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Serum albumin and α-1 acid glycoprotein impede the killing of *Schistosoma mansoni* by the tyrosine kinase inhibitor Imatinib

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ABSTRACT

In the search for new drugs and drug targets to treat the flatworm disease schistosomiasis, protein kinases (PKs) have come under particular scrutiny because of their essential roles in developmental and physiological processes in schistosome parasites. In this context the application of the anti-cancer Abl tyrosine kinase (TK) inhibitor Imatinib (Gleevec/Glivec; STI-571) to adult *Schistosoma mansoni* *in vitro* has indicated negative effects on diverse physiological processes including survival.

Motivated by these *in vitro* findings, we performed *in vivo* experiments in rodent models of *S. mansoni* infection. Unexpectedly, Imatinib had no effect on worm burden or egg-production. We found that the blood components serum albumin (SA) and alpha-1 acid glycoprotein (AGP or orosomucoid) negated Imatinib's deleterious effects on adult *S. mansoni* and schistosomula (post-infective larvae) *in vitro*. This negative effect was partially reversed by erythromycin. AGP synthesis can increase as a consequence of inflammatory processes or infection; in addition upon infection AGP levels are 6–8 times higher in mice compared to humans. Therefore, mice and probably other rodents are poor infection models for measuring the effects of Imatinib *in vivo*. Accordingly, we suggest the routine evaluation of the ability of AGP and SA to block *in vitro* anti-schistosomal effects of small molecules like Imatinib prior to laborious and expensive animal experiments.

1. Introduction

Schistosomiasis is caused by a number of schistosome species, which belong to the class Trematoda within the phylum platyhelminthes. According to recent WHO data, about 780 million people are at risk of infection, and more than 240 million patients require treatment each year (Ross et al., 2002; Steinmann et al., 2006; King, 2010; World Health Organization, 2013). Besides humans, infection in farm and wild animals induces similar pathological consequences (De Bont and Vercruysse, 1998; Wang et al., 2006; Wu et al., 2010). Therefore, schistosomiasis represents not only a medical but also a serious socio-economic problem, which affects both developing and newly industrializing countries (Huang and Manderson, 2005; King, 2010; McManus et al., 2010; Chen, 2014).

Three drugs have been available to treat schistosomiasis, metrifonate (against *Schistosoma haematobium*; no longer commercially available), oxamniquine (active only against *Schistosoma mansoni*; restricted availability), and praziquantel (PZQ). The latter is the only drug effective against all important schistosome species and consequently, as recommended by the WHO, is the drug of choice applied in preventive chemotherapy programs worldwide (Harder, 2002; Magnussen, 2003; Fenwick et al., 2006; Mathers et al., 2007; Stothard et al., 2009; Danso-Appiah et al., 2013). However, PZQ has notable failings as a drug: (i) it mainly targets the adult worm whereas the immature forms between 7 and 28 days post-infection (p.i.) are less susceptible; (ii) complete cure is rarely achieved in the single 40 mg/kg recommended dose for MDA; (iii) this drug it is not free of adverse effects (Doenhoff et al., 2008; Caffrey et al., 2009) and (iv) with the increasingly widespread and regular application, there is justified fear of emerging resistance. Laboratory experiments have shown that reduced susceptibility against PZQ is inducible upon selection pressure (Doenhoff et al., 2008; Sabra and Botros, 2008; Pica-Mattoccia et al., 2009). Clinically relevant proof of resistance has not been reported yet, however, results of

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field studies indicate decreased PZQ efficacy (Ismail et al., 1999; Black et al., 2009; Melman et al., 2009). Due to the availability of genome data for the three important schistosome species infecting humans (Berriman et al., 2009; Schistosoma japonicum Genome Sequencing and Functional Analysis Consortium, 2009; Protasio et al., 2012; Young et al., 2012), the existence of multidrug-transporters has been confirmed and initial characterizations demonstrate that a P-glycoprotein efflux pump and multidrug resistance-associated proteins of *S. mansoni* are responsive to PZQ (James et al., 2009; Kasinathan and Greenberg, 2012; Greenberg, 2013).

Due to the lack of a vaccine and limited drug availability, the WHO ranks schistosomiasis next to malaria and tuberculosis in importance as a tropical disease for which novel treatment strategies are urgently needed (Steinmann et al., 2006; Montresor et al., 2012; World Health Organization, 2013). Many research initiatives are underway, and new targets have come into focus (Caffrey, 2007; Caffrey and Selzer, 2012; Geary, 2012; Huang et al., 2012; Prichard et al., 2012). Among these, the TKs that have been extensively studied during the last decade for their pleiotropic functions in development, growth including mitosis, reproduction, tissue integrity, and survival (Swierczewski and Davies, 2010; Dissous and Grevelding, 2011; Buro et al., 2013; de Saram et al., 2013; Dissous et al., 2013; Andrade et al., 2014). The biological functions of these TKs and their roles as presumptive candidates for targeting were elucidated by *in vitro*-culture of adults and/or larval stages with small molecule inhibitors and/or RNAi.

Among the TKs studied, the *S. mansoni* orthologs of the Abelson murine leukemia (Abl) TKs, SmAbl1 and SmAbl2, have been characterized in particular detail. By *in situ* hybridization using adults, transcripts for SmAbl1 and SmAbl2 have been detected in the gonads, the area surrounding the ootype, and the parenchyma and/or the gastrodermis indicating their involvement in reproduction and other physiological processes (Beckmann and Grevelding, 2010). Comparative sequence analyses have shown that these SmTKs possess the majority of amino acid residues necessary for human Abl-kinase to bind to Imatinib (Nagar et al., 2002; Beckmann and Grevelding, 2010). Imatinib is a small-molecule inhibitor marketed as Glivec (Gleevec/STI-571), it acts as a competitive antagonist of the adenosine triphosphate (ATP) binding site of Abl-TKs, and is used to treat chronic myelogenous leukemia and other human cancers (Manley et al., 2002; Larson et al., 2008). Biochemical studies have confirmed that both schistosome Abl-TKs are targets for Imatinib (Beckmann et al., 2011; Buro et al., 2014). Studies with adult schistosomes *in vitro* demonstrated dose- and time-dependent effects of Imatinib, including body swellings, defects in locomotion, reduced pairing stability and viability. Microscopic analyses revealed degenerative changes within the gonads such as disordered apoptotic oogonia and smaller testes with defective sperm differentiation. The most remarkable effect, however, was the degradation of the gastrodermis that caused the death of the parasites (Beckmann and Grevelding, 2010).

To further analyze the potential of Imatinib as an anti-schistosomal therapy, we employed mouse and hamster infection models as well as *in vitro* studies to investigate the effect of Imatinib *in vivo* and *in vitro* with a special focus on specific host-blood components.

2. Material and methods

2.1. Parasite stocks

Adult and larval schistosome stages originated from a Liberian (Grevelding, 1995) and a Puerto Rican (Abdulla et al., 2009) isolate of *Schistosoma mansoni*, respectively. Both were maintained in snails (*Biomphalaria glabrata*) and Syrian hamsters (*Mesocricetus auratus*). Cercariae were obtained from snails after 30 days of infection. Adult parasites were obtained by hepato-portal perfusion at 42–49 days p.i. as described before (Grevelding, 1995; Abdulla et al., 2009). Experiments were approved by the regional council Giessen, Germany (V54-19 c 20/15 c GI 18/10 and V54-19 c 20/15 (1) GI 18/10 – Nr.75/2009). All animal procedures performed at the UCSF, USA, were done in accordance with protocols approved by the UCSF Institutional Animal Care and Use Committee (IACUC) as required by the Federal Animal Welfare Act and the National Institutes of Health Public Health Service Policy on Humane Care and Use of Laboratory Animals (http://grants.nih.gov/grants/olaw/references/phspol.htm).

2.2. Infection and treatment of rodents

For these experiments 12 mice (NMRI) and 12 hamsters (*Mesocrietus auratus*) were used in Giessen (Germany), and 6 hamsters (*Mesocrietus auratus*) at the UCSF (USA). In Giessen, infections with schistosomes were performed by the paddling method as described before using 2000 cercariae per hamster and 800 per mouse (Grevelding, 1995). At the UCSF, 4–6 week old, female hamsters were infected sub-cutaneously with 800 cercariae of a Puerto Rican isolate of *S. mansoni* (Abdulla et al., 2009).

Previously obtained data indicated killing of adult parasites within 1-2 days using 100 μM, or within 4–7 days using 1–10 μM *in vitro* (Beckmann and Grevelding, 2010; Dissous and Grevelding, 2011). Experiments in the mouse model have shown an elimination half-life of 1.3 h for Imatinib in mice and tissue/plasma concentration ratios of Imatinib of about 3.76 ± 1.09, and 12.0 ± 6.3 μg/ml after applications of 25 mg/kg or 50 mg/kg (Teoh et al., 2010). For liver, tissue/plasma concentration ratios between 19.2 ± 13.3 μg/g, and 61.4 ± 40.1 μg/g were determined after multiple dosages of 25 mg/kg or 50 mg/kg, respectively (Teoh et al., 2010). With respect to these data and pharmacokinetic considerations concerning the application of a drug for humans in rodents (Fabbro et al., 2002; Kretz et al., 2004; Löscher et al., 2006) different dosages of 20–100 mg/kg body weight were applied. From days 40 p.i. or 34 p.i. on, Imatinib (dissolved in 0.9% NaCl) was given at a daily basis by gavage over 4-days or 10-days periods, respectively, which was conducted by a veterinarian. The constitution of the animals was checked daily. One day after the final treatment, the animals were euthanized and the parasites recovered by perfusion.

2.3. In vitro culture of adult schistosomes

After perfusion, adult schistosomes were washed three times with M199 medium before being cultured in the same medium (Gibco; including glucose, sodium bicarbonate, 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid) supplemented with an antibiotic/antimycotic mixture (1%, Sigma) and new born calf serum (NCS) (10%, Sigma Aldrich) at 37°C and 5% CO_2 as described before (Beckmann and Grevelding, 2010; Beckmann et al., 2012). For each experiment, 5–10 *S. mansoni* couples were kept per well in 6-well or 12-well plates with 2–3 ml culture medium per well. The medium was changed every 24 h. All following experiments were done in duplicate. To investigate the influence of serum albumin (SA), adult *S. mansoni* couples were maintained in culture medium, which contained 1.93 g/L SA due to the supplemented NCS. Because the *in vivo* concentration of albumin in blood of mice or humans is much higher with 44 g/L (mice) or 35–55 g/L (human), the culture medium was supplemented with BSA or HSA in a concentration of 43 g/L to achieve a final albumin concentration of 45 g/L, reflecting the *in vivo* situation. *S. mansoni* couples were treated in these different media (without additional SA, with BSA, or with HSA) with Imatinib (0, 10, or 50 μM) for 6 days.

During this time period, pairing stability, physiology, morphology, and survival were investigated.

Correspondingly, human alpha acidic glycoprotein (AGP; Sigma Aldrich) was added to the culture medium in a concentration of 0.8 g/L. Erythromycin was used in the culture medium in a final concentration of 20 or 40 μM, dissolved from a standard solution (Sigma Aldrich). *S. mansoni* couples were treated in varying culture media with different concentrations (10–20 μM) of Imatinib (Enzo Life Sciences; dissolved in water) up to 4 days. Pairing status, egg production, morphology and vitality of treated or control parasites were documented by bright-field microscopy on a daily basis.

2.4. In vitro culture of larval schistosomes

Cercariae were harvested and mechanically transformed into schistosomula as described (Abdulla et al., 2009). Schistosomula were cultured in 96-well plates (200 parasites/well) for four days at 37°C with 5% CO_2 in Basch medium 169 (Basch, 1981) supplemented with 5% FBS (fetal bovine serum). Morphology and vitality of treated or control schistosomula were measured by bright-field microscopy on a daily basis as described (Rojo-Arreola et al., 2014). Measurements were performed in duplicate.

3. Results

3.1. Schistosomes are not affected by Imatinib treatment in rodents

Motivated by the promising results obtained *in vitro*, animal experiments were performed to investigate whether Imatinib affects the physiology and survival of adult parasites also *in vivo*. Based on previous data on the effect of Imatinib on adult *S. mansoni in vitro* and plasma- as well as tissue-concentrations of this drug in treated mice (Teoh et al., 2010) we expected that the application of 20-100 mg/kg over 4-10 days starting 34 or 40 days p.i. are sufficient to negatively affect schistosome reproduction and survival along the application period. Following perfusion and inspection of parasites perfused from mice treated over 4 or 10 days with up to 100 mg/kg, we surprisingly noted no differences of worm number, pairing stability, egg production or survival parameters (sucking capability to the Petri dish, mobility, gut peristalsis) compared to the control (data not shown).

3.2. SA decreases the detrimental effects of Imatinib on adult S. mansoni in vitro

Binding of Imatinib to SA has been reported to negatively influence the activity of Imatinib *in vivo* (Fitos et al., 2006). However, for schistosomes, albumin is also discussed as a good drug carrier for chemotherapeutic substances like anthelmintics because it appears to be an important source of energy supply for adult parasites (Bennett and Caulfield, 1991; Delcroix et al., 2006; Holtfreter et al., 2010). Therefore, it was also conceivable that albumin could positively influence Imatinib efficacy on schistosomes.

Without additional SA in the culture medium, the couples had separated by 24 and 72 h in the presence of 50 and 10 μM Imatinib, respectively (Fig. 1A). In each case, the couples were no longer attached to the bottom of the culture plates, were slower, and less vital. Also, bulges recorded previously as one of the consequences of Imatinib treatment (Beckmann and Grevelding, 2010) were distributed along the body. After 156 h, all of the parasites had died. In comparison, medium enriched with BSA or HSA (45 g/L final concentration) delayed these effects to different degrees. With BSA in the culture medium, couples exposed to 50 μM Imatinib did not become separated before 96 h; in the presence of 10 μM Imatinib couples did not separate at all (Fig. 1B). Correspondingly, with 50 μM Imatinib a reduced vitality and small bulges were first observed after 84 h, and after 156 h, these parasites were dead. Using 10 μM Imatinib and BSA, no influence on physiology or moving capacity was observed. With HSA in the culture medium, the couples separated completely after 72 h (with 50 μM Imatinib) or 156 h (with 10 μM Imatinib) (Fig. 1C). With 50 μM Imatinib, the schistosomes were no longer attached to the culture dish within 48 h under these conditions, showed reduced moving, vitality and bulges after 48–72 h, and they died after 156 h. Using 10 μM Imatinib, however, no killing was observed although the parasites displayed decreased movement and vitality after 156 h.

These *in vitro* results indicate that SA decreased the efficacy of Imatinib on adult *S. mansoni in vitro*. Furthermore, we noted that compared to HSA, BSA exerted a stronger inhibition of Imatinib.

3.3. AGP decreases the detrimental effects of Imatinib on adult S. mansoni in vitro

Like SA, AGP has also been reported to bind to Imatinib, thus negatively influencing its therapeutic activity in cancer patients (Kremer et al., 1988; Gambacorti-Passerini et al., 2000; Fitos et al., 2006, 2012; Hegde et al., 2012). To investigate the effect of AGP in our system, adult *S. mansoni* couples were maintained in culture medium, which was supplemented with human AGP at a final concentration of 0.8 g/L, similar to that found in human plasma (0.5–1.4 g/L) (Schultz and Arnold, 1990; Gambacorti-Passerini et al., 2000). In addition, the medium contained 1.93 g/L albumin due to the supplemented NCS.

Control couples (no Imatinib treatment) maintained in culture medium with and without AGP showed no differences in morphology, behavior or vitality (Supplementary data 1, 2). However, within 48–72 h clear differences were observed between schistosomes treated with Imatinib in the presence or absence of AGP. Thus, in the presence of 20 μM Imatinib but without AGP, couples had separated and detached from the culture dish, were morphologically abnormal (bulges and swellings; Fig. 2B), and died after 96 h. In contrast, in the presence of AGP, schistosomes remained paired, adhered to the petri dish (Fig. 2D), and were apparently similar to control couples (Fig. 2A, C). Using 10 μM Imatinib, similar observations were made within 72–96 h (Supplementary data 3, 4).

Previous studies have demonstrated that antibiotics such as erythromycin compete with Imatinib for binding of AGP (Gambacorti-Passerini et al., 2000, 2002; Azuma et al., 2007). As erythromycin has the potential to indirectly increase the concentration of free Imatinib, we hypothesized that this antibiotic may rescue the schistosomicidal effect of Imatinib in the presence of AGP. When erythromycin was added to culture medium at concentrations of 20 or 40 μM, the inhibitory effect of AGP on the Imatinib (20 μM) response was abolished: couples separated, detached from the culture dish, showed reduced movement and morphological perturbations (bulges and swellings along the body; Fig. 2F). These parasites had similar phenotypes as those treated in medium without AGP, and they also died within 96 h. Corresponding observations were made for the couples treated with 10 μM Imatinib (Supplementary data 5, 6). Erythromycin alone also negatively affected the vitality of the schistosome couples, but only after treatment periods exceeding five days (data not shown). In addition to direct effects on schistosomes, decreased egg production was measured. Whereas couples in medium (with and without AGP; 0, 20, or 40 μM erythromycin; 0 μM Imatinib) laid normal eggs (Fig. 3A, B), the couples treated with Imatinib in medium without AGP (0, 20, or 40 μM erythromycin) laid less eggs. Instead of eggs, a lot of vitellocytes and oocytes were detected in the culture medium (Fig. 3C, E). When the couples were treated with Imatinib (20 μM) in medium with AGP, normal eggs were laid

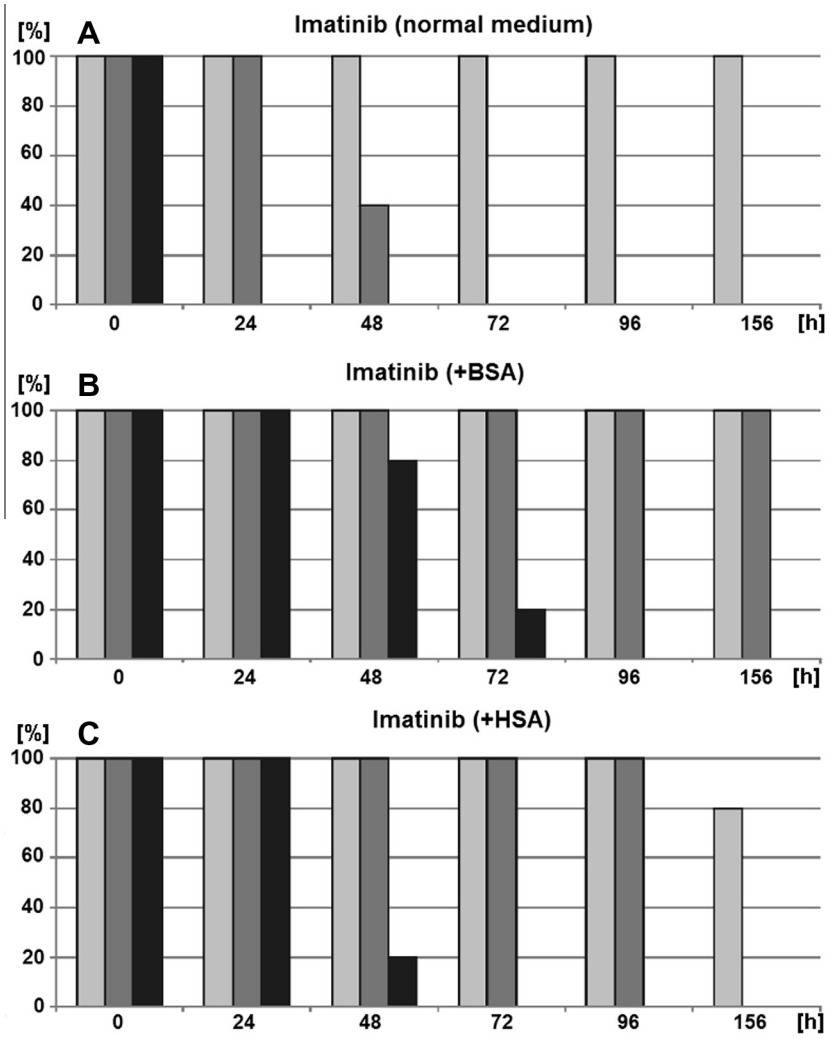

Fig. 1. Influence of serum albumin on pairing stability of Imatinib-treated *S. mansoni* couples. *S. mansoni* couples were treated for up to 156 h *in vitro* with 0/10/50 μM Imatinib in medium with and without bovine or human serum albumin. The effect of the treatment was monitored over time (0–156 h; *x*-axis) analysing pairing stability (given in % values on the y-axis). (A) treatment in normal M199 medium, (B) treatment in medium with BSA (45 g/L), C: treatment in medium with HSA (45 g/L). Unexpectedly, all schistosomes treated with 10 μM Imatinib and HSA (C) had separated after 156 h of treatment, which in this specific case is unlikely to be a consequence of inhibitor treatment alone. Light grey: 0 μM Imatinib, grey: 10 μM Imatinib, dark grey: 50 μM Imatinib.

again, and no free vitellocytes and oocytes were found in the medium (Fig. 3D). However, when erythromycin (20 μM or 40 μM) was added additionally, egg production decreased, and again vitellocytes and oocytes were deposited into the medium (Fig. 3F).

These results demonstrate the negative influence of AGP on the schistosomicidal effect of Imatinib, an influence that can be reversed by erythromycin.

3.4. SA and AGP combined exert the strongest effect on the potency of Imatinib

In a combined treatment approach, *S. mansoni* couples were treated with 10 μM Imatinib in medium containing HSA up to a concentration of 45 g/L, 0.8 mg/ml AGP and, additionally, erythromycin at a final concentration of 20 μM. In the first seven days, no changes to control couples without Imatinib in the medium were observed. After 8 days, a slightly reduced vitality of the Imatinib-treated schistosomes was observed as well as a higher number of separated couples (50%) as well as schistosomes which were no longer attached to the petri dish (70%). After 14 days, the Imatinib-treated parasites still showed reduced vitality, but did not die (data not shown). Thus, erythromycin could not completely reinstate the anti-schistosomal effect of Imatinib in the presence of both HSA and AGP.

3.5. Imatinib kills schistosomula in vitro but efficacy is decreased by BSA and AGP

In vitro experiments were performed to test whether Imatinib kills schistosomula and whether SA and/or AGP decrease the inhibitor's efficacy. When treated with Imatinib alone (10 μM and 20 μM; 24 h to 96 h), schistosomula degenerated (becoming rounded and dark) and eventually died. These effects occurred in a dose- and time-dependent manner (Fig. 4A, B).

In contrast, schistosomula treated with different concentrations of Imatinib in medium supplemented with BSA (45 g/L) were phenotypically similar to DMSO controls. Also the addition of AGP (0.8 g/L) to the medium influenced the effect of Imatinib. Although not completely reversed, the Imatinib effect was delayed by AGP. Consequently, the concurrent addition of both BSA and AGP to the medium blocked the Imatinib effect as well, but the numbers of degenerate parasites obtained by combining these blood components did not significantly differ from those of the BSA-only treatment group. The presence of erythromycin as a single

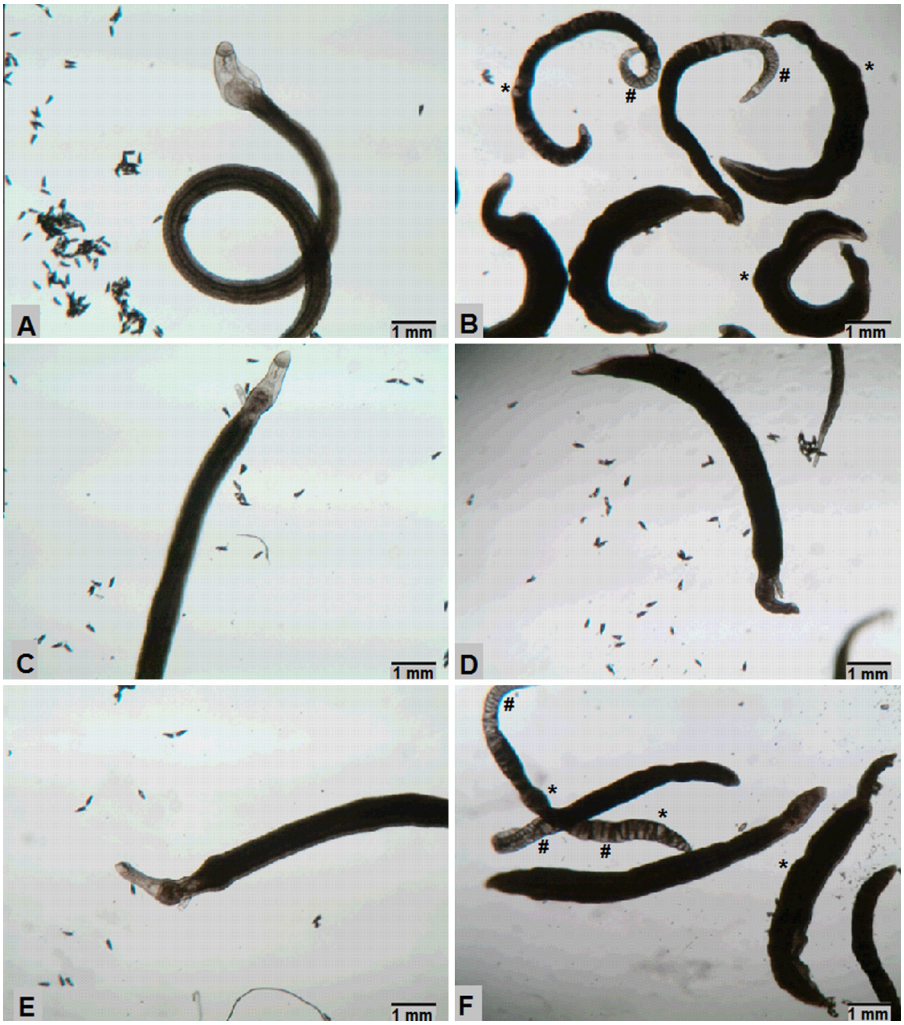

Fig. 2. Influence of alpha-1 acid glycoprotein against erythromycin addition on pairing-stability and physical integrity of Imatinib-treated *S. mansoni* couples. *S. mansoni* couples were treated for 3 days *in vitro* with 20 μM Imatinib in medium with and without 0.8 g/L AGP as well as with or without 20 μM erythromycin. (A) 0 μM Imatinib, M199; (B): 20 μM Imatinib, M199; (C): 0 μM Imatinib, M199 + AGP; (D): 20 μM Imatinib, M199 + AGP; (E) 0 μM Imatinib, 20 μM erythromycin, M199 + AGP; (F) 20 μM Imatinib, 20 μM erythromycin, M199 + AGP. Scale bars: 1 mm; *bulges and #gut alterations indicating the deleterious effect of Imatinib.

component in concentrations of 20 μM or 40 μM had no influence on vitality of schistosomula (data not shown). Erythromycin (20 μM or 40 μM) partially reversed the negative influence of AGP on Imatinib beginning at 48 h after treatment. However, when BSA and AGP were added simultaneously with the inhibitor (up to 20 μM), erythromycin was unable to reverse the negative effect of Imatinib (data not shown).

4. Discussion

4.1. SA and AGP impede the effect of Imatinib on schistosomes

Recently, the search for alternative treatments for schistosomiasis and other infectious diseases caused by parasites in humans and animals has gained momentum. Many initiatives have identified putative candidate molecules, often accompanied by the characterization of genes and/or proteins and their importance for parasite biology. Whether or not the putative target(s) is known, the literature is full of reports on the *in vitro* identification of promising anti-schistosomal compounds. Experiments involving animal (rodent) models of infection are then required to confirm the effects observed *in vitro*. However, very often *in vivo* studies fail in this regard (Keiser, 2010; Rodriguez et al., 2010; Murthy et al., 2011; Da'dara et al., 2013; Katz et al., 2013; Soeiro et al., 2013; this study). Likewise, our studies with Imatinib suggest that there is a remarkable discrepancy between the *in vivo* and the *vitro* results that registered dramatic effects on adults (Beckmann and Grevelding, 2010; Beckmann et al., 2012) and schistosomula (this study), and the lack of schistosomicidal activity *in vivo* (Katz et al., 2013; this study). This unexpected result corresponded to (i) similar treatment experiments with schistosome-infected hamsters, in which compared to the mouse model even higher plasma concentrations of Imatinib were determined (about 42 μM after 30 min and about 12 μM after 6 h following a 60 mg/kg dosage; data not shown) but also (ii) to recent data obtained in a parallel study where Imatinib treatment of adult worms showed similar *in vitro* effects but no obvious *in vivo* effects in mice treated with up to even 1000 mg/kg (Katz et al., 2013).

Although SmAbl1 and SmAbl2 were identified as targets of Imatinib (Beckmann and Grevelding, 2010; Beckmann et al., 2011), and although this small-molecule inhibitor had been successfully applied in cancer studies using the mouse (Manley et al., 2002; Waller, 2010), Imatinib has no effect on the schistosome parasite in rodents. It is known that serum levels of Imatinib in mice after administration of up to 50 mg/kg range between 3 and 12 μg/ml (6–24 μM; Teoh et al., 2010), which is in the range of those concentrations that affect vitality of adult schistosome

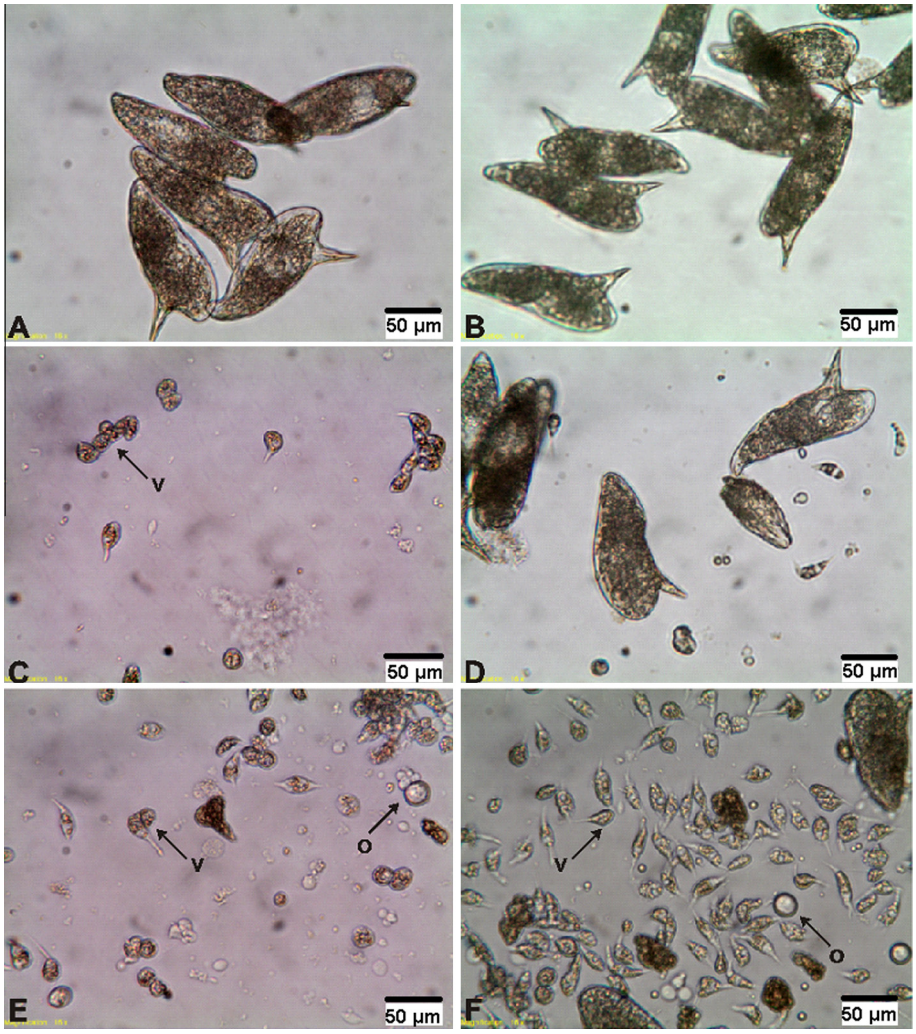

Fig. 3. Influence of alpha-1 acid glycoprotein against erythromycin on egg production of Imatinib-treated *S. mansoni* couples. Overview of egg production of *S. mansoni* couples, which were treated with 20 µM Imatinib in medium with and without 0.8 g/L AGP as well as with or without 20 µM erythromycin *in vitro* for 3 days. (A) 0 µM Imatinib, 0 µM Imatinib/20 µM erythromycin, M199; (B) 0 µM Imatinib, 20 µM erythromycin, M199 + AGP; (C) 20 µM Imatinib, 0 µM erythromycin, M199; (D) 20 µM Imatinib, 0 µM erythromycin, M199 + AGP; (E) 20 µM Imatinib, 20 µM erythromycin, M199; (F) 20 µM Imatinib, 20 µM erythromycin, M199 + AGP. V = vitellocytes, O = oocytes; scale bars: 50 µM.

in vitro (Beckmann and Grevelding, 2010; Beckmann et al., 2012). Also, in human patients Imatinib was found to be rapidly absorbed leading to mean maximal concentration of 2.3 µg/ml (4.6 µM) when 400 mg were applied once a day with a half-life of the drug in the circulation ranging from 13-16 h, and the drug level increased by a factor of 2–3 at steady state with once-daily dosing (Druker et al., 2001; Awidi et al., 2010; Ishikawa et al., 2010).

To answer the question why the *in vivo* studies using Imatinib in the mouse and hamster models failed, we focused on SA and AGP as they are known to bind to drugs negatively influencing their action (Piafsky, 1980; Kremer et al., 1988; Svensson et al., 1986). This includes Imatinib, which was reported to be bound by both of these major blood components (Gambacorti-Passerini et al., 2000; Fitos et al., 2006, 2012; Hegde et al., 2012). Whereas no evidence was reported for a negative influence of HSA for Imatinib activity (Hegde et al., 2012), there are controversial opinions about the consequences of its interaction with AGP. Some studies describe a negative influence (Gambacorti-Passerini et al., 2000, 2003; Azuma et al., 2007). Yet in another report little impact of AGP on Imatinib activity was found (Ishikawa et al., 2010). The results obtained in our study clearly indicate that both SA and AGP decrease Imatinib's efficacy on schistosomula, adult schistosomes, and egg production. Although the effect was slow, the addition of either BSA or HSA led eventually to the death of adults incubated with Imatinib. This indicates that SA as such does not decisively influence Imatiníbs action on adults in a negative way. Furthermore, we observed a difference in the anti-Imatinib activity of HSA and BSA. The latter seemed to disable Imatinib more efficiently, which may be due to structural differences.

Compared to SA, AGP exerted a stronger negative effect on the activity of Imatinib against adult *S. mansoni*. Whereas worm couples died within 96 h following exposure to 10–20 µM Imatinib, couples treated the same way but in the presence of AGP survived this time period and seemed unaffected. The combination of BSA and AGP showed the strongest negative influence on Imatiníbs efficacy in adults.

These *in vitro* findings for adults were similar to our observations made with Imatinib-treated schistosomula, which reacted less susceptible when SA and AGP were present in the medium. In schistosomula, however, SA revealed a more pronounced effect on Imatinib action than AGP. We cannot explain this observation at this stage of the analysis. In a previous study with schistosomes, SA uptake was shown for schistosomula and adults suggesting that albumin is a source of energy (Bennett and Caulfield, 1991; Delcroix et al., 2006; Holtfreter et al., 2010). With respect to the fact that SA may also serve as carrier for drugs (Tesseromatis and

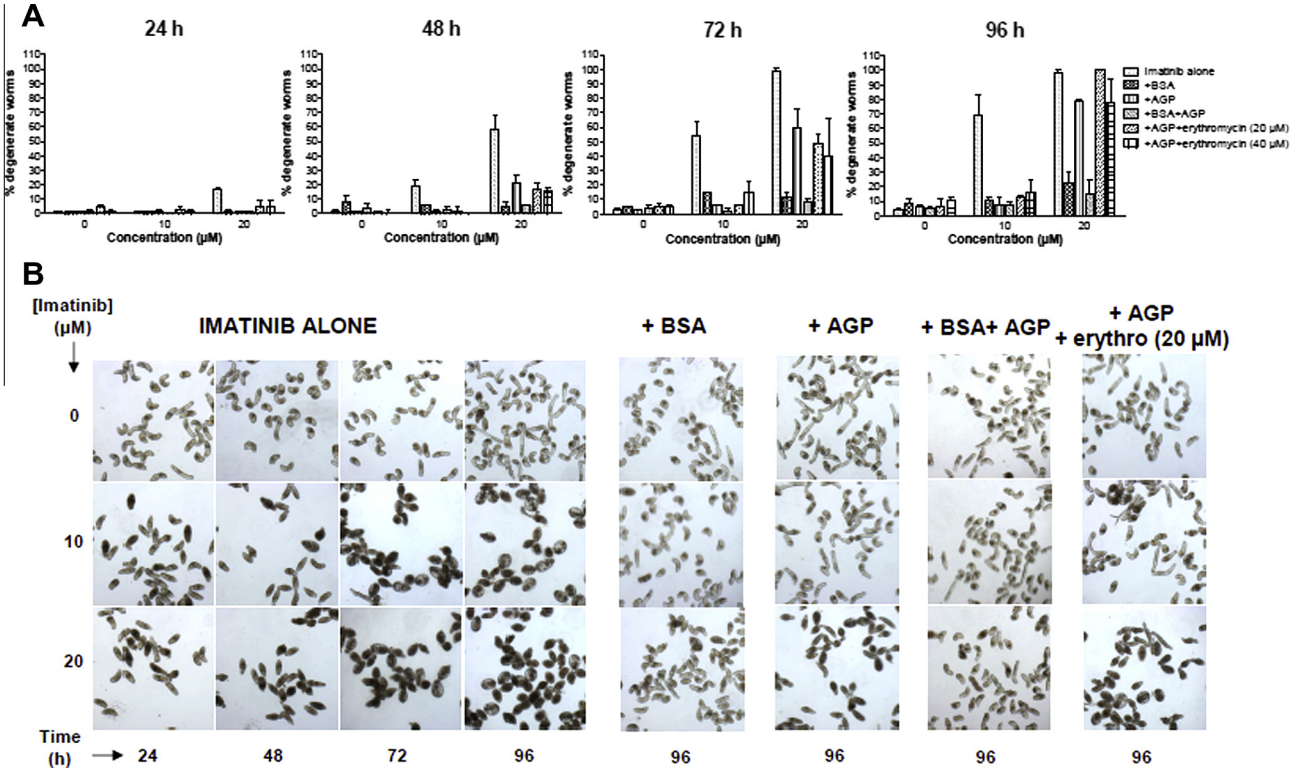

Fig. 4. *In vitro* effects of Imatinib on schistosomula treated additionally with BSA, AGP, and erythromycin. Newly transformed schistosomula were incubated with different concentrations of Imatinib (0, 10, 20 μM) and compared to the effects caused by the addition of BSA (45 g/L), AGP (0.8 g/L), and erythromycin (20 and 40 μM). (A) Graphical representation of the percentage of degenerate schistosomes (*y*-axis) in different conditions (*x*-axis). Normal versus "degenerate" schistosomes were counted manually in different conditions during 96 h. Error bars represent standard deviation of two independent experiments performed in duplicate. (B) Images display representative pictures of schistosomula treated with Imatinib (*y*-axis), with and without BSA, AGP and/or erythromycin during 96 h (*x*-axis).

Alevizou, 2008), it was concluded that SA may be a carrier for anthelmintics in drug-targeting models. With respect to our findings, a potential carrier function of SA for Imatinib did not positively influence the druǵs potential to kill schistosomes. Thus it appears questionable, whether the SA-assisted drug transport and uptake by schistosomes or other SA-importing parasites may enhance drug efficacy. This may depend on the class of drugs transported and has to be tested for each candidate compound.

As an additional test to confirm AGP's negative influence on Imatinib's efficacy, we performed competition experiments adding erythromycin to this system. This antibiotic binds to AGP, and it has the potential to enhance the efficacy of other drugs, including Imatinib, if applied in parallel (Gambacorti-Passerini et al., 2002; Azuma et al., 2007; Hegde et al., 2012). When we added erythromycin to adult parasites or schistosomula in the presence of both Imatinib and AGP we observed a partial reinstatement of Imatinib́s anti-parasite activities. On the one hand, this result is another indication of the negative role of AGP in influencing Imatinib́s efficacy. On the other, this finding may open novel perspectives on how to follow test applications of small-inhibitor drugs if they reveal AGP-binding capacity. Combinations of erythromycin and the drug of choice may positively influence druǵs efficacy. However, against both adults and schistosomula, in the used concentrations erythromycin was unable to reverse the combined effects of SA and AGP on Imatinib́s efficacy.

5. Conclusions

The results of this study allow the following conclusions:

1. AGP and SA influence the efficacy of Imatinib, which explains the discrepancy of the *in vitro* and *in vivo* results obtained in this and earlier studies. Our findings may set a precedent for testing drugs in other *in vitro* parasite systems, and future *in vitro* studies should consider including BSA/HSA and AGP competition-assays.
2. As models, the mouse and perhaps other rodents such as hamsters and rats are not suitable for compound tests if those compounds have been shown to bind AGP. Besides its role as transporter for different kinds of molecules, AGP fulfills an additional role as an acute phase protein during infections (Hochepied et al., 2003). Indeed, AGP levels in mice can increase 30–40 times during infection (Gambacorti-Passerini et al., 2000, 2003). This compares to only a 5-times increase in humans during infection (Kremer et al., 1988; Schultz and Arnold, 1990; Gambacorti-Passerini et al., 2003). Accordingly, rodents are not ideal models for "AGP-sensitive" compounds. Alternative models, such as rhesus monkeys (McMullen et al., 1967; Cheever and Powers, 1969), which may be much closer to humans with respect to AGP-levels upon infection, or even pigs may be better suited. Indeed, the pig was recently described as the first organism, in which AGP levels are negatively correlated with the acute phase of infection (Heegaard et al., 2013).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijpddr.2014.07.005.

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