Characterization of Simian Malarial Parasite (Plasmodium knowlesi)-induced Putrescine Transport in Rhesus Monkey Erythrocytes

A NOVEL PUTRESCINE CONJUGATE ARRESTS IN VITRO GROWTH OF SIMIAN MALARIAL PARASITE (PLASMODIUM KNOWLESI) AND CURES MULTIDRUG RESISTANT MURINE MALARIA (PLASMODIUM VYELII) INFECTION IN VIVO*

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A stage-dependent increase in the level of putrescine, spermidine, and spermine during intraerythrocytic growth of Plasmodium knowlesi in rhesus monkey erythrocytes was observed. Further, intraerythrocytic P. knowlesi-induced putrescine influx was found in trophozoite stage-infected erythrocytes and process was time- and temperature-dependent and showed saturable kinetics. Characteristics of induced putrescine influx appears in infected erythrocytes to be close to the normal erythrocytes in terms of affinity of putrescine to the putrescine transporter (Km = 34.6 ± 3.8 μM as normal erythrocytes and Km = 37.2 ± 5.2 μM in infected erythrocytes). However, the difference involves the significant increase in the putrescine influx rate after infection (Vmax = 4.21 nmol/min/1010 normal erythrocytes, compared with 11.6 nmol/min/1010 infected erythrocytes). Energy dependence, involvement of -SH group, and non-interference by amino acid, spermidine, and spermine in the putrescine influx process clearly demonstrate the presence of a distinct transporter for putrescine in infected erythrocytes. A putrescine conjugate N1,N4-bis-(7-chloroquinoline-4-yl)butane-1,4-diamine (BCBD) was synthesized, which inhibits the putrescine influx in the P. knowlesi infected erythrocytes (Ki of 43.2 μM) as well as in vitro growth of P. knowlesi (IC50 value, 7.6 ± 0.97 ng/ml BCBD, 10.8 ± 0.45 ng/ml chloroquine). Addition of exogenous polyamines failed to reverse the inhibitory effect of BCBD in vitro. Administration of BCBD (24 mg/kg body weight, intraperitoneal, twice a day for 4 days) cured the Swiss mice infected with multidrug-resistant infection of Plasmodium yoelii. Therefore, inhibition of putrescine transport in malaria-infected erythrocytes offers a lead in the search of a new class of chemotherapeutic molecules against malaria.

Malaria remains one of the most important parasitic disease in the tropical countries. The global expansion of the disease has been attributed mainly to the failure of vector control programs and spread of Plasmodium falciparum resistance to chloroquine and other known antimalarial drugs (1). For the development of new molecules effective against resistant strains, a new target for rational drug design is imperative (2).

The naturally occurring polyamines, spermidine, spermine, and their precursor, putrescine, are important regulators of growth and differentiation in a wide variety of cell types including protozoan parasites (3). Inhibition of polyamine biosynthesis could be a suitable target to control proliferative disorders and infectious diseases (4–6). Parasites of genus Plasmodia, during its intraerythrocytic schizogony (I-ES), undergo a complex developmental cycle resulting in 8–32 merozoites from a single parasite. However, normal erythrocytes are devoid of polyamine biosynthetic machinery, and only traces of spermidine and spermine are detectable (7). Postinvasion, as P. falciparum rapidly multiply and grow, polyamines and their peak biosynthetic enzymes like ornithine decarboxylase (ODC) and S-adenosyl methionine decarboxylase increases in parasitized erythrocytes (7, 8). These polyamines at certain stage of Plasmodium cell cycle trigger the replication of malarial DNA and stimulate synthesis of specific proteins (9). These observations were confirmed further by the presence of polyamine dependent -like DNA polymerase that catalyzes synthesis of malarial parasite DNA during I-ES (10). ODC, which catalyzes the rate-limiting step in polyamine biosynthesis, is irreversibly inhibited by a-difluoromethyl ornithine (DFMO), an ornithine analogue (11). This compound inhibits the proliferation of various cell types including parasitic protozoan (12). Addition of DFMO to P. falciparum culture prevented the accumulation of putrescine and spermidine in parasitized erythrocytes, thus resulting in the arrest of malarial parasite growth at trophozoite stage, i.e. prior to initiation of DNA synthesis (7). On the basis of the above available evidence, it appears that polyamine may also regulate the I-ES of malarial parasite. However, DFMO failed to inhibit the erythrocytic schizogony of malarial parasite in vivo (13). No possible biochemical basis has yet been established for refractoriness of DFMO against blood stage malarial parasite infection in vivo.

Our efforts to investigate the putrescine transport system in malarial parasite-infected erythrocytes are based on three spe-

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† The abbreviations used are: I-ES, intraerythrocytic schizogony; DFMO, difluoromethyl ornithine; MGBG, methylglyoxal bis-glyburide; BCBD, N1,N4-bis-(7-chloroquinoline-4-yl)butane-1,4-diamine; ODC, ornithine decarboxylase; SPD/SPM, spermidine/spermine; Mops, 4-morpholinesulphonic acid; pCMBS, p-chloromercuribenzene; N-Et, N-ethylmaleimide.
cific reasons. First, an increasing body of evidence suggests that refractoriness of tumor cells and protozoan parasites to inhibitors of polyamine biosynthesis is due to influx of polyamines across the membrane, and this transport is also subject to regulation by environmental and physiological stimuli (14–16). Changes in the influx or efflux of polyamines provides an additional mechanism to regulate the intracellular polyamine contents (17). Second, it has been well established that I-ES development of malarial parasite results in numerous biochemical, structural, and functional changes in the host erythrocytes membrane (18, 19) and increased permeability to a wide variety of structurally unrelated solutes via transport pathways that are functionally distinct from those known to operate in normal erythrocytes (2, 20). These parasite-induced pathways facilitate both entry of metabolic and biosynthetic substrates into infected erythrocytes and rapid efflux of potentially harmful catabolites (21). Finally, exogenous addition of putrescine to DFMO-inhibited in vitro culture of P. falciparum resulted in the resumption of growth (7) and DNA synthesis (9). On the basis of the above mentioned facts, we envisaged that putrescine might be available from plasma by increasing its transport across the red cell membrane in the parasitized erythrocytes to overcome DFMO inhibition of ODC.

To prove the above mentioned hypothesis, the roles of specific polyamines were studied during intraerythrocytic development of Plasmodium knowlesi (a simian malarial parasite) in rhesus monkey and P. knowlesi-induced putrescine transport system in the parasitized erythrocytes was characterized. In recent years, emphasis is on altered membrane transport in malaria-infected erythrocytes as a potential pharmacological target for antimalarial drug development (20, 21). We have designed and synthesized a putrescine conjugate, N1,N4-bis-[7-chloroquinoline-4-yl]butane-1,4-diamine (BCBD) on the basis of characteristics of induced putrescine transport system in parasitized erythrocytes and established a correlation between inhibition of putrescine influx and antimalarial activity. A summary of a part of this work has been published previously in abstract form (22).

**EXPERIMENTAL PROCEDURES**

**Materials**—Healthy rhesus monkeys of either sex weighing 4–6 kg were procured from the primate house of our institute. [14C]putrescine hydrochloride and 8-[3H]hypoxanthine were obtained from Amersham International and 6-DFMO was synthesized at Marion Merrell Dow Inc. (Cincinnati, OH) and obtained through Walter Reed Army Institute of Research. All other chemicals and biochemicals were obtained from Sigma.

**Maintenance of P. knowlesi**—The infection of P. knowlesi was maintained in rhesus monkeys by serial blood passage with 1 × 106 infected erythrocytes, and parasitemia was recorded by microscopic examination of Giemsa-stained thin blood smears.

**Enrichment of Different Developmental Stages of P. knowlesi-infected Erythrocytes**—Blood from healthy and P. knowlesi-infected monkeys (at different developmental stages of the parasite, viz. ring, trophozoite, and schizont) were collected in prechilled acid citrate-dextrose solution. The blood was passed through CF-11 column, and thereafter, erythrocytes (both normal and infected) were washed with phosphate-buffered saline (0.85% NaCl, 10 mM phosphate buffer, pH 7.2) and suspended in the same medium. Different developmental stage-specific P. knowlesi-infected erythrocytes were enriched using Ficol-Conray gradient as described earlier by Joshi et al. (18), and an enriched population of P. knowlesi-infected erythrocytes (ring-stage infected ~ 85–90% pure, trophozoite 85–88% pure, and schizonts stage 90–95% pure) were obtained.

**Extraction and Analysis of Polyamines**—The normal and infected erythrocytes were lysed by three brief cycles of freezing and thawing (at −70 and 37 °C) followed by sonication (Branson Sonifier B-12, microtip 15 s, 3 times at 15 watts). 4 to 6 volumes of perchloric acid (0.3 M) were added to sonicated medium and thereafter centrifuged at 10,000 × g for 30 min at 4 °C. Polyamines were analyzed in the clear supernatant by reverse-phase high pressure liquid chromatography after preparing their benzoyl derivatives according to method of Verkoelen et al. (23).

**Putrescine Transport Inhibitor**—Freshly prepared normal and trophozoite-infected erythrocytes were used for putrescine influx as described previously (24) with few modifications, and optimal conditions were standardized. In brief, erythrocyte (both normal and infected) were washed four times with Hepes buffer saline (134 mM NaCl, 2.7 mM KC1, 1.5 mM KH2PO4, 8.1 mM Na2HPO4, and 25 mM Hepes, pH 7.4) and resuspended in the same medium at 37 °C. Erythrocyte suspension (450 μl of 2–5 × 106 erythrocytes/ml) in combination with appropriate reagent solutions was taken in an Eppendorf tube and kept at 4/37 °C for 10 min for temperature equilibration. Influx of putrescine was initiated by adding varying concentrations of putrescine pulsed with 0.5 μCi [14C]putrescine and incubated further at 4/37 °C for the specified time. The reaction was terminated by transferring three aliquotes of 145 μl each of erythrocyte suspension to another Eppendorf tube containing 800 μl of ice-cold isotonic Mops-buffered MgCl2 solution (106 mM MgCl2, 15 mM Mops, pH 7.4) layered over 250 μl of dibutylphalate. Tubes were immediately centrifuged at 10,000 × g (15 min at 4 °C), and supernatant along with dibutylphalate layer were aspirated out. Traces of the solution on the inner wall were removed by cotton swab saturated with ethyl alcohol. The cell pellet was lyzed by brief freezing and thawing (−70 and 37 °C) and deproteinized by addition of 0.5 ml of trichloroacetic acid (5% w/v) followed by centrifugation at 10,000 × g (10 min at 4 °C). The radioactivity in the supernatant was measured after transferring 400 μl of supernatant to the 10 ml of water-based scintillation fluid (Aquasol) in LKB, 1209 Rack-beta liquid scintillation counter. For parallel control samples, [14C]putrescine influx was determined by subtracting values obtained by carrying out incubations in the presence of 1.2 mM putrescine (25).

**Synthesis of N1,N4-bis-[7-chloroquinoline-4-yl]butane-1,4-diamine**—To a stirred putrescine-free base (0.088 g, 1 mmol), 4,7-dichloroquinoline (0.396 g, 2 mmol) at room temperature was added. The reaction mixture was stirred at 140−145 °C for 4 h and then allowed to attain room temperature. The solid residue was washed with cold ether several times to remove unreacted substrates. The solid residue, free from the starting material, was triturated with 2 ml HCl (5 ml solution), cooled in an ice bath, filtered, washed with water, and air-dried. The product was finally washed with ether to give 0.262 g (64%) of colorless BCBD, m.p. 340 °C (Fig. 1).

The data are as follows: yield 64%; m.p. 340 °C; IR (KBr) cm−1: 3450, 3240, 3080, 3040, 2970, 1610, 1580, 1560; 1H NMR (DMSO-d6), 400 MHz: 1.85 (s, 4H), 3.30, 3.78 (m, 4H), 6.88 (d, 2H), 7.68 (d, J = 8 Hz, 2H), 7.92 (s, 2H), 8.45 (m, 2H), 8.65 (d, J = 8 Hz, 2H), 9.62 (br, 2H); m/z 411 (M+). Anal. Calcd for (C22H20Cl2N4) (M): 411.1 g/mol. Calc: C, 64.23; H, 4.87; N, 13.63. Found: C, 63.83; H, 4.79, N, 13.52.

**Microtiter Cultures Test for in vitro Antimalarial Activity**—The in vitro antimalarial activity of BCBD was evaluated using short term culture of P. knowlesi (quotidian periodicity in vivo as well as in vitro) (26). In brief, P. knowlesi-infected cells synchronized at predominantly ring stage were obtained from infected monkey and suspended in the culture medium (RPMI supplemented with 10% normal monkey serum) to get a final concentration of 6% hematocrit and 1.5% parasitemia. Test compounds were dissolved in ethanol:water (1:1) solution and further diluted with culture medium. After initiation of the culture, test compounds [1H]hypoxanthine (2 μC/ml) were added to each well as the culture plate was kept at 37 °C in candle jar module. After incubation for 20–22 h, the culture was harvested on glass membrane filter using automatic cell harvester (PBD™, Cambridge, MA). Filters were transferred to Triton-based scintillation fluid (333 ml Triton X-100, 5.5 g 2,5-diphenyloxazole, 0.1 g 1,4-bis-[2-(5-phenyloxazolyl)] benzene, and 100 ml of toluene), and radioactivity was counted in an LKB, 1209 Rack-beta liquid scintillation counter.

**In Vivo Antimalarial Activity**—The multidrug-resistant Plasmodium yoelii nigeriensis strain maintained in Swiss mice was used to evaluate the in vivo antimalarial activity (27). Four-week-old Swiss albino mice of either sex weighing 20–22 gm were infected with P. yoelii (1 × 106 infected erythrocytes) intraperitoneally. The BCBD was given intraperitoneally in 0.2 ml of water:ethanol mixture twice daily from days 0 to 3. Degree of parasitemia was recorded from Giemsa-stained thin blood smears (28). The animals which did not develop patent infection
was again different (crease in the polyamine levels though the pattern of increase was different (7.0 fold) and spermine (3-fold), and putrescine also appeared as a new molecule. At trophozoite stage-infected erythrocytes, significant increase was found in all the three polyamines though the pattern of increase was different (−3.5-fold for putrescine, −2.7-fold for spermine, and −2.11-fold for spermine) as compared with ring stage-infected erythrocytes. Similarly, schizont infected erythrocytes showed further increase in the polyamine levels though the pattern of increase was again different (−2.5-fold for spermidine, −2.41-fold for putrescine, and −1.21-fold for spermidine). These observations clearly demonstrate stage-specific increases in the polyamine levels during I-ES of *P. knowlesi*.

In our previous studies, increased putrescine influx was observed in *P. knowlesi*-infected erythrocytes as compared with normal erythrocytes (30). Fig. 2 showed that putrescine influx was quite low at 4 °C in normal erythrocytes (0.5 nmol/10^10 erythrocytes after 5 min of incubation) which did not increase significantly even after 30 min of incubation. In contrast, at 37 °C, putrescine influx increased linearly with time, reaching up to 2.0 nmol/10^10 erythrocytes after 20 min. In infected erythrocytes, putrescine influx was still very low at 4 °C (0.75 nmol/10^10 erythrocytes after 20 min). However, at 37 °C, there was a steep linear increase in the putrescine influx, which reached 31.6 nmol/10^10 erythrocytes after 15 min, *i.e.* more than 11-fold higher as compared with that observed in normal erythrocytes.

The concentration-dependent influx of putrescine was investigated in normal and infected erythrocytes after 2 min of incubation at 37 °C (Fig. 3). Data obtained from concentration-dependent putrescine influx curve were computed in the Lineweaver-Burk plot using SigmaPlot 5.0, and parameters obtained using axis to axis regression analyses were *K*ₐ = 34.6 ± 3.8 μM and *V*ₘₐₓ = 4.21 nmol/min/10^10 erythrocytes and *K*ₘ = 37.2 ± 5.2 μM and *V*ₘₐₓ = 11.6 nmol/min/10^10 erythrocytes for normal erythrocytes and infected erythrocytes, respectively.

**Table I** Polyamine profile in normal and different developmental stages of *Plasmodium knowlesi* infected monkey erythrocytes

| Sample                  | Polyamine | Polyamine | Polyamine |
|-------------------------|-----------|-----------|-----------|
| Normal erythrocytes     | ND        | 26.8 ± 3.4| 11.9 ± 1.6| 2.24      |
| Ring infected erythrocytes | 31.1 ± 4.9| 204 ± 10.8| 42.7 ± 5.0| 4.76      |
| Trophozoite infected erythrocytes | 111 ± 18.7| 556 ± 31.1| 90.3 ± 10.7| 6.17      |
| Schizont infected erythrocytes | 262 ± 16.2| 693 ± 59.8| 228 ± 23.2| 3.03      |

Therefore, observations from these studies indicate that parasite-induced putrescine transport system was highly specific for putrescine. Further competition studies were carried out with the –SH group interfering molecules and metabolic inhibitors to investigate the possibility of carrier-mediated process of putrescine influx in the infected erythrocytes. –SH group blockers, *p*-chloromercuri benzoate (pCMB) and N-ethylmaleimide (N-EM) inhibited the putrescine influx up to 85 and 93%, respectively, as compared with control (Table II). Involvement of –SH group in the putrescine influx process was further confirmed when pCMB and/or N-EM coincubated in presence of dithiothreitol restricted the effect of pCMB and/or N-EM in the putrescine influx process. In the presence of potassium cyanide, sodium arsenate, iodoacetic acid, and dinitrophenol in the incubation medium, the process of putrescine influx was inhibited significantly and maximum effect (82%) was observed with dinitrophenol.

Putrescine influx inhibition by BCBD was carried out by Michaelis-Menten approach. Data obtained from influx inhibition curve were computed in the Lineweaver-Burk plot using SigmaPlot 5.0, and parameters obtained using axis to axis regression analysis were *K*ᵢₐ = 43.2 μM with mixed competitive inhibitory pattern (Fig. 4).

Effect of BCBD of *in vitro* growth of *P. knowlesi* was carried out by means of ³Hhypoxanthine incorporation, and thereafter, concentration response data were fitted to generalized sigmoid function using nonlinear regression (31). Chloroquine as a reference gave IC₅₀ of 10.3 ± 0.45 ng/ml (Fig. 5A), and BCBD yielded IC₅₀ value of 7.64 ± 0.97 ng/ml (Fig. 5B).

Effect of BCBD in combination with different polyamines during short term culture of *P. knowlesi* were determined using...
Dine partially inhibited the process. Spermine inhibited \(^{3}H\)hypoxanthine incorporation by 67% compared with corresponding \(^{3}H\)hypoxanthine incorporation assay (Fig. 6). Putrescine did not affect the \(^{3}H\)hypoxanthine incorporation, while spermidine partially inhibited the process. Spermine inhibited \(^{3}H\)hypoxanthine incorporation by 67% compared with corresponding

**FIG. 3.** Concentration-dependent kinetics of putrescine influx in normal and \(P.\) knowlesi-infected erythrocytes. Values given are mean ± S.D. of three sets of experiments performed in duplicate. **Insert** shows the mean of the data obtained from substrate saturation curve plotted in Lineweaver-Burk plot 1/v (substrate concentration) versus 1/v (velocity).

**TABLE II**

| Effector (Conc. 1 mM) | Putrescine influx % of control |
|----------------------|-------------------------------|
| **Part-I**            |                               |
| Amino Acids           |                               |
| Serine (A)            | 84 ± 9.1                      |
| Leucine (L)           | 86 ± 13                       |
| Lysine (Lγ)           | 106 ± 11                      |
| Aspartic Acid (β)     | 92 ± 16                       |
| **Part-II**           |                               |
| Polyamine & related molecules |                  |
| Spermidine            | 62 ± 9.8                      |
| Spermine              | 69 ± 10                       |
| DFMO                  | 106 ± 16.2                    |
| MGBG                  | 39 ± 14.7                     |
| **Part-III**          |                               |
| -SH Group Interference|                               |
| N-Ethyl maleimide     | 15 ± 3.8                      |
| p-Chloromercuribenzoic acid | 17 ± 9.7                   |
| Dithiothreitol + N-Ethyl maleimide | 69 ± 14              |
| Dithiothreitol + p-Chloro Mercuric Benzoate | 91 ± 28            |
| **Part-IV**           |                               |
| Metabolic inhibitor   |                               |
| Dinitrophenol         | 18 ± 8.9                      |
| Potassium Cyanide     | 22 ± 7.2                      |
| Sodium Arsenate       | 37 ± 14                       |
| Iodicotic Acid        | 42 ± 16                       |

\[^{3}H\]hypoxanthine incorporation assay (Fig. 6). Putrescine did not affect the \[^{3}H\]hypoxanthine incorporation, while spermidine partially inhibited the process. Spermine inhibited \[^{3}H\]hypoxanthine incorporation by 67% compared with corresponding

control. Therefore, high concentration of spermine seems to be toxic to the parasite. BCBD inhibited the nucleic acid synthesis up to 58% at 25 μM concentration. Coincubation of putrescine or spermidine failed to reverse the effect of BCBD. However, cytotoxic effect of spermine against \(P.\) knowlesi culture in combination with BCBD produced an additive effect on nucleic acid synthesis of the parasite.

The in vivo effect of BCBD against infections with multidrug-resistant (MDR) strain of \(P.\) yoelii nigeriensis in Swiss albino mice was studied at two dose levels (Group 1, vehicle control; Group 2, 12 mg/kg body wt, twice daily, 0–3 day; and Group 3, 24 mg/kg body wt, twice daily, 0–3 day) (Table III). The results on day 4 showed that no parasitemia appeared in any of the BCBD-treated groups; whereas in vehicle control, all mice developed patent infection and average parasitemia was 9.5 ± 3.68%. All group 2 mice were negative till day 7 but subsequently developed patent infection while group 3 mice did not develop any patent infection up to observed period of day 28. Therefore, 96 mg/kg total dose treatment (group 2) suppresses the development of the parasitemia in \(P.\) yoelii-infected mice, and mean survival time was increased while 192 mg/kg total dose treatment of BCBD was found curative. LD\(_{50}\) value of BCBD determined after intraperitoneal administration in Swiss mice was 430 mg/kg body wt (confidence limit 265–698 mg/kg body wt).

**DISCUSSION**

Polyamines are known to play an important role in cell proliferation. We have, therefore, investigated how each specific polyamine is involved with stage-dependent growth and multiplication of malarial parasite during intraerythrocytic schizogony and the polyamine based regulatory mechanism of the intra-erythrocytic parasite proliferation. Analysis of elevated polyamine levels in terms of spermidine/spermine (SPD/SPM) ratio, which is indicative of cell proliferation (32), revealed that this ratio in normal erythrocytes was 2.24. However, after invasion of malarial parasite, increase in the polyamine profile was observed, and this ratio reached to 4.76 in virus-infected erythrocytes. A threshold level of SPD/SPM (6.17) was attained in trophozoite-infected erythrocytes. It is not surprising that it is maximal at trophozoite stage because major molecular synthesis occurs during this stage (33) and resembles the late G1 phase in eukaryotic cell cycle. Though maximum level of polyamines were found in schizont-infected erythrocytes, there was decrease in the SPD/SPM (3.03) due to
a specifically higher increase in spermine profile as compared with spermidine. Studies carried out with malarial parasite α-DNA polymerase and polyamines showed that in vitro addition of polyamines (1 mM putrescine, 1.5–2.0 mM spermidine, and 0.1–0.3 mM spermine) in the presence of 2 mM MgCl₂ increases the P. falciparum α-like DNA polymerase activity many-fold. But in contrast to these observations, higher concentrations of spermine (0.5–1.0 mM) inhibit the enzyme activity (more than 90%) (10). Therefore, terminal lowering of SPD/SPM in schizont-infected erythrocytes may lead to arrest of DNA synthesis at the end of the multiplication phase during the intraerythrocytic malarial parasite growth cycle and corresponds with the completion of multiplication phase during I-ES of P. knowlesi. This is further supported by our findings that at ring stage of the P. knowlesi culture, addition of spermine to the culture medium arrest the nucleic acid synthesis ([³H]hypoxanthine incorporation assay) while the same concentration of putrescine and spermidine do not interfere with the process (Fig. 6). Therefore, it can be concluded that during I-ES of malarial parasite, elevated concentrations of spermidine and spermine act as positive and negative regulator, respectively. Further, putrescine that acts as a precursor for spermidine and spermine is not detected in normal erythrocytes while it appears in ring-infected erythrocytes also as its biosynthetic enzyme, i.e., ODC. Inhibition of ODC by DFMO arrest the culture growth at the trophozoite stage (8). This indicates the importance of putrescine for proliferation of the malarial parasite.

Characterization of malarial parasite-induced putrescine transport system in trophozoite-infected erythrocytes explains the biochemical mechanism by which addition of exogenous putrescine reverses the α-DFMO inhibition of P. falciparum (7–9) and in vivo refractoriness of blood stage malarial parasite infection to α-DFMO (13). In recent years, considerable interest has been generated in elucidating the structure, mechanism, and regulation of the polyamine transport system due to its major role in the homeostasis of intracellular pool (16, 17). No molecular characterization of polyamine transport beyond influx kinetics and competition studies has yet been reported, and the exact mechanism of transport as well as variety of transport systems involved is still debated (34). We found that enhancement in the rate of putrescine influx in infected erythrocytes was due to a marked increase in the corresponding Vₘₐₓ value rather than to a significant decrease in the affinity constant. These results seem to indicate that increased requirement of polyamines for rapid growth and multiplication of intraerythrocytic malarial parasite give rise to a compensatory mechanism producing a remarkable induction of putrescine transporter synthesis. In most mammalian cell types examined for polyamine transport, it has been shown that induced polyamine transport is marked by increase in the influx rate without affecting the affinities of the substrate to the transporter (35–38). Energy dependence, involvement of –SH group, and noninterference by amino acid, spermidine, and spermine in the putrescine influx process clearly demonstrate the presence of a distinct transporter for putrescine in malarial parasite-infected erythrocytes.

The presence of a malarial parasite-induced specific transport system for putrescine in the infected erythrocytes suggests that required putrescine for growth and multiplication of the parasite can be obtained from the external milieu, and failure of biosynthetic inhibitors in such organisms is not surprising. Therefore, blockage of polyamine transport or replacement of natural polyamines by their analogues may constitute an effective strategy for the development of chemotherapeutic molecules (39). For the development of new chemotherapeutic molecules, we have used three different approaches. First, diamine [HₓN(CH₂)ₓNH₃] and structural homologs of spermidine [HₓN(CH₂)ₓNH₄(CH₂)₅NH₄] antagonize putrescine and spermidine influx in mammalian cells, and available evidence suggests that a minimum of four methylene groups (x or x + y = 4) or their equivalent are necessary for efficient interaction with the polyamine transport system (40–43). Second, conjugates of transporter substrate and other drugs have been used as a means of enhancing the intracellular access of the conjugated
drug. This approach has been applied to the polyamine transporters, polyamine-nitroimidazole conjugate, and polyamine-chlorambucil conjugate and has been shown to augment drug accumulation in Erlich-asites tumors cells with a resulting enhancement of cytotoxicity (44, 45). Finally, we have found that chloroquine shares the parasite-induced putrescine transport system in P. knowlesi-infected erythrocytes (46). We have succeeded in designing and synthesizing BCBD using the combined knowledge of all the three approaches mentioned above.

Putrescine influx in P. knowlesi-infected erythrocytes was inhibited by BCBD in a dose-dependent manner, and the cytotoxic concentration of growth of intraerythrocitic parasite (IC50 value of 7.6± 0.94 ng/ml) was found to be many-fold lower than other known polyamine analogues or biosynthetic inhibitors (47–49). The novelty of this approach is that inhibitory effect of BCBD on the parasite growth and multiplication in the culture medium is known polyamine analogues or biosynthetic inhibitors (47–49). The novelty of this approach is that inhibitory effect of BCBD on the parasite growth and multiplication in the culture medium is known polyamine analogues or biosynthetic inhibitors (47–49).

Putrescine Transport Inhibitor

| Dose mg/Kg body wt | Route | No. of mice | day 4 parasitemia | No. of mice did not develop patent infection till day |
|--------------------|-------|-------------|-------------------|--------------------------------------------------|
| Group-I Vehicle Control | i.p.  | 10          | 9.55 ± 3.68       | 0/10                                             |
| Group-II 12 mg     | i.p.  | 16          | nil               | 16/16                                            |
| Group-III 24 mg    | i.p.  | 8           | nil               | 8/8                                              |
|                   | i.p.  | 6           | nil               | 6/6                                              |

*The dose was given twice daily × 4 days (0–3 day).

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