Available online www.jocpr.com

Journal of Chemical and Pharmaceutical Research, 2014, 6(10):329-334

Research Article

ISSN : 0975-7384
CODEN(USA) : JCPRC5

Structural aspects of ozonides on lymphoma cell viability: Part B

Brandon Cullen[1], Brian Henriksen[1] and Richard Lomneth[2]

[1]*School of Pharmacy and Health Professions, 2500 California Plaza, Creighton University, Omaha, NE*
[2]*Department of Chemistry, University of Nebraska at Omaha, 6001 Dodge St, Omaha NE*

ABSTRACT

Synthetic ozonides (1,2,4-trioxolanes) are known to be effective antimalarials agents, and have been shown to have activity against lymphoma cells. In this study, conformational analysis was used on eight ozonides to see if the previously established pharmacophore would accurately predict the set's activity against lymphoma cells. Based on previous work there are at least two crucial factors in anti-lymphoma activity, an ionizable amine and the distance from the proximal peroxide to the ionizable functional group. A small follow-up library of compounds was assayed for their activity. Seven of the compounds in the new library exhibited low activity and as a result the pharmacophore has been refined with two additional factors. Two alkyl groups alpha to the ionizable amine or a carbonyl beta to the ionizable amine interfered with the anti-lymphoma activity of the compounds in this set.

Keywords: Malaria, Ozonide, Artemisinin.

INTRODUCTION

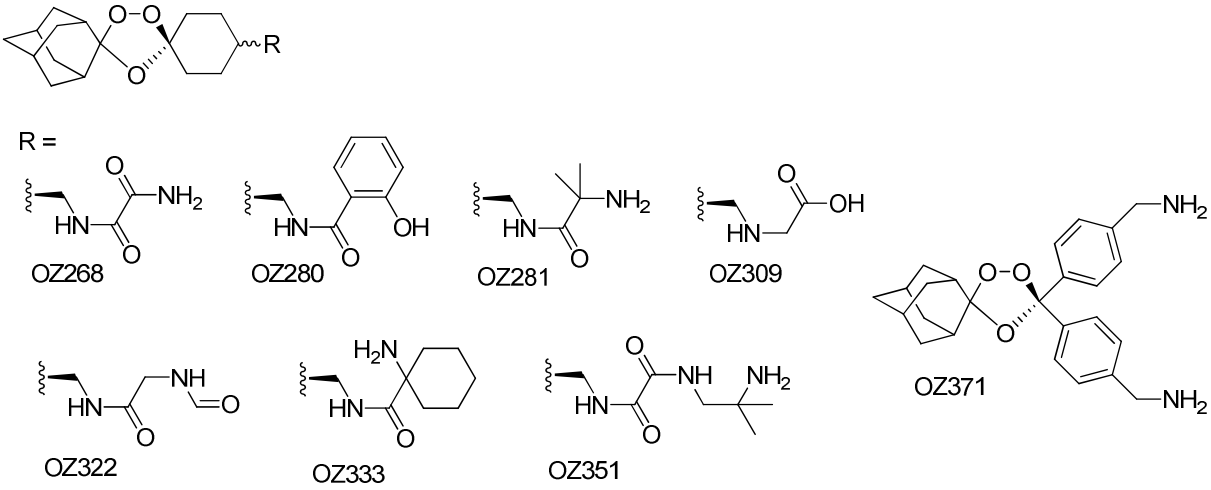
Artemisinin is a naturally found sesquiterpene lactone found in the Chinese plant, Artemisia annua, or sweet wormwood[1]. Artemisinin is characterized by a 1,2,4-trioxolane heterocycle, which is the pharmacologic foundation for drug action against malaria. It has been suggested that artemisinin and related derivatives have antiparasitic activity through the reduction of heme[2]. One of the disadvantages of semisynthetic artimisinins is that the short elimination half-life[3] of these compounds leads to longer therapeutic regimens, which can lead to non-compliance. Synthetic ozonides containing the 1,2,4-trioxolane heterocycle have been developed as an alternative treatment option to semisynthetic artemisinins[4].

Artemisinin and its derivatives have been shown to decrease viability of cancer cells in addition to their activity against malaria[4-6], although at concentrations several orders of magnitude higher than needed for anti-malarial activity[4]. There is evidence that the effect of these compounds is due induction of cellular apoptosis caused by the formation of a carbon radical from the endoperoxide bridge[6]. This carbon radical eventually leads to the depolarization of the mitochondrial membrane and ultimately to DNA degradation, causing apoptosis.

Synthetic ozonides have not been researched as extensively with regard to activity against cancer, though they have been shown to cause a decrease in lymphoma cell viability[7]. It was found that the pharmacophore for ozonide action against Raji lymphoma cells requires an ionizable amine group about nine angstroms away from the proximal peroxide of the 1,2,4-trioxolane ring. It was also found that many of the ozonideswith high activity against lymphoma cells contain phenyl rings as part of the structure, although it is not necessarily required as there were structures tested with high activity that did not contain a phenyl ring.

In this study, we have analyzed the structure of eight ozonides (Figure 1) to determine if we could use the previously reported model to predict the activity of ozonides towards lymphoma cells. The results indicate the model worked well to identify compounds of low activity while also demonstrating the model needed refinement to better predict the effect of "bulky" groups near the protonated primary amine that is critical for activity.

Figure 1: Ozonide Structures



EXPERIMENTAL SECTION

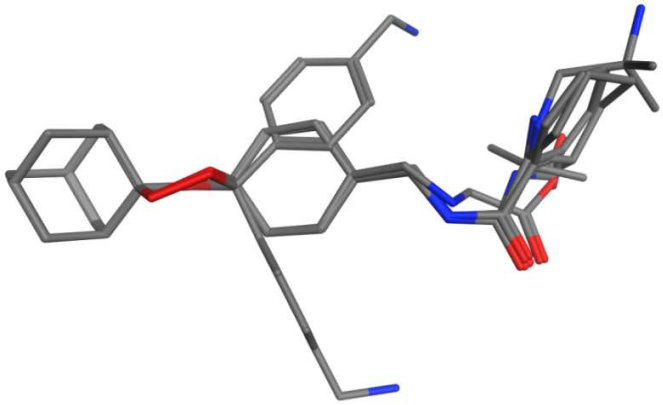
The library of compounds in Figure 1 was generated, protonated and minimized at a simulated pH 7.4 using the Protonate 3D function in MOE. Molecules were minimized using the MMFF94x forcefield and aligned using the Flexible Alignment feature in MOE.

Raji lymphoma cells were grown in T-25 flasks in RPMI 1640 media supplemented with 10% fetal calf serum (Hyclone), 2 mM L-glutamine and 100 µg/mL penicillin-streptomycin (GIBCO) at 37 ^{o}C in a humidified 5% CO_2 incubator. When cells became dense and the media started to turn slightly acidic, the cells were subcultured by removing a small volume of cell suspension (1 mL) and adding the cells to 7 mL fresh complete supplemented media.

Raji cells growing in T-25 flasks were counted using a hemocytometer and an inverted microscope. Cells (50,000 in 100 µL fresh media) were added to each well of a 96 well plate and exposed to the experimental treatment or solvent control for 2 days unless otherwise noted. Cell viability was determined using the MTT method [9,10] similar to the conditions used by Mercer, et al [8]. After exposure to the test compounds, 25 µL MTT reagent (5 mg/mL 1-(4,5-dimethylthiazol-2-yl)-5-diphenylformazan in phosphate buffered saline) was added to each well and the cells were incubated for 2 hours at 37 ^{o}C. After the 2 hour incubation, 100 µL of stop solution (pH ~4.7, 50% dimethylformamide, 20% sodium dodecylsulfate, 0.5% of an 80% acetic acid/2.5% hydrochloric acid solution) was added. Cells were covered and sealed with Parafilm (Pechiney Plastic Packaging) and incubated overnight at 37^{o}C to dissolve the cells and the purple product. Absorbance of the purple solution was measured at 550 nm using a plate-reader spectrophotometer. All cell studies were performed at least in replicates of 6 with average background absorbance (MTT reagent in wells without cells) subtracted. Cell viability was reported as a percent absorbance relative to control cells. Control cells were exposed to the same concentration of solvent as the cells treated with test compounds. Artesunate was used in each experiment as a positive control and generally 10 µM artesunate-treated cell viability was ~30-50% that of control. Compounds were dissolved in either dimethyl sulfoxide (DMSO) or ethanol to make stock concentrations 100 times the concentration studied during the experiment.

RESULTS

The compounds in this set were overlaid employing the Flexible Alignment application in MOE. Alignment of the compounds was performed as described previously[7]and is shown in Figure 2. With the majority of compounds containing an amide near the cyclohexyl ring, it is not surprising the overall geometry of these compounds is so similar



.

Figure 2: The alignment image for ozonide molecules. In this image, the nitrogens are blue, oxygens are red, and carbons are grey. Hydrogens are omitted for clarity

The distances from the trioxolane oxygens to the functional groups are given in Table 1. Distances were measured to primary amines for OZ281, OZ333, and OZ351, secondary amine for OZ309, primary amides for OZ268 and OZ322, and phenolic oxygen for OZ280. For OZ371, distances are given to each of the amine groups, labeled Top and Bottom in Table 1 in relation to the trioxolane ring.

Table 1: Distance from trioxolane oxygen to functional group of interest in angstroms (Å)

Molecule	Distance from Proximal peroxide to functional group (Å)	Distance from non-peroxide oxygen to functional group (Å)
OZ268	7.89	8.80
OZ280	9.11	9.38
OZ281	7.75	8.65
OZ309	5.77	6.70
OZ322	7.50	8.12
OZ333	9.07	9.29
OZ351	10.94	11.82
OZ371 (top)	7.30	7.18
OZ371 (bottom)	7.30	7.22

OZ281, OZ333, and OZ351 appeared to be likely candidates for high activity since they contain ionizable amines. The molecular distance from the ionizable amine to the trioxolane oxygens shows that OZ333 was within the predicted pharmacophore distance for activity against Raji lymphomas. However, none of these ozonides showed significant activity *in vitro* against Raji lymphoma cells, with cell viability greater than 70% for each of these three compounds. OZ281 and OZ351 share the dialkyl motif alpha to the ionizable amine and the distance from the proximal peroxide oxygen to the amine nitrogen in both is outside the optimal range. In addition, OZ281 and OZ333 share a beta carbonyl feature as part of an amide. To better understand why OZ333 does not have high activity against lymphoma cells, the compound was compared to a library of molecules with known activity against Raji lymphoma cells. The highly active compounds were aligned with each other according to Henriksen et. al.[7], and held constant in the alignment of OZ333. The alignment picture is given in Figure 3.

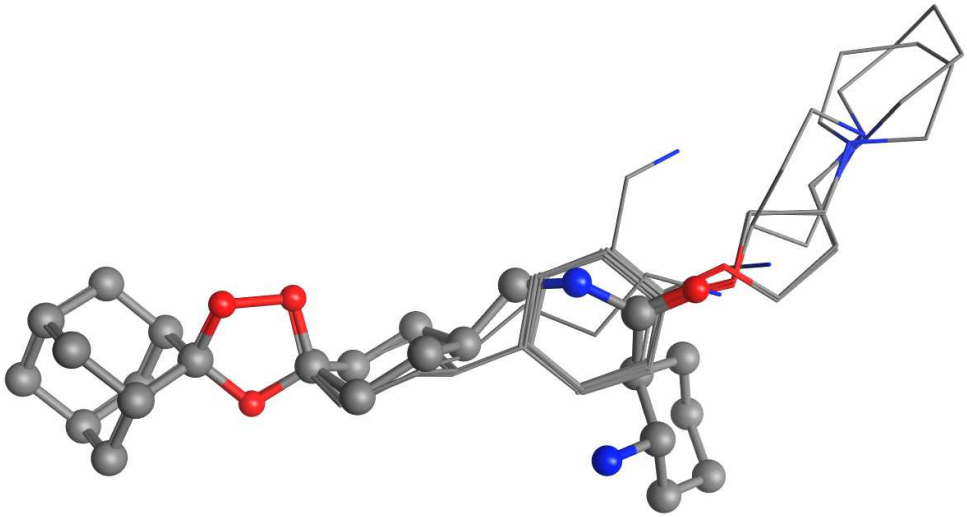


Figure 1: Alignment picture of highly active molecules with OZ333

In this alignment picture, OZ333 is rendered as ball and stick, with the carbonyl group aligning with the oxygens of the highly active molecules. Manual rotation of the sigma bonds did not result in a conformationally favorable orientation of OZ333 with the desired distance between the ionizable amine and the peroxide oxygens.

DISCUSSION

The established hypothesis for the effect of ozonides on lymphoma cell viability is that there is an optimal distance between the 1,2,4-trioxylane heterocycle and an ionizable amine group. The established distance for high activity against Raji lymphoma cell viability is given as 9.00±0.41 angstroms from the proximal peroxide of the trioxolane ring to the amine group[7]. The highly active ozonides described in Henriksen et al. had three common structural features: the adamantane and 1,2,4-trioxolane heterocycles, and an ionizable amine within a certain distance range from a peroxide oxygen, which is predicted to be predominately protonated at physiological pH.

OZ268 and OZ322 have nitrogens within the distance hypothesized as a potential pharmacophore feature, but they are both found in neutral amide groups. The hydroxyl functional group for OZ280 is the correct distance for the hypothesized pharmacophore, but the functional group is uncharged at physiological pH confirming the importance of an ionizable amine. Based on the hypothesis that the ozonide must have an ionizable amine at the correct distance away from the trioxolane ring to have action against lymphoma cells, it can be hypothesized that OZ268, OZ280, and OZ322 would have low activity.

The distance from the proximal peroxide oxygen to the secondary amine in OZ309 is much shorter than all the other compounds and much less than the value of 9.00 ± 0.41 angstroms found previously for highly active compounds[7] This is consistent with low activity towards lymphoma cells.

Three other compounds in the new library also exhibited low activity. As a result of the low activity the pharmacophore has been refined with some factors that stand out but need additional compounds to delineate the importance of each respective feature. OZ281 and OZ351 are outside the optimal distance between the proximal peroxide oxygen and ionizable amine. In addition, the presence of an amide and the ability of the ionizable amine to form a putative intramolecular hydrogen bond appears to be related to the activity of the compound. None of the highly active compounds contain an amide. Furthermore, two alkyl groups alpha to the ionizable amine as demonstrated by OZ281 and OZ351 or a cycloalkyl group such as OZ333 may influence the activity of these compounds.

The ozonide compounds from this set, and the previously published set, able to form intramolecular hydrogen bonds exhibited low activity. This low activity was unexpected since some of the intramolecular interactions would involve intramolecular seven or eight membered rings, such as OZ351. Proteases, an area of active investigation in

lymphoma cells, may be cleaving the compounds and leading to their subsequent poor activity.Since the highly active compounds contained an ionizable amine and none of those compounds contain an amide it's reasonable that proteolytic inactivation may be the primary culprit for low activity compounds that otherwise meet the previously established pharmacophore features necessary for high activity.

OZ284 and OZ348, previously published, were the two compounds in the library to exhibit intermediate activity. OZ284 contains an amide in resonance with an aromatic ring and therefore has a less nucleophilic carbonyl carbon in its amide. Its amine is also attached to an aromatic ring so its lone pair of electrons are in resonance resulting in a lower likelihood for ionization based on pK_a so its intermediate activity could be the result of either its weaker ability to act as a base, its amide being less susceptible to proteolytic cleavage or some combination of the two.OZ348 contains alkyl groups alpha to the ionizable amine as well as the ability to form an intramolecular hydrogen bond but it contains a urea functional group instead of an amide. The difference in the urea oxygen's partial negative character may account for its intermediate, instead of low, activity in the Raji cell line again to its potentially less labile amide. Once again it remains unclear what role the methyl groups alpha to the amine play in its activity and remains a site for further investigation.

OZ335 is an analog of OZ281 but only contains one alpha methyl compared to OZ281's two alpha methyl groups. Both compounds exhibited low activity but are able to form five membered rings through intramolecular hydrogen bonding. It is unclear whether the presence of alkyl groups on the alpha carbon, intramolecular hydrogen bonding involving the amine or the presence of the amide functional group contribute the most to the low anti-lymphoma activity of these compounds.However, the group of highly active compounds includes secondary amines and compounds which could theoretically form rings through intramolecular hydrogen bonds, while none of the compounds with high anti-lymphoma activity contain the amide functional group[7].

Taking the new information into consideration the pharmacophore model for this class of compounds has been refined to exclude compounds that contain amides. Whether the amides are labile to protease activity or if they provide intramolecular charge stabilization that interferes with receptor binding remains unclear. Steric bulk on the ionizable amine, or alpha to it, do not appear to be having a negative impact on the compound's activity. Although OZ371 is the only branched inhibitor in the set it exhibits high activity and follows the putative pharmacophore's rules. It maintains an ionizable amine and does not have the ability to form an intramolecular hydrogen bond.Although the distance between its ozonide and ionizable amine is shorter than the high activity compounds reported previously, having two ionizable amines may allow it to have an additional affinity interaction with the target receptor. Since the plane of symmetry of this compound is unique in the set of compounds assayed so far it provides an exciting new direction for pursuing additional highly active analogs.

CONCLUSION

In conclusion, a library of ozonides was generated and analyzed using the previous pharmacophore of ozonide action against lymphoma cells as a guide. The set included compounds that were not anticipated to have strong anti-lymphoma activity to confirm the previous pharmacophore. As expected, compounds without a predominately positively charged functional group at the proper distance from the trioxolane did not have activity. Three of four compounds with ionizable amines had no activity, but the three shared the common feature of amide bonds. As a result the pharmacophore for this class of compounds has been further refined to exclude compounds able to form intramolecular hydrogen bonds. Without further investigation it is unclear if the potentially labile amide or intramolecular hydrogen bonding results in loss of anti-lymphoma activity. OZ371's unique branched structure did highlight the importance of an ionizable amine with no ability to form intramolecular hydrogen bonds or labile functional groups in the highly active compounds. Furthermore, it demonstrated that a wide variety of constrained geometry analogs would be tolerated by the receptor as long as the distance and ionizable amine features are maintained.

Acknowledgements
We would like to thank Jonathan L. Vennerstrom, Yuxiang Dong, Alex Buga, and Shawn Brandenburg for their various and invaluable help. We would also like to thank the University of Nebraska at Omaha Chemistry Department.

REFERENCES

[1] DL Klayman. *Science* **1985**, 228(4703), 1049-1055.
[2] S Krishna, et al. *Drug Resist Update* **2004**, 7(3), 233-244.
[3] JL Vennerstrom, et al *Nature* **2004**, 430(7019), 900-904.
[4] T Efferth. *Drug Resist Update* **2005**, 8(2), 85-97.
[5] NP Singh; HC Lai,. *Anticancer Res.* **2004**, 24(4), 2277-2280.
[6] AE Mercer; JL Maggs; XM Sun; GM Cohen; J Chadwick; PM O'Neill; BK Park. *J. Biol. Chem.* **2007**, 282(4), 9372-9382.
[7] B Henriksen, et al. *J. Chem. Pharm. Res.* **2012**, 4(4), 2012-2020.
[8] AE Mercer; JL Maggs; XM Sun; GM Cohen; J Chadwick; PM O'Neill; BK Park. *J. Biol. Chem.* **2007**, 282(13), 9372-9382.
[9] TJ Mosmann. *Immunol. Methods* **1983**, 65(2), 55-63
[10] MB Hansen; SE Nielsen; KJ Berg. *Immunol. Methods* **1989**, 119(2), 203-210.