KPCRTF State Funded Projects Reporting Template

University of Kentucky
Artesunate – Treatment for Acute Myeloid Leukemia
Program Director: Jill Kolesar, PharmD, MS

Reporting Period: April 1, 2021 through June 30, 2021

Below please provide a brief summary of the status of the Project listed as well as for each Objective listed below. Include any barriers, how and if they were overcome, and successes achieved.

Project Status

Deliverable	Due Date	Completion Date	Barriers	Notes
1a) Biosafety Protocol Approval	12.31.20	7.22.20	None	
1b) Animal Protocol Approval	12.31.20	7.13.20	None	
2) Draft Clinical Protocol	12.31.20	8.21.20/6.30.21	None	Drafted update to protocol on 6.30.21
3) Obtain Regulatory Approval	12.31.20			
• CCART Approval	12.31.20	8.31.20	None	
• PRMC Approval	12.31.20			
• UK IRB Approval	12.31.20			In process of submitting modified protocol due to FDA concerns for IRB approval
• State Approval	12.31.20			CHFS IRB administrator Bob Blackburn has given notification that the CHFS IRB will accept UK IRB approval
• IND Approval	12.31.20		Not able to obtain FDA approval for artesunate with overseas manufacturer	Altering plan for clinical component of grant to establishing primary PDX models instead of phase I treatment study.
4) Establish GMP manufacturing	12.31.20	6.10.20	None	Contracted with Ipca laboratories to produce artesunate tablets
5) Fractionate and quantitate *Artemisia annua* extracts from Apollon and CNAP8001	12.31.20	11.25.20	Fractionation by HPLC requires over 25 injections through column to get enough for use in downstream testing	Methonal extraction results in highest anti-cancer activity. Processing through HPLC does not alter anti-cancer activity of extracts. Currently sub-fractionating extracts
6) Test the synergy by Bliss score of six commercially available	12.31.20	12.18.20	None	Tested for artesunate synergy with dasatinib (targets SRC family), KB

compounds that impact the NRF/SRC pathway in combination with artesunate in two AML cell model systems.				SRC4 (targets cSRC), NRF2 inhibitor ML385, NRF2 activator dimethyl fumarate (DMF), and methazolamide (an inhibitor of cytochrome C release).
7) Verify the mechanism of synergy by siRNA knockout for the most synergistic combinations with artesunate.	12.31.20		Cannot use lipid based transfection reagents	Establishing viral mediated CRISPR/Cas9 system to mediate gene knockdown
8) Determine the effect of artesunate binding on cytochrome C activity	12.31.20			Determined appropriate time point 12-24hrs to assess artesunate effects on cytochrome c and mitochondrial stability.
9) Recruit and train the graduate student outlined in this proposal	12.31.20	7.20.20	None	Heather True, PharmD, second year graduate hired. Specific trainings completed: general cell culture techniques 7.27.20; single agent dose response assays 8.10.20; synergy experiments 9.14.20
10) Conduct monthly meetings with Leadership team to discuss progress and problem-solve any potential barriers	12.31.20	Standing monthly meeting established	None	Meetings held on 7.8.20, 8.12.20, 9.9.20, 10.14.20, 11.11.20, 12.13.20
11) Submit report outlining progress and any preliminary findings through 12.31.20	1.15.21	1.15.21	None	Submitted to Janet Luttrell on 1.15.21
12) Open Clinical Trial to accrual	6.30.21		FDA and IRB approval of protocol	Purposing alternative study design for IRB approval.
13) Enroll three subjects to the clinical trial	6.30.21		Trial currently not open	
14) Evaluate single agent anticancer activity of *Artemisia annua* extracts from Apollon and CNAP8001	6.30.21		Over 25 injections required for HPLC steps to have enough extract to test	Whole extracts have been assessed to determine the best extraction solvent. Optimized HPLC based fractionation method. Currently performing final fractionation so we can move forward with the most active fraction
15) Test synergy by Bliss score of six commercially available compounds that impact the NRF/SRC pathway in combination with *Artemisia annua* in two AML cell model systems	6.30.21			Waiting for final fractionation of extracts to determine which fraction is most active
16) Evaluate the effect of artesunate and synergistic compounds in animal models of AML	6.30.21			Developed luciferase expressing AML cell line models to more accurately quantify tumor burden in animal models

17) Asses the RNA expression profile in AML cells after *Artemisia annua* exposure	6.30.21		Waiting on final fractionation	Waiting on determination of most active fraction of Aa extracts.
18) Conduct monthly meetings with leadership team to discuss progress and problem-solve any potential barriers	6.30.21	Following schedule previously established	None	Meetings held on 1.13.21, 2.10.21, 3.10.21, 4.14.21, 5.12.21, 6.9.21, and 7.14.21
19) Submit report outlining progress and any preliminary findings through 6.30.21	7.15.21	7.15.21	None	Submitted 7.15.21

Objective 1: Conduct a proof of concept clinical trial in children with AML.

A draft of the clinical trial protocol and the consent and assent forms were developed and Dr. Badgett received approval from the Pediatric CCART to move the trial forward to the PRMC on 8/31/2020. Dr. Badgett's staff is preparing all the required documents for submission to the University of Kentucky (UK) IRB. The CHFS IRB administrator Bob Blackburn has given notification that the CHFS IRB will accept UK IRB approval; the UK IRB will submit an IRB Authorization Agreement to him.

Additionally, we have contracted with a GMP manufacturer, Ipca Laboratories Limited, to produce the artesunate tablets for the study. The Markey Cancer Center Clinical Research Office has submitted the study protocol and all requested documents to the IND. The initial IND (#15311) was filed on Sept 23, 2020 and we are working with the FDA to revise the protocol to address their comments. After multiple discussions with the FDA regarding the source of artesunate for the proposed proof of concept clinical trial purposed for this project we were unable to get IND approval. Therefore we are purposing an alternative clinical study that does not involve the direct treatment of patients. We have currently drafted an IRB proposal to isolate primary AML cells from the peripheral blood or bone marrow of newly diagnosed patients for use in establishing a panel of patient derived xenograft (PDX) models of AML at the University of Kentucky. These models will be used to assess artesunate alone and in combination with the synergist drug combinations identified in this study. Ultimately the drug screens performed on the established PDX models will support future clinical trials focused on identifying new drugs and combinations of drugs that can be used to treat patients with pediatric AML.

Objective 2: Evaluate the preclinical synergy of artesunate and NRF2 and SRC inhibitors.

a. Assess synergistic combinations with artesunate in cell lines.

The initial study proposal called for the use of MV4-11 (KRAS wild-type) and M07E (KRAS mutant) pediatric AML cell lines; however, the M07E cell line was only available for purchase from Germany and due to shipping delays due to COVID-19 would not be available in time to complete the studies outlined in this project. Therefore, we substituted a different KRAS mutant pediatric AML cell line which was available from ATCC, specifically the THP-1 cell line, which is reported in the literature to have a similar IC50 for artesunate as the M07E cell line.

In order to assess the synergy of artesunate with drugs targeting the NRF/SRC pathway we first needed to determine the sensitivity of MV4-11 and THP-1 cells to these compounds as single agents. The drugs tested include Src inhibitors dasatinib (targets SRC family) and KB SRC4 (targets cSRC), NRF2 inhibitor ML385, NRF2 activator dimethyl fumarate (DMF), and an inhibitor of cytochrome C release (methazolamide). At least three independent experiments were performed for each compound in each cell line and cell viability was measured after 96hr treatment with increasing concentrations of each drug. The IC50 and 95% confidence interval of the IC50 for each compound is listed below in **Table 1.**

Table 1: IC50 and 95% confidence intervals calculated for artesunate and SRC/NRF pathway targeting compounds in pediatric AML cell lines.

	MV4-11		THP-1	
	IC50	**95% CI**	**IC50**	**95% CI**
artesunate	252.9nM	228.5-280.0nM	366.4nM	312.6-429.6nM
ML385	1.764µM	1.223-2.545µM	0.7877µM	0.6686-0.9281µM
DMF	33.24µM	21.26-51.97µM	39.51µM	13.92-112.2µM
dasatinib	1.396µM	1.073-1.806µM	31.32µM	19.94-49.19µM
KB SRC4	20.16µM	17.55-23.16µM	9.997µM	6.438-15.53µM
methazolamide	no effect		no effect	

Having calculated IC50 values for each compound we then used them to establish the concentration range for each drug which would be used to test for synergy with artesunate. To assess if drug combinations are synergistic, additive, or antagonistic we perform cell viability assays similar to what is used for single drug experiments except that we use a 6x6 matrix design. For these experiments each drug is serially diluted so five concentrations or each drug plus a 0.1% DMSO control are assessed alone or in combination with each dilution of the second drug. Cell viability was assessed following a 96 hour treatment using CellTiter-Glo 2.0. Each well was normalized to untreated control cells which were grown in media with 0.2% DMSO and the percentage of viable cells was determined. R statistical software, specifically the snergyfinder package (version 1.10.4), was used to generate a synergy score using the Bliss independence model. **Table 2** shows the mean BLISS score obtained from at least three independent experiments for each drug in combination with artesunate. Synergy is defined as a BLISS score >1 while antagonism is defined as a BLISS score <-1, scores between -1 and 1 are considered antagonistic. While antagonistic drug interactions are not desired for patient treatment they can be useful tools for identifying a drugs mechanism of action.

Table 2: Average BLISS score to assess synergy between artesunate and the indicated drugs in pediatric AML cell lines. Synergy: score >1, additive: score = 0, antagonistic score <-1

		Average BLISS score	
Drug name	target	MV4-11	THP-1
ML385	NRF2 inhibitor	6.146	0.853
DMF	NRF2 activator	2.714	0.257
dasatinib	Src family inhibitor	-7.042	-3.882
KB SRC4	cSrc specific inhibitor	0.604	-0.815
methazolamide	inhibits Cyto C release	-6.548	-2.506

Based on the BLISS score artesunate was synergistic with ML385 (NRF2 inhibitor) and DMF (NRF2 activator) in MV4-11 cells while these same drug combinations appear additive in THP-1 cells. The next step is to confirm these synergistic or additive drug combinations by using siRNA knockdown of NRF2 (to mimic ML385) or KEAP1 (to mimic DMF). Cells, such as MV4-11 and THP-1, which grown in suspension culture can be difficult to transfect with siRNA and this fact has delayed our progress towards this benchmark. Currently we are testing multiple lipid based transfection reagents identified during a literature review to be effective in transfecting adult AML cell lines using

Dharmacon siGENOME SMARTpool siRNA targeting human *KEAP1*, which we have used previously to successfully knockdown KEAP1 in non-small cell lung cancer cell lines. An initial test using DharmaFECT 1 reagent with increasing amounts of *siKEAP1* did not result in reduced KEAP1 expression. Next we will test DharmaFECT 2, DharmaFECT 4, and Qiagen HiPerFect reagents which have been reported to be more effective for transfecting various AML cell lines in the literature. After testing four different lipid based transfection reagents in MV4-11 and THP-1 cells without success we are working with collaborators to establish a viral mediated CRISPR/Cas9 knockdown system in the lab to generate stable knockdown cell lines for sue in assessing the mechanism of synergy between artesunate and NRF2 pathway targeting drugs. Additionally, knocking down Src family members which are inhibited by dasatinib but not by KB SRC4 could provide additional information on artesunate's mechanism of action and reveal additional synergistic drug targets.

Major conclusions

- MV4-11 (KRAS wild-type) cells are slightly more sensitive to artesunate than THP-1 (KRAS mutant) cells
- MV4-11 cells are more sensitive than THP-1 cells to a general Src family inhibitor (dasatinib); however, THP-1 cells are more sensitive to a cSRC specific inhibitor (KB SRC4)
- Methazolamide has no effect, on cell viability, as a single agent in either MV4-11 or THP-1 cells at the concentrations tested
- Determined the concentrations range for each drug needed to set up experiments measuring synergy with artesunate in MV4-11 and THP-1 cell lines
- Artesunate was antagonistic with dasatinib and methazolamide in both MV4-11 and THP-1 cells
- Artesunate was synergistic with both the NRF2 inhibitor (ML385) and NRF2 activator (DMF) in MV4-11 cells, while these combinations were only additive in THP-1 cells
- BLISS scores in MV4-11 cells indicate stronger synergistic and antagonistic effects with artesunate than the same drug combinations in THP-1 cells
- Lipid mediated siRNA transfection of MV4-11 and THP-1 cells failed, therefore we must utilize a CRISPR/Cas9 system for knockdown studies in these cell lines.

b. Assess the most synergistic artesunate combination in patient derived xenografts (PDX).

Before initiating animal studies to test effectiveness of artesunate alone or in combination with ML385 or DMF, which were identified in objective 2a as being synergistic, we need to establish appropriate animal models of pediatric AML. Both MV4-11 and THP-1 pediatric AML cell lines have been shown previously to grow as subcutaneous xenograft tumors in immune-compromised mice. In order to more consistently quantify tumor burden of xenograft tumors the MV4-11 and THP-1 cells were stably transfected with the gene encoding firefly luciferase and stable luciferase expressing cell lines were established. These cells have the ability to release light when exposed to D-luciferin as a result of the luciferase and ATP-catalyzed oxidation of D-luciferin into oxyluciferin. The light emission from these cells both *in vitro* and *in vivo* is able to be captured and quantified. In order to confirm that MV4-11 and THP-1 cells were stably transformed to express firefly luciferase we plated cells onto a 96-well white walled tissue culture plate and treated them with D-luciferin. Light emission from these cells was detected on a ThermoScientific Varioskan LUX plate reader and the results are reported in relative luminescent units (RLU). Two different concentrations of luciferase encoding virus (MOI-10 and MOI-15) were used to generate these cell lines both of which resulted in increased light emission compared to the non-transfected parental control cells, **Figure 1**.

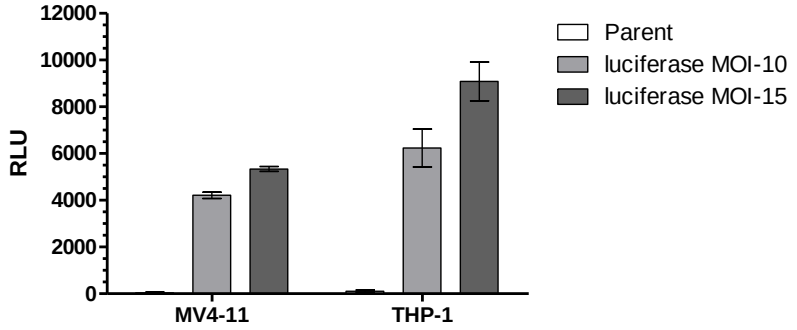

Figure 1: Relative luminescence signal from MV4-11 and THP-1 cells stably expressing firefly luciferase. Parental control and cells transformed with two different concentrations of lentivirus encoding firefly luciferase were treated with 15µg/mL D-luciferin, incubated for 10min and then the relative luminescence signal was quantified using a ThermoScientific Varioskan LUX plate reader. Graph shows mean signal in relative luminescence units (RLU) +/- SD.

c. Interrogate the dynamics and activity of cytochrome C upon artesunate binding.

Cytochrome C is a key protein in the electron transport chain and is a key link between the disruption of mitochondrial function and the induction of apoptosis. The first step in assessing in artesunate effects the activity of cytochrome C is to determine if treatment with artesunate disrupts mitochondrial function be effecting the mitochondrial membrane potential. We utilized a dye called MitoTracker orange which is concentrated in intact mitochondrial, but not in mitochondrial where the membrane potential is disrupted. MV4-11 and THP-1 cells were treated with 0.1% DMSO (control), 10µM artesunate, or 40µM Carbonyl cyanide 3-chlorophenylhydrazone (CCCP). CCCP is a compound known to disrupt mitochondrial membranes and therefore was used as a positive control for this assay. In both MV4-11 and THP-1 cells treatment with 10µM artesunate or 40µM CCCP significantly (P<0.001, Two-way ANOVA) decreased the intensity of mitochondrial staining (**Figure 2**) indicating that artesunate treatment disrupts the mitochondria.

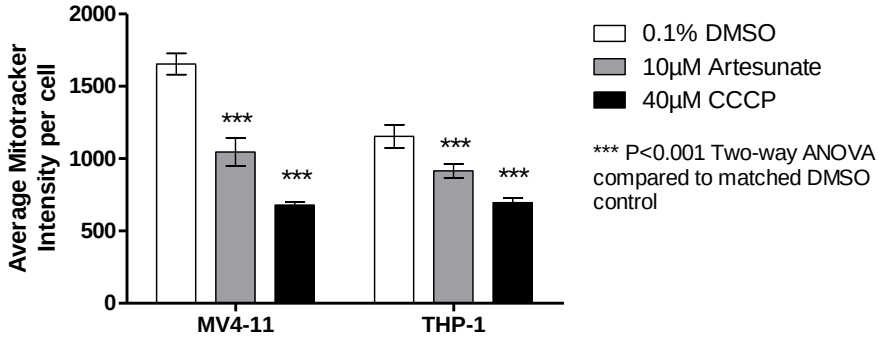

Figure 2: Treatment with artesunate decrease mitochondrial staining in MV4-11 and THP-1 cells. MV4-11 and THP-1 cells were treated with 0.1% DMSO (control), 10µM artesunate, or 40µM CCCP (positive) for 24hrs prior to staining with 25nM MitoTracker and 5µg/mL Hoechst for 45min at 37ºC. Cells were fixed and imaged using the CX7 high content imaging platform. Positive Hoechst staining was used to define each cells and the average intensity of MitoTracker orange stain per cell was reported. Graph shows average MitoTracker orange stain per cell +/- SD.

Disruption of mitochondrial membrane potential has been linked to the activation of apoptosis so we next tested if artesunate treatment induced activation of caspase 3/7 in a dose dependent manor. MV4-11 cells were treated with 0.1% DMSO, 1µM artesunate, 10µM artesunate, or 40µM CCCP for 24 hours prior to staining with MitoTracker orange or activated caspase 3/7 using CellEvent™ Caspase-3/7 Green Detection Reagent (Invitrogen). Artesunate treatment resulted in a dose dependent significant decreased in MitoTracker orange staining and an increase in caspase 3/7 staining after 24hr, **Figure 3**. To further elucidate the timing of artesunate induced apoptosis, as assessed by caspase 3/7 activation, and mitochondrial membrane disruption, as measured by MitoTracker orange staining, the next step is to assess both events after 6, 12, 18, and 24hr treatments. A preliminary study using only MV4-11 cells shows a significant increase in caspase 3/7 activation after 12hrs, while the decreased in mitochondrial staining does not occur until 24hrs when compared to a time matched 0.1% DMSO treated control. Having shown that artesunate induces a dose dependent decreased in mitochondrial staining and increase in caspase 3/7 activation, the next step we are working on is to determine the timing of these events.

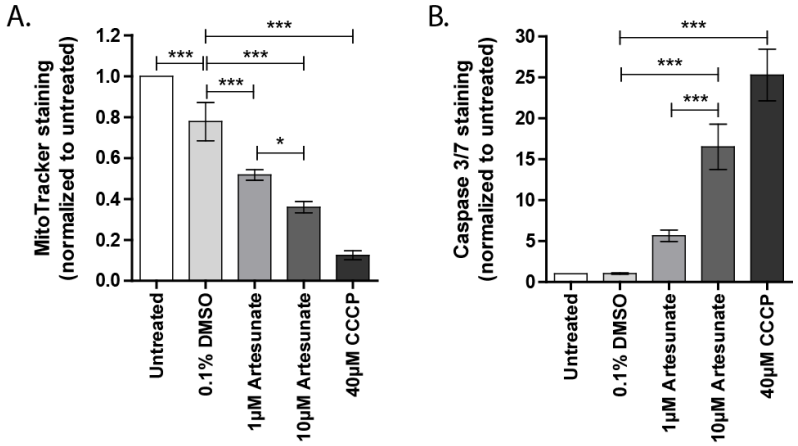

Figure 3: Artesunate induced changes in mitochondrial membrane potential and caspase 3/7 activation in MV4-11 cells. Cells were treated with 0.1% DMSO (control), 1µM artesunate, 10µM artesunate, or 40µM CCCP (positive) for 24hrs prior to staining with 25nM MitoTracker (**A**) or with 5µM Caspase 3/7 reagent (**B**). The average intensity of MitoTracker orange stain per cell was determined and results were normalized to untreated control cells. Graph shows average fold change in signal intensity per cell +/- SD. Statistical analysis using a One-way ANOVA compared each treatment to 0.1% DMSO control. (* P<0.05, ***P<0.001)

In addition to looking at the effect of artesunate on the state of the mitochondria in general, we also started to test if artesunate treatment inhibits or enhances the activity of cytochrome C oxidase. Cytochrome C oxidase accepts an electron from cytochrome C in the electron transport chain and thus functions to regulate the oxidative state of cytochrome C. There have been some delays in getting the cytochrome C oxidase activity assay up and running in our laboratory related to determining the number of cells needed to extract sufficient quantities of intact mitochondria to detect cytochrome C oxidase activity. Currently we have optimized this assay using Non-small cell lung cancer cells lines and have performed a pilot experiment in both MV4-11 and THP-1 cells. Analysis of the raw data from this pilot study indicates that treatment with 10µM artesunate alters cytochrome C oxidase activity compared to cells treated with 0.1% DMSO (control); however, the extent of this change cannot be determined until the results are normalized to the amount of mitochondria present under each condition. We are currently working to determine the amount of mitochondria isolated from each treatment and will be performing additional replicate experiments in the future.

Major conclusions

- Artesunate disrupts the mitochondrial membrane potential and thus mitochondrial function in MV4-11 and THP-1 cells
- Treatment with artesunate induces apoptosis as assessed by staining for activated caspase 3/7
- Effects of artesunate on mitochondrial membrane potential and caspase 3/7 activation are dose dependent

Objective 3: Fractionate and assess the anti-cancer activity of the constituents of Aa extracts.

Leaves from two strains for *Artemisia annua* (CNAP 8001 and Apollon) were ground down and passed through a 1mm screen prior to extraction with methanol, hexanes, or supercritical fluid extraction (SFE). The supercritical fluid used for SFE in this study was carbon dioxide. Two separate extractions were performed the first used 10g (methanol and hexane) except 20g CNAP8001 for hexanes extract or 12g (SFE) of starting material and was extracted for 2 hours while the second used 20g of starting material and was carried out overnight. A third extraction was performed using methanol or hexane were 20g of starting material was extracted in 200mL solvent solution for 2 hours. Following solvent extraction the samples were filtered to remove any remaining leaf powder. The extracts from all three solvents were then evaporated to dryness using a Genevac EZ-2 Personal Evaporator and then reconstituted by DMSO and diluted prior to quantification by HPLC/MS/MS. Of note for the third extractions samples extracted in hexane were reconstituted in 3mL DMSO, while samples extracted in methanol were reconstituted in 2mL DMSO.

The artemisinin, artemisinic acid, and dihydroartemisinic acid analysis was performed with Waters ACQUITY UPLC H-Class System equipped with Xevo TQD Triple Quadrupole Mass Spectrometry. Waters ACQUITY UPLC BEH C18 2.1 x 50 mm column with 1.7 µm particles were used. Labeled artemisinin-d_3 was used as an internal standard. The artemisinin, artemisinic acid, and dihydroartemisinic acid separation was achieved using a gradient mobile phase consisting of 0.1% acetic acid in water (mobile phase A) and acetonitrile (mobile phase B). The flow was 0.2 ml min^{-1}. Waters Xevo TQD was operated in the electrospray ionization (ESI) in the positive mode with Multiple Reaction Monitoring (MRM). The source and desolvation temperatures were set to 150 and 500 °C, respectively. The desolvation gas flow was set to 800 L Hr^{-1}. The quantities of artemisinin, artemisinic acid (AA), and dihydroartemisinic acid (DHAA) from each *Aa* strain using each solvent and extraction method is shown in **Table 3.**

In order to compare the cytotoxicity of the different extractions on pediatric AML cell lines the extractions were diluted to generate stock solutions that were normalized so the concentration of artemisinin was equal, 100mM artemisinin in each stock solutions. Therefore any differences in the sensitivity of the cells to these different extraction preparations is hypothesized to be due to the levels of AA, DHAA, or some other unquantified component present in the extracts. Dose response curves were generated for the first two extractions shown in Table 3 and cell viability was assessed after 48 and 96 hours. For all extraction solvents and preparations the IC50 values calculated after 96 hours (**Table 4**) were less than the IC50 measured after 48hrs; however, these differences were not significant. For both the 2hr extraction and the overnight extraction cells had the lowest IC50 when methanol was used as the solvent; therefore, future studies will focus on fractionation of methanol extractions of both strains (Apollon and CNAP8001).

Table 3: Quantification of artemisinin, artemisinic acid (AA), and dihydroartemisinic acid (DHAA) in *Artemisia annua* extracts.

10g starting material 2hrs (leaves collected 2019)

		Artemisinin (mg/g)	AA (mg/g)	DHAA (mg/g)
Hexane	**Apollon**	26.98	2.45	6.76
	CNAP 8001	47.44	2.46	8.30
Methanol	**Apollon**	38.64	3.96	11.07
	CNAP 8001	40.43	2.67	8.51
SFE	**Apollon**	56.74	5.18	14.89
	CNAP 8001	51.60	3.44	10.77

20g starting material overnight (leaves collected 2020)

		Artemisinin (mg/g)	AA (mg/g)	DHAA (mg/g)
Hexane	**Apollon**	56.26	5.25	15.36
	CNAP 8001	57.23	4.61	15.71
Methanol	**Apollon**	47.59	5.10	15.10
	CNAP 8001	49.22	3.13	10.55
SFE	**Apollon**	74.31	7.42	22.21
	CNAP 8001	63.96	4.17	14.58

20g starting material 2hrs (leaves collected 2020)

		Artemisinin (mg/g)	AA (mg/g)	DHAA (mg/g)
Hexane	**Apollon**	20.79	1.56	4.39
	CNAP 8001	21.55	0.87	2.82
Methanol	**Apollon**	49.20	3.51	9.69
	CNAP 8001	53.22	2.05	6.67

Table 4: IC50 and 95% confidence intervals in Mv4-11 cells treated for 96hrs with *Artemisia annua* extractions. IC50 calculations based on concentration of artemisinin in each extraction

		Extraction #1		Extraction #2	
		IC50	**95% CI**	**IC50**	**95% CI**
Hexane	**Apollon**	1.75µM	1.26 – 2.44µM	1.36µM	0.928 – 1.99µM
	CNAP8001	3.31µM	2.52 – 4.33µM	2.98µM	2.17 – 4.07µM
Methanol	**Apollon**	0.985µM	0.752 – 1.29µM	0.852µM	0.671 – 1.08µM
	CNAP8001	1.68µM	1.36 – 2.08µM	1.54µM	1.23 – 1.93µM
SFE	**Apollon**	2.59µM	1.96 – 3.44µM	1.02µM	0.756 – 1.36µM
	CNAP8001	2.41µM	1.93 – 3.01µM	1.87µM	1.41 – 2.49µM

In order to fractionate the Artemisia annua extraction 80 g *Artemisia annua* (Apollon strain) leaf material was ground and extracted with methanol for 2 hours. The sample was filtered to remove the remaining leaf powder and then evaporated to dryness using a Genevac EZ-2 Personal Evaporator. The dried extract was reconstituted in 80ml methanol and used as the stock solution for fractionation by HPLC. Fractionation of the *Artemisia annua* extract was performed with a Waters 2767 sample manager, injector and collector purification system equipped with a Waters 2996 PDA detector. A Xbridge Prep C18 column (19 x 150 mm) with 5 µm particle size was used. The chromatography profile generated during HPLC shows different retention times of artemisinin, artemisinic acid (AA), and dihydroartemisinic acid (DHAA) eluted out from the column, **Figure 4**. Each experimental preparation requires the extract stock to be injected at least 26 times into the purification system to generate a large enough amount of each time point collected for downstream cellular assays. The fractions were collected based on the different elution times. The final collections were evaporated to dryness and reconstituted with 300µl DMSO. In the first experiment, the whole eluents (everything eluted out of the column during the gradient and washing time (0-42 min)) were collected. The concentration of artemisinin, AA and DHAA in the fraction were shown in **Table 5**. In the second experiment, two fractions was collected (0 -28 min and 28-42 min). The concentrations of artemisinin, AA and DHAA in each fraction were shown in **Table 6**. Following each experiment the extracted fractions were serially diluted into media and tested for activity against pediatric AML cell lines, **Figure 5**. We are currently working to collect fractions with narrower time intervals, specifically, from 0-10min, 10-15min, and 15-28min which were selected based on the outflow times shown in Figure 4.

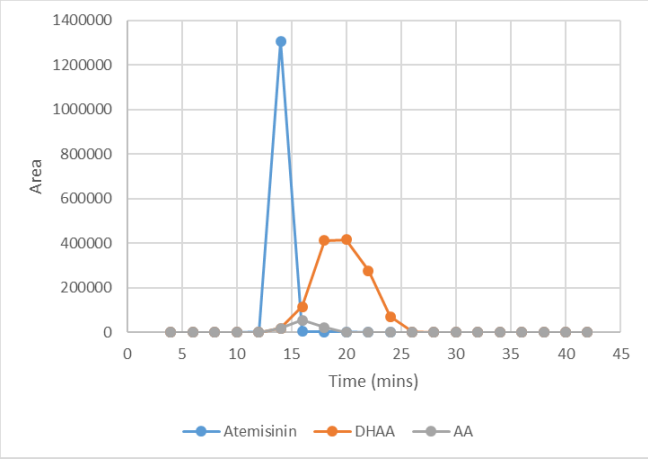

Figure 4: The chromatography profile of artemisinin, artemisinic acid (AA), and dihydroartemisinic acid (DHAA) detected at the outflow of the column during the elution of Aa extract.

Table 5: Concentrations of artemisinin, artemisinic acid (AA), and dihydroartemisinic acid (DHAA) in Artemisia annua extract fraction

Artemisinin	DHAA	AA
ug/g	ug/g	ug/g
6020	2600	1100

Table 6: Concentrations of artemisinin, artemisinic acid (AA), and dihydroartemisinic acid (DHAA) in Artemisia annua extract fraction

	Artemisinin	DHAA	AA
	ug/g	ug/g	ug/g
First fraction (0-28min)	10865	2490	845
second fraction (28-42min)	23.62	5.03	0.52

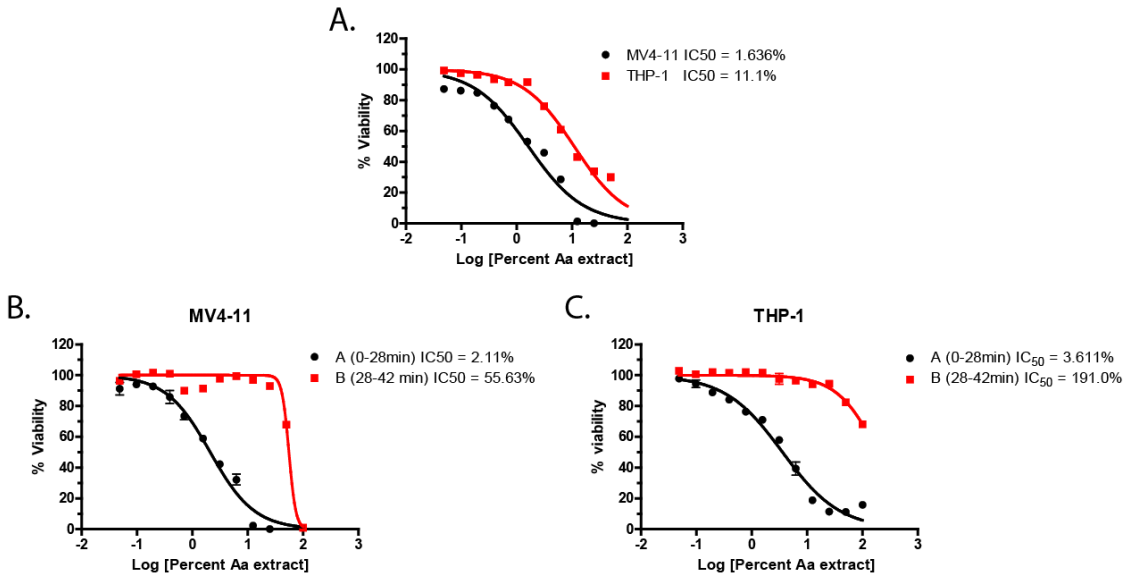

Figure 5: Dose response curves demonstrating anti-cancer activity of Artemisia annua extracts following processing through HPLC. Following HPLC extracts were dried down and reconstituted in DMSO prior to serial dilution in cell growth media. Concentrations are expressed as a percentage of the reconstituted stock. **A.** MV4-11 and THP-1 cells were treated with the whole eluent (0-42min) which included the column wash. **B** (MV4-11) and **C** (THP-1) the whole eluent was separated into two fractions A (0-28min) which was the primary eluent and B (28-42min) which was the column wash.

Major conclusions

- Artemisinin, artemisinic acid and dihydroartemisinic acid were extracted from both *Artemisia annua* stains using all three solvent methods
- Both strains of *Artemisia annua* contain similar levels of artemisinin, artemisinic acid, and dihydroartemisinic acid
- Overnight extractions of fresh plant material resulted in the highest yields of artemisinin, artemisinic acid and dihydroartemisinic acid; however, these extractions likely also contain the highest levels of contaminants
- Supercritical fluid extraction (SFE) resulted in the highest yields of artemisinin, artemisinic acid and dihydroartemisinic acid.
- MV4-11 and THP-1 cells are more sensitive to *Artemisia annua* extractions generated with methanol as the solvent

- Processing of *Artemisia annua* methanol extracts through HPLC does not inhibit their anticancer activity in pediatric AML cell lines
- The most active components of the *Artemisia annua* methanol extracts progress through the HPLC prep column within the first 28min

Objective 4: Assess the ability of artesunate to modulate downstream biomarkers in children who have completed AML therapy.

This objective relies on activation and recruitment to the clinical trial and is anticipated to begin in 2021.

Deliverables (check appropriate time period when each deliverable is completed)	**Month 1-3**	**Month 4-6**	**Month 7-9**	**Month 10-12**	**Month 13-15**	**Month 16-18**	**Month 19-21**	**Month 22-24**	√
Notify DPH when IRB approval is received or if not required									
#1 Amend the animal protocol and biosafety protocol to encompass experiments outlined in the proposal	√								7.13.2020 7.22.2020
#2 Draft the clinical protocol to conduct the clinical study outlined in the proposal	√								8.21.20
#3 Obtain regulatory approval for the clinical protocol and open to activation									
#4 Establish procedures for GMP manufacturing of clinical artesunate	√								6.10.20
#5 Fractionate and quantitate Artemisia annua extracts from Apollon and CNAP8001		√							11.25.20
#6 Test the synergy by Bliss score of six commercially available compounds that impact the NRF/SRC pathway in combination with artesunate in two AML cell model systems		√							12.8.20
#7 Verify the mechanism of synergy by siRNA knockout for the									

most synergistic combination with artesunate									
#8 Determine the effect of artesunate binding on cytochrome C activity									
#9 Recruit and train the graduate student outlined in this proposal	√								7.20.20
#10 Conduct monthly meetings with Leadership team to discuss progress and problem-solve any potential barriers	√	√							7.8.20 8.12.20 9.9.20 10.14.20 11.11.20 12.9.20
#11 Submit a report outlining progress and any preliminary finding through December 31, 2020		√							1.15.21
#12 Open the clinical trial to accrual									
#13 Enroll three subjects to the clinical trial									
#14 Evaluate the single agent anticancer activity of Artemisia annua extracts from Apollon and CNAP8001									
#15 Test the synergy by Bliss score of six commercially available compounds that impact the NRF/SRC pathway in combination with Artemisia annua in two AML cell model systems									
#16 Evaluate the effect of artesunate and synergistic compounds in animal models of AML									
#17 Assess the RNA expression profile in AML cells after Artemisia annua exposure									
#18 Conduct monthly meetings with Leadership team to discuss			√	√					1.13.21 2.10.21

progress and problem-solve any potential barriers									3.10.21 4.14.21 5.12.21 6.9.21 7.14.21
#19 Submit a report outlining progress and any preliminary findings through June 30, 2021				√					7.15.21
#20 Enroll three subjects to the clinical trial									
#21 Prepare first manuscript, assessing synergy and mechanism of artesunate in AML									
#22 Submit first manuscript									
#23 Submit first patent for Artemisia annua extracts to UK Office of Technology									
#24 Evaluate the effect of Artemisia annua and synergistic compounds in animal models of AML									
#25 Conduct monthly meetings with Leadership team to discuss progress and problem-solve any potential barriers									
#26 Submit a report outlining progress and any preliminary findings through December 31, 2021									
#27 Enroll four subjects to the clinical trial									
#28 Prepare second manuscript, assessing synergy and mechanism of Artemisia annua in AML									
#29 Submit second manuscript									
#30 Evaluate biomarkers in specimens from patients enrolled on the clinical trial									

#31 Prepare third manuscript, describing the clinical trial ***#32 Submit third manuscript*** ***#33 Prepare grant submissions to continue Artesunate research*** ***#34 Conduct monthly meetings with Leadership team to discuss progress and problem –solve any potential barriers.*** ***#35 Submit a report outlining progress and any preliminary findings through June 30, 2022***									

Quarterly Reports are due:

- October 15, 2020
- January 15, 2021
- April 15, 2021
- July 15, 2021
- October 15, 2021
- January 15, 2022
- April 15, 2022
- July 15, 2022

Reports should be returned to:
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