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ASSOCIATION OF NUCLEAR MEMBRANE PROTEIN LAMIN B1 WITH NECROSIS AND APOPTOSIS IN CELL DEATH INDUCED BY 5-FLUORO-2′-DEOXYURIDINE

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□ *We report that anticancer 5-fluoro-2′-deoxyuridine (FUdR) shows cytotoxicity against mouse cancer cell line FM3A, using a progeny clone F28-7 and its variant F28-7-A. In this process, the cell-death morphology is different between F28-7 and F28-7-A cells, that is, necrosis in F28-7 but apoptosis in F28-7-A cells. In the proteomic analysis of these cells before their exposure to FUdR, the nuclear inner-membrane protein lamin B1 is up-regulated in F28-7 but not in F28-7-A, suggesting that lamin B1 may possess a function to regulate the morphology of cell-death. A knockdown of lamin B1 expression in F28-7 cells was performed by use of the small interfering RNA technique, resulting in a decrease of the lamin B1-expression level down to the level in F28-7-A. Remarkably, the FUdR-induced death morphology of this knocked-down F28-7 was apoptosis, definitely different from the necrosis that occurs in the FUdR-treated original F28-7. Thus, the swelling feature for the necrosis was no longer observable, and instead cell shrinkage typical of apoptosis took place in almost all the cells examined. This finding suggests a new role for lamin B1 as a regulator in cell death.*

Keywords 5-Fluoro-2′-deoxyuridine; necrosis; apoptosis; lamin B1

INTRODUCTION

Two general pathways for cell death have been defined, necrosis and apoptosis. Depending on cell type, cellular context, or stimulus, a cell is destined to necrosis or to apoptosis.[1] Necrosis is characterized by swelling of the cell accompanying enlargement of the organelles in it, followed by disruption of the cell membrane, resulting in cell lysis.[1,2] In contrast,

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apoptosis is morphologically characterized by membrane blebbing, shrinking of the cell and its organelles, and internucleosomal DNA degradation, followed by disintegration of the cell.[3,4] However, previous studies of Leist and Nicotera[5] and Kakutani et al.[6] suggest that some early events in the death program may be common in the two types of cell death, and that downstream events may contribute to the guiding of cells toward necrosis or apoptosis. It is important to elucidate how a cell is guided toward either necrosis or apoptosis.

Recent studies indicate that signalling pathways, such as death receptors, kinase cascades, and mitochondria participate in both of these cell-death processes.[7] We have now explored the possibility that by modulating these pathways, a switch between necrosis and apoptosis may occur.

5-Fluoro-2′-deoxyuridine (FUdR), a potent anticancer agent, exerts its effect by inhibiting thymidylate synthase, an essential machinery for DNA synthesis in cell proliferation.[8,9] In our previous study on the action of FUdR against mouse mammary tumor FM3A cell line, using the original clone F28-7[6] and its variant F28-7-A cells,[6] we noticed that the FUdR-treatment can induce in F28-7 cells a breakdown of DNA into chromosome-sized fragments leading to necrosis, but, on the other hand, in F28-7-A a more extensive DNA cleavage into oligonucleosome-sized fragments and subsequent development of apoptosis are observed.[6] However, the process of regulation in the FUdR-induced necrosis and apoptosis are still unclear. Recently, we investigated the pattern of differentially expressed proteins in these cells by the proteomic analysis using two-dimensional gel electrophoresis and mass spectrometry. With this analysis, it was shown that at the untreated stage the nuclear membrane-constituent protein lamin B1 is 2.4-fold higher in F28-7, compared with F28-7-A cells (results to be published elsewhere). Lamin B1 is one of the nuclear lamins and a key structural component of the nuclear lamina, an intermediate filament meshwork that lies beneath the inner nuclear membrane. It is known that the nuclear lamins play a crucial role in fundamental cellular processes, including nuclear organization, chromatin segregation, DNA replication, and gene expression.[10–14]

Here, we report that lamin B1 may regulate necrotic and apoptotic morphology induced by FUdR. Thus, when a knockdown of lamin B1 expression in F28-7 cells by small interfering RNA (siRNA) was performed, the upregulation of lamin B1 disappeared in these cells. We then explored whether this lack of upregulation may affect the FUdR-induced cell death morphology. Remarkably, the cell death morphology was no longer the necrosis but it took a distinctly apoptotic feature. These findings suggest an interesting possibility that lamin B1 regulates the cell death pattern.

MATERIALS AND METHODS

Cells, Cell Culture, and Reagents

The two cell clones derived from a mouse mammary tumor FM3A cell line, the wild-type F28-7 and its variant F28-7-A cells, used in this study have been described previously.[6] The cells were cultured in ES medium (Nissui Pharmaceuticals, Japan) supplemented with 2% fetal bovine serum (Gibco, USA) and 0.03% L-glutamine (Wako, Japan) in a humidified atmosphere with 5% CO_2 at 37°C. Under these conditions, the doubling time was approximately 12 hours. Cell viability was estimated with a hemocytometer by means of trypan blue dye-exclusion.

5-Fluoro-2′-deoxyuridine was obtained from Sigma (USA). The lamin B1-siRNA was Mm_Lmnb1_1_HP siRNA, Catalog number: SI01090831, Sense; r(CGU UGU AAG AUG UGA AUU A)dTdT, Antisense; r(UAA UUC ACA UCU UAC AAC G)dTdG, and the nonsilencing siRNA was AllStars Negative Control siRNA, Catalog number: 1027280, Sense; unpublished, Antisense; unpublished. Both were purchased from Qiagen (Germany). The primary antibodies; mouse monoclonal anti-lamin-B1 and rabbit polyclonal anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were from Zymed Laboratories (USA) and Trevigen (USA), respectively. The secondary antibodies; anti-mouse IgG horseradish peroxidase-linked whole antibody and anti-rabbit IgG horseradish peroxidase-linked whole antibody were from GE Healthcare (UK). 4′,6-Diamidino-2-phenylindole dihydrochloride (DAPI) was purchased from Invitrogen (USA).

Transfection

Exponentially growing 2×10^5 F28-7 cells were suspended in 75 μl siPORT electroporation buffer (Ambion, USA) containing lamin B1 or nonsilencing siRNA (final concentration 1×10^{-7} M) and introduced into 0.1 cm gap electroporation cuvette (Bio-Rad, USA). Cells were then electroporated using the Bio-Rad Gene Pulser Xcell at voltage 0.15 kV, pulse length 1,000 μs, and number of pulse 1. After electroporation, cells were plated at 5×10^4 cells/ml in fresh ES medium in tissue culture flasks. Forty-eight hours after the electroporation, cells were used for further experiments.

Western Blot Analysis

Cells were washed in phosphate-buffered saline (PBS) and then whole cell lysates were prepared using Laemmili sample buffer (Bio-Rad). Proteins (5×10^4 cells equivalent per lane, respectively) were subsequently fractionated under reducing conditions by 7.5% SDS-polyacrylamide gel electrophoresis and blotted onto a polyvinylidene difluoride membrane

(Millipore, USA). The blotted membrane was blocked for 1 hour with 5% bovine serum albumin in Tris-buffered saline with 0.1% Tween 20 and then immunoblotted overnight at 4°C using the respective primary antibody. The membrane was then incubated for 1 hour at room temperature with a horseradish peroxidase-conjugated anti-mouse IgG or anti-rabbit IgG secondary antibody, and the protein bands were visualized using an ECL plus western blotting detection system (GE Healthcare). Protein expression was quantified using VersaDoc imaging system (Bio-Rad). The following antibodies were used: anti-lamin B1 antibody (1:1,500), anti-GAPDH antibody (1:10,000), anti-mouse IgG horseradish peroxidase-linked whole antibody (1:20,000), anti-rabbit IgG horseradish peroxidase-linked whole antibody (1:20,000).

Morphological Changes

Cells were washed with PBS and then fixed with 4% paraformaldehyde in PBS at 4°C for 30 minutes. Then, the fixed cells were washed with PBS and stained with DAPI in PBS. The stained cells were spread on a slide and mounted with coverslips. Cell morphology was observed by Olympus BX61 fluorescence microscopy.

RESULTS AND DISCUSSION

In our proteomic analysis, lamin B1 was identified as an up-regulated protein in F28-7, in contrast to the normal level in the variant cell strain F28-7-A (results to be published elsewhere). Recent studies from several laboratories indicate that lamin B1 and other lamins play a role in nuclear architecture, DNA replication, and gene expressions.[10–14] The possibility that lamin B1 may be associated with the differential patterns of cell death morphology observable in the treatment with FUdR is now investigated. The Western blot analysis of the cells before the treatment with FUdR revealed that lamin B1 protein level is 1.8-fold higher in F28-7, in comparison to that in F28-7-A cells (Figure 1A). Thus, the greater enhancement in the F28-7 over the F28-7-A observed in the proteomic analysis was confirmed by this Western blot analysis. To test if a downregulation of endogenously expressed lamin B1 in F28-7 cells can modulate FUdR-induced necrosis, we carried out a knockdown of the expression in F28-7 cells by using lamin B1 siRNA. Transfection efficiencies at higher than 80% were obtained by the electroporation (data not shown). Western blot analysis performed for cell extracts at 48 hours after the transfection indicates that the siRNA treatment resulted in a reduction of the lamin B1 protein levels, while the GAPDH protein levels as a control showed no change (Figure 1B). The level of lamin B1 protein expression in F28-7 became the same as that in F28-7-A cells. Another control experiment in which a nonsilencing siRNA

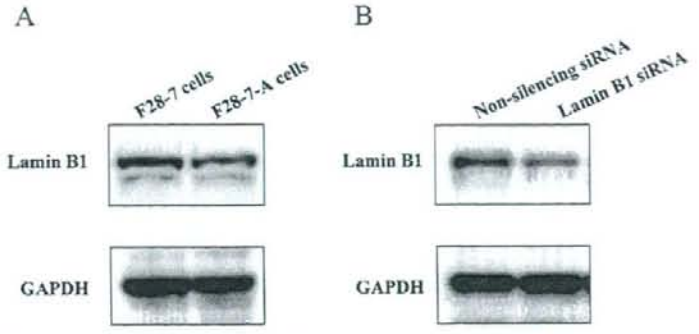

FIGURE 1 Knockdown of lamin B1 by RNA interference. A) Whole cell lysates were prepared from F28-7 and F28-7-A cells. Expression of lamin B1 and GAPDH proteins were examined by Western blot analysis. Expression of GAPDH was used as an internal control. The patterns shown are results obtained in one set of experiments. Three independent sets of experiments were performed gaving similar results. B) F28-7 cells were transfected with nonsilencing siRNA, and lamin B1 siRNA. Forty-eight hours after the transfection, the levels of expression of lamin B1 and those of GAPDH were examined by Western blot analysis.

was administered showed no effect on the expression of lamin B1 or GAPDH (Figure 1B). The cell viability at 48 hours after the transfection was 98% with the vehicle, 98% with the nonsilencing siRNA, and 96% in the lamin B1 siRNA-trensfected cells. In addition, nonsilencing siRNA and lamin B1 siRNA themselves had no impact on the cell morphology, if no FUdR administration is performed (Figure 2, upper diagram). The knockdown of lamin B1 in the F28-7 cells did not change the cell viability, if FUdR was not given.

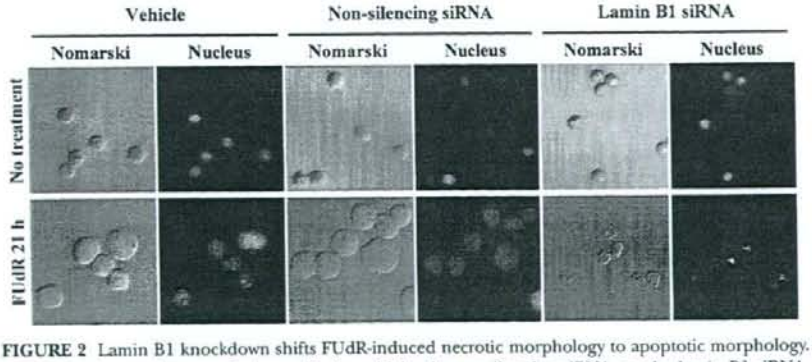

FIGURE 2 Lamin B1 knockdown shifts FUdR-induced necrotic morphology to apoptotic morphology. Forty-eight hours after transfection with the vehicle, the nonsilencing siRNA, or the lamin B1 siRNA, the F28-7 cells were treated with or without 1×10^{-6} M FUdR for 21 hours and then stained with DAPI as described under Materials and Methods. Morphological changes were analyzed under a fluorescence microscope at 400 × magnification. The results are typical of the results of three experiments that gave similar results.

Next, we explored the morphology in the lamin B1-knockdown F28-7 cells on treatment with 1×10^{-6} M FUdR. At 21 hours after the treatment was initiated, the controls given vehicle or nonsilencing siRNA showed the cytoplasmic swelling, a hallmark for necrosis. The lamin B1 siRNA transfected cells, in contrast, showed a typical apoptotic morphology; the membrane blebbing and the formation of apoptotic bodies (Figure 2, at the bottom). At this period of treatment, the cell viability was 26% with the vehicle, 24% with the nonsilencing siRNA, and 23% with the lamin B1 siRNA transfection. Furthermore, almost all the dyeing cells underwent apoptosis after the lamin B1 siRNA transfection with subsequent FUdR treatment. These observations suggest that lamin B1 participates in modulating the process of the FUdR-induced necrosis and apoptosis. It would be important to further investigate the mechanisms involved in the lamin B1-control of the necrosis and apoptosis in the process of FUdR treatment. Our present work may contribute to the understanding of the mechanisms regulating necrosis and apoptosis.

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ORIGINAL ARTICLE

Polymorphisms of cytochrome *b* gene in *Leishmania* parasites and their relation to types of cutaneous leishmaniasis lesions in Pakistan

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ABSTRACT

The exact species and/or strains of *Leishmania* parasites involved strongly influence the clinical and epidemiological features of leishmaniasis, and current knowledge of those influences and relationships is inadequate. We report that cytochrome *b* (*cyt* b) gene sequencing identified causal *Leishmania* parasites of 69 cutaneous leishmaniasis cases in Pakistan over a 3-year period. Of 21 cases in highland areas (Quetta city, Balochistan province), 16 (76.2%) were identified as *Leishmania (L.) tropica* and five (23.8%) as *Leishmania (L.) major*. Of 48 cases from lowland areas, cities/villages in Indus valley in Sindh and Balochistan provinces, 47 (97.9%) were identified as *L. (L.) major* and one (2.1%) as *L. (L.) tropica*. Statistical analysis (Fisher's exact test) revealed a significant difference ($P < 0.0001$) in the distribution of the two species by altitude; *L. (L.) major* is predominant in lowland and *L. (L.) tropica* at highland areas. The present result enriched our earlier finding, based on the first year's cultured parasite data, that only *L. (L.) tropica* was found in highland areas and only *L. (L.) major* in lowland areas. Among *Leishmania* samples analyzed, three types of *cyt* b polymorphism of *L. (L.) major* were found, including 45 (86.5%) cases of type I, six (11.5%) of type II and one (2%) of type III. We report for the first time on the presence of polymorphisms in *L. (L.) major* (types I, II and III) based on species identification using *cyt* b gene sequencing from clinical samples. Moreover, we found no correlation between clinical presentation (wet-, dry- and/or mixed-types of cutaneous lesions) and causal *Leishmania* parasites.

Key words: cutaneous leishmaniasis lesions, *cyt* b gene sequencing, *Leishmania (L.) major* polymorphisms, *Leishmania (L.) tropica*, Pakistan.

INTRODUCTION

Leishmaniasis is the result of infection with intracellular protozoan parasites belonging to the genus *Leishmania*.[1] It affects more than 12 million people in 88 countries, with 350 million people at risk; every year there are 1–1.5 million new cases of cutaneous leishmaniasis (CL) and 0.5 million cases of visceral

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leishmaniasis (VL).[2] The appearance of clinical features depends on the complex interactions resulting from the invasiveness, tropism and pathogenicity of the parasite and the immune response of the host.[3] The epidemiology of leishmaniases is extremely diverse and far from fully elucidated.[4] More than 20 species of *Leishmania* parasites are known to infect humans and approximately 30 species of sandfly to transmit the disease.[2]

Pakistan, a tropical and subtropical country located in the northwest of South Asia, is an endemic area for leishmaniasis. The country is divided into four provinces, namely the North West Frontier Province (NWFP), Punjab, Sindh and Balochistan.[5] VL and CL are more common in Pakistan than are mucocutaneous and diffuse cutaneous leishmaniasis.[5–10] VL, considered deadly if untreated, mainly occurs in the northern region of the country, in areas such as Baltistan district, Chilas district, Azad Jammu and Kashmir, although reported sporadically in other areas of NWFP, Balochistan and Punjab provinces.[11]

In Pakistan, CL is popularly known as oriental sore, Delhi boil, Baghdad boil and Quetta sore.[5–7] Most of the skin lesions are of the wet-type and caused by *Leishmania (L.) major* which is endemic in NWFP and Balochistan province.[7] Some patients from the city of Multan in Punjab province presented with dry-type lesions, which was taken to indicate that only *Leishmania (L.) tropica* was present in that area.[8] Certain districts (Jacobabad, Larkana and Dadu) in Sindh province were reported to be endemic areas for CL, with the presence of both wet- and dry-type lesions taken to indicate clinically the presence of both *L. (L.) tropica* and *L. (L.) major* in that region.[5]

The identification of *Leishmania* species is important not only from an epidemiological perspective but also on clinical grounds in order to select diagnostic methods, plan treatment, define patient prognosis and monitor clinical outcomes. Accurate identification of the parasites must be based on molecular approaches because parasitological, clinical or epidemiological features by themselves are insufficient for the task.[4,12–14] One molecular technique, polymerase chain reaction (PCR), can quickly give exact diagnoses, which can reduce working hour losses, costs and social suffering of the patients.[14]

Among the molecular methods applied in *Leishmania* species identification, DNA-based techniques have been used increasingly. One of them, PCR amplification and sequencing of cytochrome *b* gene method (*cyt* b gene sequencing) has recently been established as a useful tool for the identification and phylogenetic study of the genus *Leishmania*, able to differentiate among human-infecting species and from other trypanosomatids.[15] Previous comparison of *cyt* b gene sequencing results for the cultured parasites with the split-specimen findings of other molecular techniques such as multi-locus enzyme electrophoresis (MLEE) analysis and polymorphism-specific (PS)-PCR showed total agreement among the results.[12,16] Those findings led us to use *cyt* b procedures for exploration of polymorphism in CL-causing *Leishmania* species from Pakistan.

In this study, we report for the first time the presence of polymorphisms in *L. (L.) major* (types I, II and III) based on species identification using *cyt* b gene sequencing in two different altitudes of Pakistan over a 3-year period. Moreover, we report an association between species and/or types of *Leishmania* and clinical presentation (dry-, wet- and mixed-types) of the skin lesions.

METHODS

Study areas and collection of sample

A total of 69 *Leishmania* biopsy specimens were obtained from 69 different subjects with cutaneous leishmaniasis in Pakistan. Individuals were residing in areas around Quetta city (Balochistan province), located 1600–1800 m a.s.l. in highland or mountainous regions, and in Sukkur city, Jacobabad district, Larkana district (Sindh province) and Jhal Magsi district, Jafar Abad district, Sibi city (Balochistan province) which are approximately 100 m a.s.l. and located in lowland regions of Pakistan. We diagnosed the clinical lesions according to textbook[1,3,17,18] criteria which were compiled and are described in Table 1. Thirty-one biopsy samples were collected in 2003, 28 in 2004, and 10 in 2005. The patients involved in the surveys were treated with meglumine antimonate and/or antibiotics by local physicians, depending on clinical diagnosis and clinical indications.[5]

Table 1. Characterization of types of cutaneous leishmaniasis lesions

	Dry type	Wet type
Lesion Course	Single, grow slowly and persist for a year or more. A small, brownish nodule appears which becomes a slowly expanding plaque, reaching 1–2 cm in about 6 months.	Multiple, mature more rapidly, lasting a few months. – Red furuncle-like nodule. – After 2 weeks, a central crust forms. – Crust may fall away to reveal the underlying ulcer. The raised, red margin enlarges in 2–3 months.
Healing	After 8–12 months, lesions regress and ulcers heals.	Heal in 2–6 months, leaving scars.

Mixed-type lesions combine or mix the features mentioned.

Each lesion was examined by a dermatologist, and those found to be consistent with CL were cleaned with soap and water and swabbed with ethanol before samples were taken; each patient gave informed consent for surgical biopsy. A sterile scalpel was used to make an incision in the border of the lesion; and each biopsy specimen was put immediately into a sterile screw-capped 2-mL tube containing 70% ethanol; the tubes were stored at room temperature and brought to Japan by the dermatologist for PCR and *cyt* b sequencing analysis.

Extraction of DNA from the clinical samples

From the above biopsy samples, DNA extractions were performed according to the protocol for extraction of DNA from the tissue which was described by the company (GenomicPrep Cells and Tissue DNA Isolation Kit, Amersham Biosciences, USA).

Polymerase chain reaction amplification

From the above genomic DNA extract, PCR amplification and *cyt* b gene sequencing-based identification of *Leishmania* parasites were carried out as follows. We performed PCR using 0.2 μL DNA polymerase Ex Taq (Takara, Japan) in a total PCR solution volume of 50 μL. Each PCR solution contained 1 μL of 100 to 200 ng/μL DNA template, *Leishmania cyt* b gene consensus primer (0.25 μL of LCBF1 forward primer [100 μM], 5′-taatacgactcactataGGTGTAGGTTTTAGTYTAGG-3′; and 0.25 μL of LCBR2 reverse primer [100 μM], 5′-gggttttcccagtcacgacgCTACAATAAACAAATCATAATATRCAATT-3′), 4 μL of 2.5 mM deoxyribonucleotide triphosphate (dNTP), 5 μL of 10X buffer and distilled water 39.3 μL. PCR conditions were initial denaturation at 94°C for 1 min, followed by 39 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min, extension at 72°C for 1 min, followed by a final extension of 72°C for 5 min.

For the samples which were not amplified by DNA polymerase Ex Taq, PCR using 0.5 μL DNA polymerase Phusion (High-Fidelity DNA Polymerase; Finnzymes, Espoo, Finland) in a total PCR solution volume of 50 μL were performed. Each PCR solution contained 1 μL of 100–200 ng/μL DNA sample template, 0.5 μL *Leishmania cyt* b gene consensus primer, 4 μL of 2.5 mM dNTP, 10 μL of 10X buffer and 34 μL distilled water. PCR conditions were initial denaturation at 98°C for 30 s, followed by 39 cycles of denaturation at 98°C for 10 s, annealing at 50°C for 30 s, extension at 72°C for 45 s, followed by a final extension of 72°C for 5 min.

For the samples which were not amplified by means of the above, we performed nested PCR by amplification with COIIIF (5′-taatacgactcactataGTTTATATTGACATTTTTGTWGATT-3′) and MURF4R (5′-gggttttcccagtcacgacgAATCTCTCTCTCCCTT-3′) primers, following which the resulting 1 μL of PCR products were amplified again with *Leishmania cyt* b gene consensus primers as mentioned for Ex Taq PCR. All PCR products were visualized by gel electrophoresis with 0.7% Agarose-LE, Classic type (Nacalai Tesque, Kyoto, Japan).

DNA purification and sequencing

The amplified DNA products were harvested by gel electrophoresis on 0.4% Seakam GTG agarose gel (FMC BioProduct, Foster City, CA, USA). The visualized DNA products were excised carefully and then purified by QIA Quick Gel Extraction kit (Qiagen, Valencia, CA, USA) according to the manufacturer's recommendation. Concentration of each purified DNA product was measured by Gene Spec

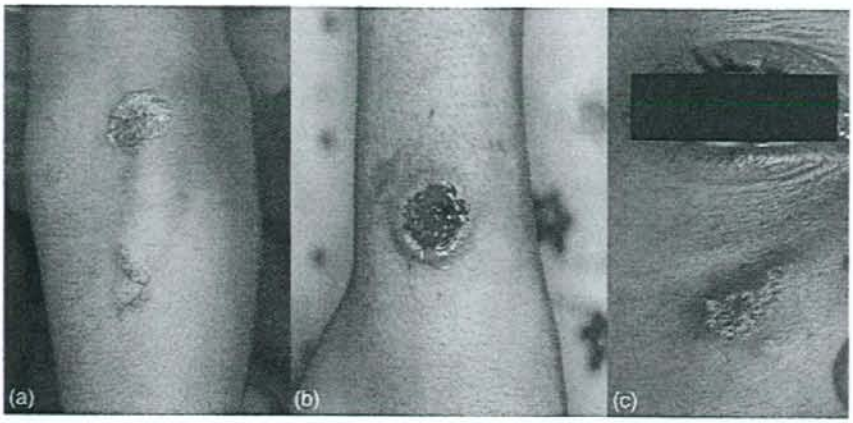

Figure 1. Clinical appearance of cutaneous leishmaniasis cases in Pakistan. (a) Dry-type (RU-51 in Table 2), (b) wet-type (RU-22 in Table 2), and (c) mixed-type (RU-12 in Table 2).

III (Naka Instruments, Ibaraki, Japan) and checked again with gel electrophoresis (0.7%) by comparing with a concentration-known DNA sample as positive control. DNA sequencing was carried out on an ABI PRISM-301 automated sequencer (Applied Biosystems, Foster City, CA, USA) by using the Big Dye terminator cycle sequencing ready reaction kit (Applied Biosystems). The following primers were used for sequencing: T7 (17 mer) 5′-AATACGACTCACTATAG-3′, U19 (19 mer) 5′-GGTTTTCCCAGTCACGACG-3′, LCYTB F4L (22 mer) 5′-TGTTATTGAATATGAGGTAGTG-3′ and LCYTBR4 (26 mer) 5′-GAACTCATAAAATAATGTAAACAAAA-3′.

Analysis of sequenced result

The sequenced results were assembled and edited by using Genetyx-Mac software ver. 11.0. (Software Development, Tokyo, Japan). They were compared with previously published reference strains, available from EMBL/DDBJ/GenBank, *L. (L.) major*, accession number AB 095970 and *L. (L.) tropica*, accession number AB 095960.

RESULTS

Clinical and epidemiological profiles of study patients

The patients were 45 males and 24 females (M : F, 2:1), ranging 1–50 years in age (mean age, 20 years). Thirteen patients were under 9 years old, 26 were 10–19 years old, 14 were 20–29 years old, six were 30–39 years, seven were 40–49 years and three were 50 years old. According to the patients' own statements, the evolution of their disease processes (in terms of the time since they had first noticed a lesion) ranged from 2 weeks to 3 years. Characteristics of the CL-patients and their clinico-epidemiological data are summarized in Table 2. Examples of the dry-, wet- and mixed-type clinical lesions of cutaneous leishmaniasis in cases included in the present study are shown in Figure 1.

Findings based on *cyt* b gene sequencing

The *cyt* b analysis showed 17 cases of *L. (L.) tropica* (16, 94.1%, in highland areas and one, 5.9%, in lowland areas) and 52 cases of *L. (L.) major* (47, 90.4%, in lowland and five, 9.6%, in highland), showing a significant difference ($P < 0.0001$, Fisher's exact test) in distribution of the two species by altitude; *L. (L.) major* is predominant in the lowlands, and *L. (L.) tropica* in highland areas (Fig. 2).

In this study, three types of *cyt* b polymorphism of *L. (L.) major* were identified. In the sequencing result of *L. (L.) major cyt* b, in what we have called *L. (L.) major* type I, cytosine (C) is replaced by thymine (T) at nucleotide positions 416 and 799. In what we call *L. (L.) major* type II, at nucleotide positions 416 and 799, C is replaced by T, and in nucleotide position 436,

Table 2. Detailed characteristics of cutaneous leishmaniasis samples and clinico-epidemiological profiles of the patients

Sample name	Species	Altitude	Skin Lesion site	Lesion no.	Lesion type	Lesion evolution (days)	Age (years)	Sex	Geographical origin of patient
RU 1	*L. (L.) mj* type I	L	Buttock	1	Dry	90	30	M	Sukkur, Arore
RU 2	*L. (L.) mj* type II	L	Rt arm	1	Dry	90	45	F	Sukkur, Khairpur
RU 3	*L. (L.) mj* type III	L	Rt arm	1	Mixed	180	38	M	Larkana, Sono Khan
RU 4	*L. (L.) mj* type I	L	Both arm	4	Dry	30	10	F	Larkana, Sono Khan
RU 5	*L. (L.) mj* type I	L	Lt arm, buttock, rt leg	3	Dry	30	12	F	Larkana, Warah
RU 6	*L. (L.) mj* type I	L	Rt arm	1	Dry	60	15	M	Larkana, Warah
RU 7	*L. (L.) mj* type I	L	Lt arm	1	Dry	NA	18	M	Sukkur, Pano Aqil
RU 8	*L. (L.) mj* type I	L	Rt leg	1	Dry	60	25	M	Jacobabad
RU 9	*L. (L.) mj* type I	L	Lt leg	1	Dry	NA	40	F	Sukkur, Khairpur
RU 10	*L. (L.) mj* type I	L	Lt upper arm	1	Dry	30	45	F	Larkana, Warah
RU 11	*L. (L.) mj* type I	L	Arm	1	Mixed	30	3	F	Larkana, Shahdadkot
RU 12	*L. (L.) mj* type I	L	Face	1	Mixed	60	7	F	Larkana, Shahdadkot
RU 13	*L. (L.) mj* type I	L	Face, rt leg	2	Mixed	60	10	M	Larkana, Kambar
RU 14	*L. (L.) mj* type I	L	Rt leg	1	Mixed	90	28	M	Larkana, Sono Khan
RU 15	*L. (L.) mj* type I	L	Both legs	3	Mixed	180	50	M	Larkana, Kambar
RU 16	*L. (L.) mj* type I	L	Ear, rt upper arms	2	Mixed	45	1	M	Larkana, Sono Khan
RU 17	*L. (L.) mj* type I	L	Face	2	Mixed	45	20	M	Larkana, Sono Khan
RU 18	*L. (L.) mj* type I	L	Both legs	3	Mixed	60	30	M	Larkana, Shahdadkot
RU 19	*L. (L.) mj* type I	L	Buttock	4	Dry	NA	7	M	Sukkur, Pano Aqil
RU 20	*L. (L.) mj* type I	L	Rt leg	1	Wet	30	2	M	Larkana, Sono Khan
RU 21	*L. (L.) mj* type I	L	Lt arm	1	Wet	60	2	M	Baluchistan, Jhal
RU 22	*L. (L.) mj* type I	H	Lt arm	2	Wet	60	10	F	Quetta city
RU 23	*L. (L.) mj* type I	H	Face	1	Wet	30	10	M	Quetta, Mari Abad
RU 24	*L. (L.) mj* type I	H	Lt leg	2	Wet	75	12	F	Quetta, Mari Abad
RU 25	*L. (L.) mj* type I	L	Both arms and trunk	3	Wet	60	15	M	Larkana, Warah
RU 26	*L. (L.) mj* type I	L	Both arms	2	Wet	15	15	M	Jacobabad
RU 27	*L. (L.) mj* type I	L	Lt arm	1	Wet	30	15	M	Jacobabad
RU 28	*L. (L.) mj* type I	L	Lt leg	1	Wet	60	20	F	Sibi City
RU 29	*L. (L.) mj* type I	L	Lt leg, buttock	4	Wet	60	20	M	Larkana, Warah
RU 30	*L. (L.) mj* type I	L	Face, rt arm	2	Wet	75	20	M	Larkana, Kambar
RU 31	*L. (L.) mj* type I	L	Both legs	2	Wet	60	20	M	Sukkur, Shikarpur
RU 32	*L. (L.) mj* type I	L	Lt leg	1	Wet	30	25	F	Baluchistan, Jhal
RU 33	*L. (L.) mj* type I	L	NA	1	Wet	90	25	M	Sukkur, Khairpur
RU 34	*L. (L.) mj* type I	L	Lt arm, both legs	4	Wet	30	28	M	Jacobabad
RU 35	*L. (L.) mj* type I	L	Chest, lt arm	3	Wet	30	40	M	Sukkur City
RU 36	*L. (L.) mj* type I	L	Lt arm, rt leg	3	Wet	60	50	F	Sukkur, Khairpur
RU 37	*L. (L.) mj* type I	L	Rt leg	1	Wet	30	8	M	Larkana, Warah
RU 38	*L. (L.) mj* type I	L	Lt arm	1	Wet	NA	10	F	Sukkur, Khairpur
RU 39	*L. (L.) mj* type I	L	Rt arm, both legs	3	Wet	NA	19	M	Sukkur, Khairpur
RU 40	*L. (L.) mj* type I	L	Lt leg	2	Wet	90	24	M	Larkana, Warah
RU 41	*L. (L.) mj* type I	L	Rt leg	4	Wet	60	25	M	Larkana, Warah
RU 42	*L. (L.) mj* type I	L	Rt leg	2	Wet	30	40	F	Larkana, Warah
RU 43	*L. (L.) mj* type II	L	Lt arm	1	Wet	NA	14	M	Baluchistan, Jhal
RU 44	*L. (L.) mj* type II	L	Both legs	4	Wet	60	18	M	Jacobabad
RU 45	*L. (L.) mj* type II	L	Both legs	4	Wet	60	19	M	Sukkur, Khairpur
RU 46	*L. (L.) mj* type II	L	Lt arm	1	Wet	NA	29	M	Sukkur, Shikapur
RU 47	*L. (L.) tr*	H	Face	2	Dry	360	3	F	Quetta, Mari Abad
RU 48	*L. (L.) tr*	H	Face	2	Dry	180	7	F	Quetta city
RU 49	*L. (L.) tr*	H	Rt ear	1	Dry	30	9	F	Quetta, Mari Abad
RU 50	*L. (L.) tr*	H	Lt leg	1	Dry	360	14	M	Quetta city
RU 51	*L. (L.) tr*	H	Both arms	3	Dry	1080	21	M	Quetta, Mari Abad
RU 52	*L. (L.) tr*	L	Lt arm	4	Dry	30	30	M	Baluchistan, Jhal
RU 53	*L. (L.) tr*	H	Lt arm	2	Mixed	180	18	M	Quetta city
RU 54	*L. (L.) tr*	H	Face	1	Wet	360	7	F	Quetta city
RU 55	*L. (L.) tr*	H	Face	2	Wet	60	14	M	Quetta, Samagali
RU 56	*L. (L.) tr*	H	Face, lt leg	2	Wet	60	16	F	Quetta, Mari Abad

Table 2. *Continued.*

Sample name	Species	Altitude	Skin Lesion site	Lesion no.	Lesion type	Lesion evolution (days)	Age (years)	Sex	Geographical origin of patient
RU 57	*L. (L.) tr*	H	Rt arm, rt leg	3	Wet	60	16	M	Quetta, Gorabad
RU 58	*L. (L.) tr*	H	Rt upper arm	1	Wet	60	30	M	Quetta, Mari Abad
RU 59	*L. (L.) tr*	H	Face, lt arm	2	Wet	360	50	M	Quetta, Mari Abad
RU 60	*L. (L.) mj* type II	L	Lt leg	4	Wet	75	10	F	Larkana, Gul Mohd
RU 61	*L. (L.) mj* type I	L	Rt leg	1	Wet	90	40	M	Larkana, Sarang Kumar
RU 62	*L. (L.) mj* type I	L	Lt arm, lt leg	2	Wet	60	12	F	Jacobabad, Dera Murad
RU 63	*L. (L.) mj* type I	H	Face, rt leg	2	Wet	360	12	M	Quetta, Mari Abad
RU 64	*L. (L.) mj* type I	L	Lt leg	1	Wet	60	17	M	Larkana, Mehar
RU 65	*L. (L.) mj* type I	H	Lt arm	1	Wet	30	30	F	Quetta city
RU 66	*L. (L.) tr*	H	Rt leg	1	Wet	360	6	M	Quetta, Mari Abad
RU 67	*L. (L.) tr*	H	Lt leg	1	Wet	360	10	M	Quetta, Mari Abad
RU 68	*L. (L.) tr*	H	Rt arm	1	Wet	180	45	F	Quetta, Mari Abad
RU 69	*L. (L.) tr*	H	Face, lt leg	3	Mixed	1080	5	F	Quetta, Mari Abad

L. (L.) tr, Leishmania leishmania tropica; L. (L.) mj, Leishmania leishmania major; H, highland; L, lowland; lt, left; rt, right; NA, not available; M, male; F, female.

Table 3. Types of *Leishmania* parasites identified in highland and lowland areas of Pakistan

Type of *Leishmania*	No. of cases identified in Lowlands					Lowland total	Highland total
	Sukkur	Jacobabad	Larkana	Jafar Abad/Jhal Magsi	Sibi		
L. (L.) major type I	10	5	22	2	1	40	5
L. (L.) major type II	3	1	1	1	0	6	0
L. (L.) major type III	0	0	1	0	0	1	0
L. (L.) tropica	0	0	0	1	0	1	16

guanine (G) is replaced by adenine (A). In the sequence we call *L. (L.) major* type III, in corresponding nucleotide position number 265, A is replaced by G and C is replaced by T at nucleotide positions 416 and 799 (Fig. 3). We identified 40 cases of *L. (L.) major* type I, six of *L. (L.) major* type II, one of *L. (L.) major* type III and only one of *L. (L.) tropica* in cases from lowland areas of Pakistan. We identified 16 cases of *L. (L.) tropica* and five of *L. (L.) major* type I in patients from the highlands around Quetta city, in a mountainous region close to Afghanistan (Table 3).

In our *cyt* b gene analysis findings, the parasites from patients who presented with dry-type lesions were identified as *L. (L.) tropica* in six cases (38%) and as *L. (L.) major* in 10 cases (62%). In those clinically presenting with wet-type lesions, nine cases (21%) were identified as *L. (L.) tropica* and 33 (79%) as *L. (L.) major*. In those clinically presenting with mixed-type lesions, two cases (18%) were identified as *L. (L.) tropica* and the remaining nine (82%) as *L. (L.) major* (Table 4). Statistical analysis for relationship between types of skin lesions (wet-, dry- and/or mixed-types) and type of *Leishmania* parasite (i.e. *L. (L.) tropica* vs *L. (L.) major*) identified yielded a χ^2 test result at $P > 0.05$ (SPSS 11.0, Chicago, IL, USA), demonstrating that clinical features are not reliable indicators of the species of *Leishmania* parasite at the present study sites in Pakistan.

Table 4. Types of *Leishmania* parasite identified by cytochrome *b* gene sequencing and their presenting types of clinical lesions

Presenting features	*L. (L.) tropica*	*L. (L.) major*				Total
		Total	Type I	Type II	Type III	
Dry type	6	10	9	1	0	16
Wet type	9	33	28	5	0	42
Mixed type	2	9	8	0	1	11
Total	17	52	45	6	1	69

DISCUSSION

Cutaneous leishmaniasis is prevalent in Pakistan and has been reported in all provinces and almost all major cities.[5–9] The disease manifests not only in

Figure 2. Types of cutaneous leishmaniasis cases identified in the two study areas. M, *Leishmania (L.) major*; T, *Leishmania (L.) tropica*.

classical presentation but also in various forms such as acute paronychial, chancriform, annular, palmoplantar, zosteriform and erysipeloid forms.[6,10] Molecular techniques are favored also for the identification of such non-classical cases.

Regarding the causative agents of CL in Pakistan, the most commonly mentioned parasites were *L. (L.) major* and *L. (L.) tropica*.[5,9,12] It had been believed that *L. (L.) major* initiates the wet or moist lesions of CL and that *L. (L.) tropica* triggers the dry lesions of CL.[1,3,17,18] However, our findings indicate that both *L. (L.) major* and *L. (L.) tropica* can present with either type of lesion and with mixed lesions, and no statistical association was found between the types of presenting lesions (dry-, wet- or mixed-types) and the *Leishmania* species identified. This study thus contradicts, for example, the previous assumption of complete absence of *L. (L.) major* in an area where patients present only with dry-type lesions.[8] Our findings show that it is not possible to determine the causal type of *Leishmania* parasite by clinical presentation alone.

In the lowland areas of Pakistan, *L. (L.) major* type II and type III were found in only seven of our cases, six of the former and one of the latter. Regarding *L. (L.) major* type I, the majority of patients (40 cases) were from the lowland areas, and five were from mountainous areas around Quetta city in Balochistan

```
L.(L.)major/5ASKH          241 TTTTGTGATATATATATTTATAGTAATAATAGGTTTTATTGGCTATGTTTTACCATGTAC 300
L.(L.)major/Friedlin       241 ************************************************************ 300
L.(L.)major/Type I         241 ************************************************************ 300
L.(L.)major/Type II        241 ************************************************************ 300
L.(L.)major/Type III       241 ************************G*********************************** 300

L.(L.)major/5ASKH          361 TGGTACTTGACTTTGTTATTGAATATGAGGTAGTGAGTATATTAATGATTTTACACTGTT 420
L.(L.)major/Friedlin       361 ************************************************************ 420
L.(L.)major/Type I         361 ************************************************************ 420
L.(L.)major/Type II        361 ************************************************************ 420
L.(L.)major/Type III       361 ************************************************************ 420

L.(L.)major/5ASKH          421 AAAATTACATGTGTTGCATGTGCTATTACCTTTTGTATTAATACTTGTAATATTTATGCA 480
L.(L.)major/Friedlin       421 ************************************************************ 480
L.(L.)major/Type I         421 ************************************************************ 480
L.(L.)major/Type II        421 ****************A*********************************************** 480
L.(L.)major/Type III       421 ************************************************************ 480

L.(L.)major/5ASKH          781 GGTTATTTTATTATTTTCCTTATTTTTGTTTATATTA                       817
L.(L.)major/Friedlin       781 ***********************************                        817
L.(L.)major/Type I         781 ********************T*******************                   817
L.(L.)major/Type II        781 ********************T*******************                   817
L.(L.)major/Type III       781 ********************T*******************                   817
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Figure 3. Cytochrome *b* gene alignment showing the sites of different nucleotide positions of *L. (L.) major* type I, *L. (L.) major* type II and *L. (L.) major* type III. *, sequence identities.

province. Only one case of *L. (L.) tropica* was identified in the lowland area (Jhal Magsi, Balochistan province), and all the remaining 16 cases were identified in highland areas. A significant difference ($P < 0.0001$, Fisher's exact test) was found in the distribution of the two species by altitude, with *L. (L.) major* predominant in lowland areas and *L. (L.) tropica* in highland areas. The present result enriched our earlier findings, based only on the first year's cultured parasite data, that only *L. (L.) tropica* was found in highland areas and only *L. (L.) major* in lowland areas.[12] The small discrepancy found between the previous and the present studies might be due to some unidentified factors such as increased sample size, migration of patients from lowland to highland and vice versa. Our finding from biopsy

samples of *cyt* b polymorphism in *L. (L.) major* and homology in *L. (L.) tropica* confirms the previous findings of polymorphism in the former species and homology in the latter identified by MLEE.[12]

In the field of PCR-based methods for detecting *Leishmania* species, it has been necessary to isolate the parasite in culture before using any of the existing high-resolution techniques such as isoenzymes, randomly amplified polymorphic DNA analysis and schizodemes. Also, recovery of parasites in culture is rarely more than approximately 70% efficient even with easily cultured *Leishmania* parasites.[19] In our study, we could explore the causal *Leishmania* parasite up to intraspecies level from the clinical samples. This finding can reduce the procedures which are necessary to isolate the parasite in culture. The technique we used, PCR followed by *cyt* b gene sequencing, will be useful for identifying *Leishmania* parasite strains from clinical samples.

Previous studies revealed genetic polymorphism in natural populations of different *Leishmania* species, which could explain the plasticity of these parasites and their ability to adapt the changing ecological conditions. One study reported finding three patterns of genetic and biological variation among *L. (L.) major* strains in rodent reservoir hosts (*Rhombomys opimus*, *Merioned libycus* and *Psammomys obesus*) from central Asia, the Middle East and Africa, explored by permissively primed intergenic polymorphic (PPIP)-PCR and single-stranded conformation polymorphism (SSCP)-PCR.[20] To link the above report and our findings, we would need to explore the role of the sandfly (vector) in polymorphism. However, our report fills a gap in the understanding of the epidemiological phenomena of cutaneous leishmaniasis.

In conclusion, we identified three types of *L. (L.) major* polymorphism in patients from two different altitude regions of Pakistan, one region in lowland areas (~100 m a.s.l.) of Sindh province (Sukkur city, Jacobabad district and Larkana district), Balochistan province (Jhal Magsi district, Jafar Abad district and Sibi city) and the other a highland area (1600–1800 m a.s.l.) of Balochistan province (Quetta city). For reasons of feasibility and accessibility, the study was confined to the above-mentioned areas. To explore the whole country's *Leishmania* parasite profile, we would need to extend the study into the other two provinces, Punjab and NWFP. To develop a more complete profile of the patterns of leishmaniasis, continual and vigilant surveillance is required. Several other pockets of infection and of vectors and reservoirs of leishmaniasis in Pakistan still need to be studied, and such studies are sure to contribute to needed knowledge concerning the clinical forms, causal agents and eco-epidemiological patterns of leishmaniasis in Pakistan.

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Anti-babesial Quassinoids from the Fruits of *Brucea javanica*

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The medicinal plant *Brucea javanica* (L.) Merr. (Simaroubaceae), grown in China, was examined for anti-babesial properties. The anti-babesial activity of the fruit was found to be attributed to its quassinoid constituents. Ten active compounds were isolated and purified from a chloroform extract. The identities of these compounds were confirmed from NMR spectroscopic and mass spectral data as brusatol (**1**), bruceantin (**2**), bruceine A (**3**), bruceantinol (**4**), dehydrobruceine B (**5**), dehydrobrusatol (**6**), dehydrobruceine A (**7**), bruceine D (**8**), bruceoside A (**9**), and yadanzioside G (**10**). When tested *in vitro* against *Babesia gibsoni*, compounds **1-10** had IC_{50} values of 0.74, 13.4, 4.0, 12.0, 308.2, 10.5, 835.0, >1000, and >1000 ng/mL, respectively. Compounds **1-4**, **6** and **7** had far higher activity than the commercial anti-babesial drug diminazene aceturate, which possesses an IC_{50} value of 70.5 ng/mL. Except for bruceine A (**3**), bruceantinol (**4**) and bruceine D (**8**), this is the first report of the anti-babesial activity of these isolated quassinoids.

Keywords: *Brucea javanica*, Simaroubaceae, anti-babesial activity, diminazene aceturate, *Babesia gibsoni*.

Canine babesiosis is a tick-borne disease caused by the protozoal parasites *Babesia gibsoni* and *B. canis*. They infect the red blood cells of dogs and typically cause hemolytic anemia. Infection with *B. gibsoni* usually results in more severe clinical manifestations than infection with *B. canis*, and may cause dysfunction of multiple organs [1]. *B. gibsoni* is distributed in many regions throughout the world, including Asia, Africa, Europe, America, and Australia [2,3]. Treatment of or spontaneous recovery from an acute infection frequently fails to clear the invading organisms from the host, which leads to it being a carrier. The animals that recover are a reservoir for tick-transmitted infections and are at risk for recrudescent infection [2]. No drugs have proven effective for the elimination of *B. gibsoni* from infected dogs. Some anti-babesial drugs reduce the severity of clinical signs and the mortality associated with the disease. The available drugs on the market are diminazene aceturate, imidocarb diproprionate, phenamidine isethionate, pentamidine isethionate, parvaquone, and niridazone [2]. However, these usually cause severe side effects [4]. Therefore, an alternative chemotherapeutic agent with fewer side effects is urgently needed for the treatment of *B. gibsoni* infection.

Brucea javanica (L.) Merr. (Simaroubaceae) is widely distributed throughout Asia, where the bitter fruits (known as "ya dan zi") are used in traditional medicine for various ailments, including cancer, amoebic dysentery, and malaria [5]. In common with other Simaroubaceae species, the bitter principles of this plant are due to quassinoids, some of which have been investigated extensively as antitumor agents [6]. Bruceantin (**2**) has been shown to possess high activity *in vitro* against *Entamoeba histolytica* [7,8]. Although *B. gibsoni* is transferred from ticks and

restricted to dogs, *Plasmodium falciparum*, one of the species of *Plasmodium* that cause malaria in humans and is transmitted by *Anopheles* mosquitoes, has a very similar life cycle and shows similar disease symptoms to those caused by *B. gibsoni*. Therefore, it was proposed by our research group that an anti-babesial component may be present in extracts of plants that are used for malarial treatment. The therapeutic value of *Brucea javanica* as an antimalarial remedy in traditional medicine is associated with the content of different bruceines existing in the plant. The quantity and the existence of these bruceines in the plant depend upon climatic, seasonal, and geographic factors [9]. Recently, our research group reported that extracts of *B. javanica* fruits show strong inhibitory activity of parasite growth at a concentration of 10 μg/mL [10]. During this study, some quassinoids were isolated and investigated for their anti-babesial activities and showed much higher activity than the standard drug, diminazene aceturate. The plant material in the previous study was purchased from a local shop in Indonesia, but the activity against *B. gibsoni* and the quassinoid content are thought to differ with fruits from other regions. The aim of this study, therefore, was to isolate and characterize the anti-babesial quassinoids from the fruits of *B. javanica* from other regions and to compare their activity. Here we report on the anti-babesial activity of compounds **1-10** isolated from fruits purchased from China and on structure-activity relationships.

Purification of the chloroform extract of *B. javanica* fruits using silica gel column chromatography followed by HPLC and PTLC led to the isolation of ten quassinoids (**1-10**). The chemical structures were deduced to be brusatol (**1**), bruceantin (**2**), bruceine A (**3**), bruceantinol (**4**) dehydrobruceine B (**5**), dehydrobrusatol (**6**), dehydrobruceine A (**7**), bruceine D (**8**), bruceoside A (**9**), and yadanzioside G (**10**) (Figure 1) based on comparison of 1D- and 2D-NMR spectral data and optical rotation values with published information [12]. Following this isolation scheme, we anticipated isolating bruceines A, B, and C as the major compounds [9]; however, bruceine A was only obtained in small amounts and bruceines B and C were not isolated. This confirms earlier observations that the yield and composition of bruceines in *B. javanica* depend on geographic and seasonal factors.

Compounds **1-10** were tested for anti-babesial activity against *B. gibsoni in vitro*. *B. gibsoni* parasites treated with the test compounds (**1-10** and diminazene aceturate) demonstrated stagnation in the ring form, including reduction in the size of the nucleus and disappearance of the cytoplasm. On the other hand, untreated parasites demonstrated typical petaloid and schizont forms after 3 days of incubation, with clear cytoplasm and nuclei. The IC_{50} values of compounds **1-10** and diminazene aceturate are shown in Figure 1.

It was obvious from our results that relatively small molecular changes produce quite large differences in the *in vitro* activity of the tested quassinoids. Compounds **1-4** differ only in the nature of the ester moiety at C-15, but have IC_{50} values ranging from 0.74 ng/mL for brusatol (**1**), the most potent compound, to 13.4 ng/mL for bruceantin (**2**). In particular, brusatol (**1**) and bruceine A (**3**) are identical, except for either the presence or absence of the unsaturated C-2′/C-3′ bond, which caused a fivefold difference in activity. Also, compounds **5-7** differ only in the nature of the ester moiety at C-15, but have IC_{50} values ranging from 10.5 ng/mL for dehydrobrusatol (**6**) to 308.2 ng/mL for dehydrobruceine B (**5**). The above-mentioned results clearly indicate the important contribution to *in vitro* anti-babesial activity of the C-15 ester function of the quassinoids.

Dehydrobrusatol (**6**) and brusatol (**1**) have the same C-15 ester function; however, the decrease in activity for **6** was about 14 times less than for **1**; also, bruceine A (**3**) is more active than its dehydrated derivative (**7**). Therefore, the decrease in the activity of **6** and **7** is due to dehydration of ring A. On the other hand, bruceoside A (**9**) and yadanzioside G (**10**) have far lower activity than their aglycones; with IC_{50} values greater than 1000 ng/mL. Thus, glycosylation at *O*-C2 has a negative effect on anti-babesial activity. Our results led to the following structure-activity relationships for quassinoids related to growth inhibition of the parasite *B. gibsoni*. Activity is influenced by (i) the nature of the C-15 side chain, (ii) the nature of the A ring modification, and (iii) the presence or absence of a sugar moiety at *O*-C2.

Considering the potentially fatal side effects of diminazene aceturate, bruceines are promising new candidates for the treatment of *B. gibsoni* infection. However, the mechanism of action of these bruceines on the growth of *B. gibsoni* is not yet known. Therefore, there is an urgent need o determine the precise mechanism of action of these compounds in order to develop new anti-babesial drugs.

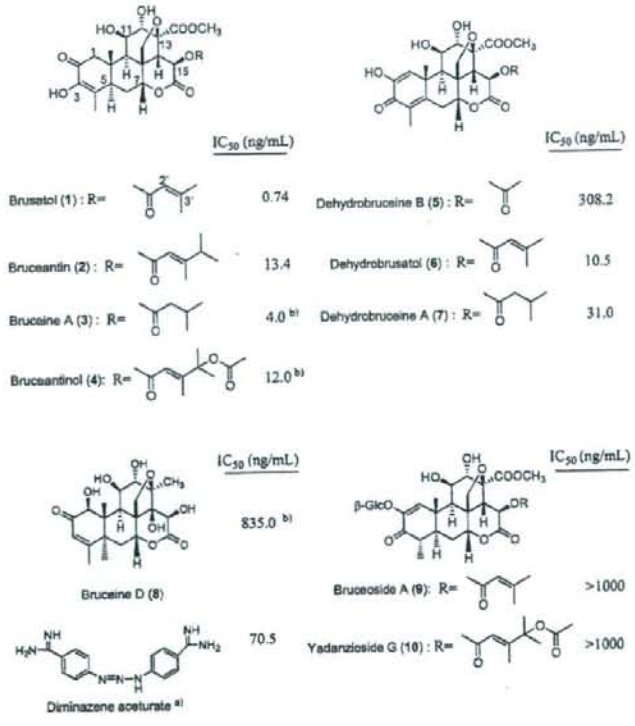

Figure1: Chemical structures and anti-babesial activity against *B. gibsoni in vitro* of compounds (**1-10**)
a) Standard drug for the symptom caused by *Babesia gibsoni*. b) Data are from reference [10]

Experimental

General experimental procedures: Optical rotations were measured with a Jasco DIP-370 digital polarimeter. Mass spectra were recorded on JEOL JMS-SX102A and JMS-AX500 spectrometers. NMR spectra were recorded on a JEOL JNM-EX 270 FT-NMR spectrometer and on a Bruker AMX-500 FT-NMR spectrometer. Column chromatography was conducted with silica gel 60 (Kanto Chemical). Analytical thin-layer chromatography was performed with silica gel 60 F_{254} (Merck).

Plant material: Fruits of *Brucea javanica* were purchased from a local shop in Huaihua City, West Hunan province, Mid-Southern China in June 2007 and identified by Dr Zhu Ming, the vice president of Huaihua Red Cross Hospital, Huaihua City, West Hunan province, Mid-Southern China.

In vitro test for anti-babesial activity: The anti-babesial assay was performed against *B. gibsoni in vitro* according to the reported methods [11].

Extraction and isolation: Dried ground fruit (500 g) was defatted with *n*-hexane (3L×3) and the marc was successively extracted with chloroform (3L×3). The combined chloroform extract was filtered and concentrated under reduced pressure. The concentrated chloroform extract (29.0 g) was chromatographed on a silica gel column (750 g) and eluted with a gradient of MeOH-$CHCl_3$ (from 5:95 to 40:60) to give three fractions, Fr. I [MeOH-$CHCl_3$ (5:95), 1000 mL], Fr. II [MeOH-$CHCl_3$ (5:95), 1000 mL), and Fr. III [MeOH-$CHCl_3$ (40:60), 1000 mL]. Fr. I was further purified by HPLC (Capcell Pak C_{18}, 5 μm, 15 mm × 250 mm, Shiseido) with CH_3OH-H_2O (6.0:4.0 *v/v*) using a flow rate of 3 mL/min and detection using a UV spectrophotometer at 254 nm. The following quassinoids were obtained: brusatol (**1**) (15.0 mg, t_R: 15.0 min) and bruceantin (**2**) (4.0 mg, tR: 27.2 min). In addition, two fractions, Fr. I-1(t_R: 21.3~ 22.2 min) and Fr. I-2 (t_R: 23.3~ 25.3 min) were collected for further analysis. Fr. I-1 was further purified by PTLC on silica gel. The plates were developed with MeOH-$CHCl_3$ (5:95). The