compounds in normoxia. All five tetraoxanes showed promising anti-cancer activity on both leukemic cell lines, MEC-1 and HL-60 **(Table 1)**. Unsurprisingly, all tetraoxanes showed greater potency against HL-60 versus MEC-1. The IC50 concentration range for the five tetraoxane compounds on MEC-1 was between 22.7-47.8 µM, with DHA also falling within this range (39.2 µM). CLB was the least potent on the CLL cell line at the 24 hour time point, resulting in an IC_{50} value of 85.1 µM. The IC_{50} concentrations of all five tetraoxanes against the HL-60 cell line ranged from 6.0 µM to 9.0 µM while clinically used CLB was 3-5 times less potent with an IC_{50} value of 29.6 µM. DHA was the most potent of all of the compounds against HL-60 cells with an IC_{50} value of 0.9 µM.

The selectivity of the compounds for tumour cells over non-tumour cells can be expressed as the selectivity index **(Table 2)**. All seven compounds are ranked in order of selectivity in **Table 2**. For the non-cancer cell line, BL-2052, IC_{50}

concentrations ranged from 8.8 µM to >50 µM for tetraoxanes 1-5.

Table 1: IC50 values calculated for novel and reference therapeutics on MEC-1, HL-60, and BL2052 cells as determined via the MTT assay and IC_{10} values for MEC-1 cells for all compounds as determined via the LDH assay and MTT assay (+/- SD).

IC_{50} (µM)	HL-60	MEC-1	BL-2052	LDH Assay IC_{10} (µM)	MTT Assay IC_{10} (µM)
DHA	0.9 (± 0.04)	39.2 (± 3.8)	16.6 (± 0.9)	23.0 (± 3.4)	N/A
1	6.7 (± 0.1)	22.7 (± 1.7)	8.8 (± 3.4)	17.3 (± 2.0)	11.0 (± 1.7)
2	6.0 (± 0.7)	33.3 (± 3.0)	9.2 (± 0.3)	36.4 (± 1.6)	N/A
3	7.4 (± 0.9)	45.5 (± 0.7)	36.8 (± 2.0)	26.0 (± 2.2)	18.8 (± 6.1)
4	7.5 (± 1.9)	37.6 (± 1.3)	>50	41.4 (± 3.1)	8.9 (± 1.6)
5	9.0 (± 1.4)	47.8 (± 4.6)	30.2 (± 5.8)	33.4 (± 6.2)	24.1 (± 9.5)
CLB	29.6 (± 7.3)	85.1 (± 5.9)	38.9 (± 3.7)	98.4 (± 1.3)	59.3 (± 5.9)

Table 2: Selectivity index (SI) calculated by non-tumour/tumour ratio of IC50 values for BL-2052 and HL-60 cell lines, and for BL-2052 and MEC-1 cell lines **(Table 3)**. Higher non/cancer/cancer ratios indicate better cancer cell selectivity. Compounds are ranked (1-7) in order of highest selectivity in both tumour cell lines .

Compound	Ranking vs HL-60	Selectivity Index (SI): BL-2052/ HL-60	Ranking vs MEC-1	Selectivity Index (SI): BL-2052/ MEC-1
DHA	1	18.4	5=	0.4
4	2	>6.7	1	>1.3
3	3	5	2	0.8
5	4	3.4	3	0.6
2	5	1.5	7	0.3
1	6	1.3	5=	0.4
CLB	7	1.3	4	0.5

All five tetraoxanes displayed higher than or equal selectivity to the currently used CLB for HL-60 over BL-2052 cell lines with tetraoxanes 3-5 performing significantly better than CLB. While none of the tetraoxanes performed better than DHA against HL60 cells, the HL60 cells proved significantly more sensitive to the Tetraoxane 4 induced toxicity compared to the CLB. However, in contrast to its high selectivity for HL-60 cells versus the non-tumour cell line, DHA performed poorly against the MEC-1 cell line.

Disappointingly, four of the five tetraoxanes also had selectivity indices of less than 1, although tetraoxanes 3-5 provided a slightly increased level of cell death than both CLB and DHA. However, Tetraoxane 4 was the only compound that to show some selectivity for MEC-1 cell line (SI>1.3) which, particularly given the small number of tetraoxanes investigated in this preliminary study, demonstrates that these compounds may indeed prove to be good candidates for development as a new class of selective anti-leukemia drugs.

Table 3: IC_{50} values calculated for novel compounds before (T_0) and after nine months (T_9) storage at -80°C in 100% DMSO, from dose-response curves from MTT assay. Concentrations calculated on HL-60 cell line at the following concentration ranges: 0 µM-50 µM (Tetraoxanes 1 to 5). Paired, one-tailed t-test, statistically significant $p<0.05$.

IC_{50} (µM)	T_0	T_9	p-value
3	7.4 (± 0.9)	14.1 (± 1.4)	0.0023
4	7.5 (± 1.9)	33.1 (± 4.5)	0.0084
2	6.0 (± 0.7)	12.1 (± 1.3)	0.0026
1	6.7 (± 0.1)	11.2 (± 1.4)	0.0125
5	9.0 (± 1.4)	19.9 (± 0.7)	0.0017

Determination of plasma membrane damage-LDH Assay

LDH present in the cell culture medium can be measured to determine the extent of plasma membrane damage as a marker of irreversible cytotoxicity. In this study, this assay was used to determine the extent of plasma membrane damage induced by each compound after 24 hours. The IC_{10} value was calculated, due to the low levels of LDH detected. These results were compared to IC_{10} values calculated from the MTT assay performed on MEC-1 cells. For all compounds analyzed, the IC_{10} values were higher in the LDH assay, indicating that the plasma membrane is not likely to be the primary target of the tetraoxanes at the concentration range analyzed, therefore, the LDH assay was not carried out on further cell lines. However, results indicate that some irreversible damage is caused to the cells.

Flow cytometric analysis: Apoptosis detection

MEC-1 and HL-60 cells were exposed to all compounds, at the IC_{50} concentrations determined from the MTT assays, for various periods of 12, 24 and 48 hours, to indicate the timeframe in which each compound induced cytotoxic effects on the cell line. These results can be seen in **Figures 2 and 3** for MEC-1 and HL-60 cell lines, respectively. The graphs illustrate the reduction of cell numbers in the live, apoptotic and death cell populations over time. For the MEC-1 cell line, a decrease in live-cell numbers can be noted across all three-time points for all compounds screened **(Figure 2)**. The tetraoxanes reduced cell numbers in the live cell population gradually over 48 hours timeframe. All compounds showed a much greater effect on the HL-60 cell line, with the IC_{50}

concentrations showing a reduction of more than 50% by the 24 hour time point. Further effects of their cytotoxic properties can be observed by 48 hours, with less than 20% viable cells in all cell populations analyzed **(Figure 3)**. The cytotoxicity induced in the 24-48 hour timeframe is notably greater on the HL-60 cell line in comparison to MEC-1.

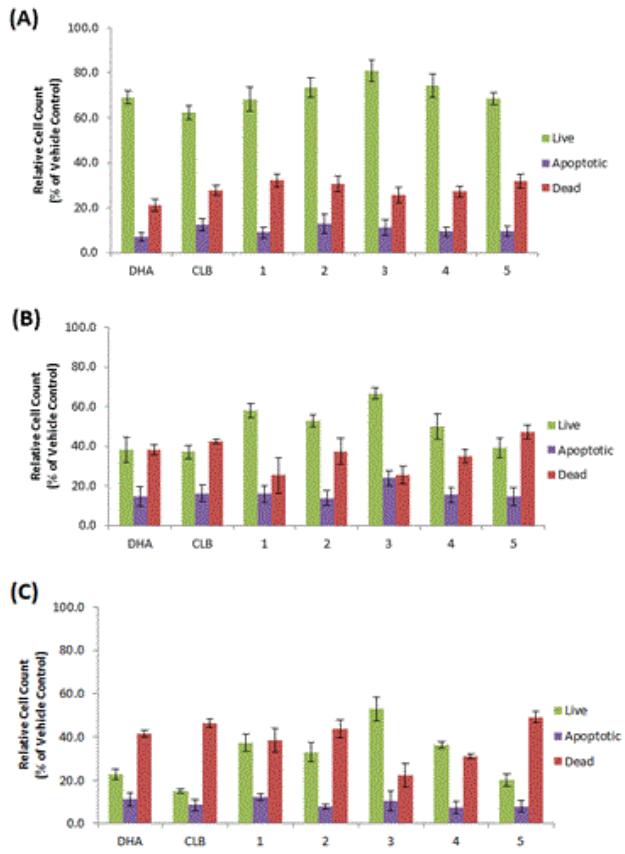

Figure 2: Flow cytometry analysis of MEC-1 cells after exposure to compounds at IC_{50} concentrations (as determined by MTT). MEC-1 cells were seeded at 100,000 cells/well, treated with compound for various periods (A=12 h, B=24 h and C=48 h) and analyzed using the Annexin-V apoptosis kit. Flow cytometric data expressed as percentage cell count relative to total cells present in the vehicle control; live (FITC-/PI-), apoptotic (FITC+/PI-), dead via apoptosis (FITC+/PI+) and necrotic (FITC-/PI+). All data is representative of triplicate experiments (± SEM).

Compound stability evaluation

Re-evaluation of tetraoxanes IC_{50} concentrations was performed after 9 months of storage of aliquots of the compounds as solutions in DMSO at -80°C. Evaluation of anti-proliferation activity using the MTT assay on HL-60s showed statistically significant degradation of all five tetraoxanes ($p \leq 0.05$, paired one-tailed t-test), with tetraoxane 4 showing the highest level of degradation with over 4-fold reduction in activity **(Table 1)**. There was a 2-fold increase in the IC_{50} values of the remaining compounds. These findings demonstrate the importance of compounds stability testing and the significance of using freshly prepared solutions for relevant and accurate screening of compounds in work of this nature.

Discussion

Cell cytotoxicity evaluation was performed following the ISO international standards, which requires that two tests be chosen for the determination of cytotoxic effects induced by test compounds. The two assays used were MTT, a measure of inhibition of cell growth, and LDH for evaluation of cell lysis and membrane integrity. All assays were carried out on cells cultured at 5% oxygen tension, conditions that more accurately reflect physiological conditions (i.e. normoxia). For this study two reference compounds were used as a comparison with the novel tetraoxanes to help identify the anti-cancer potential of each compound relative to: (a) a compound currently used in routine treatment of CLL (CLB); and (b) a pharmacophoric analog, i.e. endoperoxides with known anti-cancer activity (DHA). Screening on the non-tumour cell line, BL-2052, was carried out to evaluate the Selectivity Index (SI) of each compound.

All five tetraoxanes and DHA showed promising activity relative to the currently used chemotherapeutic agent CLB at 5% oxygen tension versus both HL-60 and MEC-1 cell lines. Conversion of IC50 values to a selectivity index for the HL-60 cell line demonstrated that four of the five tetraoxanes (2-4) show selectivity superior to CLB with the worst-performing tetraoxane (5) presenting an equal therapeutic window **(Table 2)**. DHA was the most potent of all of the compounds investigated versus HL-60 cells and also presented with the greatest selectivity **(Table 1)**.

All compounds were much less potent against the more resistant MEC-1 cell line than against HL-60 cells. This is expected since the MEC-1 cell line is derived from a CLL patient with poor-prognostic factors, and inherits the del17p mutation, associated with resistance to chemotherapeutic drugs [25,26]. The del17p mutation is associated with the disruption of the TP53 gene, responsible for the encoding of the pivotal p53 tumour suppressor protein which plays a key role in regulating signaling pathways such as cell cycle arrest, DNA repair and apoptosis [27]. While the absence of this master regulator accounts for the relatively reduced cytotoxic effects of the tetraoxanes against this cell line, it is significant that they still display good potency. Moreover, HL-60 cells are highly sensitive to the artemisinins and other endoperoxides-containing compounds. For the 'hard-to-treat' MEC-1 cell line, only one of the compounds analyzed, tetraoxane 4, was less potent to BL-2052 (SI>1.3) **(Table 2)**. Interestingly, CLB, which is routinely used to treat leukemia, was significantly more toxic on the non-cancerous cell line than MEC-1, with IC_{50} concentrations of 38.9 µM and 85.6 µM, respectively. CLB also displayed no significant difference in the toxicity to BL2052 cells versus HL-60 ($p=0.1128$, unpaired two-tailed t-test). Known side-effects of CLB include bone marrow suppression, peripheral neuropathy and hepatotoxicity [28]. Novel compounds with more selectivity to cancer cells hold the potential to reduce the adverse effects associated with currently used therapeutics such as CLB.

The anti-tumour mechanism of the artemisinins and synthetic endoperoxides is not yet fully understood although parallels can be drawn with their antimalarial action. The cytotoxic species are not the intact drugs themselves but are generated following bio-activation of the peroxide bond, which is essential for activity. Iron (II) catalyzed bio-activation probably by heme, whose level is elevated in cancer cells versus non-cancer cells- leads to cleavage of the peroxide bond and formation of cytotoxic radical species [29-31]. In the case of malaria, cytotoxic carbon-centered radicals have been shown to indiscriminately alkylate parasitic biomolecules including heme itself, numerous proteins and to result in lipid peroxidation [32-35]. Similar pathways have been proposed in tumour cells and would account for the range of cellular effects that have been reported. Research has also focused on the ability of these compounds to cause oxidative damage by the generation of Reactive Oxygen Species (ROS) and Ferroptosis, Iron (II) dependent cell death mediated by lipid peroxidation [36,37]. Superoxide radical scavengers have been shown to inhibit the production of ROS and cytotoxicity of the artemisinins and synthetic endoperoxides [10,38]. Cancer cells are known to have naturally higher levels of ROS than non-cancer cells, making them more susceptible to oxidative stress. An increase in ROS levels by these drugs can perturb the thiol redox status within cancer cells and induce cell death at lower levels than non-cancer cells [39-41].

While it is very unlikely that a Structure Activity Relationship (SAR) should emerge for these tetraoxanes from a study involving only 5 compounds, it is noteworthy even at this early stage that tetraoxanes 3-5 all contain a very weakly basic pyridyl group in their structure. It is tentatively suggested that perhaps the pyridyl group plays a distinctive role in their mechanism of action. Pyridyl groups are good sigma donor ligands and co-ordinate well to metal ions, including Iron (II), which may be significant given the likely role of heme (or other Iron (II) source) in their bio-activation. In SAR studies of related 1, 2, 4-trioxolanes, 25 compounds were ranked as high, intermediate or low for their activity against Raji lymphoma cells [3]. It was proposed that basic nitrogen approximately 9 Å (+/-0.41 Å) from the proximal oxygen of the endoperoxides bond and an aromatic group was required for optimal activity. Two of the trioxolanes contained a pyridyl group and were ranked as possessing intermediate activity; however, the selectivity for these compounds for tumour versus non-tumour cells was not reported. A much larger library of compounds is now required to establish firm SAR.

Disruption of cell membrane integrity was analyzed in this work using the LDH assay. From the results obtained IC_{10} concentrations were generated. These results indicate that within the range analyzed for the determination of IC_{50} by MTT, plasma membrane damage is unlikely to be not a primary target for the compounds screened. To identify the pathway of cell death induced by the tetraoxanes in this study, apoptosis detection was executed with the use of the Annexin-V apoptosis detection kit.

With the MEC-1 cell line, a significant decrease in viable cells was present for 1 and 2 ($p<0.05$ two-tailed, unpaired t-test), after 12 hours, with small but not statistically significant reductions evident with the remaining tetraoxanes 3-5 **(Figure 2)**.

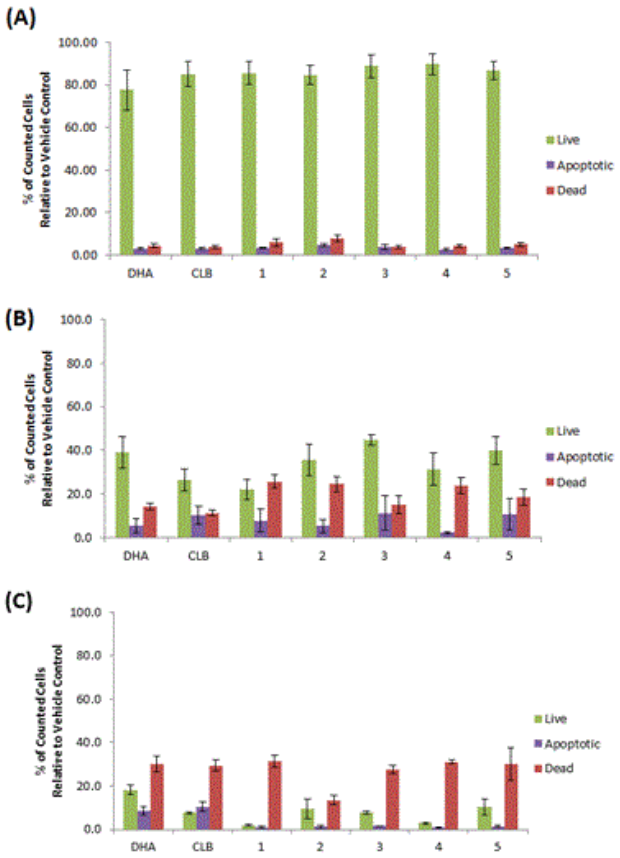

Figure 3: Flow cytometry analysis of HL-60 cells after exposure to compounds at IC_{50} concentrations (as determined by MTT). HL-60 cells were seeded at 40,000 cells/well, treated with compound for various time periods (A=12 h, B=24 h, and C=48 h) and analyzed using the Annexin-V apoptosis kit. Flow cytometric data expressed as percentage cell count relative to total cells present in the vehicle control; live (FITC-/PI-), apoptotic (FITC+/PI-), dead via apoptosis (FITC+/PI+) and necrotic (FITC-/ PI+). Data is representative of triplicate experiments (± SEM).

For HL-60 cells, no significant toxicity was detectable at the 12 hour time point **(Figure 3)**. By 24 hours, a statistically significant reduction in viable cells was detected for all compounds in both cell lines ($p<0.05$, unpaired two-tailed t-test). By 48 hours, HL-60 cells had a higher percentage of dead cells than viable cells. MEC-1 cells were also inhibited further by the 48 hour time point for all compounds screened. However, the effectiveness of the tetraoxanes is significantly less against MEC-1 than HL-60 cells during the 24 hours. These results imply compound activity after 24 hours and indicate that longer exposure times could reduce the IC50 concentrations further, depending on the cell line tested.

The detection of apoptotic cells by flow cytometry, along with low levels of membrane damage detected by LDH,

indicates the cell death pathway induced by these novel compounds is likely to be apoptosis, rather than necrosis. The detection of cell shrinkage and intracellular organelle condensation, characteristics of apoptosis, by light scatter via flow cytometry also provides added assurance of apoptotic cell death [42]. This is consistent with previously reported studies conducted on DHA and other tetraoxanes [43-45].

An important analysis parameter for all novel compounds is compound stability. Stability testing is used to evaluate expiry dates and a beyond-use date. Evaluation of the compound's stability in a solution of 100% DMSO was performed using MTT. Re-evaluation of IC_{50} values after 9 months of storage at -80°C indicates a significant reduction of compound activity for all six novel compounds **(Table 1)**. Notable reduction in compound activity suggests degradation of the compound structure, which will have a significant effect of the reproducibility of results. The significantly reduced activity of these novel compounds over this period is surprising, as tetraoxane compounds are generally well known to be thermodynamically stable [11,46]. However, DHA has been previously shown to be highly unstable under various test conditions. A study conducted by Parapini et al. confirmed a loss in biological activity and chemical degradation of DHA was pH, time and temperature-dependent [47]. These findings show that compound stability and formulation studies are critical to ensure accurate potency testing of novel compounds.

The results of this work suggest that DHA and 1, 2, 4, 5-tetraoxanes are a promising new class of anti-leukemic agents. While DHA was most effective of the compounds investigated against HL-60 cell line, it was less effective than two of the tetraoxanes against the hard-to-treat MEC-1 cell line. First-generation semi-synthetic artemisinins such as DHA also suffer from other potential drawbacks. They are only available from the plant source in low yields, despite improved methods of cultivation, and therefore they are relatively expensive to produce in comparison to their synthetic cousins. They also have short *in vivo* half-lives which have been associated with a rise in partial drug resistance of the malaria parasite. In contrast, the tetraoxanes are chiral; their synthesis is affordable and versatile-allowing incorporation of a broad range of functionality for optimization of activity and biopharmaceutical profile including prolonged half-lives [10].

Conclusion

Five novel 1, 2, 4, 5-tetraoxanes have been proven to possess excellent anti-proliferation properties, with IC_{50} concentrations significantly lower than clinically used CLB under the conditions employed in which cells were cultured in a 5% oxygen atmosphere consistent with that found *in vivo*. All five tetraoxanes and DHA showed superior selectivity toward the leukemia cell line HL-60 over the non-tumour cell line analyzed BL-2052. The higher potency and superior selectivity of these compounds for HL-60s, when compared to the currently used chemotherapeutic CLB, present the possibility of more effective treatment agents, with the potential of drastically reduced adverse side effects. When the activity in the more resistant MEC-1 cell line was compared to cytotoxicity to BL-2052, one of the tetraoxanes employed in this work displayed a selectivity index greater than 1-outperforming both DHA and CLB.

Membrane damage was not indicated as a primary target for cytotoxicity, with all compounds showing characteristic properties of apoptotic cell death, via flow cytometric analysis. Evidence of significant compound degradation while dissolved in a solution of 100% DMSO, stored at -80°C, indicates the need for further compound stability testing to be performed on the compounds, with caution needed when analyzing compounds after a period of storage.

These findings suggest further investigations and development of 1, 2, 4, 5-tetraoxanes as a new class of anti-leukemia agents are warranted and highlight the potential of these compounds in the never-ending search for better therapeutic drugs, to help vastly improve patient quality of life and morbidity.

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