Determination of Anti-Malarial Properties of Delta lactam Derivatives from Mycosynhetix Sample 232697

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Determination of Anti-malarial Properties of Delta-lactam Derivatives from MSX232697

by

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with

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Abstract:

The Medicines for Malaria Venture (MMV) seeks to obtain an alternative cure for malaria that is simple to recreate, and of lower cost margin than the current drugs available. Over 50,000 fungal samples were investigated for anti-malarial properties, and those showing promise were further analyzed. The removal and purification of compounds from sample MSX232697 was performed using methanol extractions, liquid partitions, medium-pressure liquid chromatography (MPLC), and HPLC for compound isolation. Nuclear magnetic resonance (NMR), mass spectrometry (MS), and X-ray crystallography were used to characterize and elucidate compounds. The basic structures of the two compounds of interest are known to be delta-lactam derivatives. The final purified products will be submitted for bioassay and the results will determine whether the compounds will be viable for future drug development.
Introduction:

Malaria is an infectious disease caused by the parasite Plasmodium. This parasite, a eukaryotic protist, is transmitted via the bite of an infected mosquito. (Sutherland, 2010) The type of mosquito that is responsible is the female anopheles mosquito. There are 5 different strains of the plasmodium parasite *P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae*, and *P. knowlesi*. The most deadly strain is the *p. falciparum* causing the majority of the malarial deaths globally. (Snow, 2005) This disease is the fifth leading cause of death globally and the region of sub-Saharan Africa is the greatest affected. (Murray, 2012) The people that are most at risk of dying from malaria are young children, pregnant women, people with AIDS/HIV, and international travelers. (CDC, 2010)

Figure 1: The map shows the prevalence rate of malarial cases in different regions of the world (WHO, 2004)
The symptoms for this disease are fever, shivering, joint pain, vomiting, anemia, jaundice, hemoglobinuria, retinal damage, and convulsions. (Beare, 2006)

Transmission cycle of the parasite occurs when an *Anopheles* mosquito bites an infected human victim to obtain a blood meal. The mosquito contracts the *Plasmodium* sporozoites from the human’s blood and the parasite is carried in the salivary glands. The mosquito bites the next victim and sporozoites are released into the blood stream from the salivary glands. (Bledsoe, 2005) The parasite then develops in two phases, exoerythrocyticly and erythrocyticly. Initially, the parasite performs the exoerythrocytic phase and infects the liver cells. The parasites reproduce asexually within the cell between a period of 8 to 30 days. After this period the parasite has produced thousands of merozoites and are released into the blood stream once the liver cells are ruptured. (Bledsoe, 2005) The parasite then begins its erythrocytic phase and infects blood cells. Within the red blood cells the parasite multiplies asexually, bursting the red blood cell and moving toward the next one to infect. Over time some of the merozoites then turn into male and female gametocytes. When the mosquito intakes blood from an infected person, it will uptake both male and female gametocytes. (Bledsoe, 2005) This will allow the parasite to reproduce and sexually recombine its genes for more genetic diversity. The mosquito in this case is the carrier of the parasite and the human is the intermediate host. (Bledsoe, 2005)
Figure 2: The figure shows the life cycle of the *Plasmodium* parasite (NIAID, 2010)

There are multiple treatments to combat the malaria parasite. Some of the more common drugs used are chloroquine, amodiaquine, quinine, mefloquine, halofantrine, atovaquone, artemisini, and its derivatives. (Delfino, 2002)
Figure 3: The figure shows the common anti-malarial drugs with the quinoline-containing structures on the left and the artemisinin-type compounds on the right. (Delfino, 2002)

These drugs can be taken orally or intravenously and the regimen given most commonly is artemisinin combination therapy. This type of treatment uses a combination of drugs to increase the effectiveness against the parasite. (RSC, 2006) Many of the treatments require that you take the pills before you are potentially exposed to the parasite and up to 4 weeks after the possible exposure date. This leads to excessive costs and makes these drugs inaccessible to the masses. *P. falciparum* has also recently begun to become
resistant to certain treatments. The combination of high prices and parasite resistance has driven the research of anti-malarial drugs. (RSC, 2006)

Medicines for Malaria Venture (MMV) is a non-profit organization founded to discover, develop, and facilitate delivery of new as well as inexpensive anti-malarial drugs. (MMV, 2011) This organization has aided in funding many research institutions including the project that led to this thesis.

**Methods:**

The MMV project was the starting point for this thesis project. The sample selected for this thesis needed to show activity in the initial screening performed. The initial screening process used fungal samples provided by the company Mycosynthetix (MSX). Mycosynthetix provided over 50,000 samples for this research project to extract and test. The samples tested were all sent in 20 mL vials from the supplier, making extraction of the initial samples simple.

The samples were first extracted using methanol. Methanol is used because it can dissolve a wide range of chemicals with varying polarity and it is easily evaporated. Once the sample sat in methanol for 24 hrs, the extract is then removed and plated on a 96 well plate. This plate is then submitted to our collaborator that will perform bioassay and cytotoxicity tests on each crude extract. The bioassay test checks for inhibition of growth of *f. plasmodium*, the parasite which causes malaria. Cytotoxicity testing checks for inhibition of growth of human cells when exposed to crude extracts. The combination of high parasite growth inhibition and low cytotoxicity is the idea scenario for drug development. The results from these assays varied in each sample with some having
activity against the parasite and other showing no effect. This initial screening test was performed to provide a qualitative test that would narrow down, which samples would be scaled up.

The extracts that showed activity in the initial assay were then fractioned using medium pressure liquid chromatography (MPLC). The sample must first be prepared by combining silica gel with the crude extract. The initial methanol extract from the samples has to be dried down into a pre-weighed vial in order to determine the dry weight. The methanol and 3 times the crude’s mass of silica is used. Once the sample has been completely dissolved in the methanol, the silica and the re-dissolved extract are combined into a 250 mL round bottom flask. The round bottom flask is then place onto a rotary evaporator. This machine uses a vacuum, rotation, and a warm water bath to speed up the evaporation process. The sample was then placed on the machine with a glass catch containing cotton in between the round bottom flask and the condenser. Once the sample is properly connected with safety clamps, the vacuum can then be started. The vacuum should initially be only a quarter pressure of the full vacuum. The next step is to lower the flask into the water bath, which should be at a lukewarm temperature. This low temperature insures that no organic compound is degraded during the removal of methanol. Once the flask was in the water about half way, the rotation feature can be turned on to about 100 revolutions per minute. The vacuum can now be turned up quarter turns until one sees bubbling. This process should be very slow in order to prevent over flow of sample into the catch. The drying process should not take longer than 20 min. The sample should look like loose sand in the flask before removal. Once the sample is dry, the flask is then scrapped using a spatula to remove the silica that is stuck to the
walls of the flask. This sample is now ready to be packed into a sample column for the MPLC. Once the sample column is packed it can be attached to the machine and a normal phase separation column is used for this experiment. The separation column size is based on the amount of crude extract used, so for this experiment a 4 gram flash column is necessary. Once the machine is properly set up a run must be formulated using three solvents. The solvents used in the run are hexane, ethyl acetate, and methanol. The experiment is 28 minutes in length. The initial starting concentration is 100 percent hexane for 2.5 minutes. At the point of 2.5 minutes to about 16.5 minutes is slow gradient increasing the second solvent concentration, ethyl acetate, to 100 percent. Once the gradient is reaches 100 percent at 16.5 minutes, it is kept at this concentration till about 21 minutes. Once the 21 minute mark is reached the third solvent, methanol is used. The gradient from 21 minutes to 28 minutes uses an increasing amount of methanol until no ethyl acetate is used at the end of the 28 minutes. This gradient is the standard for all samples that showed activity in the initial screening.

The results from this fractionation technique were about 50 test tubes filled with solvent and a UV spectrum. The UV spectrum was then used to combine the test tubes in about 10 fractions to be submitted for assay. The fractions are transferred to 20 mL pre-weighed vials and dried down to obtain a fraction weight. The fraction weight was used to determine how much DMSO is needed for the assays. The normal concentration of sample in each well should be about 2 mg of sample per 1 mL of DMSO. This will provide enough extract for our collaborators to perform a serial dilution on the extract and determine the samples that are of high interest.
The selection of my thesis sample was finally made after all these initial steps were taken on thousands of extracts. The sample that was chosen for my thesis was MSX232697. This sample showed primary interest on four MPLC fractions and secondary interest on another two fractions. Once the sample was selected a larger 3 liter scale up of MSX232697 was sent to the lab to be extracted. This scale up would provide enough sample mass for pure compounds to be extracted and identified. The first step for the scale up is to extract the compounds using methanol. A methanol extraction is performed three times over three subsequent days. This will ensure the full extraction of the sample. The methanol was then dried down into the crude extract. A partition containing equal parts ethyl acetate and water was performed on the crude extract. The organic layer should be drawn off and ethyl acetate should be added twice more to ensure full extraction of organic compounds. The aqueous soluble materials are many times large and difficult to separate thus the partition makes the organic compound isolation much easier. The active compounds are in the organic layer of the partition. The ethyl acetate partition obtained was then dried down, weighed, and set up for MPLC fractionation using silica. The same MPLC method used previously will be used for the ethyl acetate fraction. Using the UV spectrum the test tubes were separated into ten separate fractions from A to J. The fractions obtained were then dried down to obtain a weight. The fractions, the original crude, and the ethyl acetate partition were all then submitted for bioassay as well as cytotoxicity. The results from the testing showed to be consistent with the previous assay results with four primary hits and two secondary hits as shown in Figure 4. The next step in the purification process was to perform an HPLC on each fraction. Reverse phase LC was used with H2O and acetonitrile. Each of the
active fractions were re-dissolved with HPLC grade methanol and filtered into a 2 mL vial for injection. The experiments that were run and the UV detector was set to wavelength 225, 254, and 285nm. These wavelengths were selected because absorption at these wavelengths would indicate promising features in a compound such as double bonds and aromatic rings. The general HPLC method that was used started at 0% at 0 minutes then the concentration increased to 40% ACN in 35 minutes. The concentration of ACN was then increased to 100% of the next 5 min to the 40 minute mark. Once at 100% ACN the pumps were run until the 45 min mark. Fraction E method differed slightly initial concentration of ACN starting at 10% and increasing to 25% in 40 minutes. After the 40 minute mark was reached the concentration of ACN would increase to 100% in the next 10 minutes. Once at 100% ACN, the gradient will run until 60 minutes was reached.

These methods were run on both analytical and semi-prep columns. The method general method served as the basic method of separation for all the fractions. Fraction E had issues with the general method because the target peak would elute with smaller peaks making the separation difficult. The solution to this problem was to create the second method, making a slower gradient, which allowed for the target peak to be isolated with ease. The next step was to use the LCMS (liquid chromatography – mass spectrometry) to determine the purity and mass of the possible compounds collected. Once the mass was known, 800 MHz $^1$H NMR (Nuclear Magnetic resonance) was performed on each of the compounds. Deuterated methanol was used for all samples. The results showed impurities that further purification was needed. The peaks from fractions
E, H, and I were taken and combined into a single vial as shown in the flow chart. Combining the fraction into one vial would allow for purification in fewer injects.

The HPLC General Method was used and provided sufficient separation for collect of the peaks. The second collection provided better peak purification and crystallization. During this collect two major peaks were isolated. NMR results of the purified compounds are shown in figures 10 and 11. After the NMR data was collected, Q-TOF high-resolution mass spectrometry was used to generate a molecular formula. Q-TOF uses a time measurement to determine an ion’s mass to charge ratio. The results displayed $m/z=232.0974$ for 3-(2-(4-hydroxyphenyl)-2-oxoethyl)-5,6-dihrdropyridin-2(1H)-one and $m/z=216.1045$ for 3-(2-oxo-2-phenylethyl)-5,6-dihrdropyridin-2(1H)-one. Using the chemical formula determined by the Q-TOF, along with H-NMR data, and the chemical database AntiMarin, an initial structure was determined for peak 1 in fraction EHI. The second peak in fraction EHI also used the same determination process, but the structure could not be determined initially because there was no literature data available on the possible structure. In order to get an absolute structure for each peak, they were allowed to crystallize over the span of a week in order to obtain crystals. The crystals were then submitted for x-ray crystallography. The results showed two delta-lactam derivatives only differing by a single oxygen. The two compounds are 3-(2-(4-hydroxyphenyl)-2-oxoethyl)-5,6-dihrdropyridin-2(1H)-one and 3-(2-oxo-2-phenylethyl)-5,6-dihrdropyridin-2(1H)-one. The second compound, 3-(2-oxo-2-phenylethyl)-5,6-dihrdropyridin-2(1H)-one, has no literature data and could possibly be a new compound. This compound has a possibility to be published in the future with more structural analysis. Once the compounds had been determined, they were submitted for bioassay
and cytotoxicity at 2 mg/mL. Both the compounds did not show activity at the nano-molar range, making the compounds inactive against *P. falciparum*. 
Results & Discussion

MPLC Fractionation MSX232697

**Figure 4:** The above shows the initial MPLC fraction for MSX232967. *The fractions are the primary fractions of interest.*

<table>
<thead>
<tr>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E*</th>
<th>F*</th>
<th>G</th>
<th>H*</th>
<th>I*</th>
<th>J</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-6</td>
<td>7-10</td>
<td>11-12</td>
<td>13</td>
<td>14-17</td>
<td>18-19</td>
<td>20-25</td>
<td>26-31</td>
<td>32-34</td>
<td>35-42</td>
</tr>
<tr>
<td>35.5 mg</td>
<td>355 mg</td>
<td>9.7 mg</td>
<td>4.7 mg</td>
<td>35.6 mg</td>
<td>25.1 mg</td>
<td>31.1 mg</td>
<td>21.6 mg</td>
<td>20.4 mg</td>
<td>71.3 mg</td>
</tr>
</tbody>
</table>
**Table 1:** The red boxes indicate the fractions that were submitted for assay from my sample. The yellow highlighting indicates interest for the MMV project. The purple highlighting is of secondary interest not for the MMV project.
Figure 5: The arrow indicates peak 1 that was collected from MPLC Fraction E by HPLC purification.

Figure 6: The arrows indicate the peaks collected from MPLC Fraction F by HPLC purification.
Figure 7: The arrows indicate the peaks collected from MPLC Fraction H by HPLC purification.

Figure 8: The arrow indicates the peak of collected from MPLC Fraction I by HPLC purification.
Figure 9: The arrows indicate the fractions collected from Fraction EHI by HPLC purification. The EHI fraction is all the peaks from fractions E, H, and I combined. The black line represents 225 nm, the pink line represents 254 nm, and the blue line represents 285 nm wavelength.
Flow Chart: Fractionation Scheme of MSX232697

MPLC Fractionation
Solvents used Hexane, Ethyl Acetate and Methanol.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>35.5 mg</td>
</tr>
<tr>
<td>B</td>
<td>355 mg</td>
</tr>
<tr>
<td>C</td>
<td>9.7 mg</td>
</tr>
<tr>
<td>D</td>
<td>4.7 mg</td>
</tr>
<tr>
<td>E</td>
<td>35.6 mg</td>
</tr>
<tr>
<td>F</td>
<td>25.1 mg</td>
</tr>
<tr>
<td>G</td>
<td>31.1 mg</td>
</tr>
<tr>
<td>H</td>
<td>21.6 mg</td>
</tr>
<tr>
<td>I</td>
<td>20.4 mg</td>
</tr>
<tr>
<td>J</td>
<td>71.3 mg</td>
</tr>
</tbody>
</table>

MSX 232697 and the fractions in red showed inhibition against the parasite.

HPLC Fractionation
Solvents used Acetonitrile and Water

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>E</td>
<td>1.69 mg</td>
</tr>
<tr>
<td>F</td>
<td>25.1 mg</td>
</tr>
<tr>
<td>G</td>
<td>31.1 mg</td>
</tr>
<tr>
<td>H</td>
<td>21.6 mg</td>
</tr>
<tr>
<td>I</td>
<td>20.4 mg</td>
</tr>
</tbody>
</table>

HPLC Repurification
Same solvents used as before and all the peaks collected were combined into one fraction in order to reduce amount of purification runs.

<table>
<thead>
<tr>
<th>Combination fraction EHI</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>EHI Peak 1</td>
<td>1.3 mg</td>
</tr>
<tr>
<td>EHI Peak 2</td>
<td>0.2 mg</td>
</tr>
</tbody>
</table>

Pure Compounds

- 3-(2-(4-hydroxyphenyl)-2-oxoethyl)-5,6-dihydropyridin-2(1H)-one
- 3-(2-oxo-2-phenylethyl)-5,6-dihydropyridin-2(1H)-one
Figure 10: H-NMR spectrum on 800 MHz of 3-(2-(4-hydroxyphenyl)-2-oxoethyl)-5,6-dihdropyridin-2(1H)-one (Peak 1) using Dueterated Methanol.

Figure 11: H-NMR spectrum on 800 MHz of 3-(2-oxo-2-phenylethyl)-5,6-dihdropyridin-2(1H)-one (Peak 2) using Dueterated Methanol.
Table 2: This table compares literature H-NMR spectrum with the spectrum obtained for pure compound 3-(2-(4-hydroxyphenyl)-2-oxoethyl)-5,6-dihdropyridin-2(1H)-one (Peak 1)

<table>
<thead>
<tr>
<th>Type</th>
<th>EHI Peak 1</th>
<th>Lit. spectrum</th>
<th>Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Doublet</td>
<td>6.82</td>
<td>6.88</td>
<td>-0.06</td>
</tr>
<tr>
<td>Doublet</td>
<td>7.9</td>
<td>7.96</td>
<td>-0.06</td>
</tr>
<tr>
<td>Triplet</td>
<td>6.56</td>
<td>6.56</td>
<td>0</td>
</tr>
<tr>
<td>Multiplet</td>
<td>2.4</td>
<td>2.44</td>
<td>-0.04</td>
</tr>
<tr>
<td>Triplet</td>
<td>3.41</td>
<td>3.46</td>
<td>-0.05</td>
</tr>
<tr>
<td>Broad</td>
<td>3.89</td>
<td>3.93</td>
<td>-0.04</td>
</tr>
</tbody>
</table>

Figure 12: High resolution mass spectrum of 3-(2-(4-hydroxyphenyl)-2-oxoethyl)-5,6-dihdropyridin-2(1H)-one (Peak 1) using a Q-TOF. This figure shows the compound plus a proton (m/z = 232.0974) and also the compound plus a sodium adduct (m/z = 254.0793)
Figure 13: High resolution mass spectrum of 3-(2-oxo-2-phenylethyl)-5,6-dihrdropyridin-2(1H)-one (Peak 2) using Moffitt’s Agilent 6550 iFunnel Q-TOF LC/MS. This figure shows the compound plus a proton and the compound plus two protons.

Figure 14: Crystal structure conformation of 3-(2-(4-hydroxyphenyl)-2-oxoethyl)-5,6-dihrdropyridin-2(1H)-one (Peak 1) using x-ray crystallography.
Figure 15: Crystal structure conformation of 3-(2-oxo-2-phenylethyl)-5,6-dihrdropyridin-2(1H)-one (Peak 2) using x-ray crystallography.

**Pure compound Bioassay Results:** The concentration of the samples submitted was 2 mg/mL and a serial dilution was performed. Both compounds did not show activity at the nano molar range and thus ruling out these compounds for anti-malarial drug development.

**Conclusion:**

The two compounds identified in this thesis did not show promising anti-malarial properties thus ruling them out for further development of anti-malarial drugs. Compound 3-(2-(4-hydroxyphenyl)-2-oxoethyl)-5,6-dihrdropyridin-2(1H)-one (Peak 1) has been discovered in two different organisms the fungus, *H. grisea var thermoidea*, and the
sponge, *H. melanodocia.* (Andrioli, 2012) The literature shows that this compound has anti-allergic properties. Compound 3-(2-oxo-2-phenylethyl)-5,6-dihdropyridin-2(1H)-one (Peak 2) being a unreported in Scifinder and Marin Lit. This newly identified compound could possess these similar anti-allergic properties because of structural similarities. This type of research could be conducted in the future along with other assays to find an application for this novel compound.
References


doi:10.1097/01.smj.0000189904.50838.eb. PMID 16440920.


