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Synergism between monodesbutyl-benflumetol and artemisinin in *Plasmodium falciparum* in vitro

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Synergismus zwischen Monodesbutyl-Benflumetol und Artemisinin bei *Plasmodium falciparum* in vitro

Zusammenfassung. In 34 frischen Isolaten von *Plasmodium falciparum* wurde die Sensibilität gegenüber Artemisinin, Monodesbutyl-Benflumetol (DBB) und einer 1:1 m/m Kombination beider Stoffe ermittelt. Auf molarer Basis war die Kombination am wirksamsten, vor DBB und Artemisinin. Die geometrischen Mittelwerte für volle Hemmung lagen bei 49,25 nM für die Kombination, 279,12 nM für DBB und 494,05 nM für Artemisinin. Der Unterschied der Wirksamkeit der Kombination gegenüber Artemisinin allein und DBB allein war hoch signifikant. Die Interaktionsanalyse ergab mäßigen Synergismus bei der EC_{50} und hochgradigen Synergismus bei EC_{90} und EC_{99}. Bei den einzelnen Isolaten zeigten Höhe der EC und Grad des Synergismus signifikante umgekehrte Korrelation. Der Synergismus ist daher bei Isolaten mit reduzierter Empfindlichkeit gegenüber Artemisinin und DBB am stärksten ausgeprägt.

Summary. The sensitivity to artemisinin, monodesbutyl-benflumetol (DBB) and a 1:1 m/m combination of the two compounds was successfully investigated on 34 fresh isolates of *Plasmodium falciparum*. On a molar basis the combination was most active, followed by DBB and artemisinin. The geometric mean concentrations effecting full inhibition (GMCOC) were 49.25 nM for the combination, 279.12 nM for DBB, and 494.05 for artemisinin. The difference between the efficacy of the combination and that of its components was highly significant. Interaction between artemisinin and DBB showed moderate synergism at the EC_{50} and strong synergism at EC_{90} and EC_{99}. The individual parasite isolates showed a significant inverse correlation between the ECs and the degree of synergism. Positive specific pharmacodynamic interaction was therefore most marked in isolates with reduced sensitivity against artemisinin and DBB.

Key words: *Plasmodium falciparum*, desbutyl-benflumetol, artemisinin, synergism.

Introduction

Artemisinin (Fig. 1), a natural sesquiterpenelactone, occurs in *Artemisia annua* L., and is obtained by extraction from this plant [1]. Semi-synthetic derivatives such as artemether, Na-artesunate and dihydro-artemisinin have become important blood schizontocidal and gametocytocidal drugs in the context of artemisinin-based combination therapy of falciparum malaria [2], where the artemisinin derivatives increase the efficacy of the partner drugs.

Monodesbutyl-benflumetol (DBB, Fig. 2) is an analogue of lumefantrine (formerly benflumetol) and was originally considered a natural metabolite of lumefantrine. As compared to the latter, DBB has significantly higher activity against *Plasmodium falciparum* [3], and *Plasmodium vivax* where the difference between the activities of the two compounds spanned one order of magnitude [4].

Since synergistic interaction was reported between artemether and lumefantrine in *P. falciparum* [5], it was

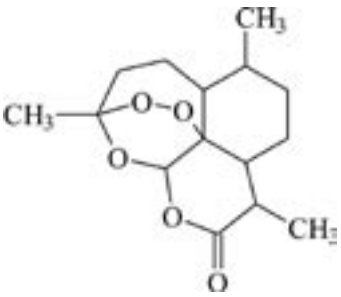

Fig. 1. Chemical structure of artemisinin

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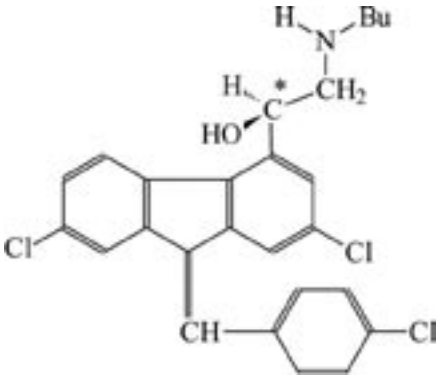

Fig. 2. Chemical structure of monodebutyl-benflumetol (DBB)

plausible to investigate the interaction between artemisinin and DBB, the more so as earlier observations indicated descending synergism with increasing EC level. Moreover, these observations were made 6 years earlier when *P. falciparum* in the study area showed significantly higher sensitivity, both to artemisinin and DBB. For the study, the use of artemisinin was given preference over that of artesunate or dihydro-artemisinin since artemisinin is more stable. Moreover the activity of artemisinin shows very high correlation with that of dihydro-artemisinin, the active metabolite of all artemisinins [6].

Material and methods

The study was carried out under auspices and as part of the programme for the monitoring of the drug sensitivity of *Plasmodium falciparum*, an activity of the Ministry of Public Health of Thailand.

Study site and time

The study was conducted between May and July 2006 at the Malaria Clinic of Mae Sot. The district town of Mae Sot is part of the Province of Tak and situated near the border to Myanmar, approximately 550 km northwest of Bangkok, Thailand. The Malaria Clinic is frequented by patients suffering from fever or other symptoms suggestive of malaria. The clientele consists mainly of patients from adjacent Myanmar. Diagnostic procedures and species-specific treatment of malaria are free of charge. Over the past years *P. vivax* has become the prevailing parasite species (~60%), followed by *P. falciparum* which was formerly the lead species. Infections with *P. malariae* or species of the *P. ovale* group are rare. While mixed infections with *P. falciparum* and *P. vivax* are quite exceptional in Thai patients, they are more frequently found in patients from Myanmar.

Malaria in Mae Sot District is hypo-endemic as a result of intensive malaria control, while it is hyperendemic in neighbouring areas of Myanmar. The terrain is dominated by forested hills and stream valleys, the preferred environment for *Anopheles dirus* (formerly *A. balabacensis balabacensis*) and *Anopheles minimus minimus*, the main malaria vectors in the area.

The timing of the study coincided with the monsoon rains and thus with the main transmission season for malaria that extends from May to September. There is usually a secondary peak of transmission in November/December. In principle, malaria transmission in the area can occur all the year round since the mean monthly temperature does not drop below 20 °C and the high relative humidity ensures long survival of vectors.

Parasite isolates

The parasite isolates were obtained from patients who suffered from mono-infections with P. falciparum at an asexual parasite density of 1000–100000/µl blood, and who had given written or verbal consent to being included in the study. Exclusion criteria were pregnancy, age < 10 years and recent treatment with antimalarial drugs or antibiotics (9 weeks for mefloquine, 4 weeks for the other medicaments).

In vitro tests

The in vitro tests followed the WHO standard methodology for the assessment of inhibition of schizont maturation in *P. falciparum* [7]. After finger-prick 200 µl of blood were taken up in sterile, heparinized capillary tubes and immediately dilued with RPMI-1640 medium (Sigma R 7388, supplemented with sodium bicarbonate) to a haematocrit of approximately 2% in the blood-medium-mixture (BMM). At the same occasion a thick blood film was prepared for the confirmation of the mono-infection with *P. falciparum* and the reading of the pre-incubation parasite count. Pending further processing within a maximum of 3 hours, the BMM was kept at 37.5 °C. After gentle re-suspension, 50 µl aliquots of the BMM were added to the wells of the scheduled test series in the 8 x 12 well test plates (Falcon 3070, BD). Each test line consisted of 8 wells, well A as drug-free control, wells B-H containing ascending quantities of the test drug. For artemisinin (Laboratory standard of the Academy of Military Medical Sciences, Beijing), DBB (Novartis TA 2256) and the 1:1 combination of both compounds the dose per well rose from 0.15 pmol in well B to 150 pmol in well H, corresponding to test concentrations of 3–3000 nM. The test plates were pre-dosed at the Institute of Specific Prophylaxis and Tropical Medicine, Centre for Physiology and Pathophysiology, Medical University of Vienna, Austria.

After dosing with the BMM, the test plates were closed with a lid (Falcon 3071, BD) and placed in a candle container. After lighting the candle, the container was tightly closed and inserted in an incubator set at 37.5 °C. Following 23.5 hours of incubation the plates were removed and the tests "harvested" by decanting the supernatant fluid from the wells and preparing thick films from the sediments, placing the 8 films of the test line in a set order on the same slide. After thorough drying, usually over night, the films were stained at pH 6.85 with a 3% dilution of a commercial Giemsa stock solution.

Test evaluation and statistical analysis

The pre-incubation slides were read for asexual parasite density by counting ≥200 leukocytes against up to 500 *P. falciparum* trophozoites, whichever count was reached earlier. The parasite density per µl was then calculated on the basis of a normal WBC count of 8000/µl [8]. In the slides from the sensitivity tests a total of ≥200 asexual parasites were differentiated into trophozoites and schizonts, i.e. parasites with ≥3 chromatin bodies. Test series with ≥10% schizonts in the drug-free control well A were considered valid [6]. As part of the reading, the lowest drug concentration was noted at which no schizont maturation had occurred ("cut-off concentration"), to serve the calculation of the geometric mean cut-off concentration (GMCOC) of schizont maturation.

Since the inhibition of schizont maturation by artemisinin and DBB follows a log-normal pattern, the log-probit method of Litchfield and Wilcoxon [9] was applied for the calculation of the key parameters of the regression, using an EDP version of the procedure [10]. Student's t-test was employed for the comparison of continuous data.

Table 1. Response parameters of *Plasmodium falciparum* observed in 34 isolates simultaneously tested with monodesbutyl-benflumetol (*DBB*), artemisinin (*ART*) and a 1:1 m/m mixture of both compounds (*DBB-ART*). *GMCOC* geometric mean concentration for complete inhibition

Parameter	DBB	ART	DBB-ART
EC_{50} nM	10.37	7.08	2.89
EC_{90} nM	67.71	62.70	15.07
EC_{99} nM	312.52	371.18	57.99
r	0.9982	0.9929	0.9963
χ^2	0.2591	0.7615	0.1667
Slope S	4.2889	5.4354	3.6061
GMCOC nM	279.12	494.05	49.25

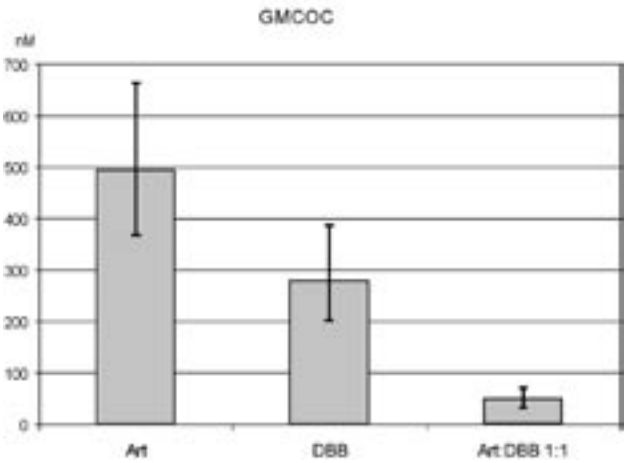

Fig. 4. Geometric mean cut-off concentrations (*GMCOC*) of schizont maturation of *Plasmodium falciparum* observed with artemisinin alone (*ART*), monodesbutyl-benflumetol (*DBB*) alone, and a 1:1 m/m combination of both compounds

Berenbaum's method [11] was used for the analysis of specific pharmacodynamic interaction between artemisinin and DBB, calculating the fractional inhibitory concentrations for both drugs (FIC_{ART} and FIC_{DBB}) and their sums (SFIC). Values of SFIC <0.5 denote strong synergism, 0.5<1.0 moderate synergism, 1.0 fully additive activity, >1–2 partially additive activity (provided none of the contributory FICs is ≥1), and >2 antagonism.

Results

Parallel tests with artemisinin, DBB and a 1:1 m/m combination of both compounds were successfully carried out with 34 fresh isolates of *P. falciparum* obtained from 5 female and 29 male patients, 11 resident in Thailand, 23 in Myanmar. The age of the patients was in the range of 12–48 years (mean 29.7 years, median 30 years). Four patients stated to have contracted the infection in Thailand, 30 in Myanmar. The asexual parasite counts were between 4273 and 100000 per µl blood, with a geometric mean of 25395/µl.

The individual EC_{50} values for artemisinin covered the range of 1.39–49.73 nM, with a mean of 10.48 ± 10.66 nM (SD). The corresponding data for DBB were 0.64–20.44 nM for the range and 5.51 ± 4.73 for mean and standard deviation. For the combination of artemisinin and DBB the range was narrower with 1.61–8.25 nM, and the mean 3.41 ± 1.89 nM (SD).

The log-probit regressions for artemisinin alone, DBB alone and the 1:1 m/m combination of both compounds showed low heterogeneity and a good fit of the data points to the regression lines (Table 1). The mean EC_{99} values were highest with artemisinin (371.18 nM), followed by those for DBB alone (312.52 nM) and those of the combination (57.99 nM), pointing to a high degree of synergism (Fig. 3). This was also evident in a comparison of the GMCOC values with 494.05 nM for artemisinin alone, 279.12 nM for DBB alone and 49.25 nM for the combination and t = 9.8210 ($p = 4.9373 \times 10^{-15}$) for artemisinin alone vs the combination, and t = 7.099 ($p = 3.5766 \times 10^{-7}$) for DBB alone vs the combination (Fig. 4). The steepest log-probit regression (slope S) was observed with artemisinin-DBB.

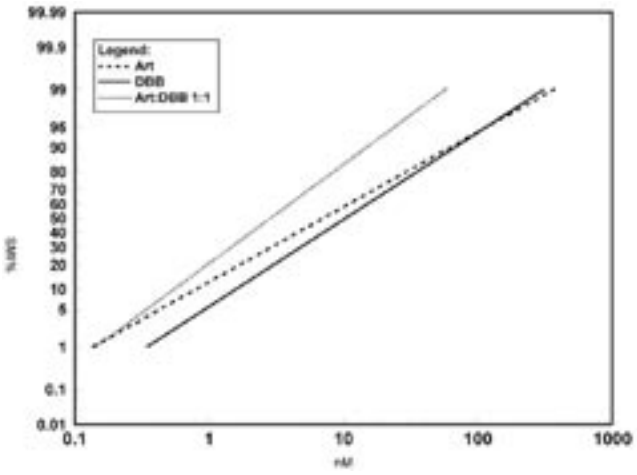

Fig. 3. Log-probit regressions reflecting the response of *Plasmodium falciparum* to artemisinin alone (*ART*), monodesbutyl-benflumetol (*DBB*) alone, and a 1:1 m/m combination of both compounds (*ART-DBB*). Results from 34 parasite isolates

Table 2. Geometric means of the fractional inhibitory concentrations for monodesbutyl-benflumetol (*FIC-DBB*) and artemisinin (*FIC-ART*) and their sum (*SFIC*) observed in 34 fresh isolates of *Plasmodium falciparum*

Group	FIC-DBB	FIC-ART	SFIC
EC_{50}	0.3663	0.2031	0.6395
EC_{90}	0.2623	0.1286	0.4447
EC_{99}	0.1975	0.0896	0.3484

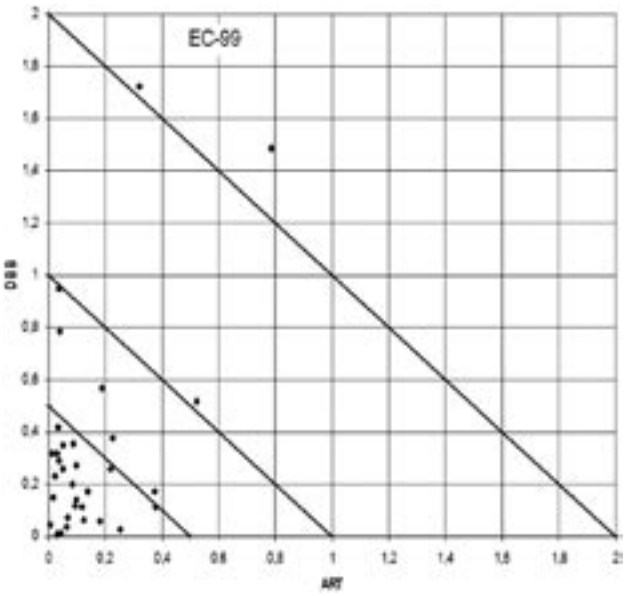

Fig. 5. Pharmacodynamic interaction between artemisinin (*ART*) and monodesbutyl- benflumetol (*DBB*) at the EC_{99} level in *Plasmodium falciparum* according to Berenbaum [11]

Analysis of specific pharmacodynamic interaction according to Berenbaum [11] indicated moderate synergism at the EC_{50} and strong synergism at EC_{90} and EC_{99}, the intensity of which is rising with increasing EC values (Table 2, Fig. 5).

Discussion

The response to artemisinin of *P. falciparum* in the study area is still within the range of sensitivity. None of the individual isolates has shown an EC_{99} commensurate with resistance. Threshold levels of resistance to DBB are yet to be determined. However, in view of the structural relationship with lumefantrine, and the higher intrinsic activity of DBB it is likely that the response of all parasite isolate was very well within the range of sensitivity.

Earlier observations pointed clearly to the presence of synergism between artemisinin and DBB (personal communication Dr. J. Raffelsberger) and this study confirmed the presence of such an interaction. The difference between the two studies was a significant reduction of the sensitivity of *P. falciparum* in the study area to both, artemisinin and DBB, indicating a potential role of the sensitivity level in the quantitative expression of synergism. Following this lead, correlation analysis of isolate-specific EC to artemisinin and the FIC_{ART} indicated a significant inverse correlation between the two parameters at the EC_{50} ($p=0.0015$) and the EC_{99} ($p=0.0184$). The same applies to the ECs for DBB and the FIC_{DBB} at the EC_{50} ($p=0.022$) and the EC_{90} ($p=0.0188$). Interestingly, the influence of the isolate-specific, drug-specific ECs on the SFIC values remained below the threshold with artemisinin, while it showed significant inverse correlations for DBB ($p=0.118$ at the EC_{50}; $p=0.0374$ at the EC_{90}), pointing to a key role of this compound. Thus, synergism between artemisinin and DBB rises obviously with a reduction of sensitivity to both drugs. Re-checking the results of the interaction analysis it was seen that the two "escapees" in the low antagonistic range of Fig. 5 represented the FICs of two highly artemisinin- and DBB-sensitive isolates. In the light of these observations the relatively moderate interaction between artemisinin and DBB in *P. vivax* [12] may be explained by a still highly sensitive response of this species to both, artemisinin and DBB.

The synergistic interaction between artemisinin and DBB seems to open up prospects for a practical improvement of the antimalarial armamentarium, especially in view of the higher intrinsic activity of DBB as compared to that of the structurally related lumefantrine. As to be expected, lumefantrine and DBB show activity correlation in *P. falciparum* [3]. Therefore the current liberal use of the combination of artemether and lumefantrine in connection with artemisinin-based therapy (ACT) in tropical Africa – largely unsupported by diagnostic evidence – may lead to the selection of lumefantrine-resistant *P. falciparum* populations and compromise the efficacy of DBB, the compound with an *a priori* greater therapeutic potential as compared to that of lumefantrine.

Conclusions

The study has confirmed the synergistic interaction between artemisinin and DBB in *P. falciparum* and shown the synergism to increase with reducing isolate-specific sensitivity to the partner drugs. This phenomenon may not be confined to artemisinin and DBB alone, but also explain the continued efficacy of treating falciparum malaria with a combination of mefloquine and artesunate in an area where 60% of the parasite populations show resistance to mefloquine.

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