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Antimalarial Activity of Granzyme B and Its Targeted Delivery by a Granzyme B–Single-Chain Fv Fusion Protein

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We present here the first evidence that granzyme B acts against *Plasmodium falciparum* (50% inhibitory concentration [IC_{50}], 1,590 nM; 95% confidence interval [95% CI], 1,197 to 2,112 nM). We created a novel antimalarial fusion protein consisting of granzyme B fused to a merozoite surface protein 4 (MSP4)-specific single-chain Fv protein (scFv), which targets the enzyme to infected erythrocytes, with up to an 8-fold reduction in the IC_{50} (176 nM; 95% CI, 154 to 202 nM). This study confirms the therapeutic efficacies of recombinant antibody-mediated antimalarial immunotherapeutics based on granzyme B.

Malaria is caused by parasites of the genus *Plasmodium* and remains one of the most widespread and dangerous infectious diseases, causing ~627,000 deaths worldwide per year (1). Among the six species that infect humans, *Plasmodium falciparum* causes the severest form of the disease (2). There is no effective vaccine against these parasites (3, 4), and resistances are emerging against a wide variety of antimalarial drugs (5). It was recently shown *in vitro* that natural killer (NK) cells can eliminate erythrocytes infected with *P. falciparum* (6) and that this is associated with the production of the serine protease granzyme B (Gb) (7). *In vivo*, NK cells are essential for protection against plasmodial infections in mice (8), and increased levels of circulating Gb are found in naturally infected humans (9).

We therefore investigated the antimalarial effect of recombinant Gb on *P. falciparum* (strain 3D7A) in a standardized 72-h drug susceptibility assay starting with synchronized ring-stage parasites (10). Gb was produced in HEK293 cells with an N-terminal protective peptide fused to an enterokinase cleavage site (EGb) to suppress the enzymatic activity in the host cells, as previously described (11). Activity was restored by the enzymatic removal of this peptide using 0.02 U of recombinant enterokinase (Novagen; Merck) per μg of protein (12). The restored enzymatic activity was confirmed using a colorimetric activity assay (13). Parasite growth was specifically inhibited by activated Gb, with a half-maximal inhibitory concentration (IC_{50}) of 1,590 nM (95% confidence interval [95% CI], 1,197 to 2,112 nM, calculated using the Hill equation in GraphPad Prism version 5). Undigested (inactive) EGb showed no inhibition (Fig. 1 and Table 1). To our knowledge, this is the first time that the antimalarial activity of Gb has been directly confirmed *in vitro*.

We developed a strategy to target Gb to the parasite and thus reduce the required dose. Targeted toxin delivery via the parasite transferrin receptor has already been reported (14, 15). Although some authors claim to have identified and characterized this receptor (16, 17), others argue that iron uptake by the parasite is nonspecific and that the *P. falciparum* transferrin receptor remains elusive (18). Promising alternative targets include the merozoite surface proteins (MSPs), especially MSP1, MSP2, MSP4, and MSP8, which bear glycosylphosphatidylinositol (GPI) anchors and therefore are not completely shed during merozoite invasion. Some also contain immunogenic epidermal growth factor (EGF)-like domains near the C-terminal GPI anchors, which serve as ideal targets for specific antibodies (19–22). Because EGF-like domains are less variable between strains and even species (23), they are ideal targets for antibody-based approaches. Recently, it was shown that MSP4 is imported into newly infected erythrocytes without significant processing, and it remains there for up to 5 h (24). MSP4-specific antibodies or their fragments are therefore attractive candidates for guiding Gb into the infected erythrocyte.

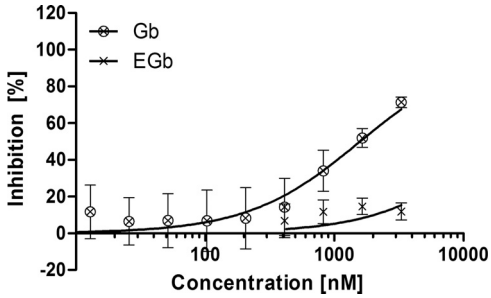

FIG 1 The 72-h drug susceptibility assay using *P. falciparum* 3D7A. The susceptibility of *P. falciparum* 3D7A toward active Gb (⊗) and inactive EGb (×) was determined in a 72-h drug susceptibility assay starting at the ring stage. The data represent the mean ± standard deviation (SD) from two experiments, using technical duplicates.

We generated a single-chain variable fragment (scFv) from an $MSP4_{EGF\text{-}like\ domain}$-specific murine antibody, 2.44IgG1 (S. Kapelski, A. Boes, H. Spiegel, M. de Almeida, T. Klockenbring, A.

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TABLE 1 IC_{50}s of all tested samples in the drug susceptibility assays

Protein[a]	Duration of drug susceptibility assay (h)	No. of replication cycles (stages)	*P. falciparum* strain	IC_{50} (nM)[b]	95% CI (nM)	Relative enzymatic activity (AU)[e]	Activity-corrected IC_{50} (95% CI) (nM)
Gb	72	1.5 (ring-schizont-schizont	3D7A	1,590	1,197–2,112	1.09	1,733 (1,305–2,302)
EGb			3D7A	>3,000[c]		0	NA[d]
2.44IgG1	48	1 (schizont-schizont)	3D7A	>25,000[c]		NA	NA
Gb-2.44			3D7A	176	154–202	1	176 (154–202)
Gb-H22			3D7A	1,103	949–1,283	0.95	1,048 (902–1,219)
Gb-Ki4			3D7A	1,500	1,180–1,905	0.85	1,275 (1,003–1,619)
Gb			3D7A	970	852–1,104	1.09	1,057 (929–1,203)
EGb-2.44			3D7A	>4,000[c]		0	NA
EGb-H22			3D7A	>4,000[c]		0	NA
EGb-Ki4			3D7A	>4,000[c]		0	NA
Gb-2.44	30	0.5 (ring-schizont)	3D7A	1,773	1,496–2,102	1	1,773 (1,496–2,102)
Gb-H22			3D7A	3,668	2,804–4,798	0.95	3,485 (2,664–4,558)
Gb			3D7A	2,143	1,426–3,220	1.09	2,336 (1,554–3,510)
Gb-2.44	48	1 (schizont-schizont)	K1	386	306–488	1	386 (306–488)
Gb-H22			K1	3,497	2,368–5,166	0.95	3,322 (2,250–4,908)
Gb-Ki4			K1	>2,000[c]		0.85	NA

[a] Gb, active granzyme B; EGb, inactive granzyme B with retained N-terminal protective peptide and enterokinase cleavage site; 2.44IgG1, MSP4-specific full-size chimeric antibody; Gb-2.44, MSP4-specific Gb-scFv fusion protein; Gb-H22 and Gb-Ki4, *P. falciparum*-unrelated Gb-scFv fusion proteins.
[b] IC_{50}s were calculated from the data presented in Fig. 1 to 3, rounded to the nearest integer.
[c] If no inhibition of parasite growth was determined at the highest applied concentration, this highest applied concentration was given.
[d] NA, not applicable.
[e] AU, arbitrary units.

Reimann, R. Fischer, S. Barth, and R. Fendel, unpublished data), by splicing by overlap extension (SOE)-PCR using a glycine-serine linker peptide and fusing it to the SerpinB9-resistant EGb_{R201K} mutant (12). This was expressed in HEK293-6E cells using a vector based on pTT5 (25) modified with an expression cassette designed for EGb-scFv fusion proteins (26, 27). Two unrelated EGb-scFv fusion constructs named EGb-H22 (targeting human CD64) (11) and EGb-Ki4 (targeting human CD30) (13) were used as negative controls. Following enterokinase-mediated activation, the Gb-scFv fusion proteins were used in a 48-h drug susceptibility assay, similar to the standard invasion inhibition assay used for the evaluation of antibodies (28). We also used 2.44IgG1 as a full-size control antibody in the assay. The proteins were added to synchronous schizont-stage *P. falciparum* 3D7A parasites growing in 96-well half-area microtiter cell culture plates at a parasitemia level of 0.05% and a final hematocrit level of 1.5%, in a total volume of 50 μl per well. After incubation for 48 h, inhibition was determined as described previously (29). The IC_{50} of Gb-2.44 was 176 nM (95% CI, 154 to 202 nM), which was 5- to 8-fold lower than that of Gb, Gb-H22, and Gb-Ki4, each of which showed IC_{50}s of ~1,000 nM (Fig. 2A and Table 1). Undigested controls (EGb-2.44, EGb-H22, EGb-Ki4, and EGb) and antibody 2.44IgG1 showed no effect on parasite growth (Table 1). Similar experiments were carried out using the multidrug-resistant strain *P. falciparum* K1, resulting in a similar IC_{50} for Gb-2.44, which were again substantially lower than that of Gb-Ki4 and of Gb-H22 (Fig. 2B and Table 1).

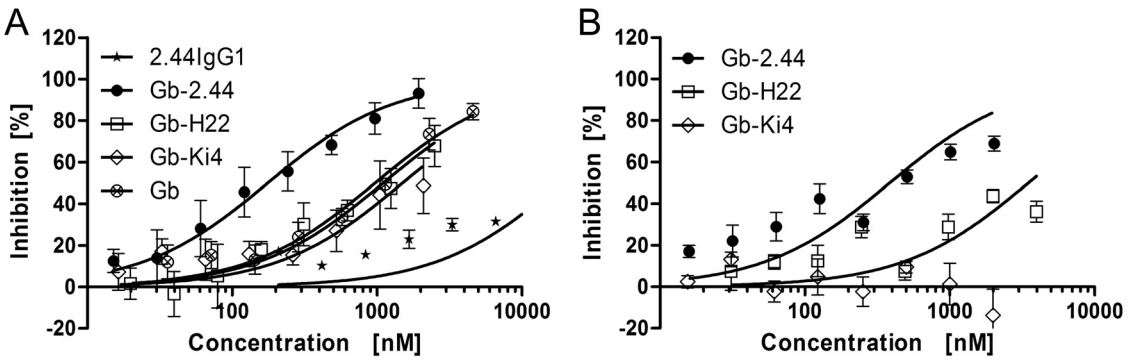

FIG 2 The 48-h drug susceptibility assay using strain *P. falciparum* 3D7A and the multidrug-resistant strain *P. falciparum* K1. The inhibition of parasite growth mediated by Gb fused to an MSP4-specific scFv was determined in a 48-h drug susceptibility assay from schizonts to schizont stage. The schizonts were incubated in the presence of the MSP4-specific fusion Gb-2.44 (●), two unrelated fusions named Gb-H22 (□) and Gb-Ki4 (◇), nonfused Gb (⊗), and the chimeric MSP4-specific antibody 2.44IgG1 (*) for 48 h. (A) The assay was carried out on a standard laboratory strain, *P. falciparum* 3D7A, and the data represent the mean ± SD from three (Gb-2.44 and Gb-H22), two (Gb-Ki4 and Gb), or one (2.44IgG1) experiment, each in technical triplicates. (B) Multidrug-resistant strain *P. falciparum* K1 was used, and the data represent the mean ± SD from two experiments, each in technical triplicates (Gb-2.44), or one experiment using technical triplicates (Gb-H22 and Gb-Ki4).

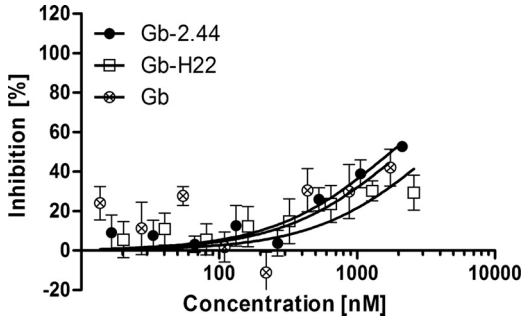

FIG 3 The 30-h drug susceptibility assay using strain *P. falciparum* 3D7A. The dependency of growth inhibition on the coimport of Gb-2.44 (●) was determined by adding the fusion protein and the unrelated fusion Gb-H22 (□) or nonfused Gb (⊗) to synchronous ring-stage parasites and incubating them for 30 h, when mature schizonts but no rings were present to exclude the potential coimport step. The data represent the mean ± SD from two experiments, using technical triplicates.

The various fusion partners of the Gb constructs might have an influence on enzymatic activity. Therefore, the enzymatic activity of each construct was determined by a colorimetric assay (13), and the relative activity to Gb-2.44 was calculated. All IC_{50}s were corrected by this factor (Table 1).

The dependency of the enhanced inhibition of parasite growth was assessed in a 30-h drug susceptibility assay. Here, the fusion proteins were added to synchronous ring-stage parasites at a 0.2% parasitemia level (to achieve an equal parasitemia level at the assay endpoint in the wells without any parasite inhibition) and 1.5% hematocrit and incubated until mature schizonts were present, omitting a schizont rupture and merozoite invasion step. Thus, without possible coimport of the *P. falciparum*-specific Gb-2.44 into the newly infected erythrocyte along with the merozoite, the Gb fusion proteins all showed similar levels of inhibition (Fig. 3 and Table 1).

Of note, the IC_{50}s determined in the various assays are not equivalent, as the drug exposure time of the parasites has a direct influence on the IC_{50}. Therefore, we strictly perform intra-assay comparisons of IC_{50}s.

We have therefore provided direct confirmation that Gb has antiparasitic activity, and we demonstrated its potential therapeutic use against malarial infections. The concept of recombinant immunotoxins has been extensively investigated in the field of targeted tumor therapy with various bacterial and human effector domains (30–32), including Gb (11, 13, 27, 33). After binding to a disease-specific cell surface antigen, these immunotoxins are internalized, e.g., by receptor-mediated endocytosis, released from endosomal compartments into the cytoplasm, and efficiently kill the malignant cell by their catalytic activity. In the case of Gb, the corresponding human cytolytic fusion protein proteolytically cleaves certain caspases to induce apoptosis (34). Comparably, *P. falciparum* bears one designated metacaspase, *P. falciparum* MCA-1 (35), which may be the target of Gb. Another hypothesis is that Gb induces eryptosis in infected erythrocytes and thus kills the parasite (7).

Taken together, we provide solid evidence that Gb-containing malaria-specific fusion proteins are valuable drug candidates acting against multidrug-resistant *P. falciparum* strains. The IC_{50} of these novel immunotherapeutic agents in the drug susceptibility assay lies within the range for a hit candidate as a new therapeutic agent, as suggested by the Medicines for Malaria Venture (MMV) (http://www.mmv.org/research-development/essential-information-scientists). The 50% effective concentration [EC_{50}] of recombinant Gb *in vitro* (using Jurkat cells) is ~8 μM (36); thus, the estimated selectivity index of our human antimalarial fusion protein is >40.

In further studies, we will investigate the underlying mechanism of its antimalarial activity in order to increase the specificity and further reduce the inhibitory concentration.

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