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Ex-vivo Evaluation of Cytotoxic potential of Proguanil

Keval R. Rathod*, Shubham Rajput, Pravin R. Tirgar

Department of Pharmacology, School of Pharmacy, R K University, Kasturbadham, Rajkot-360020, India

Abstract : Background and Objectives: Proguanil is also known as chloroguanide is medication which is used to treat malaria.Its active metabolite cycloguanil is an inhibitor of DHFR (Dihydrofolate Reductase) for both mammals & parasite. The aim of this study was to elucidate the in vitro cytotoxic activity of Proguanil.
Methodology: The cytotoxic activity of Proguanil was performed with the help of Brineshrimp Model. In which we done the Hatching of the egg of Brineshrimp. In hatching we use the premature developed cell of brineshrimp. After we adding the solution of the drug with different concentration on same number cell of brineshrimp. With the help of haemolytic study, we also see the effect of proguanil on Human blood cells. Haemolytic study done by UV-Spectrophotometer.
Result: When drug administered on the cell of brineshrimp, number of brineshrimp was decreased as per concentration of drug increased. When performing haemolytic study the %Haemolysis was significantly increase as per concentration of drug increased.
Conclusion: The cytotoxic effect of proguanil is may be useful in cancer.

Introduction:

Proguanil, also known as chloroguanide, is a medication used to treat and prevent malaria. It is often used together with chloroquine or atovaquone. When used with chloroquine the combination will treat mild chloroquine resistant malaria. It is taken by mouth.Side effects include diarrhoea, constipation, skin rashes, hair loss, and itchiness. Because malaria tends to be more severe in pregnancy, the benefit typically outweighs the risk. If used during pregnancy it should be taken with folate. It is likely safe for use during breastfeeding. Proguanil is converted by the liver to its active metabolite, cycloguanil. [1][2] Prguanil has been studied at least since 1945.[3] It is on the World Health Organization's List of Essential Medicines, the most effective and safe medicines needed in a health system.[4] The wholesale cost in the developing world is about 0.10 to 0.50 USD per day. In the United States and Canada it is only available in combination as atovaquone/proguanil. [5] When used alone, proguanil functions as a prodrug. Its active metabolite, cycloguanil, is an inhibitor of dihydrofolatereductase (DHFR). [2] Although both mammals and parasites produce DHFR, cycloguanil's inhibitory activity is specific for parasitic DHFR. This enzyme is a critical component of the folic acid cycle. Inhibition of DHFR prevents the parasite from recycling dihydrofolate back to tetrahydrofolate (THF). THF is required for DNA synthesis, amino acid synthesis, and methylation; Thus, DHFR inhibition shuts down these processes. [6]Cancer is a group of diseases involving abnormal cell growth with the potential to invade or spread to other parts of the body. [7]In 2012 about 14.1 million new cases of cancer occurred globally. [8]Tobacco use is the cause of about 22% of cancer deaths. Another 10% is due to obesity, poor diet, lack of physical activity and drinking alcohol. Other factors include certain infections, exposure to ionizing radiation and environmental pollutants. [8] Various alkylating agent, Anti-metabolite agent, Metallic compound, DHFR inhibitors are used to treat cancer. [9]

Materials & Methods

Brine shrimp model:

The brine shrimp egg containing capsule were procured from Amazon (e-market). First of all store the brine shrimp capsule in tightly closed container it must be free from moisture and stored under cool temperature below 50°F.

It's required "V" or coned shaped bottle, continuous lighting source like tungsten filament bulb, etc. continuous aeration, sea water (water containing 2/3 tablespoon NaCl and pinch of sodium bicarbonate). First fill the coned shaped bottle with sea water with continuous aeration & lighting and then add one capsule of brine shrimp. Continue this method for 24hr, after 24hr close the lighting & aeration and give time to nauplii for settle down in bottom part of bottle. Than use the egg for practical purpose.[10]

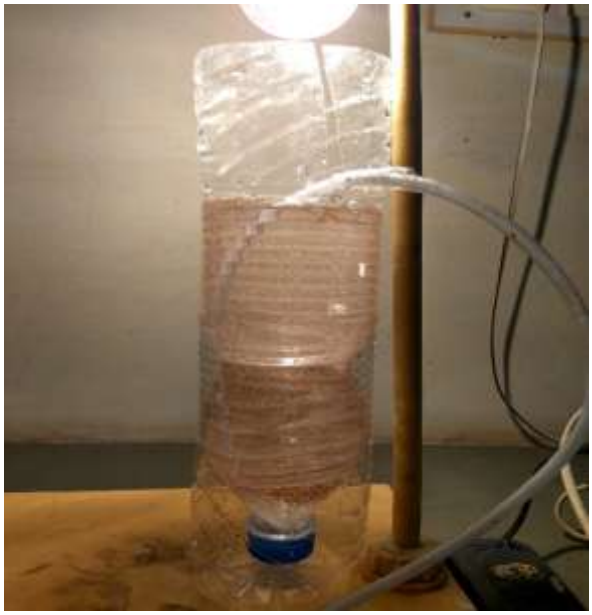

Figure: 1: Hatching Process of Brine Shrimp Egg

Haemolytic Activity:

The haemolytic assay was performed according to Daniel B. Alencar et al. Human blood cells prepared by washing them six times with 50 Mm Tris-HCl , pH 7.6, containing NaCl 0.15 M (TBS). Following the last wash, red blood cells (RBC) were diluted to 1/10 of their volume with TBS.

Haemolytic activity =

$$\frac{Abs_{sample} - Abs_{negative\ control}}{Abs_{positive\ control} - Abs_{negative\ control}} \times 100$$

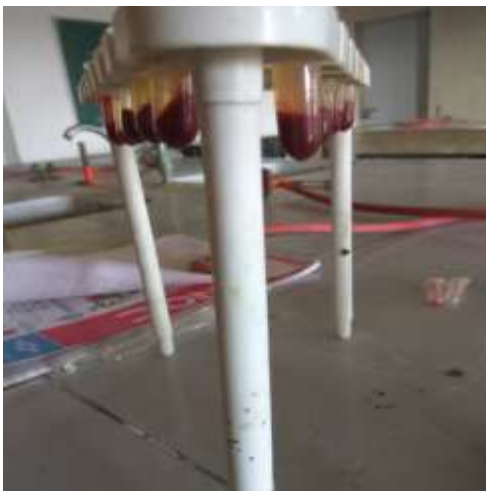

Figure: 2: Blood Sample after Centrifugation

The assay was performed by mixing 0.3 mL of the RBC solution with 0.5, 1, 2 & 5mg/ml of proguanil. 1.2 mL of distilled water was set as a positive control and 1.2 mL of TBS as a negative control. The mixtures were vortexed, left for 2h at room temperature, and then centrifuged at 4,000 x g for 10 min at 4ºC. Absorbance of the supernatants was measured at 541 nm in a UV – Visible spectrophotometer. The percentage of haemolysis of each fraction was calculated using the expression below: [11]

Result:

Effect of Proguanil on Cell Viability (Haemolytic Activity)[11]:

As the absorbance of sample increases there are more chances of haemolysis, which indicates cytotoxic activity of test samples of different concentration.

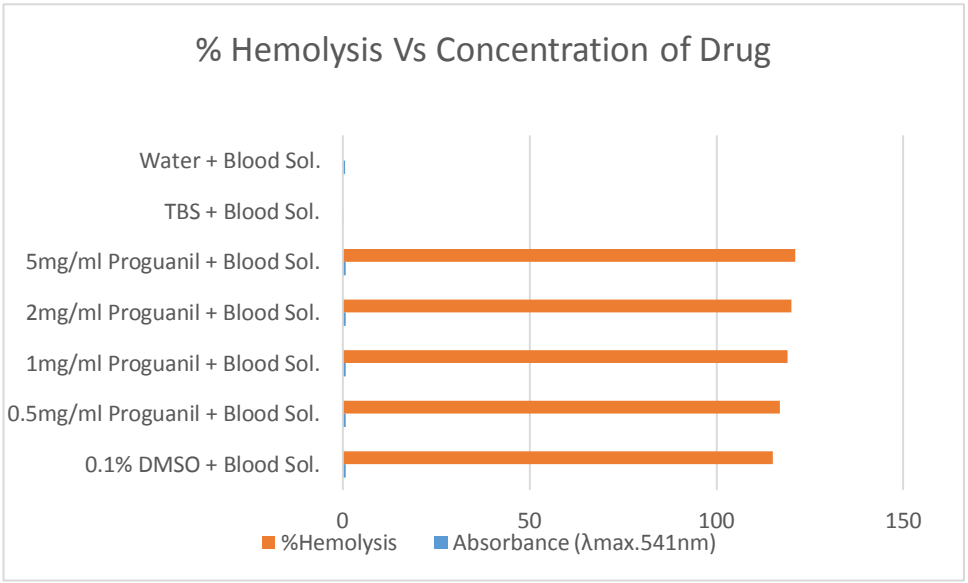

Figure: 3: Graph of %Haemolysis Vs Concentration of Drug

Effect of Proguanil on Cell Viability (Brine Shrimp Model)[12]:

After each time intervals, the viable naupliis were counted. The numbers of survivors were counted and percentage of deaths were calculated. Larvae were considered dead if they did not exhibit any internal or external movement during several seconds of observation.

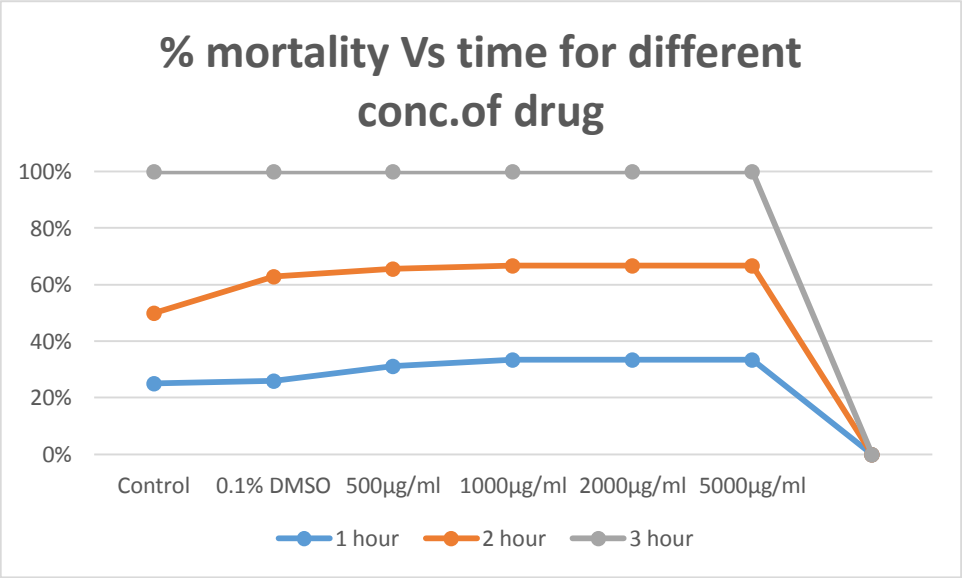

Figure:4: Graph of %Mortality Vs Time for different concentration of Drug

Table: 1: Haemolytic Activity of Proguanil

Drug concentrations	Absorbance (λ_{max}.541nm)	% Hemolysis
0.1% DMSO + Blood Sol.	0.721	115
0.5mg/ml Proguanil + Blood Sol.	0.732	117
1mg/ml Proguanil + Blood Sol.	0.745	119
2mg/ml Proguanil + Blood Sol.	0.745	120
5mg/ml Proguanil + Blood Sol.	0.759	121
TBS + Blood Sol.	0.011	(-)Control
Water + Blood Sol.	0.626	(+)Control

Table: 2: Mortality due to effect of Proguanil

% Mortality						
Time	**Control**	**0.1%DMSO**	**500µg/ml**	**1000µg/ml**	**2000µg/ml**	**5000µg/ml**
1 hour	10%	70%	90%	100%	100%	100%
2 hour	10%	100%	100%	100%	100%	100%
3 hour	20%	100%	100%	100%	100%	100%

Discussion:

Cancer is disease in which the growth of the cells in uncontrolled manner and spread to other parts of the body. Spreading of cancer cell to the other parts of the body through blood and lymph system.The normal control system that prevents the overgrowth of cells and invasion of other tissue, whereas these are disabled in cancer cell. Cancerous cells have ability to disrupt and invade the surrounding tissue. Such lesion can be arising in any parts of body, and thus cancers represent heterogeneous group of diseases, have certain biological features in common. As we show the cytotoxic potential of Proguanil. So, it's also play a role for the treatment of cancer. Proguanil is an Anti-malarial drug act as DHFR (DihydroFolateReductase) Inhibitor. The DHFR is very similar to that of the animal cell as well as mammalcell. So with the same mechanism it also kill the cancerous cell of the human. The drug powder was dissolve in 0.1% DMSO and prepare a solution. Then its different concentration was also prepared. The two Pharmacological models were incorporated to check the cytotoxic action of Proguanil, that one is In vitro haemolytic assay and the second one is Brine shrimp lethality assay. In the Haemolytic assay, as the absorbance of sample increases there are more chances of haemolysis, which indicates cytotoxic activity of Proguanil of different concentration. The liberated haemoglobin was

measured which is produced due to ruptured RCB. This is due to the effect of Proguanil. As per the analytical results obtained, the % haemolysis increases as the dose of Proguanil increases. The Brine shrimp lethality assay model is about the ability to kill laboratory-cultured Artemianauplii (brine shrimp). The assay is considered a useful tool for preliminary assessment of toxicity. The numbers of survivors were counted and percentage of deaths were calculated. Larvae were considered dead if they did not exhibit any internal or external movement during several seconds of observation. The results are indicating that as the concentration of extracts increases, the mortality also increase. So, when the higher concentrated dose comes in contact with the naupliis, they die rapidly.

Conclusion:

In-vitro cytotoxic haemolytic study for Proguanil showed concentration dependent increase in erythrocytes which support cytotoxic action of Proguanil. The highest cytotoxic activity was observed at 5000 µg/ml concentration. Hence, the haemolysis assay has proven useful to establish whether the cytotoxic activity is related to direct damage on the cell membrane or not. The brine shrimp lethality assay has proven to be a convenient system for monitoring the biological activities which shown the significant cytotoxicity on the laboratory-cultured Artemianauplii (brine shrimp). The concentration depended Death of the naupliis was observed. The Highest Lethality was observed at 5000 µg/ml concentration. Just like the haemolytic assay. So, we can conclude that the Brine Shrimp Lethality Assay is an excellent predictive tool for the toxic potential of Proguanil in humans. Proguanil is act same like Methotrexate. And Methotrexate is contraindicated in Pregnant Lady. So, it can be the next better, safer & cheaper alternate in management of chronic diseases like cancer. Thus, deserves studies that are more detailed for further evaluation .This research work shows that greater outcomes can be anticipated for further research work in this direction.

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