Research Article

Activity of anti-cancer protein kinase inhibitors against growth of different stages of *Leishmania spp.*

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Abstract: Current situation of Leishmaniasis calls for search of more effective drug. Multiplication of *Leishmania* resembles the development of mammalian tumor cells and both are regulated by the same mechanisms and having similar CDKs as cell cycle regulators. Our approach here is to test known CDK inhibitors on the growth of different stages of *Leishmania spp.* Field isolates of *L. donovani* was used in this study to check for the virulence effect (if any) on the possible inhibitory effect. We have tested the effect of Olomoucine on the growth of *L. donovani* promastigotes and Flavopiridol on the growth of *L. mexicana* axenic-amastigotes. Our data showed that *Leishmania* promastigotes (*L. donovani*) and amastigotes (*L. mexicana*) susceptibility to CDK chemical inhibitors is parasite intrinsic, stage specific, and macrophage independent. The *Leishmania* parasite has low sensitivity to chemical inhibitors, when tested *in vitro* culture. The amastigotes is more sensitive to Flavopiridol than the promastigotes. These data will be useful as baseline for macrophage infectivity and other preclinical studies. Our findings suggest the possibility of repurposing the anti-cancer drugs as anti-leishmanial and consequently reducing the cost of developing new drug.
Keywords: Flavopiridol – Olomoucine – Leishmania – drug repurposing - Sudan

INTRODUCTION

Visceral leishmaniasis (VL) is a systemic disease with highest prevalence in South Asia, East Africa, and Brazil. VL is caused by protozoan parasites of the *Leishmania donovani* complex, transmitted to humans when an infected sandfly takes a bloodmeal. Within the human host, the parasites replicate within cells, particularly of bone marrow and spleen. Without effective treatment, symptomatic VL is usually fatal. The great majority of the estimated 200,000 to 400,000 annual new cases of (VL) occurs in six countries, with Sudan having the highest in Africa (15,700 to 30,300/year) [5]. *Leishmania mexicana* can cause a wide spectrum of clinical diseases, ranging from a localized cutaneous ulcer at the infection site, which is characteristic for localized cutaneous leishmaniasis (LCL), to a disseminating disease, where intensely parasitized macrophages form nodules that spread throughout the skin and ultimately invade the oropharyngeal and nasal mucosae, which is characteristic for diffuse cutaneous leishmaniasis (DCL).

The only effective drug for treatment of leishmaniasis is the antimonial drugs, which have severe toxic side effects. Drugs such as sodium stibogluconate (Pentostam) and meglumine (Glucantime) are often used as second-line options; aminosidine has been used for cutaneous leishmaniasis. In spite of their reported toxicity, difficulty in administration, and high cost, pentavalent antimonials remain the drugs of choice in the treatment of all forms of the disease in the Sudan [17]. Unlike South American mucocutaneous leishmaniasis, mucosal leishmaniasis in Sudan is not accompanied by a cutaneous lesion and patients respond well to treatment with pentavalent antimony compounds [9, 10], except for two cases recently identified [22].

Cyclin-dependent kinases (CDKs) are conserved regulators of the eukaryotic cell cycle with different isoforms controlling specific phases of the cell cycle. Mitogenic or growth inhibitory signals are mediated, respectively, by activation or inhibition of CDKs which phosphorylate proteins associated with the cell cycle. The Central role of CDKs in cell cycle regulation makes them a potential new target for inhibitory molecules with anti-proliferative effects. Indirubin, the active constituent of a Chinese antileukaemia medicine, can inhibit CDK2 [16]. Olomoucine, is adenine derivative and bind in the adenine binding pocket of CDK2, but in an unexpected and different orientation from the adenine of the authentic ligand ATP [25], Roscovitine [8],

Staurosporine [19], Hymenialdisine [21], Paullones [30] are all investigated for CDK/inhibitors co-crystal structures. Those studies assist to uncover the molecular mechanisms of interaction. CDKs have different functions (Cell cycle regulation [CDK 1, 2, 3, 4, 6, 7], apoptosis [CDK 2, 5], transcription [CDK 7, 8, 9] and neuronal functions [CDK5]), and inhibition of CDKs is expected, therefore, to arrest cell proliferation.

Flavopiridol (HMR 1275, L86-8275) [20] is a semi synthetic flavone's derivative of the alkaloid rohitukine, derived from an indigenous plant from India, demonstrated potent and specific *in vitro* inhibition of all CDKs tested (CDKs 1, 2, 4, 6, and 7) by competitively blocking their ATP-binding pocket with clear block in cell cycle progression at the G1/S boundaries [26]. Moreover, the effect of flavones L86-8275 on cancer cells is by inhibition of histone H1 kinase activity of p34cdc2 kinase. Nuclear magnetic resonance (NMR) spectroscopy revealed that Flavopiridol binds to and likely intercalates into duplex DNA [6, 7], which explains the ability of Flavopiridol to kill noncycling cancer cells. Flavopiridol is the first CDK inhibitor to enter clinical trials and currently used in cancer clinical trials. Preclinical studies demonstrated the capacity of flavopiridol to induce apoptosis, promote differentiation, inhibit angiogenic processes and modulate transcriptional events [27]. DMSO mediates stage- regulated protein expression of some protozoa [24].

There are remarkable differences between the field isolate of *Leishmania* and the parasite that grown *in vitro* and sub-cultured several times in regards to the loss or reduced virulence. To this end, we compared the effect of inhibitors on both field isolate and sub-cultured *L. donovani*. As the disease is a consequence of multiplication of amastigotes within the macrophages, and amastigotes represent the clinically relevant stage of the parasite, analysis of drug efficacy must be carried out with this parasite stage. CDK chemical inhibitors work on cdc2-related kinases, and it was shown that CRK3 is expressed in the amastigotes stage. Therefore, we set our goal so as to test the effect of Flavopiridol on the amastigote growth of *L. mexicana* in order to find an inhibitory concentration (IC$_{50}$?) *in vitro* and thus to have a base-line data firstly; for the macrophage infectivity and secondly; for the expected dose for infected mice i.e. *in vivo* in a preclinical trial.

MATERIALS & METHODS:
1. Ethical statements:-
All protocols used in this research were approved by the Ethics Committee of the Faculty of Medicine, University of Khartoum and by the National Health Research Ethics Committee, Federal Ministry of Health, Sudan. All sampling from patients were carried out by the medical staff of Gedariff Hospital.

2. Patients and Parasitological Examination:-
Thirty-three consented confirmed positive visceral leishmaniasis (VL) patients were selected in this study. Parents signed instead of their children. Samples were taken when these patients were reported to Gedariff Hospital. They were parasitological confirmed by detecting amastigotes in the smears taken from bone marrow (adult), lymph nodes (children and adult). Samples were applied on a microscope slides, air-dried, fixed with methanol and Giemsa stained before being examined. Final conclusion was brought up after amastigotes were detected in the smear.

3. Primary Isolation and Culturing of *L donovani* parasite:-
The isolated amastigotes were transferred to NNN medium for culturing in byjo bottle and kept in ice bag. At the lab the parasite transferred to a liquid medium (RPMI - 1640) supplemented with 10% (v/v) heat inactivated bovine serum and incubated at 26 °C. Once the promastigotes growth has built up, cryopreservation process was took place to keep the isolates for further examinations.

4. Testing Olomoucine on growth of *L. donovani* promastigotes cell lines:-
The CDK inhibitor Olomoucine was a gift from Dr. Karen Grant (WCMP, Glasgow, UK). It was prepared as 50 mM stock in DMSO. 1 x 10^6 cells / ml promastigotes cultures were set up in triplicate in presence of 0.0, 250 nM, 2.5 µM, 25 µM, 250 µM, 2.5 mM, 5 mM Olomoucine in 12-well flat-bottom plate (Tissue Culture Treated, iwaki, Japan) and wrapped with Para film to prevent evaporation of the media, and were incubated at 26^0C. Cells were counted at 0, 24, 48, 72 hr time points. The average was calculated for each concentration.

5. Culturing of *L. mexicana*:
L. mexicana strain (MNYC/BZ/62/M379) promastigotes were grown at 25^0C in HOMEM medium supplemented with 10% Fetal calf serum (FCS).

6. Making metacyclic *L. mexicana*:
Mid to late log promastigote culture (5 x 10^6 cells / ml – 1 x 10^7 cells / ml) were subcultured in Schneiders' medium at 5 x 10^5 cells / ml and incubated at 25^0C for 6 days. However, the cell density was checked until stationary phase of metacyclics occurs, usually on days 4 and 5.

7. Making axenic *L. mexicana* amastigotes:
The stationary phase of metacyclics were sub-cultured into schneiders' medium at 1 x 10^6 cells / ml or diluted 1/10 (5 x 10^5; into Schneiders' media) and was incubated at 32^0C for 4 / 5 days. The culture was maintained until the amastigotes-like forms were obtained. Axenic *L. mexicana* amastigotes-like forms were then grown in Schneiders' medium at pH 5.5 and temperatures of 32^0C, and were harvested in the mid-

log phase of growth $5 - 10 \times 10^6$ cells / ml. They were cryopreserved, thawed and revived under appropriate growth conditions.

8. Testing Flavopiridol on growth of *L. mexicana* amastigotes cell lines:

For drug experiments, parasites at mid-log phase (5×10^6 cells / ml) were transferred into microplate 24-well, flat-bottom (Tissue Culture Treated, iwaki, Japan) 1 ml of fresh complete Schneiders' culture medium in presence or absence of Flavopiridol (Sigma). Briefly, 1×10^6 cells / ml cultures were set up in triplicate in presence of 0.0, 12.5, 25, 50, 100, 250, 500, 1000 nM Flavopiridol (NSC 649890) in 24-well plate and wrapped with Para film to prevent evaporation of the media, and were incubated at 32^0C. Cells were counted at 0, 24, 48, 72 hr time points. The average was calculated for each concentration.

9. Data and Statistical analysis:

The dose/response relationship was determined as described previously [2], in which it is assumed that response forms a linear relationship with the dose / log dose. Therefore, to calculate IC_{50}, we have generated a series of dose-response data (e.g., drug concentrations x_1, x_2, ...,x_n and growth inhibition y_1, y_2, ...,y_n). To estimate the IC_{50} we plot x-y and fit the data with a straight line (linear regression). IC_{50} value was then estimated using the fitted line, i.e., $Y = a * X + b$, $IC_{50} = (0.5 - b)/a$. The data were plotted using Microsoft excel Software and all relevant statistical analysis were performed using this software.

RESULTS

At concentration of 0.25μM, Flavopiridol inhibited growth of *L. mexicana* amastigotes cell lines by 50% 24 hrs after addition of Flavopiridol. As shown in figure 1, the growth of the amastigote of the *L. mexicana* was inhibited, as compared to the un-treated parasite, when incubated with Flavopiridol to a final concentration of 25 nM. This inhibition profile was observed after 3 days of growth. During this study, it was found that the IC_{50} for the amastigotes is 10 fold less than that reported for the promastigotes; 0.25 μM. This finding gave the base-line data for the next step; macrophage infectivity.

We have used here propidium iodide staining and fluorescent activated cell sorting (FACS) to investigate the cell cycle position of the treated cells. DAPI staining showed a sign of chromatin condensation (Data not shown). Facs analysis showed the cell cycle arrest of *L. mexicana* amastigotes at the G2/M phase (data not shown).
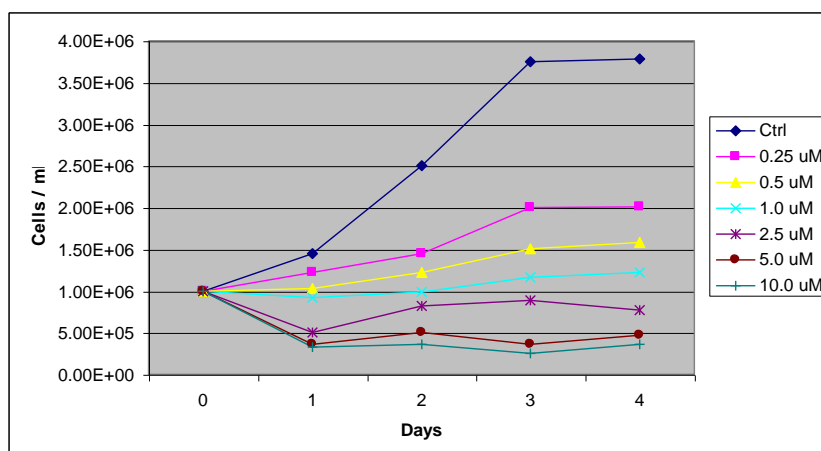

Fig-1: Inhibition of *L. mexicana* amastigotes by Flavopiridol.

Mid-log wild-type amastigotes of *L. mexicana* were incubated with the indicated amount of flavopiridol at 32^0C, and cells were counted every 24hr for 4 days. As shown by the pink line, the growth of the amastigotes was reduced 50% as compared to the un-treated ones (blue line), when the flavopiridol was added to a final concentration of 0.25 μM. Results shown are representative of a minimum of three experiments.
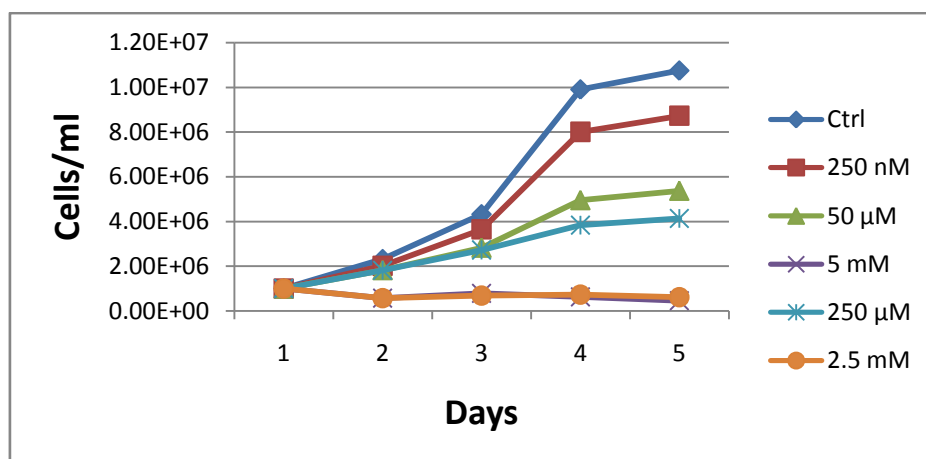
Fig-2: Inhibition of *L. donovani* promastigotes by Olomoucine.

Mid-log promastigotes of *L. donovani* were incubated with the indicated amount of Olomoucine at 26⁰C, and cells were counted every 24hr for 4 days. As shown by the green line, the growth of the promastigotes was reduced 50% as compared to the un-treated ones (blue line), when the Olomoucine was added to a final concentration of 50 µM. Results shown are representative of a minimum of three experiments.
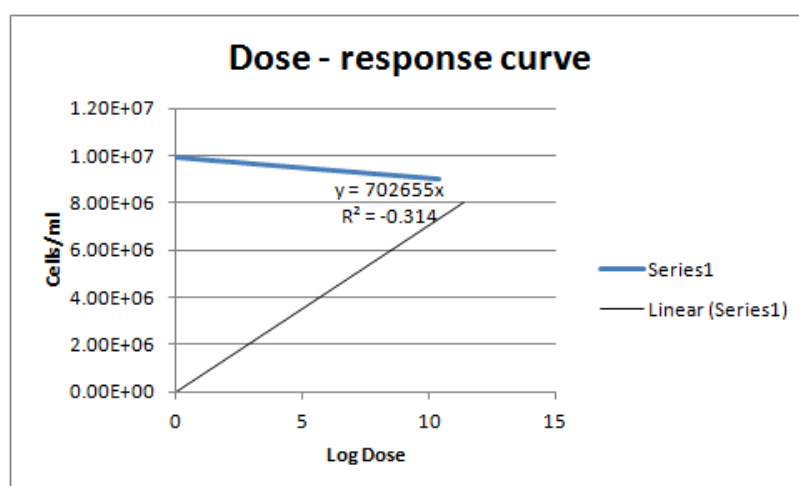

Fig-3: Dose – response curve of effect of Olomoucine on *L. donovani* promastigotes.

All transformation of data was done according to the statistical tables previously provided [12]. From figure 3, the regression line equation is Y = 702655 x, by replacing Y with 50: IC_{50} = X = 50 / 702655 = 0.00007116 = 71.16 X 10-6 = 71 µM. From the tested Olomoucine concentrations, it is very close to the 50 µM in figure 2. The regression coefficient R^2 =-0.314.

The 32 *L. donovani* isolates were confirmed by PCR analysis using species specific primers. Thirty isolates showed band size consistent with the control one, whereas, two showed band of different size (Data not shown). The homogeneity of the populations of *L. donovani* isolates used was tested using Chi square test that equal to 24.50 (> 3.841), significant at P-value = 0.05. Therefore, the null hypothesis will be rejected and that all collected isolates are *L. donovani*.

DISCUSSION

Clinical studies with Flavopiridol have already been started and clinical tumor responses could be observed in most of the phase I and phase II studies on different types of progressive tumors refractory to conventional treatment [26]. Flavopiridol and UCN-01 have showed promising results with evidence of antitumor activity and plasma concentrations sufficient to inhibit Cdk-related functions [28]. However, in spite of the accumulating evidence of its efficiency that revealed by several works, for example, it has been shown that the Flavopiridol can interfere with glycogen degradation, which might be responsible for Flavopiridol's cytotoxicity and explain its resistance in some cell lines [18], using human colon carcinoma cells in an *in vitro* model, it has been shown that no cross-resistance to either other anticancer agents or other chemical CDK inhibitors such as Roscovitine,

Purvalanol A and 9-nitropaullone [29]. Flavopiridol inhibits cycling cells as well as resting ones, which suggests other mechanisms of action [26]. It was also found to be cytotoxic to noncycling cells [6]. Our previous studies confirmed the expression of protein kinase CRK3 in the promastigotes of *L. donovani* [1, 3] and also its activity at the promastigotes of *L. mexicana* [13, 4]. All those studied carried out with purified protein and transgenic *Leishmania*. Here, we focusing on testing the chemical inhibitors on pure parasites, bearing in mind that a possible inhibition will be due to inhibition of the CRK3 protein kinase.

A previous study has reported the effect of the protein kinase inhibitor, Flavopiridol on the promastigotes growth of the *L. mexicana mexicana* wild type (WT) as well as transgenic expressing-CRK3his [15]. That inhibition was due to the inhibition of the CRK3 Histone HI activity. The IC_{50} was reported as 2.5 µM & 1.0 µM for the promastigotes and the purified enzyme, analysis of drug efficacy must be carried out with this parasite stage. Those results were consistent with the idea that sequence divergences between parasite CRKs and human Cdks are likely to result in different affinities for inhibitory molecules.

L. mexicana, axenic cultures of amastigotes has been developed, promastigotes were subjected to 32^0C plus 5% CO_2 for 24 hr, and pH was shifted to 5.5. The round shaped organisms were counted and comprised nearly 90% of the total population. CRK3 has been shown to be expressed in the amastigote stage. Therefore, we have tested the effect of Flavopiridol on the growth of the axenically-grown amastigotes of *L. Mexicana.* Although wild-type *L. mexicana* amastigotes were used in this study, wild-type *L. mexicana* promastigotes have shown a similar inhibition as the promastigote of the transgenic *L. mexicana* expressing *CRK3* (WT). Therefore, one can speculate similar effect on amastigote-like forms expressing *CRK3* gene. Moreover, it has been reported [15] that exposure of *L. mexicana* promastigote (Wild-type) and CRK3his-expressing cell lines to Flavopiridol resulted in G2/M arrest, which was associated with the loss of CRK3 activity as it judged by inability to phosphorylate histone H1. DNA analysis using FACS have revealed that those promastigotes have underwent cell cycle arrest as 95% of cells were accumulated in G2 or G2/M. It was suggested that CRK3 has an essential role in the control of the cell cycle progression at the G2/M phase transition in *L. mexicana* promastigotes [15].

On the other hand, the observation of the inhibitory effect of Flavopiridol on *Leishmania* growth has been recently supported by the finding that natural plants extracts among them, is the flavonoid-the original source of the synthetic Flavopiridol, has similar effect on *Leishmania* spp growth [23]. Several studies have investigated the effect of some chemicals on the parasite *Leishmania*, for instance, there is an evidence

of difference in susceptibility of *L. donovani* to sodium stibogluconate and meglumine antimoniate [11]. Amastigotes were 73-271 times more susceptible than were promastigotes.

Along with CRK3 inhibition investigation, Flavopiridol effect on the morphology of the *L. mexicana* promastigotes were investigated by DAPI staining of these cell lines [15]. In another study, the effect of purine analogue Olomoucine on *L. mexicana* promastigotes (tagged CRK3) was found to be with an IC_{50} of 42µM, which was 5 – 6 folds higher than human p34cdc2 / cyclin B, which indicates the less sensitivity of leishmanial kinase to the CDK inhibitor [14]. IC_{50} of 71µM obtained in this study for *L. donovani* promastigotes is higher than that reported for *L. mexicana* promastigotes which suggests that *L. donovani* is less sensitive for Olomoucine. Another possible reason is that *L. donovani* used in this study are field isolates which expect to be more virulent than the *L. mexicana* that are sub-passaged several times and some parasitic factors may interfere with the response to the chemical drug, which resulted in some tolerance. However, growth of *Leishmania* in the presence of inhibitor was resulted in cellular changes due to the loss of function of the kinase as shown by the result of the Facs analysis (data not shown) and DAPI staining where small round spots instead of elongated ones (controls) were observed (data not shown), which may be as a result of DNA fragmentation brought up by the Flavopiridol.

CONCLUSIONS
The effect of the inhibitors on parasite cell growth and division *in vitro* was determined using existing assays. Taken altogether, these data show that *Leishmania* promastigotes (*L. donovani*) and amastigotes (*L. mexicana*) susceptibility to CDK chemical inhibitors is parasite intrinsic, stage specific, and macrophage independent. The *Leishmania* parasite has low sensitivity to chemical inhibitors, when tested *in vitro* culture. This work suggests the possibility of repurposing the anti-cancer drugs as anti-leishmanial and consequently reducing the cost of developing new drug.

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