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Authors: Julie A. Naughton, Sima Nasizadeh, Angus Bell

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Downstream Effects of Haemoglobinase Inhibition in *Plasmodium falciparum*-Infected Erythrocytes

Julie A. Naughton*†, Sima Nasizadeh* and Angus Bell‡

Department of Microbiology, School of Genetics & Microbiology, Moyne Institute of Preventive Medicine, Trinity College Dublin, Dublin 2, Ireland

Our data suggest that the likely primary downstream effect of inhibition of hemoglobin degradation in erythrocytic *Plasmodium falciparum* is blockade of protein synthesis rather than premature host cell lysis.

Downstream Effects of Haemoglobinase Inhibition in *Plasmodium falciparum*-Infected Erythrocytes

Julie A. Naughton*†, Sima Nasizadeh* and Angus Bell‡

Department of Microbiology, School of Genetics & Microbiology, Moyne Institute of Preventive Medicine, Trinity College Dublin, Dublin 2, Ireland

*These authors made an equal contribution.

‡Corresponding author. Tel.: +353 1 896 1414; fax: +353 1 679 9294.

E-mail address: abell@tcd.ie (A. Bell).

Abbreviations: DMSO, dimethylsulphoxide; E-64, L-*trans*epoxy-succinyl-leucylamido-(4-guanidino)-butane; IC_{50}, 50% inhibitory concentration; iRBC, infected red blood cell (erythrocyte); MACS, magnet-activated cell sorting; PBS, phosphate-buffered saline; p.i., post-invasion; PM-I, plasmepsin inhibitor I; SSC, salt sodium citrate; *Z*-FA-FMK, N-*CBZ*-phenylalanyl-alanyl-flouromethyl ketone.

†Present address: School of Medicine & Medical Science, Health Sciences Centre, University College Dublin, Belfield, Dublin 4, Ireland.

Abstract

Blood-stage malarial parasites (*Plasmodium falciparum*) digest large quantities of host haemoglobin during their asexual development in erythrocytes. The haemoglobin digestion pathway, involving a succession of cleavages by various peptidases, appears to be essential for parasite development and has received much attention as an antimalarial drug target. A variety of peptidase inhibitors that have potent antimalarial activity are believed to inhibit and/or kill parasites by blocking haemoglobin digestion. It has not however been established how such a blockage might lead to parasite death. The answer to this question should lie in identifying the affected physiological function, but the purpose of excess haemoglobin digestion by *P. falciparum* has for many years been the subject of debate. The process was traditionally believed to be nutritional until Lew VL *et al.* [Blood 2003;101:4189-94] suggested that it is linked to volume control of the infected erythrocyte and is necessary to prevent premature osmotic lysis of the host cell. Their model predicts that sufficient inhibition of haemoglobin degradation should result in premature haemolysis. In this study we examined the downstream effects of reduced haemoglobin digestion on osmoprotection and nutrition. We found that inhibitors of haemoglobinases (plasmepsins, falcipains and aminopeptidases) did not cause premature haemolysis. The inhibitors did however block parasite development and this effect corresponded to a strong inhibition of protein synthesis. The effect on protein synthesis (i) occurred at inhibitor concentrations and times of exposure that were relevant to parasite growth inhibition, (ii) was observed with different chemical classes of inhibitor, and (iii) was synergistic when a plasmepsin and a falcipain inhibitor were combined,

reflecting the well-established antimalarial synergism of the combination. Taken together, the results suggest that the likely primary downstream effect of inhibition of hemoglobin degradation is amino acid depletion, leading to blockade of protein synthesis, and that the parasite probably degrades globin for nutritional purposes.

Keywords: malaria; *Plasmodium falciparum*; antimalarial drug; haemoglobin; peptidase; protein synthesis.

1. Introduction

Erythrocytic stages of the malarial parasite, *Plasmodium falciparum*, have developed a complex proteolytic machinery for haemoglobin digestion [1]. This is a highly energy-consuming process and presents the parasite with a serious toxic waste disposal problem. Why these parasites exert such efforts to ingest and digest haemoglobin and detoxify haem has been the subject of considerable investigation and debate [2, 3]. This question is particularly important because interference with haemoglobin digestion is presumed to be the basis of action of a number of antimalarial agents in use or in development [4].

After invading erythrocytes, *P. falciparum* ingests erythrocyte cytoplasm at the cytostome and transports it to the digestive (food) vacuole (DV) [5-7]. The *P. falciparum* DV is optimised for haemoglobin digestion. This catabolic process results in the digestion of approximately 75% of infected red blood cell (iRBC) haemoglobin [5]. The

haemoglobin tetramer is broken down to two components: globin and haem [1]. A cascade of different haemoglobinases degrades globin into its constituent amino acids [4]. Haem, which is toxic to parasites, is detoxified by the formation of haemozoin (malaria pigment) [8]. The peak of haemoglobin degradation occurs in the trophozoite and early schizont stages of parasite development.

In the DV, four different groups of peptidases have been shown to play a role in haemoglobin degradation [4]: aspartic peptidases (plasmepsins I, II, IV and histoaspartic peptidase [HAP, plasmepsin III]) [9, 10], cysteine peptidases (falcipains 2, 2' and 3) [11, 12], a metallopeptidase (falcilysin) [13] and a dipeptidylpeptidase (dipeptidyl aminopeptidase I) [14]. Aminopeptidases are proposed to be involved in the terminal stages of haemoglobin degradation [15-19], though there is scant evidence for this contention in studies of intact cells. Genetic knock-out [15, 20, 21-25] and inhibitor [4, 9, 11, 26-29] studies suggest that some of these peptidases are essential for parasite growth but in other cases there is a high degree of redundancy between them. In spite of the substantial literature on the antimalarial effects of haemoglobinase inhibitors, it has not been established how blockage of haemoglobin digestion might lead to arrest of intraerythrocytic development and/or death of the parasite. Presumably the answer to this question relates to the normal physiological function of haemoglobin digestion.

It has long been considered that the likely function of haemoglobin digestion is to supply malaria parasites with amino acids, especially since they have limited ability for *de novo* amino acid synthesis [4]. This assumption relies on different observations (as reviewed

in [1]), particularly (i) the detection of amino acids from radiolabeled haemoglobin in parasite proteins and (ii) the increased susceptibility to cysteine and aspartic peptidase inhibitors of parasites grown in medium with just the 5 amino acids that are absent (isoleucine) or in low numbers (cysteine, glutamine, glutamate and methionine) in haemoglobin compared with parasites grown in full medium. Recent observations have however questioned the nutritional significance of haemoglobin hydrolysis. *P. falciparum* can import all the amino acids from the culture medium (or serum *in vivo*) and have a significant though limited capacity for *de novo* amino acid synthesis [1]. A large proportion of the amino acids resulting from haemoglobin hydrolysis are exported to the erythrocytic cytoplasm [5] and measurements by Krugliak et al [30] indicated that the proportion of amino acids derived from hydrolyzed haemoglobin that was incorporated into parasite proteins was only ~16%.

Lew et al [2, 31, 32] have integrated the question of the function of haemoglobin degradation with that of the mechanism by which iRBCs retain their osmotic stability and integrity during the 48-h asexual cycle despite a significant increase in the permeability of the erythrocyte plasma membrane to different ions and nutrients (see also the article by Allen & Kirk [33]). Using a mathematical model that included different factors known to influence erythrocyte volume and homeostasis of iRBCs, they predicted volume changes of the iRBC at different stages of parasite growth. The predicted volume changes were experimentally tested and their results supported the model's predictions. They concluded that excess haemoglobin consumption, which reduces the colloid-osmotic pressure within the iRBC, is essential for maintaining the osmotic stability of the infected

cell for the 48 h of parasite development in the erythrocyte. An important implication, as shown in Lew et al [31] (Fig. 5), is that inhibition of haemoglobin digestion should increase the osmotic stress on the iRBC resulting in premature lysis of the iRBC. This should be readily demonstrable using relevant concentrations of the peptidase inhibitors previously shown to block haemoglobin digestion. Moreover, we contend here that if the primary purpose of haemoglobin digestion is osmotic protection, the osmotic lysis effect should be apparent at lower concentrations of inhibitors than those that affect intraerythrocytic development.

In this work, we have investigated the downstream effects of inhibitors of plasmepsins, falcipains and aminopeptidases as inhibitors of haemoglobin digestion. Under our experimental conditions, no premature osmotic lysis was observed but protein synthesis was significantly reduced at inhibitor concentrations and times of exposure that were relevant to the known antimalarial effects. Our results support the notion that the primary function of haemoglobin degradation is to provide amino acids for protein synthesis.

2. Materials and Methods

2.1. Reagents

All inhibitors were purchased from Sigma Aldrich (Dublin, Ireland) except for the plasmepsin inhibitor PM-I (Fig. 1), which was kindly provided by Drs. C. Binkert and C.

Boss (Drug Discovery, Chemistry & Biology, Actelion Pharmaceuticals Ltd., Allschwil, Switzerland). PM-I, bestatin ([(2S, 3R)-3-amino-2-hydroxy-4-phenylbutanoyl]-L-leucine), N-*CBZ*-Phe-Ala-flouromethyl ketone (*Z*-FA-FMK) and taxol were prepared in dimethylsulphoxide (DMSO) and stored at –20^{o}C. L-*trans*epoxy-succinyl-leucylamido-(4-guanidino)-butane (E-64) was dissolved in purified water (Millipore Synthesis AQ, Carrigtwohill, County Cork, Ireland) and sterile filtered. Inhibitors were diluted in complete medium freshly before each experiment. As a control for inhibitors prepared in DMSO, the same amount of DMSO solvent was diluted in complete medium and added to the parasite culture. L[^{35}S] Methionine (specific activity of 1110 Ci/mmol) and [^{3}H] isoleucine (99 Ci/mmol) were obtained from Amersham (GE Healthcare Ltd., UK).

2.2. Parasite Culture

P. falciparum clone 3D7 (obtained from M. Grainger, National Institute of Medical Research, London, UK) was cultivated in human O^{+} erythrocytes as previously described [34]. Age-selection of the parasites was performed by two steps of magnet-activated cell sorting (MACS: Miltenyi Biotec, Surrey, UK) [35] which isolated late trophozoite- and schizont-infected erythrocytes from uninfected erythrocytes and those infected with less mature forms. The MACS operations were timed to give parasites of an age range of ~8–10 h. After age-selection the parasites were re-cultured to the desired approximate ages for the experiment (see individual experiments below). The age ranges of the parasite cultures were estimated by measuring the time between the appearance of the first rings and the disappearance of the last segmenters. The parasites were continuously cultured in

human erythrocytes in RPMI 1640 supplemented with HEPES (25 mM), Albumax® II (0.5% w/v) (Gibco, Auckland, New Zealand), hypoxanthine (50 µg/ml), glucose (0.08% w/v), $NaHCO_3$ (0.18% w/v) and gentamicin (0.02 mg/ml) [36]. The parasites were cultured at 2.5% hematocrit in candle jars at 37°C. Cultures were examined microscopically with the aid of Giemsa staining. The different stages of erythrocytic parasites were defined as previously described [37].

2.3. Inhibitor Susceptibility Assays

Fifty-% inhibitory concentrations (IC_{50}) after 72 h were determined on asynchronous parasites in 96-well plates by using the parasite dehydrogenase assay [38] and were determined graphically.

For all other inhibitor assays, cultures consisting mainly of young or young–mid trophozoites (estimated ages as shown in the figure legends and text, obtained by MACS as described above) at *circa* 8% parasitemia and 2.5% hematocrit were treated with different concentrations of inhibitors (1X, 5X and 25X IC_{50}) in 96- or 24-well plates. Controls were parasite cultures incubated in inhibitor-free medium with or without solvent (DMSO). Growth and survival of parasites was assessed by microscopic examination of Giemsa-stained smears.

2.4. Haemoglobin Release Assay

P. falciparum parasites were cultured and treated as described in section 2.3 in 24-well plates (with a total volume of 500 µl) except that phenol-red-free medium was used. Parasites were harvested after specific incubation times by taking samples from wells to Eppendorf tubes, followed by centrifugation (800 *g*) for 5 min at room temperature. The supernatant was removed to another tube and the above steps were repeated until no pellet was observed. Two hundred µl of the supernatant were used to measure the absorbance at 405 nm in 96-well plates.

2.5. Protein Synthesis Assay

As described in section 2.3, *P. falciparum* parasites were cultured and treated with inhibitors in 96-well plates and incubated for different periods. At the end of designated time points, cultures were metabolically labeled with L[^{35}S]methionine (50–100 mCi/ml) for 1 h. After labeling and three steps of washing with unlabelled medium, crude extracts of iRBCs were prepared by three cycles of freezing-thawing. The extracts were stored at –70^{o}C until further use.

Proteins from labeled cell extracts were precipitated with trichloroacetic acid/acetone. Samples were then mixed with scintillation cocktail (ICN Biomedicals Inc., Aurora, Ohio, U.S.A.) and a liquid scintillation spectrometer (Packard Tri-Carb model 2100TR) was used to count radioactivity incorporated into macromolecules.

2.6. Assay of Haemozoin Formation

Haemozoin was purified and quantified as described previously [23, 39]. Briefly, parasites were isolated from iRBCs using 0.1% (w/v) saponin in ice-cold salt sodium citrate (SSC) [17] and washed in SSC. Harvested parasites were stored at –70^{o}C in PBS. The thawed, harvested parasites were centrifuged for 20 min at 10,000 *g* at 20^{o}C. The pellet was resuspended in 100 µl buffer A (Tris-HCl (100 mM, pH 8.0) containing 2.5% SDS) and incubated for 30 min at 37^{o}C with vortexing after 10 min. After a centrifugation step as above, the pellet was resuspended in 100 µl alkaline bicarbonate (100 mM, pH 9.2). The pellet obtained after centrifugation was dissolved in 10 µl 1 M NaOH and 90 µl of buffer A and incubated for 1 h at 50^{o}C. The haemozoin content was determined by measuring the absorbance at 405 nm (Multiskan Ex, ThermoScientific, Basingstoke, UK) in 96-well plates. The amount of haem was calculated using the extinction coefficient of 91,000 $cm^{-1}M^{-1}$.

2.7. Measurement of isoleucine uptake

Isoleucine uptake experiments were carried out as described previously [3] with some modifications. Briefly, *P. falciparum* parasites were cultured and treated as described in section 2.3 in 6-well plates (with a total volume of 3 ml). At specific time points (5.5 and 12.5 hours) cells were depleted of amino acids by repeated washing in PBS and incubation for 30 minutes at 37°C in Solution A (130 mM NaCl, 25 mM HEPES, 5 mM KCl, 20 mM glucose, 0.2 mM hypoxanthine, 25 mg/l gentamicin sulphate, RPMI 1640 vitamins [Sigma Aldrich] and 3.25 µM glutathione) with the appropriate inhibitor concentration. Cells were then magnet purified and resuspended to a parasitaemia of 80–

90% and haematocrit of 5% in Solution A supplemented with amino acids (alanine (356 µM), arginine (88 µM), asparagine (13 µM), aspartate (13 µM), cysteine (37 µM), glutamate (57 µM), glutamine (476 µM), glycine (217µM), histidine (85 µM), hydroxyproline (8 µM), [^{3}H] isoleucine (70 µM, 6.93 mCi/ml), leucine (100 µM), lysine (163 µM), methionine (17 µM), phenylalanine (100 µM), proline (165 µM), serine (128 µM), threonine (112 µM), tryptophan (50 µM), tyrosine (62 µM), and valine (190 µM) and incubated at 20°C for 5 minutes. Duplicate 200-µl samples were taken and the reaction terminated upon centrifugation of the cells (14,000 *g* for 2 min) through silicon oil (Dow Corning) and processed for scintillation counting as described previously [42]. Parasite-specific influx was calculated by subtracting the accumulation into an equal number of uninfected erythrocytes.

3. Results

3.1. Effects of Peptidase Inhibitors on Osmotic Stability of iRBCs

According to Lew et al [31] haemoglobin hydrolysis is necessary to maintain the osmotic stability of iRBCs during the maturation of parasites. By using peptidase inhibitors, the occurrence of premature lysis of iRBCs due to inhibition of haemoglobin degradation was tested. Inhibitors of three different classes of peptidases were used. PM-I (Fig. 1), which is closely related to the plasmepsin inhibitors previously described [40], is a non-peptidic aspartic peptidase inhibitor that is potent against three of the DV plasmepsins (I,

253 II and IV). Given that parasites can survive without these three enzymes [21, 23, 41] but
254 PM-I nonetheless has potent antimalarial activity, this compound is likely to target other
255 plasmepsins/aspartic peptidases. E-64 and *Z*-FA-FMK are cysteine peptidase inhibitors
256 and bestatin is an aminopeptidase inhibitor. The IC_{50} of the compounds used were as
257 follows: PM-I (0.8 µM), E-64 (3 µM), Z-FA-FMK (5 µM) and bestatin (2 µM).
258
259 Young–mid-trophozoite parasite cultures (21–29 h p.i.), which were at the early stage of
260 haemoglobin degradation, were treated with different peptidase inhibitors. The method
261 of age-selection used in this experiment and all other experiments reported here was
262 magnet-activated cell sorting (MACS). This method was chosen in preference to the
263 more prevalent method using sorbitol lysis of mature stages in order to diminish the
264 possible negative effects on the iRBC membrane. Following incubation for various time
265 periods, the medium of the cultures was analyzed spectrophometrically to quantify the
266 amount of released haemoglobin. According to the graph presented in Fig. 5 in [31], if
267 haemoglobin degradation decreased from 70% to ~50%, then premature lysis should take
268 place at ~45 h p.i. Since this is an estimate and it would be hard to predict the exact time
269 of maximal osmotic lysis, treated and control cultures were harvested at different time
270 points. Cultures were harvested at 14, 16, 18 and 20 h after drug treatment (39±4-, 41±4,
271 43±4- and 45±4 h p.i., respectively). Fig. 1 shows that the amount of haemoglobin
272 release in the control cultures increased as the parasites were maturing (it more than
273 tripled from 16 h to 20 h of incubation), as expected from the increasing lysis of the host
274 erythrocytes as the parasites completed their ~46-48-h cycle and released new
275 merozoites. In most of the inhibitor-treated cultures, the amount of haemoglobin release

276 remained constant during the total 20 h incubation period and in no case did it exceed that
277 of the control. In none of our experiments, regardless of concentration of inhibitor or
278 time of incubation, did we observe premature iRBC lysis (data not shown). Significant
279 differences in haemoglobin release from iRBCs in the control- and inhibitor-treated
280 cultures were however already observable after 16 h of treatment. Microscopic analysis
281 of these cultures showed that progression of parasite development was blocked. After a
282 further 13 h of incubation, ~65% of the control population consisted of rings and
283 schizonts while the inhibitor-treated cultures contained mainly what looked like mid-
284 trophozoites (i.e. the same as the starting culture). The results of control experiments
285 indicated that parasites treated with any of the three haemoglobinase inhibitors for 13 h
286 could not subsequently be revived (data not shown). These observations indicated that
287 not only was there no premature osmotic lysis, but levels of normal lysis at the time of
288 merozoite release were considerably lowered.
289
290 *3.2. Effects of Peptidase Inhibitors on Parasite Protein Synthesis*
291
292 Protein synthesis was determined by measuring the incorporation of L[^{35}S]methionine
293 into newly synthesized proteins (erythrocytes themselves lacking the capacity for protein
294 synthesis). The negative effects of peptidase inhibitors on cultures with initial ages ~16–
295 24 h p.i. were observed after 6 h (26±4 h p.i.) treatments at the 5X- and 25X IC_{50} for E-64
296 and all concentrations for bestatin and PM-I (Fig. 2). PM-I at concentrations 5X and 25X
297 IC_{50} also caused inhibition of protein synthesis after only 4 h incubation (~45% and
298 ~70%, respectively) (results not shown). Inhibition of protein synthesis was observed for

all concentrations of the inhibitors after 13 h treatment (33±5 h p.i.). For the most part the inhibition of protein synthesis was progressive with time, but in a few cases (e.g. 1X IC_{50} bestatin) the effect was greater at 6 h than at 13 h. This observation may be connected with the upregulation of protein degradation by the ubiquitin/proteasome pathway in response to amino acid starvation [43].

In order to confirm that the effect of the peptidase inhibitors was not just peculiar to the specific inhibitors discussed above, Z-FA-FMK (another cysteine peptidase inhibitor) was applied to *P. falciparum* cultures as described in Materials and Methods and its effects on protein synthesis were tested. Treatment of parasite cultures with Z-FA-FMK for 6 and 13 h resulted in a decrease in protein synthesis at concentrations 5X- and 25X IC_{50} (~50%) (Fig. 2). As a negative control, taxol (a drug whose antimalarial action is apparently unrelated to haemoglobin digestion or protein synthesis ([44] and see section 3.3) had no or only marginal effects at 5X IC_{50} in parallel experiments (Fig. 2), indicating that the effects of the haemoglobinase inhibitors on protein synthesis were unlikely to be merely secondary consequences of growth inhibition by some other mechanism.

Plasmepsin and falcipain inhibitors have been reported to display strong synergistic effects on growth of *P. falciparum* [26, 45-47]. The data in Fig. 3 confirm that the combination of E-64 and PM-I also had synergistic antimalarial activity. If the effects on protein synthesis shown here are relevant to growth inhibition, they should also be synergistic. The combined effect of E-64 and PM-I on the inhibition of protein synthesis was therefore tested by comparing parasite cultures treated with 2.5X IC_{50} of E-64 and

2.5X IC_{50} PM-I in combination with those treated with 5X IC_{50} concentrations of each agent alone. If the effect were merely additive, levels of protein synthesis in cultures treated with the combination would be expected to be intermediate between those in cultures treated with 5X IC_{50} of E-64 alone and those treated with 5X IC_{50} of PM-I alone. In fact, they were much lower than this (Fig. 2), indicating a synergistic effect.

3.3. Effects of Inhibitors on Haemozoin Formation

To confirm that the inhibitors at the concentrations and for the times used were inhibiting haemoglobin degradation, the haemozoin contents in the treated and untreated cultures were measured. After 6 hours of incubation (final ages 26±4 h p.i.), the higher concentrations of bestatin, E-64 and PM-I had slight negative effects on haemozoin formation in the parasites (~30% decrease) (data not shown). When the parasites were treated for 13 h (33±4 h p.i.), there were more significant decreases in haemozoin formation in those cultures treated with 5X and 25X IC_{50} bestatin (~20% and ~45%, respectively) and E-64 (~50% and ~65%, respectively) and all concentrations of PM-I (~70–80%) (Fig. 4). Treatment of parasite cultures with Z-FA-FMK for 6 and 13 h resulted in a decrease of haemozoin formation at all concentrations (~40–60%) (Fig. 4 and data not shown). These results show that the peptidase inhibitors used did inhibit haemozoin formation and therefore imply that haemoglobin digestion was inhibited. These were important control observations and, interestingly, we were unable to demonstrate a significant effect of relevant concentrations of the aspartic peptidase inhibitor pepstatin A on haemozoin accumulation (data not shown) so were therefore

unable to include it in the experiments above. Moreover, the degrees of inhibition obtained with the various inhibitors were the same as or greater than that expected to induce osmotic lysis of iRBCs according to Lew et al [31]. The negative control agent, taxol, had a negligible effect on haemozoin accumulation (92.6±2.2% of control at 5X IC_{50} (0.25 μM), not shown in Fig. 4).

3.4. Effect of bestatin on isoleucine uptake, a surrogate for free leucine concentration

The results described above suggested that the haemoglobinase inhibitors were not acting by increasing osmotic lysis of iRBC but that they affected protein synthesis. The most likely explanation for this effect was that inhibition of haemoglobin digestion reduced the supply of amino acids required for protein synthesis. We were unable to prove this contention because of the technical difficulties associated with measuring (possibly transient) changes in the free amino acid concentrations within parasites. The work of Martin & Kirk [3], showing the dependence of uptake of isoleucine on free intraparasitic leucine, did however suggest a measurable 'surrogate' for free leucine, one of the most abundant amino acids in haemoglobin. We therefore measured radiolabelled isoleucine uptake in parasites treated with bestatin. As an aminopeptidase inhibitor, bestatin would be expected to act most closely to the point of release of free amino acids from globin. The data in Fig. 5 show that bestatin at 5X and 25X IC_{50} reduced the uptake of isoleucine by ~20-25% and ~50% respectively, while the apparently irrelevant inhibitor taxol had no such effect.

4. Discussion

The peptidase inhibitors active on haemoglobin digestion in *P. falciparum* are attractive targets for designing anti-malarial drugs and have been explored extensively by several groups [4, 9, 11, 26-29]. It is therefore surprising that the downstream consequences of inhibition of haemoglobin digestion, and the mechanism by which this metabolic inhibition actually kills parasites or blocks their development, have not been elucidated. We addressed this question with reference to the two main theories regarding the primary biological purpose of haemoglobin degradation: maintaining the osmotic stability of iRBCs or providing amino acids for protein synthesis.

The haemoglobin release experiment did not show any premature iRBC lysis in cultures treated with the peptidase inhibitors used in this study (Fig. 1 and data not shown). Rather, the peptidase inhibitors caused a blockade of the growth and development of the parasites and eventual death before they could reach the later stages of parasite growth. This was also observed by Lew et al [2] when they treated *P. falciparum* at the age range of 22–28 h p.i. with E-64. They concluded that using peptidase inhibitors as a tool for testing the osmotic protection hypothesis of excess haemoglobin digestion was not appropriate. However, if the primary purpose of haemoglobin digestion is to prevent premature iRBC lysis and the model of Lew et al [31] (Fig. 5) is correct, it should be possible to induce this lysis with concentrations of peptidase inhibitors lower than those that affect progression through the cycle. Our data show that at a range of concentrations

above and below IC_{50}, including those that are capable of inhibiting haemoglobin degradation (as measured by haemozoin accumulation, Fig. 4) by 50% or more, no measurable premature lysis was detected. We conclude that the primary target of the inhibitors lies at an earlier stage of parasitic development. It is relevant to mention here that since inhibition of DV plasmepsins alone is unlikely to be sufficient to arrest parasite growth [48], the primary target of PM-I is likely to be an aspartic peptidase that resides outside the vacuole and therefore is not directly involved in haemoglobin digestion. Yet PM-I is a powerful inhibitor of haemoglobin degradation and is in fact the most potent of the peptidase inhibitors, relative to its IC_{50}, in this respect (Fig. 4). A possible explanation is that PM-I may inhibit one or more peptidases catalysing proteolytic activation of one or more of the haemoglobinases.

Given the proposed nutritional role of haemoglobin degradation and the fact that globin-derived amino acids are incorporated into parasite proteins, we explored the possibility that the reduced amino acid supply might cause arrest of parasite protein synthesis. This was investigated by measuring the incorporation of L[^{35}S]methionine into newly synthesized proteins (Fig. 2). Protein synthesis inhibition was observed for all four peptidase inhibitors tested after 6 hours' incubation (26±4 h p.i.) and was in most cases increased after 13 h incubation (33±4 h p.i.). The inhibitor concentrations and times of exposure were sufficiently low relative to their IC_{50} to persuade us that the effect on protein synthesis was relevant to the antimalarial action of the inhibitors. This conclusion was further strengthened by the observed synergistic effect of E-64 and PM-I on protein synthesis inhibition (Fig. 2), which mirrors that seen on parasite growth (Fig. 3).

The most logical interpretation of our results would be that protein synthesis is a downstream effect of inhibition of haemoglobin digestion, resulting from reduced supply of amino acids from globin, and perhaps the primary mechanism by which haemoglobinase inhibitors block the development of trophozoites and early schizonts. We have not been able to establish here that amino acid depletion actually occurs as a result of inhibition of haemoglobin digestion. The reduction of isoleucine uptake, which is known to depend on free leucine, following treatment with bestatin is however suggestive of such a connection. If this idea is correct, our results also imply that the primary role of the haemoglobinases and haemoglobin digestion is to provide the parasite with free amino acids, supplementing those available via uptake and *de novo* synthesis and allowing protein synthesis to proceed at the level required for normal development. Whatever the cause of the reduced protein synthesis, it appears that the role if any of haemoglobin digestion in maintaining the osmotic stability of iRBCs is a secondary one.

An important caution is however that the inhibitors employed may have effects on targets other than those involved in haemoglobin digestion. Therefore it is conceivable that the effect on protein synthesis is working by some hitherto unknown mechanism. We consider this unlikely, because (i) the effect was seen with four different chemical types of compound directed to three different classes of peptidase, but not with control compounds, and (ii) haemoglobin digestion is unquestionably the major peptidase-dependent metabolic event occurring in trophozoite-stage parasites. Nonetheless we have to admit the possibility of a hitherto unsuspected pathway, and for this reason it would be

useful to repeat these experiments with new haemoglobinase inhibitors as they become available, and in different genetic backgrounds such as parasites lacking specified DV peptidases.

One notable, related finding in the present study is the effect of the aminopeptidase inhibitor bestatin on haemozoin accumulation (Fig. 4). Previous studies [15-17] have been highly suggestive of a role of bestatin-susceptible aminopeptidases in haemoglobin digestion but the experiments have been performed in cell-free systems. Moreover, relevant concentrations of bestatin did not visibly affect any aspect of haemogobin metabolism as judged by electron microscopy [17]. The data in Fig. 4 are therefore the first demonstration that bestatin affects haemoglobin digestion in intact parasites. That this effect leads to a reduction of supply of amino acids such a leucine for protein synthesis remains to be proven, but the effect of bestatin on isoleucine uptake (Fig. 5) is consistent with this idea.

The presumed nutritional role of haemoglobin degradation has been demonstrated here in parasites in culture with medium containing high concentrations of amino acids. Since the levels of amino acids in blood plasma of malaria-infected patients in poverty-stricken areas are significantly lower [48] it can be postulated that this role of haemoglobin digestion may be of even more importance *in vivo*. If true, this would strengthen the argument that *P. falciparum* haemoglobinases or the enzymes regulating them are promising targets for new anti-malarial drugs.

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Figure legends

Fig. 1. Effects of peptidase inhibitors on osmotic stability of iRBCs. Parasites age-selected to young-mid trophozoites (21–29 h) post invasion were cultured in the presence of different protease inhibitors and harvested after periods of 14, 16, 18 and 20 h of incubation. iRBC lysis was analyzed specrophometrically by measuring the amount of haemoglobin released into the medium (at 405 nm). These results are representative of at least three separate experiments. ***Inset:*** Structure of Plasmepsin Inhibitor I (PM-I).

Fig. 2. Effects of inhibitors on protein synthesis. *P. falciparum* cultures consisting of young trophozoites (16–24 h post invasion) were treated with peptidase inhibitors for periods of 6 h (white bars) and 13 h (black bars). Taxol (IC_{50} 50 nM) was included as a control. L[^{35}S]Methionine was then added and the cultures were harvested after 1 h. The results are shown as % of control and are an average of two to three independent experiments (6 h and 13 h, respectively) and n=2 in each experiment. The error bars show SEMs.

Fig. 3. Isobologram showing interaction between E-64 and PM-I on growth of *P. falciparum* in culture. Fifty-% inhibitory concentrations of E-64 alone and in the presence of various concentrations of PM-I, and of PM-I alone in the presence of various concentrations of E-64, were determined as described in section 2.3. The solid diagonals in the isobolograms represent the theoretical line of additivity (i.e., no interaction), while the values below this line indicate a synergistic effect between the two compounds. The

630 concave isobole (dashed line) was fit by inspection. Each point is a geometric average of
631 four separate experiments and error bars represent the SEM.
632
633 **Fig. 4. Effects of peptidase inhibitors on haemozoin accumulation.** Parasite cultures
634 consisting of young trophozoites (16–24 h p.i.) were incubated for 13 h with protease
635 inhibitors. At the end of the incubation time parasites were isolated and haemozoin was
636 purified as described in section 2.6. The amount of extracted haemozoin was quantified
637 spectrophometrically at 405 nm. The results from inhibitor-treated cultures were plotted
638 as % of control (inhibitor-free culture). The amount of haemozoin in the control was 0.3–
639 1 fmol/parasite, assuming a trophozoite volume of 74 fl [49]. The data are averages from
640 two independent experiments (n=2 per experiment). The bars show SEMs.
641
642 **Fig. 5. Effect of bestatin on [^{3}H] isoleucine uptake.** Parasite cultures consisting of
643 young trophozoites (16–24 h p.i.) were treated with bestatin or taxol at 1X, 5X or 25X
644 IC_{50} (20X IC_{50} for taxol because 25X IC_{50} caused substantial parasite destruction) and
645 after 5.5 and 12.5 h cells were depleted of amino acids as described in section 2.7.
646 Infected erythrocytes were then purified by MACS, resuspended in [^{3}H] isoleucine plus
647 other (unlabelled) amino acids, and incubated at 20°C for 5 min. Duplicate 200-µl
648 samples were taken and labelled isoleucine uptake determined as described in section 2.7.
649 The data are averages from three independent experiments (n=2 per experiment). The
650 bars show SEMs.
651
652

Figure 1

Fig. 1

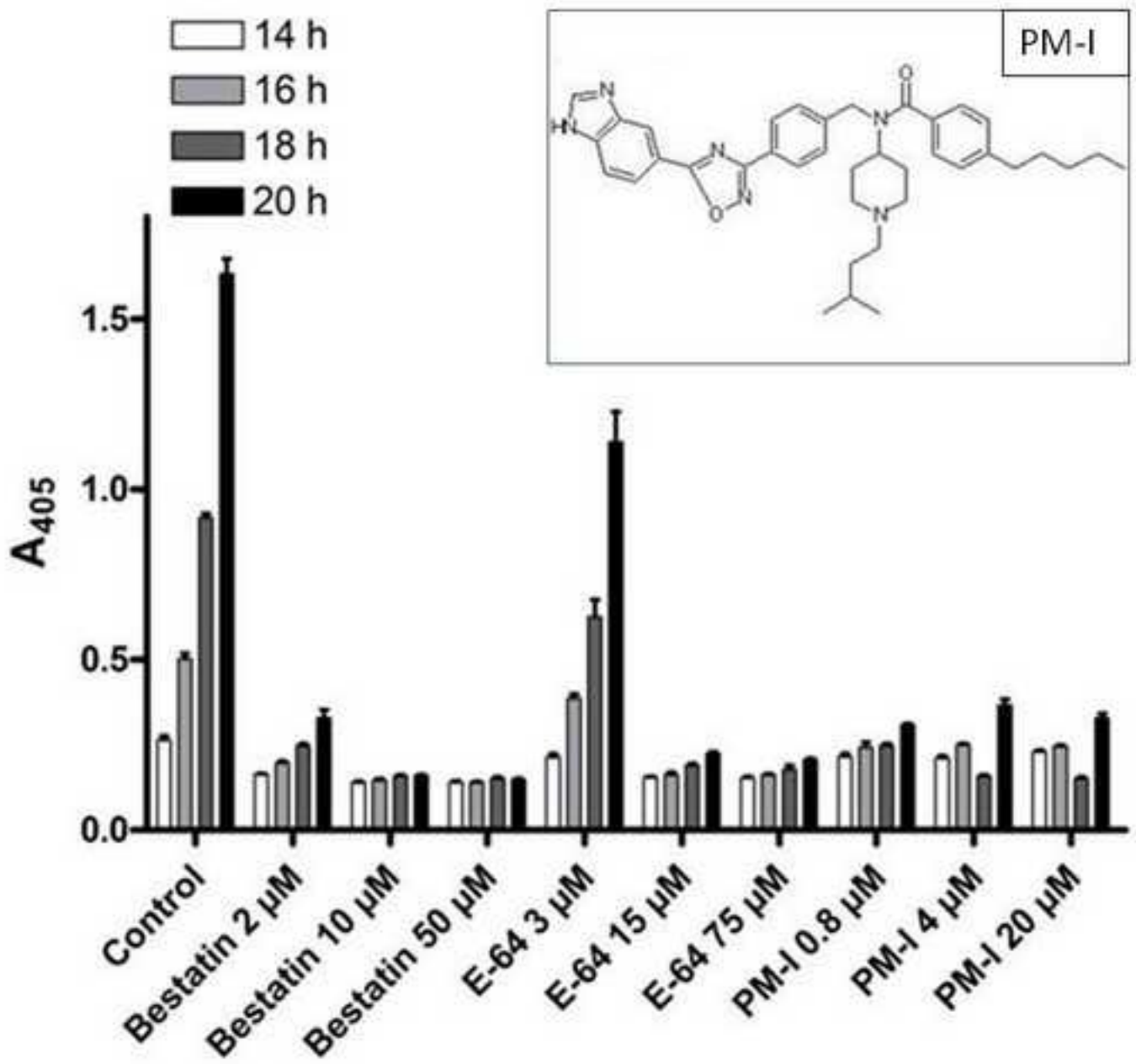


Figure 2

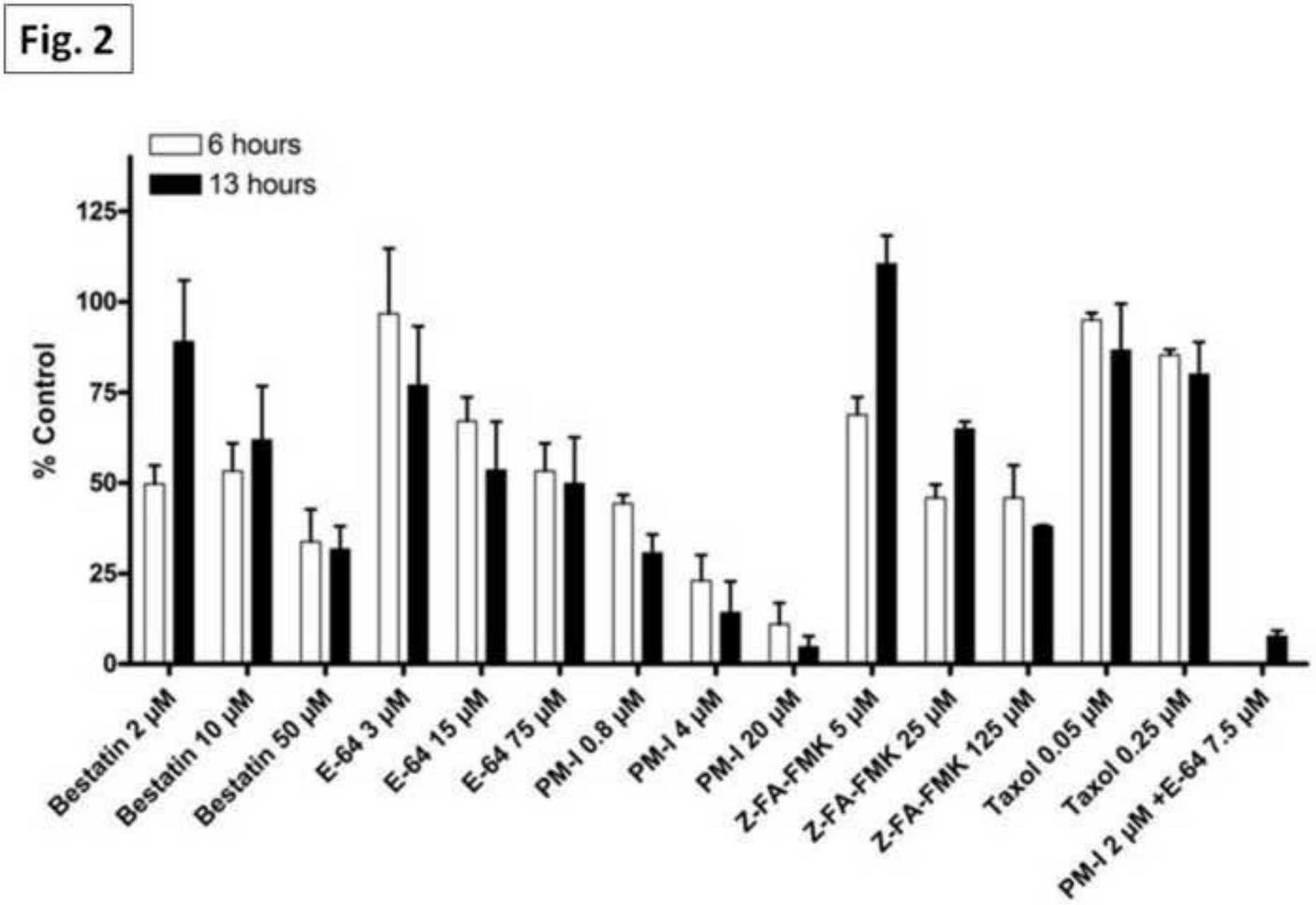


Fig. 2
6 hours
13 hours
% Control
125
100
75
50
25
0
Bestatin 2 µM
Bestatin 10 µM
Bestatin 50 µM
E-64 3 µM
E-64 15 µM
E-64 75 µM
PM-I 0.8 µM
PM-I 4 µM
PM-I 20 µM
Z-FA-FMK 5 µM
Z-FA-FMK 25 µM
Z-FA-FMK 125 µM
Taxol 0.05 µM
Taxol 0.25 µM
PM-I 2 µM +E-64 7.5 µM

Fig. 3

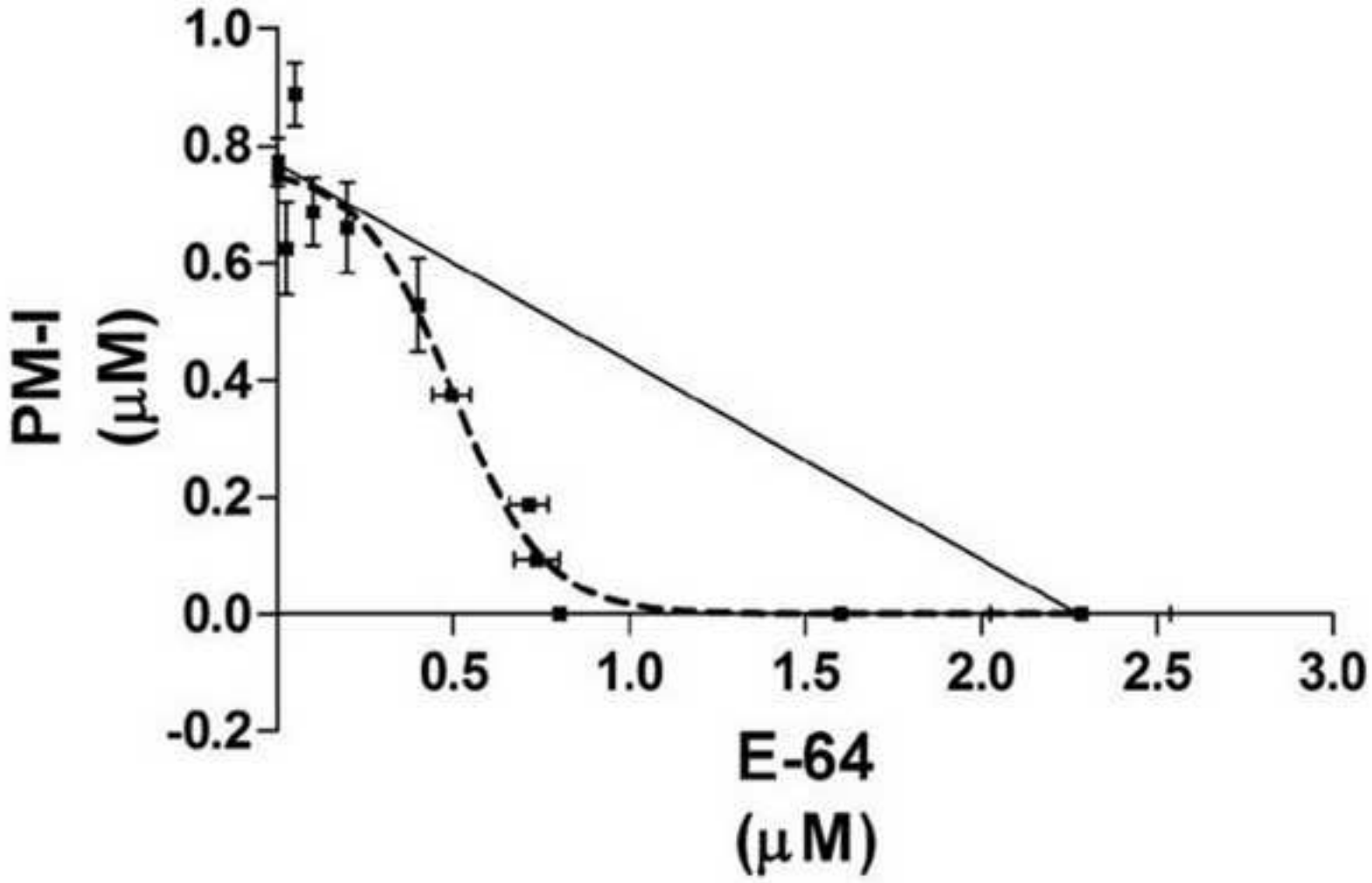


Figure 4

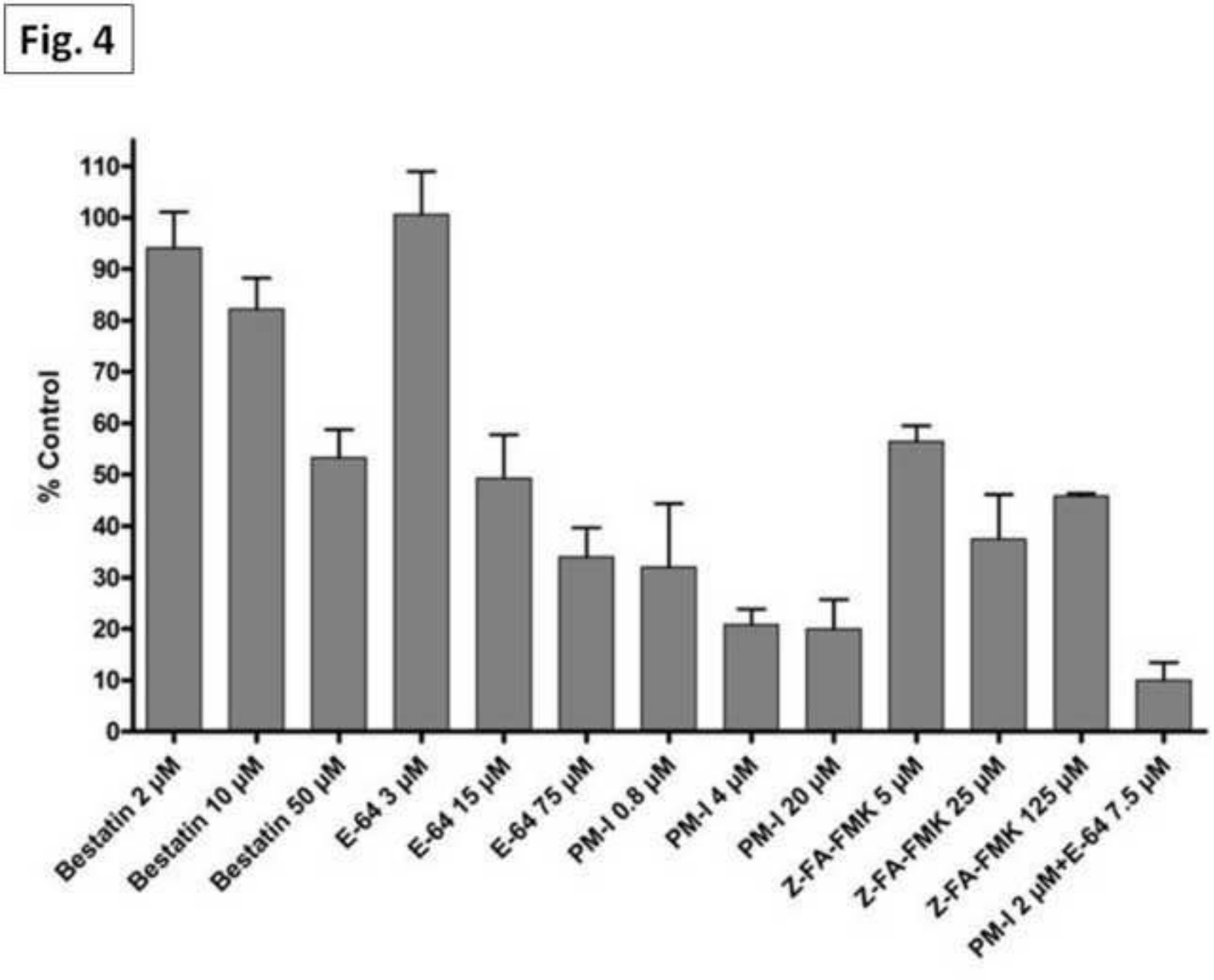
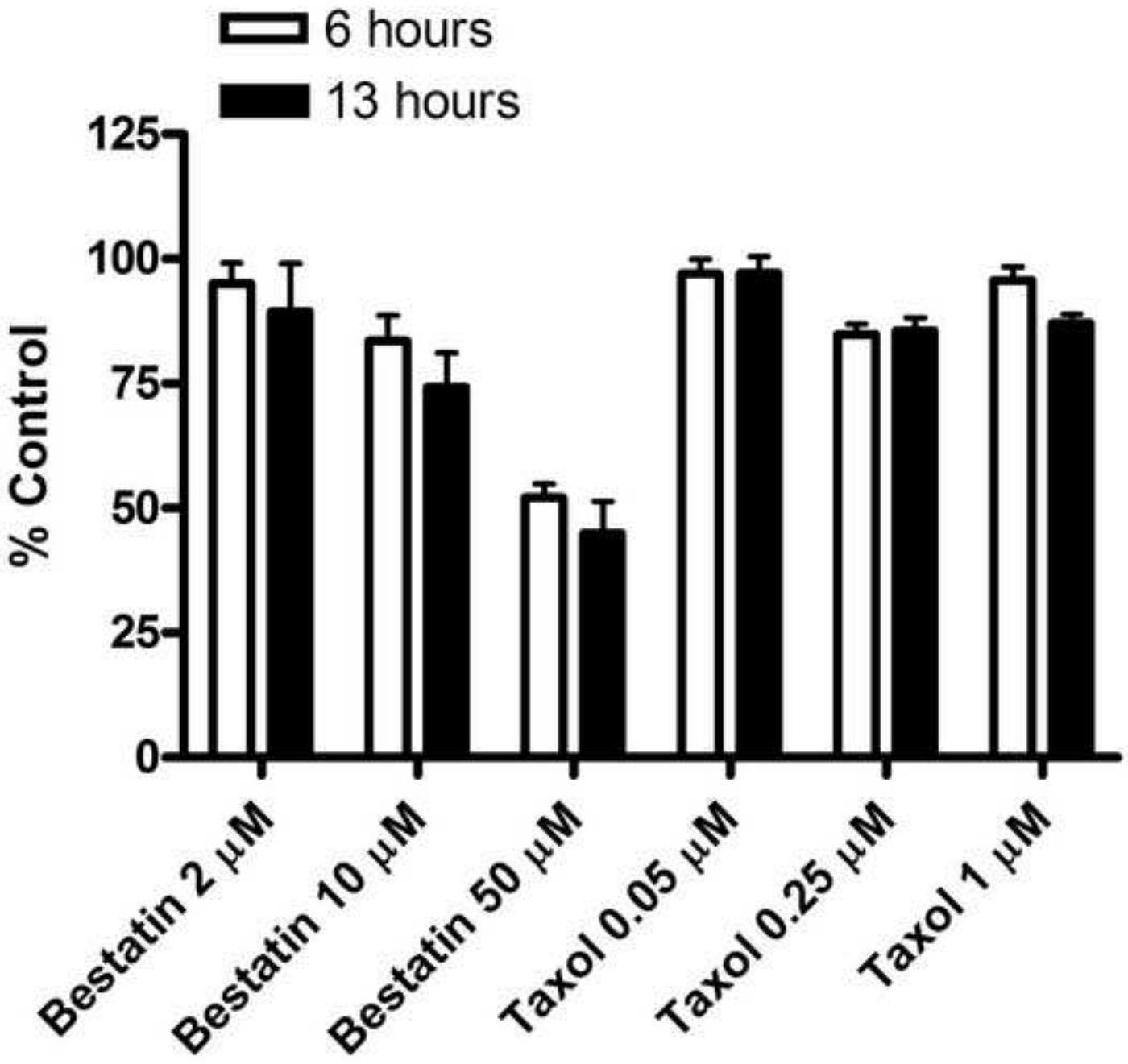


Fig. 4
% Control
110
100
90
80
70
60
50
40
30
20
10
0
Bestatin 2 µM
Bestatin 10 µM
Bestatin 50 µM
E-64 3 µM
E-64 15 µM
E-64 75 µM
PM-I 0.8 µM
PM-I 4 µM
PM-I 20 µM
Z-FA-FMK 5 µM
Z-FA-FMK 25 µM
Z-FA-FMK 125 µM
PM-I 2 µM+E-64 7.5 µM

Figure 5


6 hours
13 hours
% Control
125
100
75
50
25
0
Bestatin 2 μM
Bestatin 10 μM
Bestatin 50 μM
Taxol 0.05 μM
Taxol 0.25 μM
Taxol 1 μM