CHAPTER 3

Screening Inhibitors of *P. berghei* Blood Stages Using Bioluminescent Reporter Parasites

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Abstract

We describe two improved assays for *in vitro* and *in vivo* screening of inhibitors and chemicals for anti-malarial activity against blood stages of the rodent malaria parasite, *Plasmodium berghei*. These assays are based on the determination of bioluminescence in small blood samples that is produced by reporter parasites expressing luciferase. Luciferase production increases as the parasite develops in a red blood cell and as the numbers of parasites increase during an infection. In the first assay, *in vitro* drug luminescence (ITDL) assay, the *in vitro* development of ring-stage parasites into mature schizonts in the presence and absence of candidate inhibitor(s) is quantified by measuring luciferase activity after the parasites have been allowed to mature into schizonts in culture. In the second assay, the *in vivo* drug luminescence (IVDL) assay, *in vivo* parasite growth (using a standard 4-day suppressive drug test) is quantified by measuring the luciferase activity of circulating parasites in samples of tail blood of drug-treated mice.

1. Introduction

Antimalarial drug screening and validation is relatively time-consuming and complicated. The first phase of drug screening usually comprises the following two steps. Initially it involves the use of whole cell (infected red blood cell) assays that are used to determine the efficacy of drugs on *in vitro* growth of the human parasite *Plasmodium falciparum* in erythrocytes. The second step involves the testing of the *in vivo* efficacy of the most promising drug candidates in small animal models of malaria, principally using the rodent parasite *P. berghei* in laboratory mice. Compared to progress in the development for automated drug screening assays using the human parasite *P. falciparum* [1–7], the development of simple and sensitive assays for drug-screening in small animal models has been slow in part because the new technologies developed for *P. falciparum* cannot be directly applied in drug screening using rodent malaria parasites [8]. Usually *in vivo* drug screening is performed using the standard '4-day suppressive drug test' [9], in which inhibition of parasite growth (*P. berghei*) in drug-treated mice is determined by manual counting the parasitemia in Giemsa-stained smears from small blood samples. Analysis of *in vitro* drug susceptibility has only been reported for one of the four rodent parasites, i.e. *P. berghei*. Since *P. berghei* blood stages can be cultured for only one developmental cycle, drug potency can only be determined during the development of ring forms into mature schizonts, which is established by determination of schizont maturation in Giemsa stained smears or by FACS analysis [10,11]. The availability of a *P. berghei in vitro* drug susceptibility assay is important since it permits to determine whether a discrepancy between *in vitro P. falciparum* drug-sensitivity and *in vivo P. berghei* drug-sensitivity is the result of intrinsic differences between the two parasites or is caused by pharmacokinetic and/or pharmacodynamic characteristics of the drug in a live animal. Because of the limitations of manual counting of rodent parasites in Giemsa stained slides and automated counting of rodent parasites stained with fluorescent, DNA/RNA-specific dyes, possibilities have been explored of using transgenic rodent parasites expressing reporter proteins, such as GFP or luciferase, for drug screening [12,13]. Herein we describe simple and sensitive *in vitro* and *in vivo* screening assays to test inhibitors and chemicals for antimalarial activity against blood stages of a reporter *P berghei* parasite [8]. These assays are based on the determination of luciferase activity (luminescence) in small blood samples containing transgenic blood stage parasites that express luciferase under the control of a promoter that is either schizont-specific (*ama-1*) or constitutive (*eef1a*). The reading of luminescence assays is rapid, requires a minimal number of handling steps and no experience with parasite morphology or handling fluorescence-activated cell sorters, produces no radioactive waste and test-plates can be stored for

prolonged periods before processing. Both tests are suitable for use in larger-scale *in vitro* and *in vivo* screening of drugs.

2. Materials

2.1. Reporter parasites

For the *in vitro* drug luminescence (ITDL) assay, reporter parasite line *Pb*GFP-Luc$_{ama1}$ (1037m1f1m1cl1; see **Note 1**) is used, which expresses a fusion protein of GFP (mutant3) and firefly luciferase (LUC-IAV) under the control of the schizont-specific *ama-1* promoter [14]. For details of *Pb*GFP-Luc$_{ama1}$, see RMgm-32 (http://www.pberghei.eu/index. php?rmgm=32).

For the *in vivo* drug luminescence (IVDL) assay, reporter parasite line *Pb*GFP-Luc$_{con}$ (676m1cl1; see **Note 1**) is used. This line expresses a fusion protein of GFP (mutant3) and firefly luciferase (LUC-IAV) under the control of the constitutive *eef1α* promoter [13]. For details of *Pb*GFP-Luc$_{con}$, see RMgm-29 (http://www.pberghei.eu/index. php?rmgm=29).

2.2. Laboratory animals

In our laboratory, we use Swiss mice (OF1 ico, Construct 242; age, 6 weeks (25–26 g); Charles River). However, other mouse strains such as C57BL/6 and BALB/c can also be used (see Note 2).

2.3. Reagents

1. Fetal bovine serum, heat inactivated (FBS; Invitrogen, cat. no. 10108-165); store at -20°C.
2. Sörensen staining buffer: 2.541 g KH_2PO_4 and 8.55 g $Na_2HPO_4.2H_2O$ in 5 L distilled water, pH adjusted to 7.2 with NaOH). Store at room temperature.
3. Giemsa solution working solution: 10% Giemsa solution (Merck, cat. no. 1666 789) in Sörensen staining buffer.
4. Phosphate-buffered saline (PBS): PBS stock solution (10×), 0.01 M KH_2PO_4, 0.1 M Na_2HPO_4, 1.37 M NaCl, 0.027 M KCl, pH 7.4; for a working solution, dilute the stock solution with nine volumes of distilled water, adjust the pH to 7.2 with 1.0 M HCl and sterilize by autoclaving for 20 min at 120 °C.
5. Heparin: Grade I-A, cell culture tested, 140 mUSP units/mg (Sigma, cat. no. H3149): dissolve the heparin powder in distilled water to a concentration

of 25,000 units/mL; filter sterilized (0.2 µm) and store at 4 °C. For a working solution, add 0.2 mL of the stock solution to 25 mL RPMI1640 culture medium without FBS to create a final solution of 200 units/mL. Store at 4°C.

6. Neomycin: (Gibco, cat. no. 21810-031): stock solution of 10.000 I.U./mL.
7. Insulin syringes: MicroFine +, 0.5 mL; 0.30 mm (30G) × 8 mm (Becton Dickinson, cat. no. 324870).
8. RPMI1640 culture medium (Invitrogen; cat. no. 13018-015): add the RPMI1640 powder (with L-glutamine and 25 mM HEPES) to 1 L distilled water. In addition, add 0.85 g $NaHCO_3$ and 5 mL neomycin-sulfate stock solution (10.000 I.U./mL). Filter sterilize (0.2 µm) and store in 100 mL aliquots at -20 °C.
9. Complete RPMI1640 culture medium: RPMI1640 culture medium supplemented with FBS to a final concentration of about 20% (v/v) (see **Note 3**).
10. Gas mixture: 5% CO_2, 5% O_2, 90% N_2 (in a gas bottle or cylinder).
11. Dimethylsulfoxide (DMSO, Merck, cat. no. 1.16743.1000): store in room temperature in dark.
12. Inhibitors/antimalarial drugs: dissolve the powder in DMSO, sterile Milli-Q water or culture medium in high concentration as stock solution (see **Note 4**); store at 4 °C or -20 °C. For serial dilutions, dilute the working stock solution with DMSO or culture medium (see **Note 5**; **Subheading 3.1.2**, **step 7**).
13. Chloroquine diphosphate salt (CQ; Sigma, cat. no. C6628): dissolve the powder in Milli-Q water to 10 mM as stock solution.
14. Cell culture lysis reagent (CCLR): Luciferase Assay System Kit® (Promega, cat. no. E1500). For working solution, dilute the 'Cell Culture Lysis 5× Reagent' provided in the kit with Milli-Q water.
15. Luciferase assay substrate solution: Luciferase Assay System Kit® (Promega, cat. no. E1500). For working solution, mix 1 vial of Luciferase Assay Substrate and 1 vial of 10 mL Luciferase Assay Buffer together. The mixed solution can be stored at -20 °C and can be subsequently freeze/thawed multiple times without a significant loss of activity, however, the solution must be kept in the dark at all times.

2.4. Equipment

1. Vortex mixer (IKA Labortechnik).

2. Table-top centrifuge (Beckman Coulter Allegra); most table-top centrifuges with a swing-out rotor are suitable.
3. Eppendorf microcentrifuge (13,000 rpm or 16,000 × *g*); most microcentrifuges are suitable.
4. Eppendorf Centrifuge 5810 (equipped with 96-well plate holders).
5. Light microscope, Carl Zeiss Standard 25 (Zeiss); all light microscopes with an oil-immersed × 100 objective are suitable.
6. Incubator or water bath (37 °C).
7. Sonicator bath.
8. Fluovac isofluorane-halothane scavenger (Stoelting Co., see **Note 6**).
9. 24-well and 96-well cell culture plates, sterile, with lids.
10. Biohazard Class II safety cabinet (see **Note 7**).
11. Glass desiccator (e.g., candle jar).
12. Heparinized capillary pipettes.
13. 96-well optical flat-bottomed and black-framed microplates (Nalge Nunc Intl.): all 96-well microplates with black frames and clear flat bottoms are suitable for luminescence measurement (see **Note 8**).
14. Luminescence microplate reader: Wallac Multilabel Counter 1420 (PerkinElmer, NL). Other microplate readers that can measure bioluminescence are suitable.

2.5. Software

1. Microsoft Excel is used to conduct preliminary data analyses.
2. GraphPad Prism software (Graph-Pad software, Inc., US) is used for statistical analyses (best-fit) effective concentration (EC_{50}) calculation.

3. Methods

3.1. The *in vitro* drug luminescence (ITDL) assay

In the ITDL assay, the *in vitro* development of ring-stage parasites into mature schizonts in the presence of drugs/inhibitors is quantified by measuring the luciferase activity in cultured mature schizonts; the luciferase activity has been shown to directly correlate with the number of schizonts [8]. The ITDL assay generates standard *in vitro* inhibition curves and EC_{50} of the inhibitors. The reporter parasite *Pb*GFP-Luc_{ama1} (see **Note 1**) is used in ITDL assay (see **Fig. 1** for the workflow).

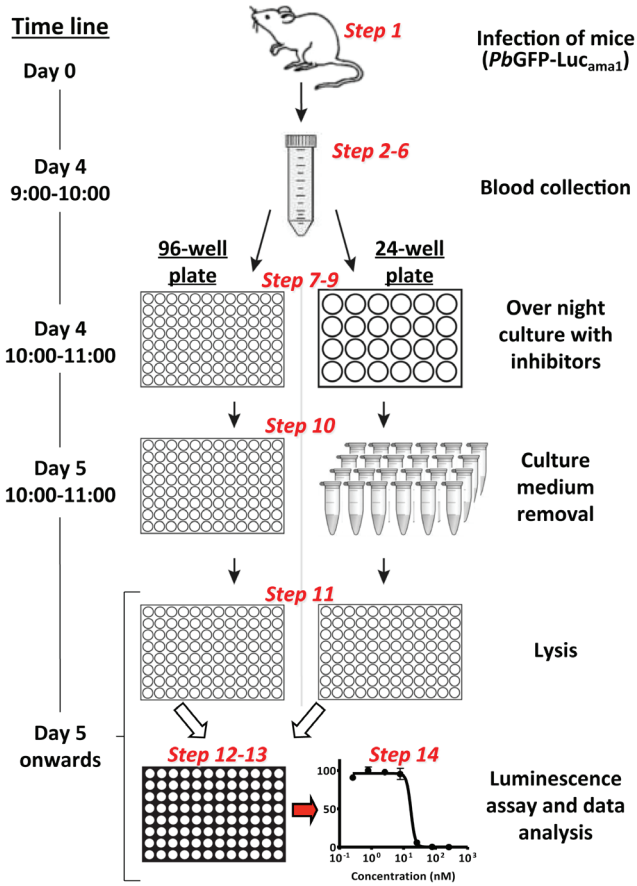

Figure 1. Workflow scheme of the ITDL assay

3.1.1. Collection of P. berghei ring forms for in vitro cultivation

1. On Day 0, infect 2 mice with 1 × 10^6 parasites of line *Pb*GFP-Luc$_{ama1}$ as follows:

   1) Collect one to four droplets (4–16 µL) of tail blood in 0.4 mL PBS from a mouse infected with parasite line *Pb*GFP-Luc$_{ama1}$. The parasitemia (i.e. the percentage of infected erythrocytes) in this mouse must be in the range of 5–15% (see **Note 9**).

   2) Immediately inject the suspension intraperitoneally into two mice, 0.2 mL per mouse.

   After infection of the two mice, it will take several days before the parasitemia reaches the required level for the transfer to *in vitro* culture for the ITDL assays. Usually blood is collected from the infected mice on day 4

after infection when the parasitemia reaches 1–3%.

2. Between 8:00 am and 9:00 am (see **Note 10**) on day 4, make a thin blood smear from one droplet of tail blood from the two mice infected on day 0. If the parasitemia ranges between 1 and 3%, proceed to the next step. If the parasitemia is lower than 1%, it is best to wait one more day when the parasitemia has increased to 1–3% (see **Note 11**).

3. Prepare complete RPMI1640 culture medium by adding 25 mL freshly thawed FBS to 100 mL RPMI1640 culture medium.

4. Collect a total of 0.8–1.0 mL blood from the infected mice by cardiac puncture under anesthesia between 9:00 am and 10:00 am (see **Note 10**). Immediately add the blood to a 50-mL tube containing 5 mL complete culture medium supplemented with 0.3 mL heparin stock solution.

5. Harvest the (infected) red blood cells (RBC) by centrifugation (table-top centrifuge) for 8 min at 450 × *g* and discard the supernatant.

6. Resuspend the (infected) RBC in complete culture medium into a final concentration of (infected) RBC of 0.5–1% (v/v) in complete culture medium. The total volume is dependent on how many serial dilutions are required for testing the inhibitors. For other details see the section below.

3.1.2. Overnight In vitro cultivation of ring forms in the presence of serial dilutions of inhibitors/antimalarial drugs

7. Preparation of dilution serials of inhibitors/antimalarial drugs:

   The inhibitors in stock solution (see **Subheading 2.3**, **item 12**) can be diluted with DMSO or culture medium according to their properties (see **Note 5**). For additional information about preparation of serial dilutions of the inhibitors, see **Note 12**. We routinely use chloroquine (at a concentration of 100 nM) as a control for complete inhibition of blood-stage development (see **step 14**).

8. Add the suspension of infected RBC in complete culture medium to wells of 24- or 96-well culture plates and subsequently add the serial dilutions of inhibitor to the wells (in triplicates) resulting in a total volume of 1 mL (24-well plates) or 75 µL (96-well plates) in each well.

9. Incubate the culture plates for a period of 24 h under standard *in vitro* culture conditions [15] (see **Note 13**), allowing the ring forms/young trophozoites to develop into mature schizonts.

1) Put culture plates into a small glass desiccator (e.g., candle jar) placed on a shaker in an incubator at 37 °C (see **Note 14**).

2) Flush the desiccator for 2 min at 1.5–2 bar pressure with the following gas mixture: 5% CO_2, 5% O_2, 90% N_2, and then either switch to lower gas flow and continuously gas overnight, or seal the dessicator once the air inside has been replaced with the gas mixture.

3) Switch on the shaker at a speed that is just fast enough to keep the cells in suspension and incubate the cultures (shaken) at 37 °C until the next day (day 5) for a period of 24 h.

3.1.3. Quantification of development of ring forms into mature schizonts in the presence of inhibitors/antimalarial drugs

10. Between 10:00 am and 11:00 am on day 5, the overnight cultures are collected and cells pelleted in the following ways:

    24-well plate cultures: transfer 500 µL of cell suspension of each well to 1.5-mL Eppendorf tubes and pellet the cells by centrifugation (microcentrifuge) at full speed (13,000 × *g*) for 1 min. Remove the supernatant (culture medium). Samples can be stored at -80 °C until you are ready to perform the luciferase assay (see **step 12**).

    96-well plate cultures: centrifuge the plates at 1000 × *g* for 5 min (Eppendorf Centrifuge 5810) and remove the supernatant (culture medium). These plates can be stored -80 °C until you are ready to perform the luciferase assay (see **step 12**).

11. Lyse RBC with 1 × cell culture lysis reagent (CCLR) as follows:

    24-well plate cultures: add 100 µL of 1× CCLR into each Eppendorf tube, mix by pipetting until all the cells are lysed, and then transfer solution to wells of new 96-well plates (see **Note 15**).

    96-well plate cultures: add 50 µL of 1× CCLR into each well and shake the plate for 5 min until the lysis is complete (see **Note 15**).

12. Luminescence assay:

    24-well plate cultures: add 100 µL of luciferase assay substrate solution and 10 µL of lysed cell samples into wells of a black-framed 96-well plate (see **Note 8**).

    96-well plate cultures: add 50 µL of luciferase assay substrate to the 96-well plate with lysed cells, mix them well and transfer the solution to a black-framed

96-well plate (see **Note 8**).

Wells containing PBS or lysed uninfected RBC are used as negative controls.

13. Luminescence spectra measurement:

    Measure the light reaction of each well of the plates for 10 s using a microplate luminometer. The luciferase activities are expressed as relative luminescence units (RLU) for each sample. The RLU for each drug concentration is calculated from the same experiment performed in triplicate.

14. Data analysis:

    1) The mean RLU value of 'complete inhibition control' (i.e. cultures with 100 nM chloroquine) is subtracted from the mean RLU values of all the other wells/concentrations.

    2) The mean RLU value of wells without drug ('no inhibition control') is taken as the maximal RLU value and given to indicate normal parasite development. All RLU values of experimental wells (i.e. parasites in the presence of inhibitors) are divided by the mean value of the 'no inhibition control' in order to calculate the percentage of inhibition.

    3) Growth inhibitory curves are plotted as a percentage of growth against concentration on a semi-log graph using the GraphPad Prism software. The non-linear regression function for sigmoidal dose-response (variable slope) is used to calculate the (best-fit) effective concentration (50% of the maximal inhibition; EC_{50} values) (see **Fig. 2** for examples).

3.2. The *in vivo* drug luminescence (IVDL) assay

In the IVDL assay, the *in vivo* parasite growth in mice is quantified by measuring the luciferase activity of circulating *Pb*GFP-Luc_{con} parasites (see **Note 1**) in samples of tail blood. The IVDL assay generates growth-curves that are identical to those obtained by manual counting of parasites in Giemsa-stained smears [8]. The IVDL assay can be applied to the standard 4-day suppressive drug test [9] or other assays in which the course of parasitemia is monitored in groups of mice (see **Fig. 3** for the correlation between luciferase activity and number of parasites). This assay has been used to determine the growth/multiplication of asexual blood stages of (genetically modified mutant) parasites (see **Fig. 4** for examples), and in other assays where infected mice have been drug-treated.

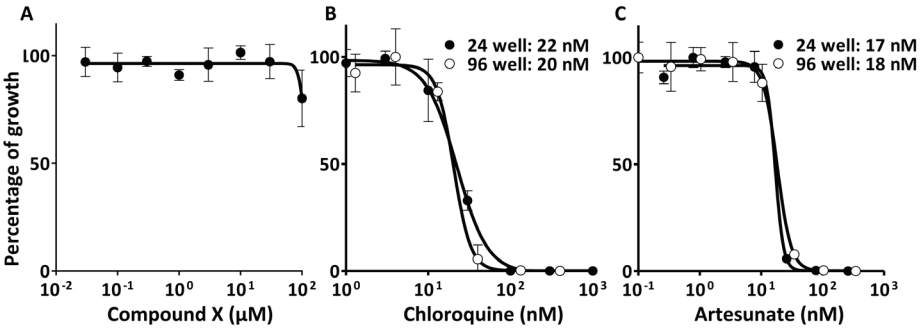

Figure 2. ITDL assay in 24- and 96-well plate cultures

Inhibition of the *in vitro* development of blood stages of *P. berghei* by compound X, chloroquine and artesunate. Inhibition of development was determined by measuring (the inhibition of) luciferase expression in parasites during development from ring forms into mature schizonts in over night culture (parasites of line *Pb*GFP-Luc_{ama1}). (A) The ITDL assay of compound X in 24-well culture plate, no inhibition was observed (unpublished data JWL and SMK). The ITDL assay of well-known inhibitors chloroquine (B) and artesunate (C) performed in both 24-well and in 96-well culture plates with the EC_{50} values shown (adapted from Ref. [8]).

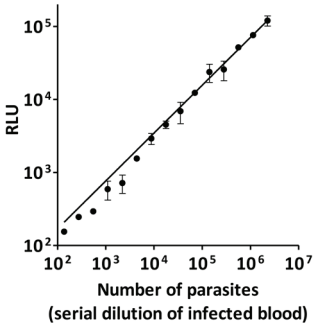

Figure 3. Correlation between luciferase activity (luminescence intensity) and the number of parasites or parasitemia.

Relationship between luminescence intensity (expressed as Relative Light Units, RLU) and numbers of parasites (*Pb*GFP-Luc_{con}). The infected blood was serially diluted with PBS (adapted from Ref. [8]).

3.2.1. The IVDL assay in combination with the standard 4-day suppressive drug test (see **Fig. 5** for the workflow).

1. On Day 0, infect 3 mice with 1×10^6 parasites of line *Pb*GFP-Luc_{con} (see **Subheading 3.1.1**, **step 1**) for every inhibitor/drug concentration that is used.
2. Administer the first drug at 3 h after parasite inoculation and then subsequently every 24 h for 3 more days (day 1–3 post infection, p.i.), which is comparable to the standard 4-day suppressive drug test.
3. Every morning (10:00 – 11:00 am, from day 4 to 15 p.i.), using heparinized capillary pipettes, collect 10 µL of tail blood from each mouse and transfer the blood into separate 1.5-mL Eppendorf tubes.

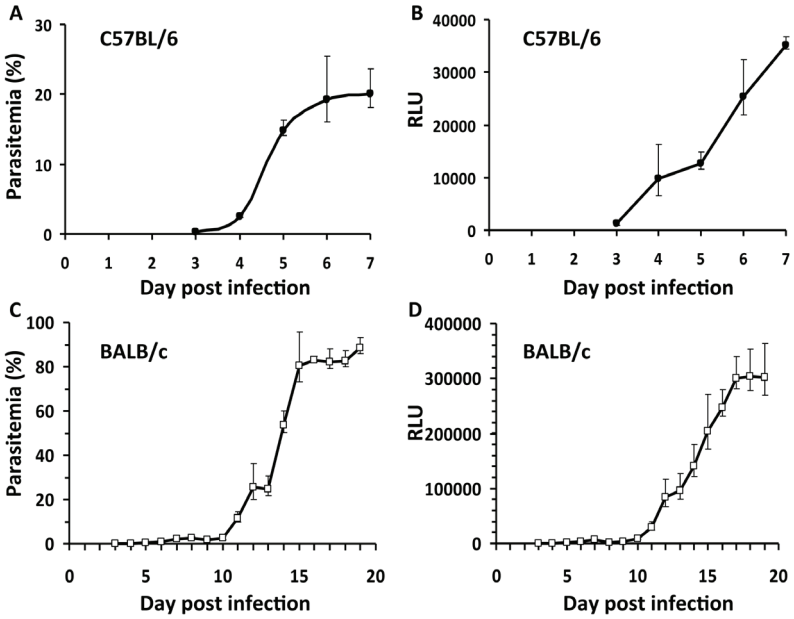

Figure 4. The course of blood stage infections in mice determined by counting parasites in Giemsa-stained blood films or measuring luminescence activity (RLU).

(A) (B) C57BL/6 mice infected with 10^6 *Pb*GFP-Luc$_{con}$ parasites. (C) (D) BALB/c mice infected with 10^6 blood stages of mutant Δ*nt1*, made in the *Pb*GFP-Luc$_{con}$ background (unpublished data JWL, SMK).

4. Briefly spin down the droplet of blood to the bottom of the Eppendorf tubes and store them at -80°C, until all samples are collected for the luminescence assay.

5. On day 16 p.i., all stored blood samples are collected and ready for the luminescence assay:

    1) Lyse blood samples with 100 µL of 1× cell culture lysis reagent (CCLR) and transfer the lysed cells to wells of 96-well plates (see **Note 15**).

    2) Add 100 µL of luciferase assay substrate solution and 10 µL of lysed cells samples into wells of a black-framed 96-well plate. Samples containing lysed uninfected red blood cells are used as negative controls.

    3) Measure the light reaction of each well for 10 s using a microplate luminometer. The luciferase activities are expressed as relative luminescence units (RLU) for each sample.

6. Data analysis: growth inhibitory curves are plotted as RLU against day p.i. using Microsoft Excel or GraphPad Prism.

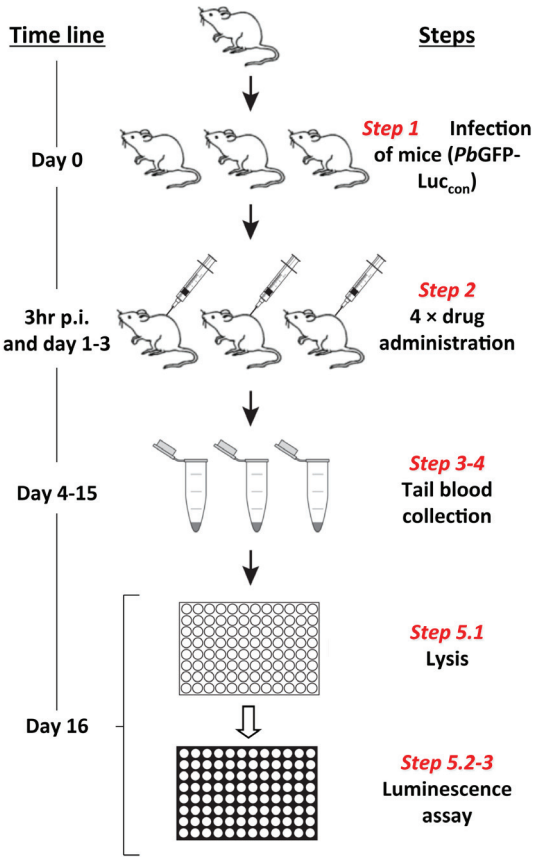

Fig. 5. Workflow scheme of the IVDL assay

3.2.2. The IVDL assay in combination with standard growth tests

1. On Day 0, infect mice with 10^4–10^5 parasites of line *Pb*GFP-Luc$_{con}$ as follows: collect 10 µL of tail blood in 10 mL PBS from a mouse infected with line *Pb*GFP-Luc$_{con}$ and with a 5−15% parasitemia (see **Note 9**); immediately inject the suspension intraperitoneally into 3–6 mice, 0.2 mL per mouse.

2. Each day (from day 3 p.i. onwards), using heparinized capillary pipettes, collect 10 µL of tail blood from each mouse and transfer the blood into separate 1.5-mL Eppendorf tubes. Spin the droplet of blood to the bottom of Eppendorf tubes and store them at -80°C for until all samples are collected (see **Note 16**) and proceed to step 3.

3. When all samples are collected, proceed to the luminescence assay and data analysis as described under **Subheading 3.2.1**, **steps 5–6**.

4. Notes

1. The two reporter parasite lines (*Pb*GFP-Luc$_{ama1}$ and *Pb*GFP-Luc$_{con}$) are used that stably express a fusion protein of GFP and luciferase (GFP-luc) without the expression of a drug-selectable marker. In both lines the *gfp-luciferase* gene is integrated into the 'phenotypically neutral' *230p* genomic locus by double cross-over integration.

   To quantify schizont development in the ITDL assay using luciferase as a reporter, a schizont-specific promoter is required. For this purpose we have used the *ama-1* promoter to generate the reporter line as this promoter drives expression of the reporter protein only in the late schizont stage [16]. In previous papers the use of GFP-Luc-expressing reporter lines 354cl4 and 875m2cl1 [8] has been described for the ITDL assay. However, now we recommend using the reporter line 1037m1f1m1cl1 (*Pb*GFP-Luc$_{ama1}$) because of the absence of a drug-selectable marker and stable integration of the reporter gene into the *230p* locus. The *Pb*GFP-Luc$_{ama1}$ line is available from the Leiden Malaria Research Group (http://www.lumc.nl/con/1040/8102 8091348221/810281121192556/).

   Line 676m1cl1 (*Pb*GFP-Luc$_{con}$) has GFP-Luc under the control of *eef1α* promoter which permits the expression of the reporter in all blood stages [13]. This constitutive expression of luciferase is essential for a reporter parasite line used for the quantitative analysis of parasitemia or parasite densities in infected blood in the IVDL assay. The *Pb*GFP-Luc$_{con}$ line is available from MR4 (http://www.mr4.org/).

2. All experiments using mice must be performed according to the applicable national guide lines and regulations. Diets of laboratory rodents with low content of total protein, energy and/or *p*-aminobenzoic acid (PABA) can negatively influence *P. berghei* infections [17]. In our laboratory, we therefore provide diets with high protein content (20–25% of total and gross energy content; 18,000–20,000 kJ/kg).

3. For optimal *in vitro* parasites growth a relatively high percentage (~20%) of FBS is used.

4. It is important to dissolve inhibitors/antimalarial drugs completely; vortexing and/or sonication and/or 37 °C incubation can help to dissolve compounds.

5. It is better to make drug serial dilutions fresh, though they can also be stored at -20 °C or -80 °C.

6. Mice are anesthetized in the 'induction chamber,' which is prefilled with the anesthetic vapor (a mixture of isofluorane and air) via the vaporizer unit. The injection of parasites/ drugs or collecting blood by heart puncture is performed in mice that are kept under anesthesia by holding their muzzles to the small mask that is connected to the vaporizer unit.

7. Most manipulations with blood infected with genetically modified *P. berghei* parasites are performed in a Class II safety cabinet.

8. For luminescence measurements, we routinely use black-framed microplates as they best reduce light scattering between wells, which can artificially increase the signals detected in neighboring wells and thereby calculated EC_{50} values.

9. The start of the procedure at day 0 requires a mouse infected with *P. berghei* reference line (*Pb*GFP-Luc_{ama1} in ITDL or *Pb*GFP-Luc_{con} in IVDL) which has a parasitemia of 5–15%, obtained either by mechanical passage or initiated from a cryopreserved parasite stock.

10. It is important to perform steps 2–4 early in the morning (8:00 – 10:00 am). *P. berghei* has a 22–24-h asexual blood-stage cycle which is partly 'synchronized' in mice with the normal day-night light regime. In these mice, the rupture of schizonts and invasion of RBC mainly occur between 02:00 and 04:00 am every day. This results in the presence of mainly young ring forms in blood of infected mice at 8:00 – 9:00 am. Most inhibitor tests are performed with these partly synchronous parasites. However, if pure populations of young ring forms are required for drug-susceptibility testing, these can be obtained by standard techniques of schizont culture and purification procedure and intravenously injecting purified schizonts into tail veins of mice to set up highly synchronized infections [15].

11. A parasitemia higher than 3% is suboptimal because many erythrocytes will become multiply infected or parasites will reside in the 'older' erythrocytes (normocytes) and not in reticulocytes. In both cases, the development of schizonts in culture is greatly impaired.

12. In case of inhibitors that are difficult to dissolve in water, dilutions need to be made in DMSO. We prepare stock solutions at a 100 times the final concentration required in the well so that when the drug is added to the well the concentration of DMSO in overnight culture is $\leq$1%, which is not harmful to the schizont development. No inhibition controls (i.e. culture without inhibitors) also contain $\leq$1% DMSO.

In our laboratory we usually prepare 10–12 different concentrations of the inhibitors (including no inhibition control). Two different 10-fold dilution serials are prepared, one is diluted into 1, 10, 100…. serial and the other 0.3, 3, 30… serial. For example, for an inhibitor with observed EC_{50} ~50 nM in *P. falciparum*, we prepared a final dilution serial of 0, 0.3, 1, 3, 10, 30, 100, 300, 1000, 3000, 10,000 nM for the *P. berghei* ITDL assay.

13. In the overnight cultures, the ring forms and (young) trophozoites develop into schizonts that reach maturity in the morning of the next day. The mature schizonts do not rupture spontaneously and remain viable for several hours. For optimal development of the schizonts, the correct gas conditions (lowered oxygen concentration compared to air), the pH of the culture medium (7.2–7.4) and the temperature are crucial. The temperature is crucial because it influences the rate of development of the schizonts. Above 38.5 °C, parasites will degenerate. Lower than 37 °C, the parasites will develop into healthy parasites but the developmental time of one complete cycle will be longer than the standard 22–24 hrs. A temperature of between 36 and 37 °C is optimal to collect viable, mature schizonts between 09:00 and 11:00 am on day 5.

14. It is very important to transfer the cultures to the CO_2 gassed glass desiccator as soon as possible, as parasites easily degenerate in O_2-rich environment.

15. The lysed samples can also be stored at -20 °C or -80 °C until being used in luminescence assay. Transferring lysed samples from Eppendorf tubes to 96-well plates is not essential but helpful for using multi-channel pipettes in later steps.

16. The period of collecting infected tail blood is dependent on the experiment, the mouse strain used and the growth rate of the parasites. Parasites of reference lines of *P. berghei* ANKA induce cerebral complications (Experimental cerebral malaria; ECM) in ECM-sensitive mouse-strains such as C57BL/6, Swiss-OF1, CBA/J or Swiss-CD1. These mice usually die at day 6 to 9 after infection with 10^4 to 10^6 parasites at a parasitemia of 10–25%. ECM-resistant mice (e.g., BALB/c or NIH Swiss mice) usually die in week 3 after infection with a parasitemia of >60% and die from complications such as severe anaemia and multiple organ failure [18,19].

References

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