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Malaria-related studies on enzymopathies, methemoglobin, and methylene blue

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In sub-Saharan Africa, *Plasmodium falciparum* malaria demands an enormous toll in lives, medical costs, and in days lost for labour and education. Children are the most vulnerable ones, with one death caused by malaria every 20 seconds. With respect to standard *affordable* chemotherapy of malaria, resistance against the mainstay drugs chloroquine and sulfadoxin/pyrimethamine is spreading. Thus there is an urgent need for affordable antimalarial drugs within five years.

Under this perspective, methylene blue in combination with chloroquine (BlueCQ) is of interest. Methylene blue is an inhibitor of the enzyme glutathione reductase of *P. falciparum*. Referring to a completely different target, the leukoform of methylene blue most efficiently reduces methemoglobin (Hi) *in vivo*; thus methylene blue is the standard drug against methemoglobinemia. Enzymopathies like certain forms of G6PD deficiency represent a contraindication against methylene blue. Consequently the thesis faced three problems:

- As methemoglobin formation is a serious complication of malaria-induced anemia, a sensitive method for determining Hi in blood of anemic children had to be established.
- What is the prevalence of X-chromosome-linked G6PD deficiency and chromosome 8-linked glutathione reductase deficiency in the five ethnias of rural Burkina Faso? Reliable and simple tests had to be developed in order to check for these enzymopathies prior to treatment. Can these tests be conducted along with other malaria-related clinical tests? Above all, is the African form of G6PD deficiency (G6PD A$_{minus}$) a contraindication for agents like methylene blue?

- What are the unwanted and wanted side effects of methylene blue treatment?

To answer these questions, the author developed laboratory procedures at the Biochemie-Zentrum der Universität Heidelberg (BZH) and tested them around the Centre de Recherche en Santé de Nouna (Burkina Faso) in five villages where malaria is holoendemic. Blood samples from more than 1000 persons were examined.

The results of the biochemical studies can be summarized as follows. Since there was no suitable method for determining methemoglobin in anemic patients, the author developed three sensitive modifications of the HiCN-based Evelyn-Malloy procedure, including a non-cyanide method. The procedures are robust photometric tests at 630 nm which require only 50 µl fingerprick blood with a total hemoglobin concentration of 5 g/dl. The tests were applied to blood samples of adult persons with mild malaria at the CRSN. 28 samples exhibited cHi/cHbtotal values below 1%, 14 between 1% and 2%, and 15 showed values between 2.0 and 3.3%. While developing these methods, I revisited Hi formation by sodium nitrite *in vitro* and prepared erythrocytes that contained defined amounts of Hi, the range for the ratio of methemoglobin/total hemoglobin being 1% to 95%.

By miniaturization of Beutler´s methods, paper fluorescence (“dry”) tests for identifying persons with G6PD deficiency and glutathione reductase deficiency were developed. For the latter enzyme, the EGRAC test was applied in order to distinguish between nutritional vitamin B2 deficiency and hereditary apoenzyme deficiency. For standardizing the EGRAC procedure - and for future studies on the glutathione reductase-methylene blue complex – *P. falciparum* glutathione reductase was purified and crystallized, using published procedures in modified form.

The first trial, conducted in the village of Bourasso with 750 participants, revealed the presence of *P. falciparum* parasitemia in 79.3% of the individuals. G6PD deficiency prevalence was found to be 16.9% in the male population and 7.6% in females; the latter value is twice as high as expected for an X-chromosome linked polymorphism. When differentiating according to age, the frequency for degree 1-G6PD deficiency was 3.7% in females and 17.7% in males under 5 years of age; for persons over 5 years, the values were found to be 9.0% for females and 16.7% for males. The diagnosis of G6PD deficiency was confirmed for 62 persons using the Brewer test, again in miniaturized version. In this test which is based on challenging the cells with methylene blue, no tendency towards immediate

hemolysis was observed for the G6PD-deficient erythrocytes.- The prevalence of glutathione reductase deficiency in Bourasso was found to be 4.1%. In all cases but one, this was due to riboflavin deficiency.

In a safety study conducted with about 100 adult male G6PD-sufficient participants representing the ethnias Fulani, Marka, Samo, and Mossi, the verum group (BlueCQ group) received chloroquine plus methylene blue, and the control group chloroquine. Side effects reported or observed in the BlueCQ group included blue urine up to 4 days after the last dose, fatigue, increased appetite, and a tendency towards rise in hemoglobin on day 5 of treatment. Although in the BlueCQ group parasitemia dropped more rapidly, 8 out of 51 persons had detectable parasitemia on day 5 (with 8 – 120 parasites per µl). In the chloroquine group there were 4 out of 41 patients with residual parasitemia of 24-480 parasites on day 5. (For comparison, the diagnosis malaria is made when parasitemia is above 5000 per µl.) The lack of complete parasite clearance on day 5 is discussed in terms of compliance, drug dosages, drug resistance, and improved recognition of parasites when stained by methylene blue *in vivo*.

The appendix of the thesis contains an extensive list of symptoms, diseases, and therapies reported by the patients and may be used for malaria- and BlueCQ-related epidemiological analyses.

Conclusions. The gene frequency for *G6PD-Aminus* is approximately 15% in rural Burkina Faso but does not appear to be a contraindication for methylene blue. Glutathione reductase deficiency is observed in 4.1% of the population but it is caused by inadequate vitamin B2 intake. Methemoglobin formation as a potential complication of malaria - and thus as an additional target of methylene blue – can now be assessed in *anemic* patients using the procedures introduced here. Methylene blue (3 mg/kg per day) was well tolerated by adult male G6PD-sufficient persons. The side effects of methylene blue appear to be mild and include also positive aspects such as the concomitant therapy of methemoglobinemia, a rise in haemoglobin levels, and increased appetite.

Literature. Schirmer RH, Coulibaly B, Stich A, Scheiwein M *et al.* (2003). Methylene blue as an antimalarial agent. *Redox Rep* 8:272-275. Eubel J, Coulibaly B, Davioud-Charvet E, Becker K, Schirmer RH (2004) Interactions of methylene blue with the glutathione redox system of *Plasmodium falciparum*. *Intern J Med Microbiol* 293 Suppl.38: 84-85 (Abstract). Ziebuhr W, Xiao K, Coulibaly B, Schwarz R, Dandekar T (2004) Pharmacogenomic strategies against resistance development in microbial infections. *Pharmacogenetics*, in press. Mandi G, Witte S, Meissner P, Coulibaly B et al. (2004) Safety of the combination of chloroquine and methylene blue in healthy adult men with G6PD deficiency from rural Burkina Faso, *submitted*