Purification of *P. c. chabaudi* aminopeptidase, and antimalarial effects of the aminopeptidase inhibitor bestatin.

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Thesis presented for the degree of
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I hereby certify that this material, which I now submit for assessment on the programme of study leading to the award of Ph.D., is entirely my own work and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my work.

Signed: _____ Date 7/02/97

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Abstract.

The malarial parasite utilises amino acids drawn from the host haemoglobin for it's intraerythrocytic development. The endopeptidases described to date will only produce peptides from host haemoglobin. In order to obtain free aminoacids for incorporation in malarial proteins the malarial parasite requires exopeptidases (i.e. aminopeptidases and carboxypeptidases).

Aminopeptidase activity was described in human malarial parasites *Plasmodium falciparum* rodent malarial parasites *Plasmodium chabaudi chabaudi* and *Plasmodium berghei*. These enzymes were shown to be inhibited by the aminopeptidase inhibitor bestatin. In light of these findings, the antimalarial effects of bestatin were investigated both *in vitro* and *in vivo*. Bestatin inhibited the *in vitro* growth of *P. c. chabaudi,* of chloroquine sensitive *P. falciparum* clone FCH5.C2 and chloroquine resistant *P. falciparum* clone K1/Thailand. *P. chabaudi* infection is naturally lethal to male mice whereas female mice develop protective immunity against a similar infection. *In vivo* investigation of the effects of bestatin showed that this aminopeptidase inhibitor could induce self-healing of *P. c. chabaudi* infection in male CD1 mice.

The aminopeptidase was purified by a combination of size-exclusion HPLC and affinity chromatography and, alternatively, by a combination of size-exclusion HPLC and anion exchange chromatography. The aminopeptidase activity appears to be composed of four different aminopeptidases with an apparent monomeric molecular weight range of 60 to 70 kDa.

Comparative studies of the inhibition of the *P. c. chabaudi* and porcine kidney aminopeptidase by bestatin indicate that the active site of the malarial aminopeptidase could differ to the mammalian aminopeptidase. Nucleotide probes/primers to clone this aminopeptidase(s) have been designed from the sequences of bestatin and metal ions binding site of several leucine aminopeptidases.

This thesis shows that the malarial aminopeptidase is a potential chemotherapeutic target with bestatin and/or bestatin analogues as potential antimalarial drugs. The difference between the binding site of bestatin on mammalian and malarial aminopeptidase could be exploited for the design of drugs that would be specific to the malarial parasite enzyme.

Abbreviations.

AMC	Amino methyl coumarin
BCIP	5'-bromo-4-chloro-3-indoyl phosphate
BSA	Bovine serum albumin
DEPC	Diethyl pyrocarbonate
DMF	Di-methyl fluoride
DNA	Deoxy ribonucleic acid
dNTP	Deoxy nucleotide triphosphate
EDTA	Ethylene-diamine-tetraacetic acid
FCS	Foetal calf serum
HCl	Hydrochloric acid
HI-FCS	Heat inactivated foetal calf serum
IgG	Immunoglobulin G
IPTG	Isopropyl-b-D-thiogalactopyranoside
iv	intravenous
K_2HPO_4	Di-potassium hydrogen phosphate
KH_2PO_4	Potassium di-hydrogen phosphate
LB	Lauria Bertani
Leu	Leucine
LF	Lambda forward
LR	Lambda reverse
Na_2HPO_4	Di-Sodium hydrogen phosphate
NaCl	Sodium chloride
NaH_2PO_4	Sodium di-hydrogen phosphate
NaOH	Sodium hydroxide
NBT	Nitro blue tetrazolium
PAGE	Poly acrylamide gel electrophoresis

PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
sc	subcutaneous
SDS	Sodium dodecyl sulphate
Tris	Tris (hydroxymethyl) amino methane
X-gal	5-bromo-4-chloro-indoyl-βgalactoside
RBC	Red blood cells
WHO	World Health Organisation

Chapter 1
General introduction

1.0. Human malaria.

Malaria, meaning "bad air" was so named because of association of the disease with the odorous air of swamps, particularly at night. Although historians and economists have largely failed to recognise it, malaria must have played a large part in the history of the world and the progress of nations (Bruce-chwatt, 1988). While the disease is tolerated as a relatively uncommon infection in most economically rich countries it still ranks as a very important disease in various tropical countries in Asia, Africa and South America.

A programme to eradicate malaria in the economically rich countries came into operation in 1947, and by 1958 seventy six countries were planning, carrying out or had completely eradicated malaria (WHO, 1955, 1957 1967; Young, 1957; Williams; 1958; Bradley, 1966). The most successful eradication of malaria was obtained by the use of the organochlorine insecticide DTT which was subsequently banned in 1972 for ecological reasons. The use of insecticides thus targeting the *Anopheles* malarial vector is still recognised as one of the most efficient methods to eradicate malaria in the tropical countries which still suffer enormous deaths (up to two million each year) from malaria (WHO, 1955, 1957, 1967, 1996).

For centuries natives of regions where malaria is endemic have obtained remedies of malaria from a specific bark of a tree which was first introduced in Europe as "fever bark". Analysis of this bark showed that this remedy contained quinine and allied alkaloids. These alkaloids, especially quinine, remain among the standard treatments for

malaria to this date. However, the increasing spread of *Plasmodium falciparum* resistant to nearly all existing anti-malarial drugs together with the absence of an efficient vaccine against malaria emphasises the urgent need for alternative eradication and treatment strategies (Maurice, 1995 and Tanner *et al.*, 1995).

A "return to basics" strategy has been adopted by the WHO which is now promoting the use of bed nets as the major means of malarial control (WHO, 1996). The other strategy adopted is the search for new parasite targets and anti-malarial molecules. In this respect the past 20 years have witnessed a very impressive increase in our understanding of the biochemistry and molecular biology of the malarial parasite, with attention focused on specific parasite molecules that are keys to the parasite life cycle or the induction of it's pathogenesis. Directed pharmacology research has involved the identification and characterization of targets that can be specifically, pharmacologically affected, they include the replication machinery (Smeijsters *et al.*, 1994; Chakrabati *et al.*, 1993; Rubin *et al.*,1993; Deharo *et al.*, 1995), protein and nucleotide synthesis (King *et al.*, 1987; Queen *et al.*, 1989; Coomber *et al.*, 1994; Rathod *et al.*, 1994; Eakin *et al.*, 1995), lipid metabolism required for membrane biogenesis (Holtz, 1977; Ancelin *et al.*, 1985; Beaumelle *et al.*, 1988; Vial *et al.*, 1990; Vial & Ancelin 1992) and protease inhibitors of metabolic pathways such as those involved in haemoglobin degradation (Charet *et al.*, 1980; Vander jagt *et al.*, 1983, 1984; Gyang *et al.*, 1982; Schrevel *et al.*,1990; Goldberg, 1992; Breton *et al.*, 1993; McKerrow *et al.*, 1993; Curley *et al.*,1994; Rosenthal, 1995). The latter pathway has already been validated as a susceptible target to antimalarials (Slater & Cerami1992). Complete characterization of the molecular structure of the target should allow attack of these molecules at novel

and distinct sites with new drugs concepts. In addition, the design of structurally analogous molecules to new and existing antimalarials that are specifically active against the parasite and tolerable to the host, will be facilitated.

1.1. Classification.

The malaria parasite belongs to the subkingdom of protozoa's, it is classified as a microscopic unicellular organism although it undergoes schizogony to form multinucleated particles known as segmentors or schizonts during the intra-erythrocytic asexual life cycle. This Protozoa is also a Sporozoa as the formation of spores are included in it's life cycle.

Class: *Telosporidae* : The zygote undergoes sporogony to produce sporozoites which after entering a host become trophozoites, which multiply by schizogony and eventually produce gametocytes.

Subclass : *Haemosporidae*: Schizogony takes place in fixed tissue cells such as the spleen and liver in *P. falciparum*, with subsequent schizogony in erythrocytes. Gametocytes are found in red cells and fertilisation takes place in arthropod host (mosquito). The oocyst grows and produces many sporozoites. The asexual phase takes place in mammals and the sexual phase takes place in arthropods (*Anopheles* mosquito).

Order or family: *Plasmodiida:* Gametocytes are found in the red blood cells of vertebrates. Formation and syngamy of gametes takes place in arthropod host, the mosquito. Pigment granules (hemozoin) are deposited when the red cell is invaded.

Genus: *Plasmodium* : Parasites of lizards, birds and mammals, gametocytes in red blood cells; erythrocytic schizogony; extra erythrocytic forms in various tissues, but always solid or at most vacuolated bodies; arthropod hosts mosquitoes.

1.2. Mammalian species of *Plasmodium*.

Typical species of malaria transmitted by mosquitoes, are found among mammals only in man and monkeys with the exception of three species, *Plasmodium berghei, Plasmodium chabaudi chabaudi,* and *Plasmodium vinckei* which are found in rats and various rodents. Both the primate and rodent parasites are transmitted by species of *Anopheles* only.

Apes and monkeys harbour a number of species which are closely related to the species found in man. *Plasmodium rhodhaini* of chimpanzees has identical pathology with *Plasmodium malariae* in humans and *Plasmodium cynomolgi* is very similar to *Plasmodium vivax*. *Plasmodium knowlesi*, which causes rapid, fatal infections in rhesus monkeys, causes relatively mild and temporary infections in man. These animal models have proved very useful in studies on the intra-erythrocytic stages of malarial parasites.

Rodent malaria *P. berghei* is similar in it's life cycle to *P. falciparum* and is widely used as an animal model for *P. falciparum* (Maegraith *et al.*, 1952; Thurston, 1953; McNally, PhD. thesis 1994). Unlike *P. falciparum*, *P. c. chabaudi* has a synchronous life cycle and has proved very fruitful for stage specific study of the intraerythrocytic parasites. It has now been accepted as a suitable animal model for *P. falciparum* infections of human malaria (Taylor-Robinson, 1995).

1.3. Species of *Plasmodium* which cause malaria in man.

P. falciparum is very prevalent in the tropics but does not thrive as far north as *P. vivax* malaria. It has a 40-48 hours intra-erythrocytic cycle in man. *P. falciparum* causes a much more dangerous disease than the other species but runs a shorter course without relapses, seldom lasting more than 8-10 months without re-infection (Shortt *et al.*, 1951).

P. vivax is the most widely distributed species, being prevalent in both tropical and temperate zones. It has an intra-erythrocytic cycle of 48 hours in man and is particularly likely to cause relapse (Coateney *et al.*, 1948; Shortt *et al.*, 1948)

P. malariae is also widely distributed in both tropical and temperate climates, but is much less common than either *P. vivax* or *P. falciparum*. It has a 72 hours intra-erythrocytic cycle in man and causes infections of many years duration.

P. ovale is very rare, though it has been found in such widely separate parts of the world as West Africa, South America, Russia, Palestine and New Guinea.

There is no animal reservoir for any of these human parasites except possibly chimpanzees for *P. malariae*. The *Anopheles* mosquito is the only vector that propagates malaria.

1.4. Malaria the disease.

In general each malaria attack begins with a shivering chill, sometimes accompanied by convulsions so severe that the teeth chatter, goose flesh stands out and the bed rattles. The temperature will be found to be several degrees above normal and still going up. In the wake of the chill comes a burning and weakening fever, with violent headache and nausea and a temperature up to 42°C or higher. The fever period is followed by a period of sweating so profuse that the clothes or bedding may become wringing wet. The sweating gradually subsides, the temperature drops rapidly, often below normal, and after 6-10 hours the patient rests fairly easily until the next attack. The paroxysms of fever and chills which are caused by merozoite lysis of red blood cells continue every other day for up to 2 weeks or more. Then they become less pronounced, the parasites become sparse, the patient feels well and the temperature becomes normal. Sooner or later there are relapses, in which the intermittent chills and fevers begin again. When the relapses begin after the long latent period they occur repeatedly at intervals of a month or so. After these have subsided some strains apparently die out, but others may persist for years. The spacing and probability of relapses are, however, apparently influenced by dosage of sporozoites, acquisition of immunity, and administration of drugs (Coatney & Cooper, 1948). It is commonly accepted that relapses after a long period may be brought on by such physiological shocks as exhaustion, childbirth, operations, alcoholic binges, etc.

In *P. falciparum* malaria the disease is quite different, primary infections are common in visitors to the hyper endemic localities. The

paroxysm of chills and fevers are irregular due to the asynchronous life cycle of parasite, they last 12 or 14 to 36 hours, are severe in nature, and often occur daily, a fresh attack sometimes beginning before the previous one has entirely subsided. The attacks usually last 8-10 days and then the temperature declines. In just a few days there is a series of paroxysms, perhaps even more severe, and these recrudescence then continue in declining severity every 10 or 12 days for about 6 or 8 weeks, after which they become more irregular, although the blood continues to be infective. In tropical malaria countries the re-infection is more or less continuous. Under these circumstances the infected persons become "carriers" harbouring a few parasites, possibly too few to be found in blood smears, and showing few symptoms or none at all.

A number of pernicious conditions may develop, in *P. falciparum* malaria. The tendency of corpuscules infected with *P. falciparum* to cling together results in clogging capillaries and preventing the proper flow of blood in vital organs (MacPherson *et al.,* 1985; Kern *et al.,* 1989). In the brain this, as well as a direct toxic effect, leads to numerous symptoms, among them coma, hypoglycemia (White *et al.,* 1993) and sometimes sudden death by a stroke. This cerebral malaria is responsible for a large fraction of malarial deaths. In some cases violent gastrointestinal symptoms resembling cholera, typhoid, or dysentry develop, and in others heart failure or pneumonia. The commonest complications in patients who die of malaria are renal failure, hyperparasitaemia, acidaemia, jaundice, cerebral malaria, and severe anaemia due to merozoite release from erythrocytes (Phillips *et al.,* 1994; Molyneaux *et al.,* 1989; Soni *et al.,* 1996).

In typical cases of primary *P. vivax* infections, after completion of one or two pre-erythrocytic cycles in the tissues, the parasites invade

the blood stream about 7-14 days after infection. The time of appearance of parasitised erythrocytes is usually a little shorter in *falciparum* infections and a little longer in *P. malariae* infections.

P. ovale infections are not prone to relapses and are more susceptible than any of the other species to drug treatment.

In *P. malariae* infections the paroxysms occur at 72 hour intervals, are milder and of shorter duration, recur more regularly, and the infections persist for a longer time.

The fact that the attacks most commonly occur between midnight and noon, instead of in the evening, is often useful in distinguishing malaria from other intermittent fevers, for *P. vivax* and *P. malariae* infections.

Some of the characteristic symptoms of malaria will be elaborated in this thesis, which will also show that some of these symptoms are correlated with the life cycle of the species of the malarial parasites in question. This thesis will only emphasise on the intra-erythrocytic stages of malaria parasites. For a more detailed reading on the liver stage, mosquito stage of malaria parasites and diagnosis see Chandler and Read (1960) and O'Donovan (1993).

1.5. Natural resistance to malarial infection.

In 1949 J. B. S Haldane suggested that heterozygotes for red cell disorders such as thalassemia might be protected against death from malaria. Since then it has been demonstrated that individuals with anaemias linked to genetically modified haemoglobin such as Hb S, Hb E and Hb C and other red cell genetic disorders such as the deficiency in glucose-6-phosphate dehydrogenase (Livingstone, 1985) display

protection against clinical and severe malaria. Most red cell genetic defects represent, in effect, polymorphic genetic resistance to malaria.

The malarial parasite has an obligatory erythrocyte stage that generates gametocytes that complete the life cycle of the plasmodia parasite, which begins and ends in the *Anopheles* vector (fig. 1). Genetic resistance to *P. falciparum* may involve the merozoite invasion into the red cell to begin the erythrocytic stage; the intracellular growth of the parasite and the erythrocytic lysis that occurs in coincidence with the maturation and subsequent dispersion of merozoites.

World distribution of the red cells abnormalities which confer innate resistance to malaria are mostly found in malaria endemic regions (Weatherall, 1987), suggesting that these genotypes are naturally selected due to exposure to malaria

1.5.1. Red cell genetic defects involving resistance to merozoite invasion of the red blood cells.

(a) Red cells with mutations in surface blood group antigens.

Natural resistance to *P. vivax* infection has been linked to the Duffy blood group antigen (Miller *et al.*, 1973). When human volunteers were exposed to mosquitoes infected with *P.vivax* (Miller *et al.*, 1976) the results demonstrated that, Duffy negative individuals were resistant to the infection, while all the other volunteers fell sick 11 to 15 days after exposure. The Duffy antigen is a 35-43 kDa glycoprotein located on the surface of erythrocytes (Hadley *et al.*, 1984). Duffy negative genotype individuals are resistant/ refractory to *P. vivax* infection

because erythrocyte invasion is prevented by the absence of this glycoprotein.

The Duffy positive genotype is predominantly encountered in the Caucasian population and very rare in African descent populations among whom resistance to *P. vivax* infection is commonly encountered (Boyd & Stratmann-Thomas, 1993)

Polymorphic forms of glycophorins identified as blood groups also exhibit resistance to *P. falciparum* invasion. The cells showing complete absence of A-glycophorin (A) can be demonstrated to reduce invasion of *P. falciparum* significantly but not completely (Pasvol *et al.,* 1982). This is a rare blood group (only about 10 individuals are known to carry this genotype world wide). Red cells expressing hybrids of the genes specifying A and B glycophorins, show 50% reduction of invasion (Pasvol *et al.,* 1982, 1989). The red cells that lack A and B glycophorins were tested with two *P. falciparum* strains 7G8 and Camp (Hadleys *et al.,* 1987). The former invaded with 50% efficiency, while the latter only at 20% of the control. These results raise the possible heterogeneity among *P. falciparum* malaria variants as to their red cell binding determinants (Mitchell *et al.,* 1986). However none of the red cells containing different polymorphic forms of the glycophorins that have been demonstrated to reduce invasion emulate the Duffy system.

(b) Red cells with cytoskeleton abnormalities.

Cytoskeleton proteins are active participants in the process of merozoite invasion. The only known highly polymorphic red cell cytoskeleton abnormality is the Melanesian ovalocytosis. Ovalocytosis is characterised by red cells with axial ratios smaller than that of

elliptocytes and there is no hemolysis associated with it. The underlying molecular defect is unknown at present, although the thermal deformation profile of these cells is abnormal (Kidson *et al.*, 1981) and the expression of surface blood groups is altered (Booth *et al.*, 1977. Deformability of these cells is deminished (Saul *et al.*, 1984). These red cells are resistant to *P. falciparum* (Kidson *et al.*, 1981) and *P. knowlesi* (Hadley *et al.*, 1983) invasion although these two parasites bind to different receptors.

Another cytoskeleton defect that has been found to be resistant to malaria invasion is elliptocytosis due to either the absence of glycophorin C or band 4.1 as described by (Hadley *et al,* 1988; Shear *et al.*, 1986). They have found dramatically decreased invasion of *P. berghei* and *P. c. chabaudi* in ankyrin/spectrin deficient mice.

1.5.2. Red cell abnormalities that involve the intracellular parasite growth period.

(a) Haemoglobin S-containing red cells.

Epidemiological and clinical studies suggest that heterozygote β^S gene carriers could acquire *P. falciparum* malaria but that they were partially protected from dying of the infection (Allison *et al.*, 1954; Livingstone *et al.*, 1971). Luzzato *et al.* (1970) showed that the sickle trait increased the probability of parasitised AS (heterozygotes for HbS) red cells being removed from the circulation. Accelerated morphological sickling occurs in AS red cells containing parasite ring forms. At conditions of low pH, which are particularly prevalent in the cytosol of late parasitised AS red cells (Friedman *et al.*, 1978), HbS polymerises, generating many nucleation events, and thus assuring that

none of the polymer domains becomes large enough to deform the cell; hence there is no apparent morphological sickling (Roth *et al.*, 1988). Friedman *et al.* (1978) demonstrated that As cells at 17% oxygen sustained normally the growth of *P. falciparum* whereas an oxygen environment of 3% after two days of normal growth in 17% oxygen inhibited parasite growth and parasites subsequently died in a couple of days. These events were even more pronounced in Sc and SS cells showing a deoxygenated dependency parasite growth inhibition. Similar experiments by Pasvol *et al.* (1980) confirmed these findings. These results suggest that there are at least two resistance mechanisms in AS cells. First the parasites may die during the ring stage if they induce sickling. Secondly their existence may be seriously hampered during deep vascular schizogony; venules bedecked with adherent parasitised cells become partially or totally obstructed, a situation that leads to hypoxia and low pH, conditions that favour sickling and in which the parasite does not thrive (Friedman *et al.*, 1978).

The mechanism of parasite death in sickled AS and SS cells is not fully understood. Friedmann *et al.* (1978), first proposed two possible mechanisms applicable to AS cells: The loss of K^+ accompanying sickling was detrimental in itself to parasites growth and the loss of water, concomitant with the loss of K^+, would progressively increase HbS polymerisation (Friedman *et al.*,1979a).

Two mechanisms have been proposed for the killing of *P. falciparum* by increased intra-erythrocytic polymerisation of HbS. The sickle polymer is not a good substrate for the proteases of the parasites and the plasmodium will starve to death (Eaton *et al.*, 1987) or/and the polymer interferes directly with some critical function of the parasite. The AS carriers might benefit from the retardation in the HbF to HbS and A switch (Weatherall *et al.*, 1981) observed, in the critical first

five years of life in which no immune defence mechanisms against malaria are in place.

(b) Haemoglobin E-containing red cells.

HbE is the most frequent single-base mutation (26 Glu-->Lys) that leads to an abnormal haemoglobin. It is likely to be the most frequent haemoglobin mutation in the world. An intriguing observation has been reported by Bunaratvej *et al.* (1986) that EE and EA erythrocytes are phagocytised to a greater extent by human monocytes than are infected normal erythrocytes, suggesting that the surface of HbE-containing red cells is modified by the parasite differently from that of normal red cells. This mechanism, conceivably based on increased oxidative stress damage of the red cell membrane, could render the carriers resistant to malaria. Increased antimalarial antibodies in these individuals might signal frequent but benign infection, conducive to early immunological resistance and survival through a double protection mechanism.

(c) Haemoglobin F-containing red cells.

Red cells containing 100% HbF cord blood cells, blood cells from adults with hereditary persistence of fetal haemoglobin (HPFH) and in HbF containing cells from infants (1-30% HbF) retard *P. falciparum* growth *in vitro* (Pasvol *et al.*, 1977). This HbF retardation of growth is mediated by an increase in oxidative stress. These findings may explain the decrease in parasitaemia observed in the first six months of life and could be a contributing factor to the advantages offered to the carriers of β^S, β^C and, particularly the thalassemia genes, which exhibit a significant retardation of the HbF switch-off that occurs in the first

five years of life. This period is critical in the protection against infection, since immune mediated resistance is not yet effective.

(d) Haemoglobinopathies due to abnormal haemoglobin chain synthesis.

Disorders of globin synthesis are genetically and physiologically more complex than the single aminoacid substitution of haemoglobins. β-thalassemias have variations in the levels of haemoglobins A2 and F as well as a variable decrease in the synthesis rate of the β chain. α-thalassemias exhibit a spectrum of changes: a clinical silent form (-a/aa), a form with slight or moderate anaemia and red cells hypochromia and microcytosis (-a/-a and --/aa), a form with moderate to severe anaemia (-a/--) and, finally a form that is lethal in-utero or at birth (--/--) (Pasvol *et al.*, 1977). A number of theories concerning the manner in which thalassemias may inhibit malaria parasite growth include three groups of mechanisms: those that impair the nutrition of the parasite as intraerythrocytic iron deficiency in thalassemia (Nurse *et al.*, 1979) interacting nutritional deficiencies (Brockelman *et al.*, 1983), and low cell indices (Allison *et al.*, 1982). Those that involve increased susceptibility to oxidative stress damage (Allison *et al.*, 1982), increased vulnerability to cell mediated phagocytosis (Allison *et al.*, 1983), and low pyridoxane-phosphate oxidase activity in thalassemias (Clements *et al.*, 1981). Finally the elevation and persistence of fetal haemoglobin in infancy and early childhood (Clements *et al.*, 1981; Lehmann *et al.*, 1972)

1.5.3. Resistance involving erythrocyte lysis

(a) Haemoglobin C-containing red cells.

Epidemiological evidence of the connection between HbC and malaria is largely lacking, except for the fact that this abnormal haemoglobin has been found in malaria endemic regions.

Friedmann *et al.* (1979a) found significantly decreased growth curves of *P. falciparum* in oxygenated CC cells but not AC cells. This finding was confirmed by Olson and Nagel (1986), who, working on the synchronised cultures in addition demonstrated that *P. falciparum* growth in CC cells is not significantly modified in low oxygen conditions nor is normal development restored when the cells are suspended in high intracellular K^+ which interferes with K^+ efflux and dehydration. The parasitised CC cells are very resistant to lysis compared to parasitised AA (normal) cells. Degenerative late schizonts are observed in day 4, an observation compatible with an incapacity of parasitised CC cells to complete their schizogony with merozoite release

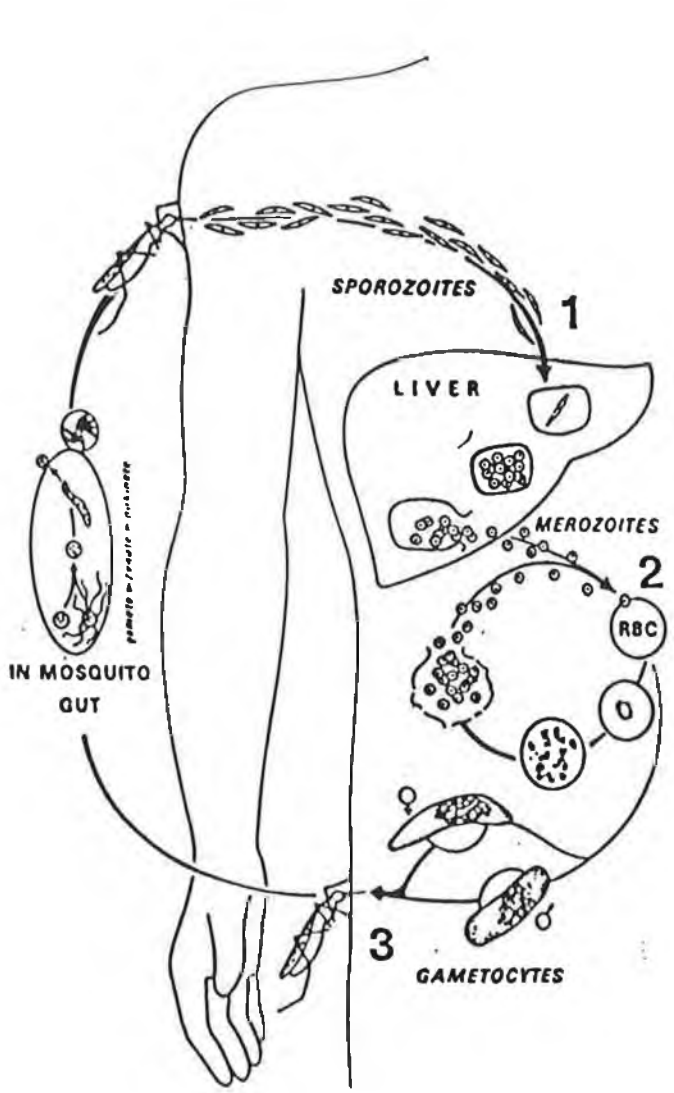


Figure 1. Life cycle of the malarial parasite in man and mosquito. (Drawing courtesy of Dr. Louis miller.).

1.6. Immunological response to malarial parasites.

1.6.1. Acquired immunity to malarial infection.

The immunity against malaria is highly specific and not only fails to protect against attack by other species, but sometimes even strains of the same species. In many cases as long as the parasites persist in the body in an exoerythrocytic reservoir, as in *vivax* and *malariae* infections, or are constantly being re-inoculated as in *falciparum* infections in the tropics, the immunity once developed is restimulated as soon as it begins to fail, and no clinical relapses can occur. However in many cases rapid formation of antibodies or phagocytes in response to a renewed stimulus does not occur and these individuals suffer a relapse before the body reacts.

Infants in hyper-endemic areas do not suffer as much as is sometimes thought from malaria infections, as they are passively protected by the presence of HbF and their mothers antibodies until they begin to develop some of their own.

Natural resistance to malarial infections include the absence of the Duffy antigen on erythrocytes against *P. vivax* or the possession of genetically modified haemoglobin against most known species of *Plasmodium* (see Section 1.5). Other host defences include non specific immunological defences which include direct cellular responses by macrophages and granulocytes and natural killer cells. In addition a humoral component is provided through the alternate complement pathway, which is antibody independent. (Roitt *et al.*, 1989)

However, the type of T cells involved and the mechanisms involved are still unclear. These T cells might control malaria by the production of cytokines such as IL-1. IL-1 subsequently activates cytotoxic immune responses which include the acute and chronic inflammatory response through production of acute-phase proteins by the liver, and stimulates the action of IgE on mast cells and fibroblast to produce acute-phase proteins which in turn can stimulate the alternative and classical complement pathways to produce vasoactive substances (fig. 2). The vasoactive substances alter vascular permeability and induce immigration of leukocytes and other effector cells such as, polymorphonuclear phagocytes, and granulocytes. This diapedesis induces T cytotoxic activation which is important in resistance to malaria.

T helper and delayed-type hypersensitivity mediator cells play central roles through their recruitment and activation of non-specific cytotoxic cells, such as phagocytes and natural killer cells, and by augmentation of the humoral response. Cytokine-activated macrophages display much more active and efficient responses to intraerythrocytic malarial parasites. They migrate to this area by chemotactic factors derived from parasite antigens or from antibody activated mast cells. Hence, the inflammatory response appears to be the main cell-mediated immune response by which intraerythrocytic malarial parasites are eliminated from the infected host.

Nitric oxide inhibits the development of the exo-erythrocytic cycle of *P. berghei* (Mellouk *et al.,* 1991), *P.yoelli* (Nüssler *et al.,* 1991) and *P. falciparum* (Rocket *et al.*, 1991) *in vivo*. Nitric oxide has been reported to play an important role in host control of the primary patent parasitaemia but not on recrudescent or re-infection parasitaemia of *P. c. chabaudi in vivo* (Taylor-Robinson *et al.,* 1996).

Cells in which nitric oxide can be produced in amounts sufficient to cause a physiological effect are either macrophages activated by INF-γ (Marletta *et al.*, 1988), TNF-stimulated endothelial cells (Kilborn *et al.*, 1990), or the T helper 1 subset (Taylor-Robinson *et al.*, 1994). The secretion of nitric oxide over short range distances in a localised environment, such as the spleen and the liver may kill parasites directly. Alternatively, nitric oxide may have an indirect effect through its well-documented property of vasodilation (Moncada *et al.*, 1991)

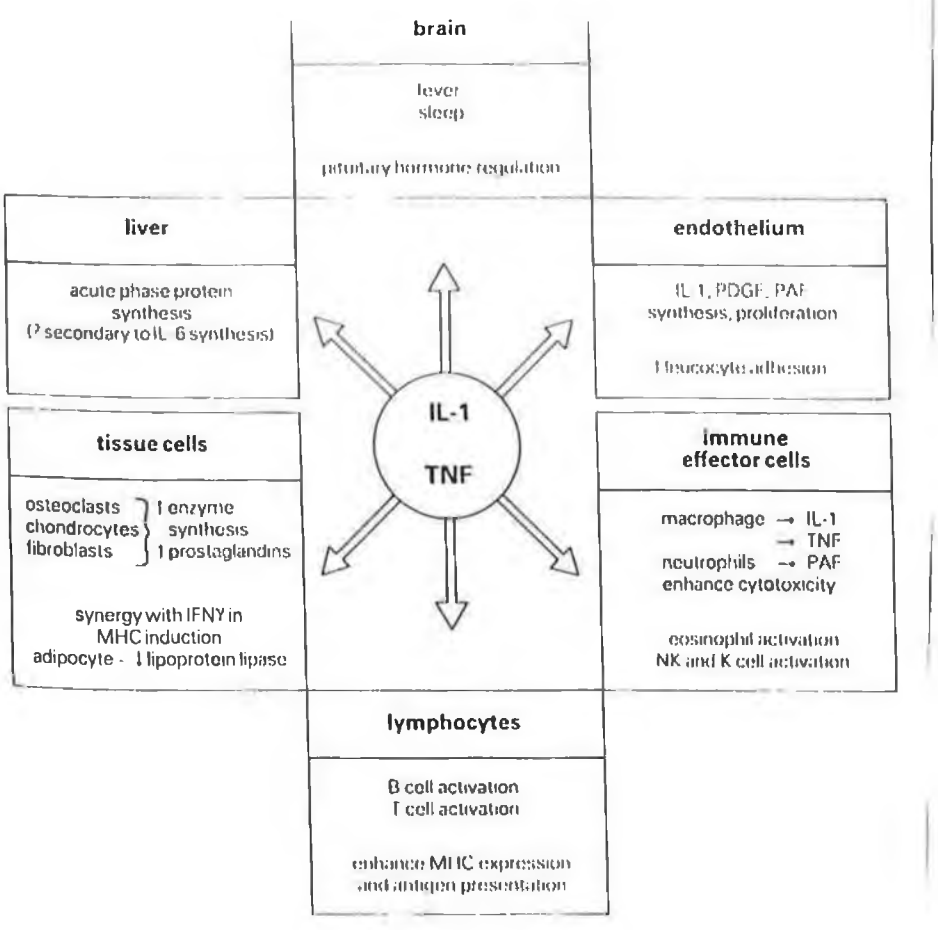


Figure 2. Actions of IL-1 and TNF. (Reproduced from Roitt *et al.*, 1989).

1.7. Treatment of malaria.

Malaria treatment chiefly involves the use of aminoquinolines, such as chloroquine and it's analogues, and a number of additional drugs, for the erythrocytic stage and chiefly primaquine for the exoerythrocytic stage. Newer compounds such as mefloquine, ghinghaosu (also known as artemisinine, artesune and artemether) and halofantrine are also used (fig. 3 & 4).

Only asexual erythrocytic parasites produce clinical illness. Hypnozoites (hepatic parasites) and gametocytes do not produce recognisable clinical symptoms. Antimalarial agents that inhibit the replication of the asexual erythrocytic cycle provide the most rapid clinical improvement.

Although toxic side effects are a major draw back in the use of primaquine it remains the most effective agent for the radical cure of *P. vivax* malaria (Werndorfer *et al.,* 1991; White, 1992) This drug interferes with the mechanism of chloroquine and should be administered subsequent to chloroquine treatment for *P. vivax.* (Van, 1990). Recent electrocardiographic studies indicate that halofantrine is potentially deleterious because of its dose-related cardiac toxicity. However new prescription rules have been introduced since this discovery (Giudiceilli *et al.,* 1996). Open comparison of artemether and mefloquine in uncomplicated *P. falciparum* hyperparasitaemia show that artemether produced a significantly higher parasite reduction in 24 hours and significantly shorter parasite clearance than mefloquine. Fever clearance times are similar. Although both drugs are well tolerated, mefloquine produces more episodes of abdominal

pain with or without diarrhoea and vomiting (Sowunmi *et al.*, 1996; Looareesuwan *et al.*, 1996; Cardoso *et al.*, 1996). Artemether may also reduce the length of coma in severe malaria (Watkins *et al.*, 1996). Several antibiotics that inhibit protein synthesis (e.g tetracycline and doxycyclin and clindamycin) are often administered in combination with the antimalarial agents. Older agents that have wide use include quinine and quinidine.

Malaria pathogens are highly adaptable to chemical changes in their environment. Following introduction of a new drug, it can be anticipated that *P. falciparum* will develop resistance within a few years. At the moment, there is no agent that can offer protection against malaria in all regions of the world hence the need for novel chemotherapeutic drugs is acute. This thesis will suggest bestatin or it's analogues as one of these novel antimalarial agents.

1.7.1. Mechanism of action of some drugs used in the treatment of malaria.

(a) 4-aminoquinolines.

1. Chloroquine

The mechanism of antimalarial action of chloroquine and other 4-aminoquinolines is still unclear. Several mechanisms have been proposed. In one, binding of chloroquine to ferriprotoporphyrin-IX, released from haemoglobin in infected erythrocytes, produces a complex that is toxic for plasmodial and RBC membranes (Chou *et al.*, 1980).

In a second, uptake and concentration of chloroquine into the parasites raises the pH of the intracellular acidic food vacuole. This pH

increase damages the ability of the parasite to degrade haemoglobin and causes the characteristic morphological changes seen microscopically (Yayon *et al.*, 1985).

The third suggestion was that the chloroquine target is a heam polymerase responsible for detoxification of the food vacuole by polymerising toxic heam into non toxic hemozoin. Chloroquine inhibits this heam polymerase causing toxic heam to accumulate in the acid vacuole thus killing the parasites (Slater & Cerami 1992).

2. Quinine, quinidine and mefloquine.

The mechanisms of action of quinine and mefloquine are thought to be similar to that of chloroquine. Quinidine also has some antimalarial properties. Both quinidine and quinine display potentially hazardous cardiac toxicity.

(b) Diaminopyrimidines.

1-Pyrimethamine and trimethoprim..

The diaminopyrimidines, such as pyrimethamine and trimethoprim, inhibit dihydrofolate reductase in malarial parasites. These agents are effective at concentrations far below those needed to inhibit the mammalian enzyme, so selectivity can be attained. Inhibition of parasite dihydrofolate reductase blocks the synthesis of tetrahydrofolate, a precursor necessary for the formation of purines, pyrimidines, and certain amino acids (Gero & O'Sullivan, 1990). Parasites exposed to these agents do not form schizonts in the red blood cells or liver. When a diaminopyrimidine is used with sulfonamide or sulfone, a synergistic effect is achieved by blockade of two steps on the same metabolic pathway (Triglia *et al.*, 1994). Sulfonamide inhibits the

synthesis of aminobenzoic acid (McConkey *et al.,* 1994) to dihydropteroic acid while the diaminopyrimidine blocks the reduction of dihydrofolate to tetrahydrofolate (Cowman *et al.,* 1991).

The molecular mechanisms of the newer experimental ghinghaosu and halofantrine are not known. However, ghinghaosu and it's derivatives are endoperoxide-containing compounds which represent a promising new class of antimalarial drugs. In the presence of intraparasitic iron, these drugs are converted into free radicals and other electrophilic intermediates which then alkylate specific malarial target proteins (Meshnick *et al.,* 1996).

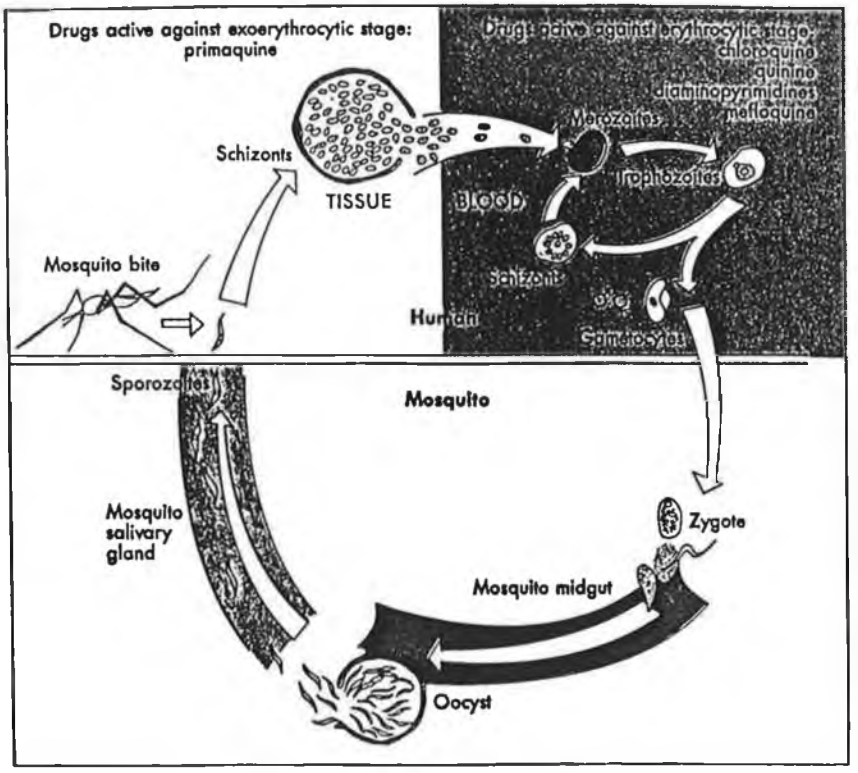


Figure 3. Drugs used to treat infection are effective against either erythrocytic or exoerythrocytic stages of the parasite. (Reproduced from Wingard *et al.*, 1991).

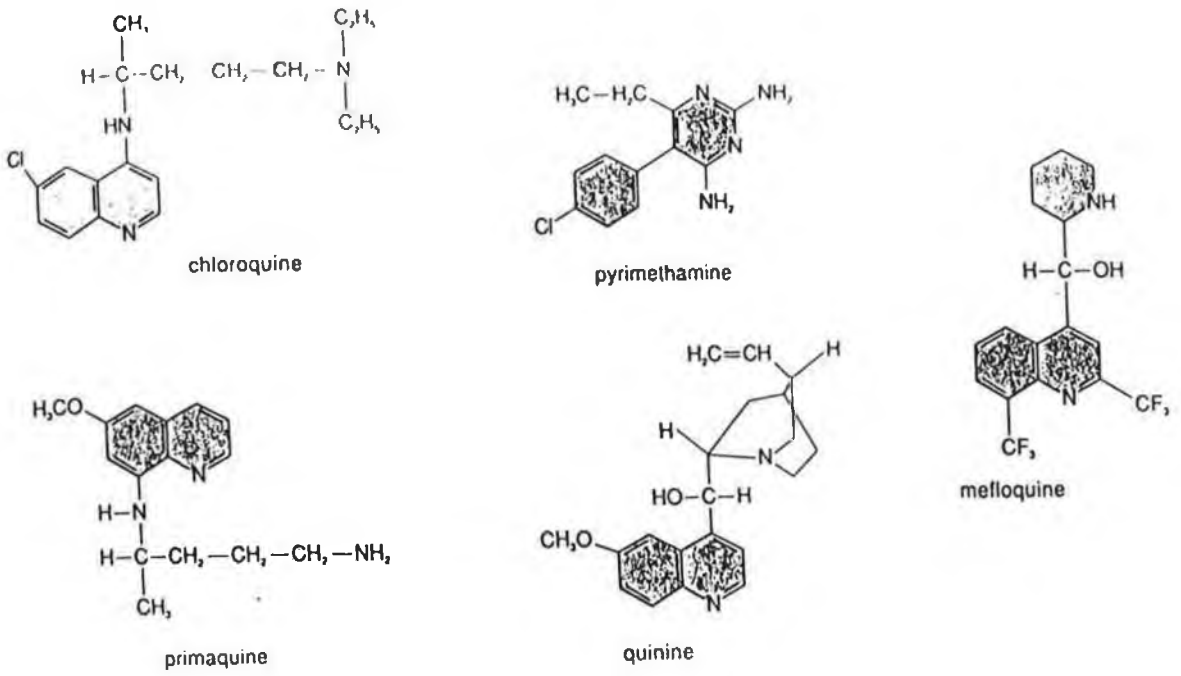


Figure 4. Structure of some drugs used in the treatment of malaria. (Reproduced from Wingard *et al.*, 1991).

1.8. Haemoglobin and malarial parasites.

Haemoglobin comprises 95% of the soluble red blood cell proteins and is present at 340 mg/ml of blood. During the trophozoite stage the malarial parasites digest up to 75% of haemoglobin for use as a nutrient source in their growth and development.

A mouth like structure the cytostome sucks red blood cell cytosol into the parasites by a process resembling pinocytosis. These vesicles pinch off and carry their contents to an acidic digestive vacuole. The outer membrane of the double membrane bearing transport vesicle appears to fuse with the digestive vacuole and release a single membrane-containing vesicle inside. The vesicle is then rapidly lysed. Globin is degraded to aminoacids for use as the main source of amino acids for synthesis of parasite proteins (Moulder, 1962 & 1985) as shown by ^{14}-C incorporation in parasite proteins from cell haemoglobin (Sherman *et al.* 1970). The heam is co-ordinated into a crystalline matrix called hemozoin or malaria pigment by a heam polymerase (Slater & Cerami, 1992).

Haemoglobin digestion is initiated in the acidic digestive vacuole by the aspartic proteases Plasmepsin I by cleaving native haemoglobin in the α chain between Phe33 and Leu34. This site is in the hinge region of haemoglobin which is involved in maintaining the integrity of the molecule as it binds oxygen (Perutz, 1987). It is a highly conserved stretch of aminoacids in all vertebrate haemoglobins (Dickerson *et al.*, 1983). None of the hundreds of characterised human variant haemoglobins have a homozygous defect in this region (Beutler, 1990) consistent with the notion that the parasite has found a way to attack the haemoglobin molecule at a site that cannot be altered

without deleterious consequences to the host (Goldberg *et al.*, 1991; Francis *et al.*, 1994). Cleavage by Plasmepsin I appears to unravel the substrate after which major early secondary cleavages occur between aa105 and aa106 by Plasmepsin I and aa108 and aa109 by Plasmepsin II. Plasmepsin II possibly cleaves native haemoglobin but cleaving activity was more enhanced on the denatured molecule. A cysteine protease degrades the unravelled haemoglobin molecule in both the α and β chains of haemoglobin. The cleavages that these enzymes make are infrequent enough that there must be exopeptidases to finish the proteolysis, generating free amino acids for the parasites nutrition. (Goldberg *et al.*, 1991) This thesis describes malarial aminopeptidases that might be involved in proteolysis of haemoglobin derived peptides and shows that inhibition of these aminopeptidases by bestatin inhibits parasite growth.

1.9. Aminopeptidases.

Aminopeptidases are exoproteases which catalyse the cleavage of polypeptide substrates near the N-terminal extreme thus releasing free aminoacids or dipeptides. They can be subdivided into aminopeptidases which hydrolyse the first peptide bond (aminoacyl-peptide hydrolase and iminoacyl-peptide hydrolase) and those which remove dipeptides from polypeptides chains (dipeptidyl-peptide hydrolase). Some peptidases act only on dipeptides or tripeptides and may also be considered as aminopapetidases. They are widely distributed throughout the animal and plant kingdom. Generally, aminopeptidases are metalloenzymes and most of them require zinc as the metal cofactor for enzymatic activity. These enzymes generally have a broad

specificity and can be monomeric or associated in protein subunits. Their specificities overlap, so that many natural and synthetic substrates may be hydrolysed by more than one enzyme.

They are involved in major regulatory process such as cell division enabling cells to pass critical check points by participating in proteolytic events essential for cell growth and viability (Constam *et al.*, 1995) and in the regulation and metabolism of neurotransmitters (which has been associated to aminopeptidase-N) within the neural cells and the gastrointestinal tract indicating the implication of aminopeptidases in pain transmission (Akasaki *et al.*, 1995; Konkoy *et al.*, 1995, Stevens *et al.*, 1995). Down regulation of cell surface molecules such as interleukin receptors on neutrophils has been associated with aminopeptidase N (Manna *et al.*, 1995) and hydrolysis of leukotriene A4 into chemotactic leukotriene (Baker *et al.*, 1995, Watterholm *et al.*, 1995) has been associated with aminopeptidase activity indicating the role of aminopeptidases in immunomodulatory activities. Aminopeptidase B-type activity has been localised in rat testis indicating a role in spermatogenesis (Cadel *et al.*, 1995). Alanine aminopeptidase activity has also been associated with transport of oligopeptides into kidney brush border membrane vesicles (Daniel *et al.*, 1995) and in the regulation of transcription in tomato leaves (Schaller *et al.*, 1995).

(a) Classification.

Aminopeptidases are classified by the number of amino acids cleaved from the NH_2-terminus of substrates and are given the numbers EC.3.11-3.4.13. Classification is also performed with respect to the relative efficiency with which residues are removed. Hence Leucine

aminopeptidase prefers leucine peptide substrates although activity with other substrates is observed. Arginyl, methionyl, aspartyl, cystynyl, prolyl, glutamyl and alanyl aminopeptidases have been described. With respect to location of the aminopeptidase, some peptidases are secreted. but most are not. Aminopeptidases are also classified by susceptibility to selective aminopeptidase inhibitors, according to the metal cofactors and/or the residues that bind the metal co factor to the enzyme and finally according to the pH at which maximum activity is observed to denominate acidic, basic, and neutral aminopeptidases.

Zinc aminopeptidases constitute the largest, or at least the most studied type, of aminopeptidases. They constitute a relatively large subdivision of the mammalian cell surface peptidase family. Identification of a new family of aminopeptidases evolved after recent work demonstrated that aminopeptidases from diverse organisms show extensive primary sequence homology (Burley *et al.*, 1992; Wood *et al.*,1993)

1.9.1. Aminopeptidases in malarial parasites.

As described above (Section 1.8) the malarial parasite requires aminopeptidases in order to finish proteolysis of haemoglobin derived peptides into free aminoacids for protein synthesis. Aminopeptidase activity has been described in *P. falciparum* and rodent malarias by different research groups. However, some very important physical-chemical properties differ to such an extent that it would be best to describe the aminopeptidases characterised by different groups separately.

a) Aminopeptidases in rodent malarial parasites.

Aminopeptidase activity was first described in rodent malarial parasites by Charet and co-workers (1980) from *P. c. chabaudi* and *P. yoelii nigeriensis* early trophozoite cytosolic extracts. The aminopeptidase from the *P. c. chabaudi* trophozoite extracts had an apparent molecular mass of 90 kDa. determined by non-reducing gel filtration and a K_M = 0.7 mM ± 0.2 with L-alanyl-4-nitroanilide. This enzymatic activity was highly inhibited by Zn^{2+} and more slightly by Co^{2+} and Mn^{2+} ions. Iodoacetamide and cysteine were without effect, but PCMB and Hg^{2+} were strong inhibitors. The optimal pH activity and temperature were 7.2 and 50°C respectively. Electrofocusing studies revealed that the aminopeptidase activity peak was composed of four different pI values (5.85, 5.7, 5.6 and 5.5) suggesting four different aminopeptidases with the same apparent molecular mass. The aminopeptidase activity was also strongly inhibited by chloroquine, quinacrine and primaquine whereas quinine had a smaller effect. Aminopeptidase from *P. yoelii nigeriensis* showed the same apparent molecular mass of 90,000, K_M = 1.4 mM ± 0.2, pH optimum of 7.2, pHi 5.2, 5.3 and 5.4. The optimal activity for this enzyme was observed at 37°C (Charet *et al.*, 1980).

Curley *et al.*,(1994) described aminopeptidase activity observed in cytosolic cell-extracts from each intra-erythrocyte stage of *P. c. chabaudi* and *P. berghei* with most activity being detected in early trophozoite. Comparative study of the aminopeptidase activity in each extract revealed that the enzymes had similar specificities and kinetics with substrate L-leu-AMC, K_M = 56 and 26 µM, Vmax = 26 and 56 nMol/min/mg protein for *P. berghei* and *P. c. chabaudi*, respectively a near neutral pH optimum of 7.2 and are moderately thermophilic (*P.*

chabaudi 50°C and *P. berghei* 45°C). The molecular weight of the enzyme from *P. c. chabaudi* and *P. berghei* extracts was estimated at 80 ± 10 kDa determined by HPLC. Both aminopeptidases were shown to be metallo-proteases but zinc did not appear to be the metal cofactor as with other aminopeptidases. However Ca^{2+} restored up to 50% of the original activity when added to the apoenzyme at 50 µM. The preferred synthetic substrate for the aminopeptidases was L-leu-AMC and L-ala-AMC. The enzymes were completely inhibited by bestatin but not leupeptin. *O*-phenanthroline was a strong inhibitor while aspartic-cysteine- and serine protease inhibitors had little or no effect, Mn^{2+}, Hg^{2+} and Cd^{2+} were strong inhibitors whereas Zn^{2+} and Mg^{2+} were weak inhibitors (Curley *et al.*, 1994).

Ultrastructural localisation studies in *P. c. chabaudi* by Slomianny *et al.*,(1983) detected aminopeptidase activity first at the level of cytoplasmic ribosomes where the aminopeptidase is synthesised. The activity then migrates and is found associated to the membrane of pinocytotic vesicles as soon as these were formed. The labelling then extends to the vesicle contents where it becomes very prominent. This enzyme activity was detected in the ring stage and early trophozoites In late trophozoites enzymatic activity decreased and was no longer detected (Slomianny *et al.*, 1983).

(b) Aminopeptidases in *P. falciparum*.

Gyang *et al.* (1982) described *P. falciparum* aminopeptidase activity with an apparent molecular mass of 186 kDa by gel filtration, pH optimum of 7.5, pI = 6.05 and apparent K_M = 2.2 mM with L-alanyl-p-nitroanilide. The aminopeptidase was completely inhibited by 1 mM Hg^{2+}, Zn^{2+} and Cd^{2+}. Leupeptine inhibited the aminopeptidase.

Vander jagt and co-workers described aminopeptidase activity from *P. falciparum* chloroquine resistant and sensitive trophozoite extracts. The purified enzyme had a molecular weight of 100 kDa (Vander jagt *et al.*, 1984). However a combination of gel filtration and hydroxylapatite revealed aminopeptidase activity at approximately 63 kDa. (Vander jagt *et al.*, 1987). This enzyme also preferred L-leu- and L-ala- peptide substrates and was inhibited by bestatin and phosphoramidone but not leupeptine. pH-rate studies indicated the presence of a sulfhydryl group on the free enzyme, pKa = 6.6, which must be in the conjugate base form for activity. This essential sulfhydryl group at the active site (Fulton *et al.*, 1956) was rapidly modified by Hg^{2+} and Zn^{2+}, and was slowly modified by p-hydroxymercuribenzoate, but was not accessible to iodoacetamide or N-ethylmaleide. This aminopeptidase activity was inhibited noncompetitively by chloroquine (K_I = 410 and 535 μM) for the resistant and sensitive strains respectively, mefloquine and quinacrine (K_I = 280 and 20 μM, respectively), but was not inhibited by quinine or primaquine.

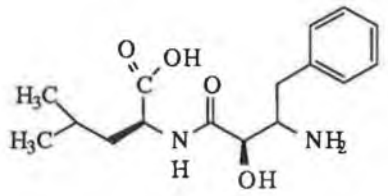
Curley and co-workers (1994) showed that the aminopeptidase from *P. falciparum* extracts had a slightly higher molecular weight than the enzyme from *P. c. chabaudi* and *P. berghei* extracts but presented the same physical properties. The enzyme was not inhibited by Mg^{2+} unlike the rodent aminopeptidases which showed up to 50% inhibition. All the *P. falciparum* aminopeptidase activities described so far show maximum activity at near neutral pH of 7.2 and the optimum temperature for activity is 50-60°C.

Aminopeptidases are increasingly being investigated as therapeutic targets in various diseases, some results of these investigations will be reported in section 1.10.3-1.10.9. The *P. c.*

chabaudi aminopeptidase described by Curley *et al.*, (1994) will be purified and characterised in this thesis. It will be compared to the other aminopeptidases to try and determine whether there is a single or four aminopeptidases. The binding kinetics of the aminopeptidase inhibitor bestatin and nitro-bestatin will be investigated and compared to the binding kinetics of bestatin to malarial aminopeptidases, cloning of this aminopeptidase will also be investigated. The purification and characterisation will enable a better understanding of the mechanism of action of malarial aminopeptidases, allow chemotherapeutic studies directed at this enzyme and vaccine trials using the purified aminopeptidase.

1.10. Bestatin.

Bestatin [(2S,3R)-3-amino-2-hydroxy-4-phenyl-butanoyl]-L-leucine (fig. 5) was discovered by Umezawa *et al.*, in 1976 from culture filtrates of *Sreptomyces olivoreticuli* while in search for low molecular weight inhibitors of aminopeptidase-B on the surface of cells. Bestatin has attracted considerable attention since it's discovery due to it's numerous biological activities, most notably as an immune response modifier (inflammatory response) and a potential analgesic. It is now widely known that bestatin inhibits most known aminopeptidases with variable rates and affinity but not aminopeptidase-A (Nishizawa *et al.*, 1977). This characteristic of bestatin offers new opportunities to identify and characterise the bestatin-binding site which is presumably the active site of susceptible aminopeptidases. There are three reactive functional groups, NH_2, OH, and COOH, in the bestatin molecule.



Bestatin

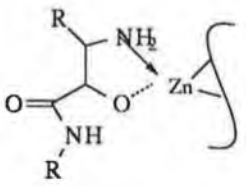
Figure 5. Structure of bestatin (Suda *et al.*, 1976).

1.10.1. Inhibition of aminopeptidases by bestatin and bestatin analogues.

Bovine lens leucine aminopeptidase is the aminopeptidase of which the bestatin binding site has been researched the most. Studies investigating the active site using bestatin show that the bestatin-binding sites of bovine lens leucine aminopeptidase were found within the unique, large tryptic and the small hydroxylamine polypeptide, both of which are located within the carboxylic third of the subunit. The peptide chain length also is an important contributor to both the slow binding properties of bestatin and the related inhibitor amastatin and to the preferential binding of bestatin and amastatin to different aminopeptidases. This was corroborated by NMR and crystallographic data, which indicated that the phenylalanyl side chain of bestatin is bound to a hydrophobic pocket comprised of met-270, Thr-359, Gly-362, Ala-451 and Met-454 and that the leucyl side chain binds to another hydrophobic cleft formed by Asn-330, Ala-333 and Ile-421 (fig. 7, Taylor A, 1993). When bound to the enzyme, the backbone of

bestatin is stabilised by hydrogen bonds involving Lys-262, Asp-273 and Leu-360 (Taylor, 1993). Bestatin is a slow tight-binding inhibitor for most of the aminopeptidases tested to date. The slow binding involves rapid formation of the enzyme-inhibitor (EI) complex slow transformation of the EI to the tighter complex EI*, and even slower deformation of that complex. There is little conformational change resulting from the binding of bestatin to blLAP. Structure modification studies of bestatin have established that the presence of the 2(s)-hydroxyl group is important for tight binding to aminopeptidases (Rich *et al.,* 1984). The structural relationship between bestatin's critical 2(s)-hydroxyl group and a probable tetrahedral intermediate for amide bond hydrolysis led to the idea that bestatin might be a transition state analogue inhibitor of aminopeptidases in which the sp3 geometry and alcohol at the C-2 carbon of the inhibitors mimics the tetrahedral intermediate formed during substrate hydrolysis. Nishizawa *et al.,* (1977) suggested that an essential, active-site zinc ion is chelated by the 2(s)-hydroxyl group and the 3-amino group when bestatin is bound to aminopeptidases (fig. 6 A). An alternative in which the C-2 hydroxyl group and the amide carbonyl bound to the active site zinc was postulated (fig 6. B; Nishino *et al.,* 1979). Both mechanisms suggest an interaction between the 2(s)-hydroxyl group and the zinc ion presumed to be present in the active site of aminopeptidases. Another point that needs clarification is why some substrates, or substrate components stimulate rather than inhibit aminopeptidase activity.

A



B

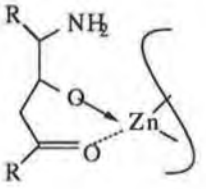


Figure 6. (A) Mechanism of inhibition of aminopeptidase by bestatin as proposed by Nishizawa *et al.*,(1977). (B) Mechanism of inhibition of aminopeptidase by bestatin as proposed by Nishino & Powers, (1979).

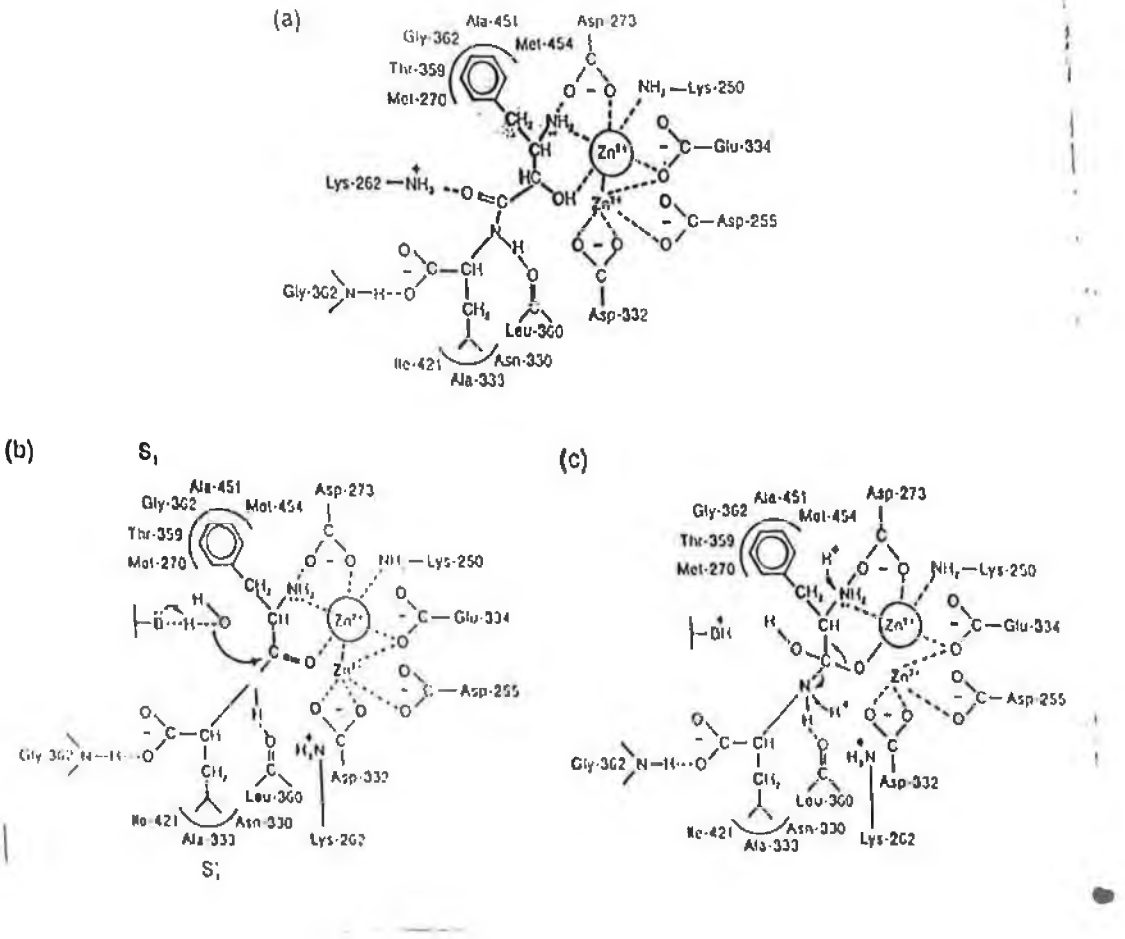


Figure 7 Proposed mode of inhibition of bovine lens leucine aminopeptidase by bestatin. (a). and peptide hydrolysis (b) & (c) as proposed by Burley *et al.*, (1992)

1.10.2. Structure-activity relationship of bestatin and analogous compounds.

Structure activity relationship of bestatin and some related compounds are an important factor in the investigation of the antimalarial activity of bestatin. Bestatin analogues modified on the amino, hydroxy and carboxylic (bestatin amides, N-benzyloxycarbonyls) groups were weak inhibitors of aminopeptidase suggesting that the free amino hydroxy, and carboxylic groups were essential for the inhibiting activity of bestatin. Aminopeptidase activity of the eight stereoisomers of bestatin indicate that the configuration at C-2, but not that of C-3 is the most important factor for the manifestation of activity and the stereochemical requirements for the other asymmetric carbon atoms with regard to activity are not very strict (fig. 8; Nishizawa *et al.*, 1977). It was also shown that the effects of different bestatin analogues varies with different aminopetidases. The activity of the stereoisomers against aminopeptidase-B did not correlate exactly with that against leucine aminopeptidase (Nishizawa *et al* , 1977). Other experiments showed that the S configuration at C-2 is a requirement for enzyme inhibition, hence the hydration of the carbonyl group is stereospecific. When the leucine moiety of bestatin was substituted, the compound in which the amino group was α to the carboxyl group (**2**) showed most activity. This result suggests that the distance between the amino and carboxylic groups is important for enzyme inhibition. The importance of the S configuration of C-2 was confirmed by substituting the benzyl group of bestatin (Nishizawa *et al.*, 1977).

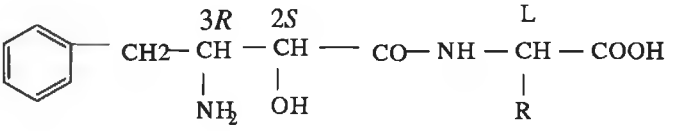
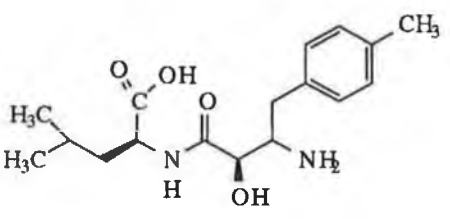
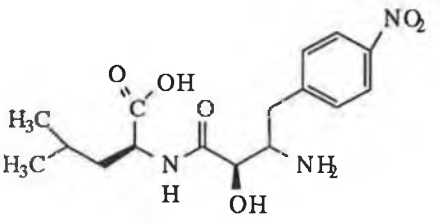


Figure 8 Structure of [(2*S*,3*R*)-3-amino-2-hydroxy-4-phenylbutanoyl]-L-leucine, R= $CH_2CH(CH_3)_2$.

The importance of synthetic analogues of bestatin was demonstrated when the activity of *p*-nitro-bestatin IC_{50} = 0.01 µg/ml (fig. 9) was found to be five times stronger than bestatin IC_{50} = 0.05 µg/ml with (2S,3R)-*p*-methylbestatin IC_{50} = <0.01 µg/ml being the most active of the compounds (Nishizawa *et al.*, 1977). Both these compounds contain a single addition of a nitro and methyl group on the phenyl ring respectively.



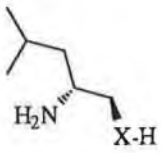
p-nitro-bestatin



p-methyl-bestatin

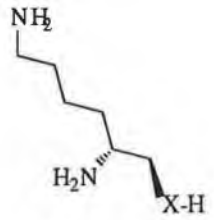
Figure 9. Structure of nitro-bestatin and methyl-bestatin.

Ocain *et al.* (1988) replaced the C-2 hydroxyl group in the 3-amino-2-hydroxy-4-phenyl-butanoic acid (AHPBA) with a thiol group in order to capitalise on the high affinity of sulfhydryl groups for zinc ions (fig. 10). Aminothiols, L-leucine thiol (**2**) and L-lysinethiol (**3**) and the corresponding alcohols (**2a** & **3a**) were synthesised for these studies. Additionally, replacement of the bestatin amide carbonyl with a thiocarbonyl group was investigated for it's affinity for the active-site zinc ion. Thiol bestatin (**4**) and thiolamides (**5**) were synthesised for this study.



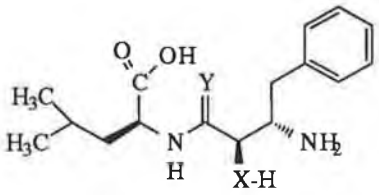
L-leucinethiol 2, X = S

3a, X = O



L-lysinethiol 3, X = S

3a, X = O



C-2 thiolbestatin 4, X= S; Y = O

C-2 thiolamide bestatin 5, X= O; Y = S

Figure 10. Replacement of the C-2 hydroxyl group in AHPBA.

The 2-thiol bestatin (**4**) was only 2-fold more potent than bestatin for the inhibition of aminopeptidase-M (AP-M) and only 13 times more potent than bestatin for inhibition of aminopeptidase-B

(AP-B). Thiol-bestatin (**4**) was significantly weaker as an inhibitor of LAP. It is the aminothiols, leucinethiol (**2**) and lysinethiol (**3**), that show the most pronounced benefit for AP-M and AP-B. The thiol compounds were actually poorer inhibitors of LAP. All thiolamides were significantly poorer inhibitors than the amide counterparts for all three enzymes (Ocain *et al* , 1988)

This thesis will investigate the binding kinetics of bestatin to malarial aminopeptidase extracted from *P. c. chabaudi* early trophozoites and compare the results to the porcine kidney mammalian aminopeptidase. The effects of some bestatin analogues on malarial aminopeptidase activity will also be investigated.

1.10.3. Immunomodulatory effects of bestatin *in vitro*.

Bestatin has been shown to have immunomodulatory effects, presumably through the inhibition of multiple aminopeptidases, including the augmentation of humoral and cell mediated immune responses and the activation of macrophages and natural killer cells. The activation of macrophages was dose dependent *in vivo* , cytotoxicity of macrophages was observed at concentrations of bestatin less than 50 μg/ml with this concentration being the optimum. At concentrations greater than 100μg/ml macrophage, cytotoxicity was inhibited. A dose dependent adjuvant-like activity was also observed (Talmadge *et al.*, 1986). IL-8, a neutrophil chemotactic agent, is known to have an active role in the induction of the inflammatory response in a number of diseases. The IL-8 receptor on the surface of neutrophils are very quickly down regulated, up to 85% of the receptor's activity is lost within 90 min of incubation at 37°C. This down regulation is almost completely prevented by bestatin at 40μM (Manna *et al.*, 1995).

Bestatin at 0.1 μm/ml was found to increase the number of the nucleated mature cells and that of the myeloid progenitors in long term bone marrow cultures (LTBMC) from the non-adherent layer versus controls. The total number of colonies and the number of myeloid progenitors but not that of erythroid progenitors from the adherent layer was also increased following treatment with bestatin at 1.0 μM/ml. This stimulation of the production of mature cells and myeloid progenitors on normal hematopoiesis by bestatin in LTBMC was mediated by an indirect action via IL-6 production (Fujisaki *at al.*, 1995). The mechanisms involved in the anti-tumour effects of bestatin were investigated on breast cancer cells line YMB-S. These cancer cells proliferate in a floating manner without aggregation in normal complete medium. Bestatin induced cell-cell and cell-surface adhesion of the cells accompanied with growth suppression. No increase in E-cadherin localised at cell-cell contact sites was observed between bestatin treated cancer cells and control cancer cells. Indicating that bestatin inhibits the proliferation of YMB-S cells and augments cell-cell adhesion through the induction of E-cadherin-mediated adhesion resulting from the functional activation of pre-expressed but inefficient E-cadherins (Fijioka *et al.*,1995).

1.10.4. Therapeutic effects of bestatin *in vivo* .

Bestatin has been shown to activate peritoneal macrophages *in vivo* . This *in vivo* activation of macrophages was confirmed using tumour cells as targets (Schrlemmer *et al.*,1983). Bestatin has been shown to have some therapeutic effects against muscular dystrophy in mice (Satoyoshi *et al.*,1992) although clinical trials with duchenne muscular dystrophy patients failed to prove these effects (Hirano *et al.*, 1994).

Tsuruo *et al.*, (1981) found bestatin to suppress the gradually-occurring lymph node metastasis of P388 leukaemia in CDF1 mice when administered intra-peritoneally at doses of approximately 30 μg/mouse. Bestatin could not, however, suppress the established large metastasis of P388 leukaemia. Bestatin prolonged survival time in *Candida albicans* infected normal mice in a dose dependent manner. In immunosuppressed mice, bestatin at 5 mg/day significantly increased the number of survivors. These results suggested that bestatin was a potent biological response modifier for prevention against opportunistic infections (Coppi *et al.*, 1994). The combination of IL-8 with bestatin induced cervical ripening in rabbits by providing approximately regular levels of neutrophil numbers, collagenase and elastase activities. It has been suggested that this regulatory mechanism also takes place *in vivo* through the inhibitory effects of bestatin on aminopeptidase N activity (el Maradny *et al.*, 1995).

1.10.5. Clinical trial using bestatin as a therapeutic tool.

Several trials have been carried out using bestatin as a therapeutic tool in various diseases where aminopeptidases were thought to play a critical role. Clinical trials show that in order to obtain a therapeutic effect bestatin must be administered in large doses. This requirement is shared with other polypeptides such as muramyl dipeptide, enkephamids, tuftsin and small proteins such as interleukin 2 and the interferons. Presumably these small proteins and peptides are subject to proteolysis by plasma and tissue proteases. Pilot clinical studies with bestatin orally administered at 150 mg/body twice per day, twice per week, for at least 8 weeks showed that the plasma half life time of bestatin was 2.1 ± 0.7 hours and the total urinary recovery was 71.4%

in 48 hours. Among 8 patients with myelodysplastic clinically studied, one achieved good response and another achieved minor response (Ueda *et al.* 1994). Bestatin was suggested as a new approach to treat chronic myelogeneous leukaemia (CML) in combination with busulfan. Twenty three patients with Phase 1 positive CML, including 20 in the chronic phase and 3 in the accelerated phase, were treated with the combination therapy. Complete hematologic remission was obtained in all patients, complete cytogenetic response was obtained in 6 patients, partial cytogenetic response in 1 patient and minor cytogenetic response in 6 patients. Cytogenetic conversion to a normal diploid state persisted for 6-41 months. Three year survival was 86.6%. Therefore bestatin effectively controls chronic myelogeneous leukaemia (Uzuka *et al.*,1993) and is now being used for treatment of acute non-lympocytic leukaemia (Takeuchi *et al.*, 1995). Bestatin is a potentially useful treatment in patients with rheumatoid arthritis (Aoyagi *et al.*, 1981 & 1990).

This thesis investigates the antimalarial potency of bestatin on *P. c. chabaudi* and on both chloroquine resistant and chloroquine sensitive *P. falciparum* strains in culture. An *in vivo* antimalarial potency of bestatin will also be investigated with *P. c. chabaudi* infected CD1 mice. This thesis also investigates the effects of some bestatin analogues on aminopeptidase activity. Indeed, a non-hydrolysable bestatin analogue could prove a better antimalarial as it would not need to be administered in such large doses and as frequently as bestatin has to be.

1.10.6. Induction of analgesia by bestatin.

Aminopeptidases are thought to play a critical role in the metabolism of endogenous opioid peptides. Inhibition of the proteolysis of opioid peptidases by aminopeptidase inhibitors has been reported to induce analgesia. Aminopeptidase M activity has been reported to be implicated in the proteolysis of endogenous opioid peptides such as Dynorphin A (Muller *et al.,* 1995) and met-enkephalin. A cocktail of aminopeptidase inhibitors including bestatin protected met-enkephalin from degradation in caudate putamen (Konkoy *et al.,* 1995). The peptide drug met-enkephamid is an analog of natural met-enkephalin. When gastrointestinal absorption of this drug was investigated, most of the met-enkephamid administered to the jujunal loop was degraded in the lumen by enzymatic hydrolysis and only 0.3 to 1.2% of the drug was absorbed into the vascular flow. Co-administration of the peptide drug Met-enkephamid with bestatin effectively increased gastrointestinal absorption of this peptide drug by supressing it's degradation (Taki *et al.,* 1995). Counterirritants such as 1-menthol which show a major effect in the early phase of pain response are widely used in the treatment of mild pains and itches by topical application. The induction of this counterirritant was potentiated by bestatin (Tanaguchi *et al.*, 1994).

The aminopeptidase inhibitor bestatin inhibits proteolysis of endogenous and synthetic opioid peptides thus increasing the half-life time of the opioid peptides and hence their absorption in the gastrointestinal tract (Muller *et al.,* 1995; Konkoy *et al.,* 1995). It can be suggested that bestatin induces analgesia by increasing the bioavailabilty of synthetic and endogenous opioid peptides. Bestatin

could also induce analgesia via the modulation of endogenous, exogeneous and synthetic opioid receptors. This induction of antinociceptive stimuli by bestatin could prove beneficial in the treatment of malaria symptoms.

1.11. *Plasmodium chabaudi chabaudi.*

P. c. chabaudi was first described in thicket rats as a rodent malaria species similar to *P. vinckei* (Landau, 1965). Ten years later Carter & Williker (1975) characterised *P. c. chabaudi* parasites as having a synchronous development, with a 24 hours asexual life cycle and schizogony occurring around midnight. This life cycle is biologically linked to light exposure.

Analysis of parasitaemias in infected mice when reticulocytosis was induced by phenylhydrazine treatment showed that the course of *P. c. chabaudi* infection was altered, in that lower parasitaemias were reached in mice treated with phenylhydrazine prior to infection (Ott, 1968). Erythrocyte invasion of the parasites was hence first attributed to a preference of mature erythrocytes as reticulocytes were invaded by *P. c. chabaudi* parasites when the circulating normocytes population had been depleted by rupturing parasites (Carter & Walliker 1975). However Jarra & Brown (1989) again using phenylhydrazine treatment of infected mice, found that the invasive behaviour of the parasites *in vivo* reflected the availability of erythrocytes of different ages, with no preference for either mature erythrocytes or reticulocytes.

Since the first description of *P. c. chabaudi* a number of wild strains have been isolated and new clones have been created. *P. c. chabaudi* AS strain is one of the cloned lines. This strain has a 24 hours

asexual synchronous cycle with schizogony beginning around midnight The asexual cycle will be described in detail in this thesis. The beginning of schizogony can be altered by reversing the night and daylight times to which the infected mice are exposed.

1.11.1. Sequestration of *P. c. chabaudi* parasites.

P.c.chabaudi AS strain shows partial sequestration, the major site of sequestration being the hepatic sinusoid (Cox *et al.*, 1987). This feature is shared by *P. falciparum* the only human malaria parasite to sequester, undergoing deep vascular schizogony. Significant proportion of this sequestration takes place in the post capillary veinular endothelium of the brain, where the parasitised erythrocytes can cause obstruction in the blood capillaries resulting in cerebral malarial (McPhearson *et al.*, 1985)

1.11.2. Recrudescence of *P. c. chabaudi* infections.

Recrudescence is another feature of *P. c. chabaudi*, and has been associated with a decline in the effector arm of the immune response and also with the capability of the parasite to undergo antigenic variation (McLean *et al.*, 1982a & b). This recrudescence arises from erythrocytic parasites which have survived after the host's immune response has cleared the majority of the parasites. Phenotypic variation is a feature shared by all human malarial parasites as a means to avoid the host's immune system, resistance to chemotherapy and vaccination (Cox, 1962; Brown & Brown, 1965; Voller & Rosan, 1969; Hommel *et al.*, 1983).

1.11.3. Protective immunity against *P. c. chabaudi* infection.

It is known that protective immunity is predominantly directed against the blood stages of the malarial parasite (Cohen & Lambert, 1982; Martinez, 1987). The spleen represents one of the major defence organs against blood stages of *P. falciparum* (Wyler, 1983). *P. c. chabaudi* is a convenient model to study protective immune mechanisms directed against blood stages of malaria (Schmitt-Wrede *et al.*, 1991) There is documented evidence that these mechanisms are critically dependent on the sex of mice due to the circulating levels of testosterone (Wunderlich *et al.*, 1988; This thesis).

Testosterone (Te) has been documented to increase susceptibility of vertebrate hosts towards many different protozoan parasites. A crucial role of Te has been documented in the outcome of murine malaria caused by *P. c. chabaudi* (Wunderlich *et al* 1988) and *P. berghei* (Kamis & Ibrahim, 1989). Female mice of the inbred strain C57Bl/10 are capable of self-healing *P. c. chabaudi* infections. However this same infection is lethal to male mice. The capability of self-healing *P. c. chabaudi* infection in male mice becomes evident after castration. On the other hand, *P. c. chabaudi* infection can be rendered lethal to female mice by pre-treatment with testosterone (Wunderlich *et al.*, 1988). Female mice thus exhibit protective immunity against *P. c. chabaudi* infection. Once this protective immunity is established it is no longer responsive to Te-treatment. This Te-unresponsive protective immunity is mediated by spleen cells, presumably non-T-cells involving IgG secreting B cells (Wunderlich *et al.*, 1992).

Host immunity to *P. c. chabaudi* shows sequential appearance of first Th1 type cells early in infection followed by Th2 type cells later

in the infection (Langhorne *et al.*, 1989a&b). It has also been showed that macrophages from *P. c. chabaudi* AS-infected A/J susceptible mice exhibit defects in oxygen metabolism and Ia antigen expression which may contribute to the susceptibility of these hosts to this intra-erythrocytic parasite (Stevenson *et al.*, 1992).

1.11.4. *P. c. chabaudi* as an animal model for human malaria.

P.c.chabaudi is now widely accepted as a model for *P. falciparum* human malaria (Taylor-Robinson, 1995). The synchronous development of *P. chabaudi* enables research to be performed with homogenous parasite populations obtained directly from the mice without recourse to *in vitro* culturing, and parasites can be obtained at a specific time of day to suite the requirements of the researcher. Stage-specific drug targeting studies and comparative studies of lethal and non lethal infections of *P. chabaudi* AS were performed. The species is not contagious to humans hence it is safe to manipulate.

Chapter 2 & 3
Materials and methods.

2.0-Materials.

Aldrich chemical Co.

Triton®X-100.

Becton-Dickinson and Co.

Microlance needles,

Plastipak syringes.

Biological Laboratories

CD1 and Balb/c mice , New Zealand white rabbits.

Chancer Propper

Gold Star microscopic slides.

Dupont

^{3}H-Hypoxanthine, ^{35}S-Methioninine.

Flow Laboratories

Foetal Calf Serum, L-glutamine

Greiner

Sterile disposable plastic 50ml and 10ml tubes.

Hamilton

HPLC syringes.

Labscan

Chloroform, Ethanol, Methanol, Propanol.

Medical Supply Company

Latex exam gloves.

Merck

Acetic acid glacial.

Milipore

Filtering membranes.

Nunc

All tissue culture flasks and plates.

Oxoid

Bacto-tryptone,Bacto-yeast extract, Bacto-agar no.3.

Pharmacia

EAH-Sepharose, QAE sepharose A50.

Phenomenex

HPLC gel filtration column BioSep S3000, 30x0.78., Sample loading loops.

Promega

RiboClone cDNA Synthesis System, RiboClone Linker Ligation System, Sephacryl S-400 spin columns, Packagene λgt11 Packaging system, Taq Polymerase, T4 Ligase and all restriction enzymes with

corresponding 10X reaction buffers, dNTP's, agarose, *E. coli* LE392, *E. coli* Y1090.

Reidel-de-Haen

Ammonium persulphate, Bromophenol blue, DMF, Azur-eosin-methylene blue (Giemsa stain), EDTA, Glycerol, HCl, Magnesium chloride-6-hydrate, KH_2PO_4, K_2HPO_4, NaCl, NaOH, NaH_2PO_4, Na_2HPO_4, TEMED.

Rhyodyne

HPLC sample loops and injectors.

Schleicher and Schull

Nitrocelulose filter disks 0.45mm, nitrocellulose membranes.

Sigma Chemical Co.

Freund's complete and incomplete adjuvants, acrylamide, ammonium sulphate, bis-acrylamide, BCIP, BSA, gentamycin, heparin, hypoxanthine, magnesium sulphate, maltose, NBT, sodium azide, sodium citrate, SDS, SDS-PAGE prestained molecular weight markers, Tween®-20, alkaline ahosphatase conjugate anti-rabbit IgG, bestatin, nitro-bestatin, Leu-AMC, AMC, EDC carbodiimide, X-gal, *p*-nitrophenyl-phosphate tablets.

S*terilin Bibby sterilin*

Sterile disposible plastic pipettes.

Whatman International

CF11 columns, 3 mm blotting paper.

2.1. Buffers and solutions.

2.1.1. Animal work solutions.

Heparin: 1000 units/ml

Qs to desired volume with sterile water

Giemsa stain.

4 mM Na_2HPO_4

2.5 mM NaH_2PO_4

10% (v/v) giemsa azur-eosin methylene-blue

Qs to desired volume with sterile water

Filter with Whatman paper filters.

2.1.2. Culture media.

2 mM L-glutamine

20 mg/l gentamicin

50 mg/l hypoxanthine or 12.5 mg/l if ^{3}H-hypoxanthine was to be used

Qs to desired volume with HI-FCS

2.1.3. ELISA solutions.

Glycine buffer

0.1 M glycine

1 mM $MgCl_2$

1 mM $ZnCl_2$

Qs to desired volume with deionised water

pH was adjusted to 10.4 with 19 N NaOH and the buffer was filter sterilised

2.1.4. PAGE solutions.

Bis -acrylamide (30:0.8) Stock solution

30%(w/v) acrylamide

0.8% (w/v) N,N-methylene bis-acrylamide

Qs to desired volume with deionised water

Filter sterilised and stored a 4°C in a dark bottle.

Non-reducing, non-denaturing sample buffer

0.125 M Tris-HCl pH 6.8

20%(v/v) glycerol

0.005%(w/v) bromophenol blue

Qs to desired volume with deionised water

filter sterilised and stored at room temperature

10% separating polyacrylamide gel

6.6 ml bis-acrylamide stock

5.0 ml 1.5 M Tris-HCl pH 8.8

8.4 ml deionised water

333 µl 10% (w/v) freshly prepared ammonium persulfate (APS)

36.6 µl Temed

Gel sets in 1-3 hours

12% separating polyacrylamide gel

8 ml bis acrylamide

5 ml 1.5 M Tris-HCl pH 8.8

6.9 ml deionised water

333 µl 10% (w/v) freshly prepared ammonium persulfate (APS)

36.6 µl Temed

Gel sets in 1-3 hours.

4% Stacking gel

0.65 ml bis acrylamide stock

1.25 ml 0.5 M Tris-HCl pH 6.8

3.1 ml deionised water

250 µl 10% (w/v) freshly prepared ammonium persulfate (APS)

36.6 µl Temed

Gel sets in 1-3 hours

Running buffer for PAGE.

25 mM Tris

192 mM Glycine

pH 8.3

Qs to desired volume with deionised water, filter sterilise

2.1.4 (a) SDS-PAGE solutions (prepare native gels but run in reducing-denaturing buffersand use denaturing sample buffer)

Reducing-denaturing sample buffer

0.125 M Tris-HCl pH 6.8

0.1%(w/v) SDS

3% (v/v) β-mercaptoethanol

20%(v/v) glycerol

0.005%(w/v) bromophenol blue

Qs to desired volume with deionised water

filter sterilised and stored at room temperature

Running buffer for SDS-PAGE

25 mM Tris

192 mM Glycine

0.1% (w/v) SDS

pH 8.3

Qs to desired volume with deionised water, filter sterilise

2.1.5. Electroblotting solutions.

Running buffer for SDS-PAGE-electroblotting purposes:

25 mM Tris

192 mM Glycine

0.1% (w/v) SDS

10% (v/v) methanol

pH 8.3.

Qs to desired volume with deionised water, filter sterilise.

Running buffer for PAGE-electroblotting purpose

25 mM Tris

192 mM Glycine

10% (v/v) methanol

pH 8.3.

Qs to desired volume with deionised water, filter sterilise.

2.1.6. Staining solutions.

Fixing solution:

10% (v/v) methanol,

10% (v/v) acetic acid

Qs to desired volume with deionised water

Commassie stain:

0.1%(w/v) commassie blue R-250

Qs to desired volume with fixing solution

Destaining solution.

30% (v/v) methanol,

10% (v/v) acetic acid

Qs to desired volume in deionised water.

2.1.7. Western Blotting and immunoscreening solutions.

PBS-tween® 0.5 %:.

Tween 0.5% (v/v)

Qs to desired volume with PBS

Blocking solution .

1% (w/v) BSA

Qs to desired volume with PBS

Alkaline phosphatase (AP) buffer

100 mM Tris-HCl, pH 9.5

100 mM NaCl

5 mM $MgCl_2$

Qs to desired volume with sterile water

Filter sterilised and stored at room temperature

NBT stock .

75 mg/ml Qs to desired volume with 70% DMF

Filter sterilised and stored at 4°C in a dark bottle.

BCIP stock.

50 mg/ml

Qs to desired volume with DMF

Filter sterilised and stored at 4°C in a dark bottle.

Alkaline phosphatase development solution

0.66% NBT stock

0.33% BCIP stock

Qs to desired volume with AP buffer

Solution was protected from light and used immediately

2.1.8. PCR programmes.

Standard programme

stage 1	95°C 5 min
1 cycle	
stage 2	95°C 1 min
	55°C 2 min
	74°C 2 min

40 cycles

stage 3 74°C 4 min

1 cycle

Touch down programme

stage 1 95°C 5 min

1 cycle

stage 2 95°C 1 min

t°C 2 min

74°C 2 min

5 cycles with t = 65, 63, 61, 57, 55, 50, 48, 46, 44, 42, 40, and 38

stage 3 74°C 4 min

1 cycle

Touch up programme

stage 1 95°C 5 min

1 cycle

stage 2 95°C 1 min

t°C 2 min

74°C 2 min

5 cycles with t = 39, 41, 43, 45, 47, 49, 51, 53 , 55 and 57

stage 3 74°C 4 min

1 cycle

3.0. Methods.

3.1. Maintenance of parasites and preparation of cell free extract.

3.1.1. Maintenance of parasites.

P. c. chabaudi AS strain was maintained in CD1 mice as described previously (McNally *et al.*, 1992). *P. c. chabaudi* infected mice were adapted to reversed lighting, the artificial daylight beginning at 12 midnight and ending at 12 noon. The parasitaemia was monitored on Giemsa's stained blood smears (see section 3.1.1a.). Parasitised blood was drawn from anaesthetized infected mice by cardiac puncture into heparinized syringes. Parasites were passaged to non-infected mice or used to prepare cell free extract. The passaging was always done with ring stage parasites which typically appear in the blood stream between 7 pm and 8 pm. Cell-free extract was prepared from late trophozoites which typically appear in the blood stream between 9 am. and 10 am. In collaboration with Angus Bell (Department of Microbiology, Trinity College Dublin) *P. falciparum* parasites were maintained in human erythrocytes as described previously (Bell *et al.* 1993) with the exception that the culture dishes were incubated in candle jars.

(a) Giemsa staining.

Infected mice were bled from the tail and blood smears were prepared on microscopic slides. The smears was fixed by pouring a few drops of methanol on the smears, washed under running sterile water and stained by covering the blood smear with giemsa staining solution for 15 min. The stained slides were washed under running sterile water, air dried and observed under a microscope. Parasitised erythrocytes were counted and parasitaemia was defined as the percentage of parasitised erythrocytes.

3.1.2. Preparation of cell-free extract.

When the *P. c. chabaudi* parasitaemia reached 15-25% mice were anaesthesied, and, blood containing early trophozoites was obtained as described in section 3.1.1. The blood was then chromatographed on a CF11 column to remove white blood cells and platelets which bind to the column. The collected red blood cells were washed in phosphate buffered saline (PBS), pH 7.4, the pellet was aliquoted in 500 µl fractions and lysed with 500 µl of 0.1%(w/v) saponin for 30 sec on ice to obtain free parasites. The free parasites were washed in PBS at 4°C to remove haemoglobin by centrifugation (15000 g for 20 min at 4°C). This centrifugation step was repeated until the extract was free of haemoglobin. Cell-free extracts were then prepared by three cycles of freeze-thaw lysis followed by centrifugation (15000 g for 20 min at 4°C). The cell-free extract containing aminopeptidase activity was stored at 4°C and the pellets stored at -20°C.

3.1.3. Direct fluorogenic substrate analysis in polyacrylamide gels.

P. c. chabaudi aminopeptidase activity was located in polyacrylamide gels using the fluorogenic peptide substrate L-leucine-amino-methyl-coumarin (L-leucine-AMC). Cell-free extracts were prepared as described in section 3.1.2. and separated by electrophoresis in non-denaturing, non-reducing 10% polyacrylamide gels as described previously (Laemmli, 1970). Following electrophoresis, the gels were washed for 3 x 10 min at room temperature in PBS pH 7.4 each time. The gels were then incubated in 50 μM L-leucine-AMC for 30 min at 37°C. Fluorescent bands locating the substrate cleaving enzyme within the gel were visualised by placing the gel on a UVP chromatove model TM-20 UV-Transilluminator and immediately photographed using a Polaroid 667 Kodak film.

3.2. *In vitro* drugs tests.

3.2.1. *In vitro* culturing of *P. c. chabaudi.*

P. c. chabaudi infected mice (~10% parasitaemia) were bled when parasites had developed to the ring stage (typically 8 pm) or to the trophozoite stage (typically 9 am). The cells were washed three times in PBS and subjected to chromatography over a CF11 column. The parasitised cells collected from the column were then washed in heat inactivated foetal calf serum (HI-FCS) which is obtained by placing foetal calf serum (FCS) at 55°C for 30 min. The cells were counted in

a hemocytometer under a microscope and 1 x 10^8 cells were added to 1 ml of HI-FCS culture media (HI-FCS with 2 mM L-glutamine, 20 mg gentamycin/l and 50 mg/l or 12.5 mg hypoxanthine/l) containing either 2.5 μCi/ml ^{35}S-methionine (ring stage parasites) or 1.0 μCi/ml ^{3}H-hypoxanthine (trophozoite stage parasites) in a 24 wells culture plate and maintained in a candle jar at 37°C for 16 and 10 hours respectively. The cells were removed, and transferred to micro-centrifuge tubes, washed three times in PBS, lysed in 1%(v/v) triton®-X 100, homogenised by sonication and protein or DNA-incorporated isotopes determined as described previously (O'Donovan *et al.*, 1993).

3.2.2. *In vitro* culturing of *P. falciparum.*

P. falciparum parasites were cultured in human erythrocytes as described previously (Bell *et al.*, 1993). Cultures of 2% haematocrite and 0.8% initial parasitaemia were incubated with various concentrations of bestatin, nitrobestatin and chloroquine. Parasite growth was monitored by measuring lactate dehydrogenase (LDH) activity according to the method of Malker (Malker *et al.*, 1993).

3.3. *In vivo* drugs tests. .

3.3.1. Chloroquine

P. c. chabaudi malarial parasites were maintained as described in section 3.1.1. CD1 mice were infected with *P. c. chabaudi* ring stage malarial parasites subcutaneously. When the parasitaemia reached 5-

6%, chloroquine was administered subcutaneously (sc) at 5, 2, or 1 mg / injection / mouse every second day for the first week and every day for the following week. Parasitaemia was monitored as described in section 3.1.1a.

3.3.2. Bestatin trial 1.

P. c. chabaudi malarial parasites were maintained as described in section 3.1.1. CD1 mice were infected with *P. c. chabaudi* ring stage malarial parasites subcutaneously. When the parasitaemia reached 5-6%, bestatin was administered subcutaneously (sc) at 5, 2, or 1 mg / injection / mouse every second day for the first week and every day for the following week. Parasitaemia was monitored as described in section 3.1.1a.

3.3.3. Bestatin trial 2.

P. c. chabaudi malarial parasites were maintained as described in section 3.1.1. CD1 mice were infected with *P. c. chabaudi* ring stage malarial parasites subcutaneously. Bestatin was administered subcutaneously (sc) or intravenously (iv) at 2 mg / injection / mouse, at the first sight of parasites (< 0.5 % parasitaemia) every day for the first week and every second day for the following week. Parasitaemia was monitored as described in section 3.1.1a.

3.4.- Effects of novel bestatin analogues on *P. c. chabaudi* aminopeptidase activity.

In collaboration with Dr. Paul Mc Cormack and Dr. Joshua Howarth (School of Chemical Sciences, DCU) bestatin analogues were synthesised using modified procedures of Krepski *et al.*, 1986 and Duboudin, *et al.*,1982.

These bestatin analogues were then assayed for their effects on aminopeptidase activity. Aminopeptidase was partially purified as described in section 3.6.1d. The enzyme assays were carried out by incubating the bestatin analogues at various concentrations with the purified *P. c. chabaudi* aminopeptidase for 45 min at room temperature prior to adding 50 mM of L-leucine-AMC in a final volume of 1 ml di-methyl sulphur oxide (DMSO), pH 7.4. The reactions were then incubated at 37°C for 30 min and stopped by the addition of 200 µl of 10% (v/v) acetic acid. Aminopeptidase activity was measured as described in section 3.5.1. Bestatin was used as a comparative control for aminopeptidase activity inhibition.

3.5. Inhibition kinetics of *P. c. chabaudi* aminopeptidase by bestatin.

3.5.1. Enzymatic fluorogenic assay for aminopeptidase activity.

P. c. chabaudi cell-free extract was assayed for aminopeptidase activity using a standard assay as described previously (Curley *et al.*, 1994). Aminopeptidase activity was determined by measuring the release of amino-methyl-coumarin (AMC) from the synthetic fluorogenic substrate, L-leucine-AMC. Enzyme was added to 50 µM substrate in a final volume of 1 ml PBS and incubated 30 min at 37°C. The reaction was stopped by addition of 200 µl of 10% acetic acid. The amount of AMC released was determined using a Perkin Elmer luminescence spectrometre LS50 fluorimeter (excitation 370 nm, emission 440nm).

3.5.2. Inhibition of *P. c. chabaudi* aminopeptidase activity. by bestatin and nitro-bestatin.

Crude cell-free extracts of *P. c. chabaudi* aminopeptidase and, was incubated with various concentrations of bestatin and nitrobestatin for 45 min at room temperature prior to adding 50 µM L-leucine AMC. Incubation was continued for 30 min at 37°C and aminopeptidase activity was measured as described in section 3.5.1. In collaboration with Dr. P. Curley, inhibition of purified *P. c. chabaudi* aminopeptidase by the same aminopeptidase inhibitors was investigated.

3.5.3. Determination of the inhibitor constants (K_I).

Partially purified or crude cell-free extract of *P. c. chabaudi* aminopeptidase was incubated with a known concentration of bestatin in the presence of known concentrations of L-leucine-AMC for 30 min at 37°C. This assay was repeated with 2 other concentrations of bestatin and aminopeptidase activity was measured as described in section 3.5.1. Line Weaver-Burk plots were prepared, one for each concentration of inhibitor reaction. The inhibitor constants were determined according to the method of Dixon (Dixon *et al.*, 1953).

3.5.4. Time course studies of bestatin inhibition on *P. c. chabaudi* and porcine kidney aminopeptidase activity

Partially purified *P. c. chabaudi* aminopeptidase and mammalian porcine kidney aminopeptidase were pre-incubated with bestatin prior to adding 50 μM L-leucine-AMC. Incubations were stopped at various times by the addition of acetic acid and the aminopeptidase activity was measured as described in section 3.5.1.

3.5.5. Dissociating rates of bestatin from *P. c. chabaudi* and porcine kidney aminopeptidase.

Partially purified *P. c. chabaudi* aminopeptidase and porcine kidney aminopeptidase were incubated with 2 μM bestatin for 1 hour at 37°C. The mixture was diluted 50-fold (0.04 μM bestatin) and incubated for various time periods prior to the addition of L-leucine-AMC.

Aminopeptidase activity was measured as described in section 3.5.1 Partially purified *P. c. chabaudi* aminopeptidase and porcine kidney aminopeptidase were pre-incubated in the same way but without bestatin and diluted into PBS containing 0.04 μM bestatin to serve as controls.

3.6. Purification and characterization of *P. c. chabaudi* aminopeptidase.

3.6.1. Purification of *P. c. chabaudi* aminopeptidase.

(a) Affinity chromatography.

The coupling reaction of bestatin to EAH-sepharose 4B and the preparation of the affinity column was done as described by the manufacturer (Pharmacia affinity chromatography manual). Briefly, the swollen gel provided by the manufacturer was washed with 0.5 M NaCl. Bestatin was dissolved in sterile water and the pH adjusted to 4.5. A slurry was made by mixing 2 volumes of dissolved bestatin with 1 volume of washed gel at 5 mg bestatin / ml of swollen gel.

Carbodiimide was added in powder form to a final concentration of 0.1 M. The suspension was mixed gently and the mixture was rotated end-over-end for 4 hours at room temperature. During the first hour of coupling the pH was adjusted to pH 4.5. The bestatin/EAH-sepharose 4B was washed with a three cycles acid/base wash. Each cycle consisted of 0.1 M acetate/ 0.5 M NaCl, pH 4, followed by 0.1 M

Tris-HCl/ 0.5 M NaCl, pH 8.0. Finally, the coupled slurry was washed in sterile water and then with 0.05 M Tris-HCl, pH 8.0.

An affinity column was prepared by pouring the slurry into a 1 ml syringe carefully avoiding air bubbles. Affinity chromatography was performed at 4°C. The affinity column was packed and equilibrated by running ten volumes of 0.05 M Tris-HCl, pH 8.0, through the column at gravity flow rate or at 0.5 ml / min when a flow rate monitor pump was available. At this moment the UV protein monitor was set at zero (A_{280nm}= 0.000 units of optical density (O.D.)).

Crude *P. c. chabaudi* cell-extracts (approx. 1 mg/ml protein) were prepared as described in section 3.1.2. and dialysed against 0.05 M Tris-HCl, pH 8.0, or 0.5 ml of concentrated partially purified extracts from gel filtration were applied to the affinity column and left to incubate for 45 min or recycled three times over the column . One ml fractions were then collected until A_{280nm} returned to 0.000 O.D. The bound proteins were eluted with a step-wise NaCl gradient (0.1-1 M) in 0.05 M Tris-HCl, pH 8.0. After all the proteins were eluted, (A_{280} returned to 0.000 O.D. with 1 M NaCl), the affinity column was washed with 10 volumes of 0.1 M Tris-HCl, pH 8.0, and then 5 volumes of 0.05% (w/v) sodium azide. The column was stored in 0.05% (w/v) sodium azide at 4°C. The collected fractions were assayed for aminopeptidase activity (section 3.5.1) and concentrated as described in section 3.6.1e

(b) Gel filtration.

Gel filtration was performed by size-exclusion high purification liquid chromatography (HPLC). Care was taken to ensure air bubbles were

not introduced onto the gel filtration column or in the apparatus tubing during manipulations. All the buffers for HPLC were sterile filtered and degassed before use. All protein samples applied to the gel filtration column were sterile filtered.

The HPLC apparatus (Shimadzu LC-9A) was washed in HPLC grade methanol until A_{280} reading (Shimadzu SPD-6AV) was constant (stable baseline on the screen (Axxion chromatography)). The buffer was changed to 0.1 M KH_2PO_4, pH 6.8, until A_{280} was stable again. The gel filtration column (Biosep S3000, 30 x 0.78 cm) was then connected to the apparatus and, in order to ensure that the column was free of any protein, the column was equilibrated with 0.1 M KH_2PO_4, pH 6.8, overnight at a flow rate of 0.1 ml/min. The following morning the flow rate was set at 0.5 ml/min and when the A_{280} was stable, the UV protein monitor was set at zero (A_{280} =0.000 O.D.).

A calibration curve of the gel filtration column was obtained using proteins of known molecular weight (IgG, 150,000 BSA, 66,000 ovalbumin, 45,000 tubulin and β-lactoglobulin, 18,400). Crude *P. c. chabaudi* cell-extracts (approx. 1 mg protein) prepared as described in section 3.1.2. and dialysed against 0.1 M KH_2PO_4, pH 6.8 or 0.5 ml of concentrated partially purified extracts from ion-exchange or affinity chromatography, were applied to the gel filtration column. 250 µl fractions were collected until all the proteins were eluted off the column i.e. A_{280} returned to 0.000 O.D. The column was left to run for a further 40 min in 0.1 M KH_2PO_4, pH 6.8, and during this 40 min the A_{280} reading remained at 0.000 O.D. The column was washed in 0.05% (w/v) sodium azide, disconnected from the apparatus and stored in 0.05% (w/v) sodium azide. After disconnecting the gel filtration column from the HPLC machine the apparatus tubing was washed with HPLC grade methanol and stored in methanol. The collected fractions

were assayed for aminopeptidase activity and concentrated as described in sections 3.5.1. & 3.6.1e.

(c) Ion-exchange chromatography .

QAE-Sepharose which is an anion exchanger was swollen overnight in 0.1 M Tris-HCl, pH 8.0, at 4°C. The ion-exchange column was prepared by pouring 4 ml of the slurry into a 5 ml syringe carefully avoiding air bubbles. The ion-exchange column was packed and equilibrated by running ten volumes of 0.1 M Tris-HCl, pH 8.0, through the column at gravity flow rate or 0.5 ml/min when a flow rate monitor pump was available. The UV protein monitor was then set at zero (A_{280nm} = 0.00 O.D.)

Crude *P. c. chabaudi* cell-extracts (approx. 1 mg/ml protein), prepared as described in section 3.1.2., were dialysed against 0.1 M Tris-HCl (pH 8.0) or 0.5 ml of concentrated partially purified extracts from gel filtration were applied and incubated on the ion-exchange column for 45 min. One ml fractions were collected until A_{280nm} returned to 0.00 O.D. The bound proteins were eluted with a step-wise NaCl gradient (0.1-1 M) in 0.1 M Tris-HCl, pH 8.0. During the elution process the column shrinks as the concentration of salt increases hence the gravity flow rate was maintained constant by moving the buffer container upwards away from the column to increase the gravity flow rate or downwards closer to the column to decrease the gravity flow-rate. (unless a flow rate monitor pump was available). After all the proteins were eluted (A_{280} returned to 0.00 O.D. with 1 M NaCl) the ion-exchange column was regenerated and washed with 10 volumes of 0.1 M Tris-HCl, pH 8.0, and then 2 volumes of 0.05% (w/v) sodium

azide. The column was stored in 0.05% (w/v) sodium azide at 4°C. The collected fractions were assayed for aminopeptidase activity and concentrated as described in sections 3.5.1 & 3.6.1e.

(d) Preparation of partially purified cell free extract of P. c. chabaudi aminopeptidase.

P. c. chabaudi cell-free extracts were prepared as described in section 3.1.2 and assayed for aminopeptidase activity as described in section 3.5.1. The cell-free extract was partially purified to a high degree of purification using a combination of size exclusion-HPLC and bestatin affinity chromatography as described in sections 3.6.1a & 3.6.1b..

(e) Concentration of collected fractions.

The collected fractions were assayed for aminopeptidase activity as described in section 3.5.1. Fractions containing aminopeptidase activity were pooled and concentrated with an Amicon 8400 concentrator, fitted with an Amicon YM3 membrane (3000 MW cut off), down to 2.0 ml. The concentrator was refilled with either: 0.1 M Tris-HCl, pH 8.0, (when the next purification step was ion-exchange chromatography); 0.05 M Tris-HCl, pH 8.0; (when the next purification step was affinity chromatography); or 0.1 M KH_2PO_4, (when the next purification step was gel filtration). Concentration was continued down to 2.0 ml. This latter process was repeated three times to make sure the sample was free of salt and fully equilibrated in Tris or Phosphate buffer depending on the following purification step. Further concentration (to 500-200 µl) was performed in a Centricon

and the sample was stored at 4°C. Protein concentration of the samples from each purification step was analysed as described in section 3.6.1f.

(f) Determination of protein concentration.

Protein concentration was measured in a microtitre plate using the BCA standard kit according to the method described by (Redinbaugh, *et al.*, 1986). The standard curve was obtained using bovine serum albumin (BSA) at 1:4 increasing dilutions, ranging from 2mg/ml down to 0.00075 mg/ml. Ten µl of BSA standards and 10-40 µl of the test samples whose concentration was sought, were mixed with the BCA dye solution provided with the kit. The microtitre plate was incubated at 37°C for 1 hour or left at room temperature for 1-3 hours. The protein concentration was determined by spectrophotometry at 600 nm. A plot of absorbance versus the concentration of BSA standards provides a protein concentration standard curve from which the concentration of the test samples can be extrapolated.

3.6.2. Characterisation of *P. c. chabaudi* Aminnopetidase.

(a) Silver nitrate staining.

Gloves were worn at all times especially when handling gels for silver staining. Silver nitrate staining was done as described (Sambrook *et al.* 1992). The proteins from each purification step were separated by electrophoresis on 12% reducing SDS-polyacrylamide gels (Laemli, 1970). The proteins were fixed by incubating the gel overnight at room temperature with gentle shaking in five volumes of fixing solution. The fixing solution was discarded and replaced with five volumes of 30%

methanol and the incubation was continued for 30 min at room temperature with gentle shaking. This step was repeated once. The gel was washed 3 times in 10 volumes of deionised water for 10 min at room temperature with gentle shaking each time. Five volumes of a freshly diluted 0.1% silver nitrate ($AgNO_3$) solution was added, and the gel was incubated for 30 min at room temperature with gentle shaking. The gel was washed for 20 sec on each side under a stream of running deionised water. Five volumes of a freshly made aqueous solution of 2.5% (w/v) sodium carbonate in 0.02% (v/v) formaldehyde was added. The gel was incubated at room temperature with gentle shaking until stained bands of protein, with an adequate contrast, were obtained. The reaction was quenched by washing the gel in 1% (v/v) acetic acid. The gel was stored at room temperature and photographed using an image analyser (UVP white/UV Transilluminator and Image store 7500).

(b) Production and analysis of polyclonal antisera.

1. Production of polyclonal antisera

P. c. chabaudi aminopeptidase was partially purified by using Ion-exchange chromatography and analysed by the direct fluorogenic substrate assay as described in section 3.6.1.c and 3.1.3. The fluorescent band was cut out of the gels and thinly mashed up. This preparation containing approximately 150 µg of total protein together with sonicated Freund's complete adjuvant (FCA) were used to immunise rats for the first immunisation. The same preparation together with sonicated Freund's incomplete adjuvant (FIA) were used for the following 4 immunisations, four weeks apart. Blood was taken

from anaesthetised rats by cardiac puncture into non heparinized syringes. The blood was left at room temperature to allow it to separate into serum and plasma. The serum was collected after centrifugation at 2000g for 10 min at room temperature, aliquoted and stored at -20°C.

Another immunisation was carried out using partially purified *P. c. chabaudi* aminopeptidase (approx. 150 µg of total protein) prepared as described in section 3.6.1d. In this immunisation rabbits were used and unlike the gel slicing method the partially purified sample was directly mixed with FCA (v/v) and sonicated, for the first immunisation and with FIA for the following four immunisations, four weeks apart. Blood was taken from the rabbits (extravenously from the vein behind the ear lobe) into non heparinized syringes. Rabbit serum was collected and stored as described above.

2. Analysis of polyclonal antisera.

***a.** Quantitative analysis of serum antibodies.*

Quantitative analysis of polyclonal antisera obtained from rabbits and rats as described in section 3.6.2b. was performed after every immunisation, using the standard enzyme linked immuno-sorbent assay (ELISA). The wells of microtitre plates were coated overnight at 37°C with 6 µg of *P. c. chabaudi* aminopeptidase extract from different purification steps. The excess binding sites in the wells were blocked with 1% (w/v) BSA in 0.5% (v/v) Tween®-PBS (TPBS), washed in TPBS, then incubated with various dilutions of polyclonal antisera for 1 hour at 37°C. The wells were then washed as above and alkaline-phosphatase conjugated anti-rabbit or anti-rat IgG added at various

dilutions for 30 min at room temperature. The plates were washed as above and then incubated with a colorigenic substrate *p*-nitrophenyl-phosphate (pNPP). The quantity of bound serum antibodies present was determined by spectrophotometry at 405 nm using a spectrophotometer fitted with an ELISA plate reader.

b. *Qualitative analysis of polyclonal antisera*

Qualitative analysis of serum antibodies obtained from rabbits and mice as described in section 3.6.2b. was performed using the standard method of Western blotting and Dot blotting. For Western Blotting, *P.c.chabaudi* protein extracts from different purification steps were separated electrophoretically in 12% reducing SDS-gel or 10% non reducing gels as described (Laemli, 1970). The proteins were transferred to a nitrocellulose membrane using a semi-dry electroblotter and transfer buffer (see section 2.5) containing methanol. The excess sites on the nitrocellulose were blocked with 1%(w/v) BSA in TPBS. The nitrocellulose was then incubated with various dilutions of rabbit serum for 45 min with gentle shaking. After removing the primary antibodies, the nitrocellulose membrane was washed 3 x 10 min in TPBS, then incubated with alkaline phosphatase conjugated anti-rabbit or anti-rat IgG for 45 min with gentle shaking. The nitrocellulose membranes were washed as before and the bound serum antibodies were revealed by incubating the membrane with a colorigenic substrate solution 5'-bromo-4-chloro-3-indoyl phosphate (BCIP). The colour reaction was stopped when the desired background was obtained by rinsing the membrane in deionised water several times.

For Dot blotting analysis an aliquot of the sample was deposited on a nitrocellulose membrane and left to dry. The membrane was then probed as for Western Blotting. The Dot blotting method was ideal for analysing denatured and non-denatured samples rapidly at various dilutions of serum antibodies.

3.7. Construction of cDNA expression library.

In collaboration with Dr. Paul Curley, a *P. c. chabaudi* c DNA expression library was constructed in the laboratory as described (McNally, PhD. thesis, 1994). In brief, the library was constructed as described in section 3.7.1-3

3.7.1. Isolation of total RNA and mRNA .

P. c. chabaudi early trophozoites were cultured until parasites developed to mature trophozoites (6-8 hours) and harvested as described previously (O'Donovan *et al.* 1993). The pellet was resuspended in DEPC-PBS and total RNA isolated using RNAzol according to manufacturer's instructions. From total RNA, mRNA was isolated using the BRL mRNA isolation system according to manufacturer's instructions.

3.7.2 Generation of double stranded cDNA.

The RiboClone® cDNA synthesis kit was used to generate double stranded cDNA according to the manufacturers instructions. *EcoR*1

linkers were then added to the cDNA in order to ligate into the unique *EcoR*1 site of λgt11 arms, this was done using the RiboClone® Linker Ligation Kit and Sephacryl® S-400 spin columns. After adding the *EcoR*1 linkers the cDNA was ligated and packaged using the ProtoClone® and Packagene® systems. A control ligation and packaging without a cDNA insert was done in parallel to allow determination of the background level religated λgt11 arms. This control also provided a supply of wild type λgt11. The packaged phage was then amplified by transfection into LE392 bacteria.

3.7.3. Amplification of phage stock.

The entire packaged phage stock in 100 µl aliquots was used to transfect LE392 competent cells. After incubation at 37°C the plates were overlaid with 5 ml SM buffer and left at room temperature for 2 hours with gentle shaking. The SM buffer was then transferred to a centrifuge tube and chloroform was added to 5%(v/v) mixed gently, and centrifuged at 3000 g for 10 min at 4°C. The supernatant containing the amplified phage was removed and chloroform added to 0.3%(v/v) and stored at 4°C until use. The pellet containing cell debris and chloroform was discarded.

3.8. Preparation of *P. c. chabaudi* cDNA expression library and rabbit antisera for immunoscreening.

3.8.1. Preparation of *P. c. chabaudi* cDNA expression library for immunoscreening

(a) Phage competent bacterial cells.

E. coli LE392 bacterial strain was used to amplify and titrate the cDNA expression library. This strain was maintained on Luria Bertani (LB) plates. *E. coli* Y1090 bacterial strain was used to screen the cDNA expression library with polyclonal serum antibodies, it was maintained on LB plates supplemented with 100 µg/ml ampicillin and 15 µg/ml tetracycline.

(b) Cloning and preparation of phage competent bacterial cells.

All the work involving culture media and cells was carried out in a sterile environment, created by working beside a Beck Bunsen burner or in a laminar flow. All glassware and pipettes were autoclaved for 20 min at 105°C and the reagents were either filter sterilised or autoclaved for 20 min at 105°C

E. coli LE392 bacterial strain was streaked on LB plates, pH 7.5. and incubated at 37°C overnight. One colony from these plates was then used to innoculate 5 ml of LB media supplemented with

0.2%(w/v) maltose, 10 mM $MgSO_4$ and cultured overnight at 37°C on an orbital shaker. Fifty µl of the freshly grown culture was used to innoculate 5 ml of supplemented LB media as above and the culture was grown at 37°C on an orbital shaker until the $A_{600\ nm}$ reached between 0.6 and 0.9 O.D.

E. coli Y1090 bacterial strain was streaked out on LB plates pH 7.5, supplemented with 100 µg/ml ampicillin and 15 µg/ml tetracycline and incubated at 37°C overnight. A single colony was then used to inoculate 5 ml of LB media and grown overnight at 37°C in an orbital shaker.

(c) Transfection of phage competent cells.

For each plate to be poured 100 µl of the required dilution of phage was added to 100 µl of fresh phage competent cells and incubated at 37°C for 10 min. This incubation can also be done at room temperature for 20 min. During this 10-20 min incubation period 3 ml molten top agar supplemented with 10 mM $MgSO_4$ was placed at 48°C and LB plates prewarmed at 37°C. The transfected cells were then added to the molten top agar, gently vortexed and poured onto the prewarmed LB plates. The plates were left for 5-8 min to harden. They were then covered and incubated upside down, overnight at 37°C.

(d)Titration of phage stock.

One hundred µl of tenfold increasing dilutions of phage stock were used to transfect LE392 competent cells as described. The efficiency of packaging for both wild type λgt11 and λgt11 containing cDNA insert

(rλgt11) was determined as the number of plaque forming units (pfu) per mg DNA packaged.

3.8.2. Preparation of rabbit antisera for immunoscreening.

(a) Absorption of antisera with E. coli extract and λgt11 wild type.

In order to reduce interference caused by cross reacting IgG components and to improve signal-to-noise ratios, the antisera was treated with *E. coli* infected with λgt11 wild type. *E. coli* Y1090 was cultured overnight in 50 ml supplemented LB media as described, section 3.7.1b. The culture was centrifuged for 5 min at 2000 g and the pellet containing the cells was resuspended in 5 ml PBS. The resuspended cells were then sonicated (3 x 20 sec bursts, duty cycle 0.7 sec). Nitrocellulose filter disks were then saturated with *E. coli* extract by incubating the nitrocellulose filters for 16 hours at 4°C with the sonicated solution. The filters were then air dried. The excess binding sites on the nitrocellulose filters were blocked by incubating the filters for 1 hour at room temperature with 1% (w/v) BSA in TPBS. These nitrocellulose filters were then washed 3 x 5 min in TPBS and stored at 4°C or room temperature until used to preabsorb antisera.

The polyclonal rabbit antisera obtained from rabbits, as described in section 3.6.2b, was diluted to 10X the concentration at which it would be used for immuno-screening. The diluted antisera was then incubated with nitrocellulose filters treated with *E. coli* extract and λgt11 wild type at 4°C overnight. The incubation was then transferred to room temperature. The filters were removed, replaced

with new treated filters and the incubation was continued for onother 6 hours. This process was repeated twice.

3.8.3. Immunoscreening of *P. c. chabaudi* cDNA expression library.

Immunoscreening of the cDNA expression library was performed according to the Promega Protocols and applications guide with some modifications. Phage competent *E. coli* Y1090 was prepared and transfected with rλgt11 *P. c. chabaudi* cDNA expression library and λgt11 wild type as described earlier in section 3.7.1c. In order to ensure that the full cDNA expression library was plated out, 20 tubes containing 100 µl of phage competent *E.coli* Y1090 were each transfected with 100 µl of λ diluent containing 2.6 x 10^6 pfu cDNA library. One extra tube was transfected with λgt11 wild type to serve as a negative control. The plates were incubated at 42°C for 5 hours and then transferred to a 37°C incubator. A nitrocellulose filter disk pre-saturated in 10 mM IPTG was gently overlaid on each plate avoiding air bubbles. The plates were then incubated at 37°C for 3.5 hours. The plates were moved to room temperature and the position of each filter on the plate was carefully marked with a needle and labelled before it was removed. A second filter pre-saturated in 10 mM IPTG was then overlaid on each plate after the first one was removed and incubated overnight at room temperature.

After the filters were removed from the plates they were rinsed in TPBS briefly to remove any residual agar and then incubated overnight in 1%(w/v)BSA in PBS to saturate nonspecific protein binding sites. The filters were then washed 3 x 5 min in TPBS and incubated for 45 min with 10 ml primary antibodies (preabsorbed with

E.coli extract transfected with rλgt11) at various dilution. The primary antibodies were diluted in TPBS. The filters were washed 3 X10 min in TPBS and then incubated for 30 min with 10 ml of a 1:7000 dilution of alkaline phosphatase conjugated anti-rabbit IgG. The filters were washed in TPBS as described above and blot dried on Whatmann Blotting paper, transferred to freshly prepared colorigenic substrate solution (BCIP). Clones expressing *P. c. chabaudi* protein reacting to primary antibodies were identified as purple dots. Only the clones which appeared purple during the first 3 hours of incubation with developing substrate were picked from the corresponding plate. These clones were re-screened at 600, 300 and 100 pfu until all the colonies from a single clone, tested positive.

(a) Picking positive clones.

The tip of a sterile pipette tip was cut with a sterile scalpel and the cut pipette tip was attached to a rubber bulb. This assembly was then used to take agar plugs containing the phage colonies of interest. The agar plug was incubated in 1 ml of lamda diluent with 0.3% (v/v) chloroform and stored this way at 4°C until used for re-screening or amplification.

3.8.4. Isolation of rλgt11 DNA.

One hundred µl of a 1:10 dilution of lamda diluent containing phage particles was boiled for 10 min to release phage DNA from phage capsids. This method yielded enough DNA for PCR analysis using 1-5 µl of the boiled sample (Sambrook *et al.*, 1989). When more than 500

ng/ml of DNA was required rλgt11 DNA was isolated as described in Promega's protocols and applications guide.

3.8.5. Screening of *P. c. chabaudi* cDNA expression library by PCR.

λgt11 recombinant (rλgt11) DNA was isolated from λ phage as described earlier. The DNA was then analysed by PCR using universal primers LF (5'-GGTGGCGACGACTCCTGGAGCCCG-3') and LR(5'-TTGACACCAGACCAACTGGTAATG-3'). The standard thermocycling programme was used for amplification with the universal primers. An internal primer BB10 (5'-GGWACWTTRACWGGTGCT-3') was designed by Dr. J.P. Dalton as a consensus sequence of the bestatin binding site of *E. coli* and Bovine lens leucine aminopeptidases. Because this internal primer contains wobble sequences, the PCR was performed using various concentrations of $MgCl_2$ (0.3-4 mM) and DNA (up to 7 µl). The Touch-up, Touch-down and standard thermocycling programmes were used to amplify the DNA preparations.

PCR reaction mix with universal primers LF and LR.

sterile H_2O	qs to 50 µl.
PCR reaction buffer 10X	5 µl
$MgCl_2$ (50 mM)	1.5 µl or 3 µl
dNTPs (1 mM each)	5 µl
LF (200 ng/ml)	1 µl
LR (200 ng/ml)	1 µl
rλgt11 DNA(10-500 ng/ml)	1-5 µl

Taq polymerase	0.25 µl

PCR reaction mix with primers LF and BB10

sterile H_2O	qs to 50 µl.
PCR reaction buffer 10X	5 µl
$MgCl_2$ (50 mM)	0.3-4 µM
dNTPs (1 mM each)	5 µl
BB10 (200 ng/ml)	1 µl
LF (200 ng/ml)	1 µl
rλgt11 DNA(10-500 ng/ml)	1-5 µl
Taq polymarase	0.25 µl

PCR reaction mix with primers LR and BB10

sterile H_2O	qs to 50 µl.
PCR reaction buffer 10X	5 µl
$MgCl_2$ (50 mM)	0.3-4 µM
dNTPs (1 mM each)	5 µl
BB10 (200 ng/ml)	1 µl
LR (200 ng/ml)	1 µl
rλgt11 DNA(10-500 ng/ml)	1-5 µl
Taq polymerase	0.25 µl

The PCR reaction mix was vortexed gently and topped with 50 ml mineral oil to avoid evaporation during thermocycling and placed in a Hybaid Omnigene thermocycler preheated at 95°C. The Standard, Touch-up or Touch-down amplification programme were executed. (see appendix).

3.8.6. Sequencing of amplified PCR fragments.

The PCR reactions products were phenol chloroform extracted. One volume of phenol chloroform pH 8.0, was added to 1 volume of PCR reaction products, vortexed and centifuged 10 min at room temperature. The phenol chloroform phase was removed and the same procedure was repeated once. The nucleic acid fractions were concentrated by precipitation in 3 volumes 100% ethanol and 1/10 volumes 3 M NaAc overnight at -20°C followed by two washes in 70% ethanol. The pellet was vaccum dried and resuspended in 30 µl sterile TE buffer or water. The fragments were separated electrophoreticaly in a 2% TAE agarose gel before being sliced from the gels using a separate sterile scalpel for each fragment. The individual DNA fragments were eluted from the gel slices by spinning the sliced gels through glass beads and subcloned into the PROMEGA pGEM-T® vector according to manufacture's instructions. Sunbcloned fragments were revealed by digestion of the vector with restriction enzymes Apa1 and Sac1. The vectors containing ligated PCR fragments were sent for sequencing to Trinity College Dublin using M13 universal primers and an ABI automatic sequencer.

Alternatively the phenol chloroform extracted fragments were Gene clean purified (Sambrook *et al.*, 1992) and sent for direct sequencing without subcloning, using λF and BB10 primers.

Chapter 4
Results

4.0. *P. c. chabaudi* AS strain infection.

4.1. *P. c. chabaudi* AS strain infection in CD1 mice.

P. c. chabaudi AS strain was maintained in male or female CD1 mice. This strain undergoes a 24 hour synchronous, intra-erythrocytic asexual life cycle, which is biologically adapted to light exposure. In order to obtain trophozoites in the morning the mice were maintained in reverse light with light exposure beginning at midnight to 12.00 noon.

4.1.1. Intra-erythrocytic cycle of *P. c. chabaudi* AS strain.

Early trophozoites can be observed in Giemsa's stained blood smears at approximately 7.30 am these develop into mature trophozoites by 10.30 am and are recognisable by their large vacuoles. Early trophozoites can be distinguished from late trophozoites as the former do not have large vacuoles and their cytoplasm is not well developed. The mature trophozoites begin to devide by schizogony and segmentors or schizonts can be seen in Giemsa's stained blood smears by 12.00 noon and are recognisable by the presence of several nucleii in a single parasite. These schizonts are also recognisable by the crystalline deposits of hemozoin which appear as small dark granules in the parasite vacuoles. By 1.00 pm merozoites formed by schizogony are visible in Giemsa's stained blood smears. These merozoites rupture the red blood cells causing anaemia and sequester to the endothelial cells. During this sequestration period (approx. 3.30-6.30 pm) no parasites

are visible in Giemsa's stained blood smears. The merozoites re-invade the red blood cells to begin a new schizogony cycle, as small rings and are visible on Giemsa's stained blood smears at approx. 6.00 pm, by 8.00 pm mature large rings are visible and they continue developing into trophozoites through the night.

4.1.2. Symptoms of *P. c. chabaudi* infection in mice.

P. c. chabaudi AS strain was lethal to male CD1 mice in practically 100% of the mice infected. Parasitaemia increased in male mice to over 60%, at this stage several lysed erythrocytes were visible in blood smears and the overall number of erythrocytes decreases, free parasites were visible as well. The mice trembled with fever, they showed pilo erection, depression, closed eye lids, lack of spontaneous movement and were very pale and anaemic (blood in urine). These paroxysms were regular in *P. c. chabaudi* infected mice due to the synchronous life cycle of the parasite. The fevers and chills are the consequence of disrupted erythrocytes and other blood corpuscles due to the liberation of successive broods of merozoites. These paroxysms are very irregular in *P. falciparum* infections due to the asynchronous development of *P. falciparum* infections. Death occurred in the next 12 to 24 hours. This diagnostic and symptomatic study was only done once in triplicate, usually the mice were bled before they reached this stage.

Female CD1 mice infected with the same strain of rodent malaria in the same conditions survived the *P. c. chabaudi* infection. Parasitaemia visibly increased at the same rate as in male mice except that when parasitemia exceeded 50-60% the number of parasitised erythrocytes begun to decrease and continued to decrease (fig. 11).

Within the following week all parasitised erythrocytes were cleared from the blood stream and the self healed female mice remained very healthy for a further 6 months until euthenased.

In over 400 male mice only two male mice survived *P. c. chabaudi* infection. Both these mice had been castrated during a fight with other mice.

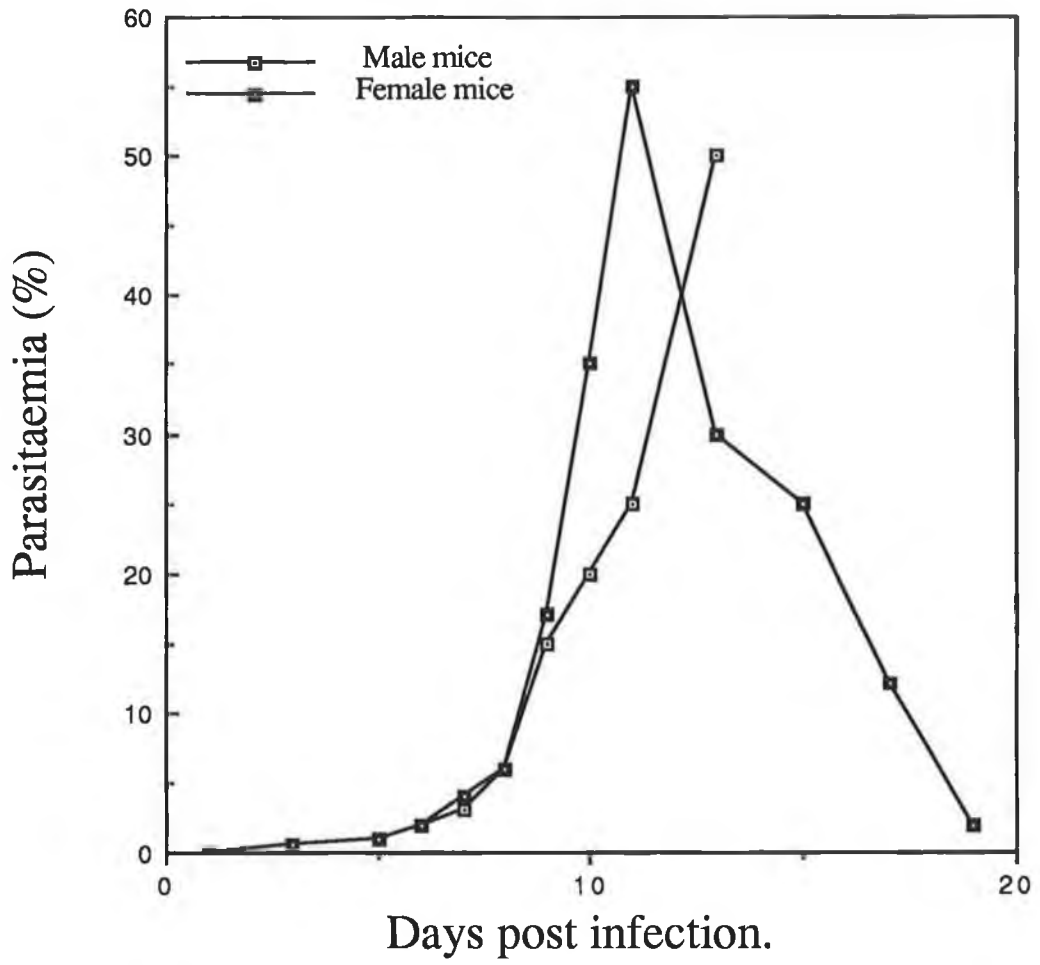


Figure 11. Development of *P. c. chabaudi* infection in CD1 mice. Mice were infected with ring-stage *P. c. chabaudi* parasites and parasitaemia was monitored daily on Giemsa's stained blood smears.

4.2. Observations on the immunological response to *P. c. chabaudi* infection.

A few days after infection circulating lymphocytes were visible on Giemsa's stained blood smears although no parasites were visible yet indicating an immunological response to an infection. Most of the leukocytes observed at this stage were mononuclear phagocytes (monocytes or macrophages) and polymorphonuclear granulocytes (neutrophils eosinophils and basophils). On Giemsa's stained blood smears all these lymphocytes stained blue with darker shades of blue for the nucleus. Monocytes were recognised as having a horseshoe shaped nucleus; neutrophils as having multilobed nucleii and neutrophilic granules in the cytoplasm. Following this appearance of immune cells, parasites became visible in the blood stream 3-4 days later. The number of circulating lymphocytes then decreased during infection. When the number of parasitised erythrocytes reached 50-60% another brusque appearance of circulating lymphocytes became visible only in female mice. This second wave of circulating immune cells was followed by a decrease in parasitised erythrocytes and the immune cells remained visible in great numbers while the number of non-parasitised erythrocytes increased. In approximately a week following this large increase in circulating lymphocytes no more parasitised erythrocytes were visible.

When infected blood from female mice was passaged to male mice, for two generations of passages, the treated male mice survived the infection in nine out of ten cases (n = 10). When infected blood from the same male mice at the third generation was passaged to both

male and female mice, only the female mice survived the infection. The same infected blood was lethal to male mice.

These observations appeared to suggest that survival/death after *P. c. chabaudi* infection was under hormonal control. The female mice appear to be able to develop protective immune mechanisms. This self-healing behaviour was transferable from female to male mice via infected blood from females. Similar observations were made with BalbC mice, therefore these results do not appear to be restricted to CD1 mice.

4.3. Inhibition of *P. c. chabaudi* aminopeptidase activity by bestatin and nitro-bestatin.

P. c. chabaudi crude cell-free extract aminopeptidase was incubated with increasing 1:2 dilution of bestatin and nitro-bestatin ranging from 1.25-0.0097 μM for 30 min prior to addition of 20 μM L-leucine-AMC and assayed for aminopeptidase activity. Aminopeptidase activity decreased towards an asymptote with increasing concentrations of bestatin and nitro-bestatin suggesting that the aminopeptidase was inhibited in a Michaelis-Menten manner by bestatin and nitro bestatin. Nitro-bestatin was approximately six times more potent an inhibitor of *P. c. chabaudi* aminopeptidase activity than bestatin (fig. 12). Inhibitor constants for bestatin and nitro-bestatin (K_I= 0.03 μM and K_I = 0.0018 μM) respectively were determined by the Dixon mathod (figs. 12 B & C).

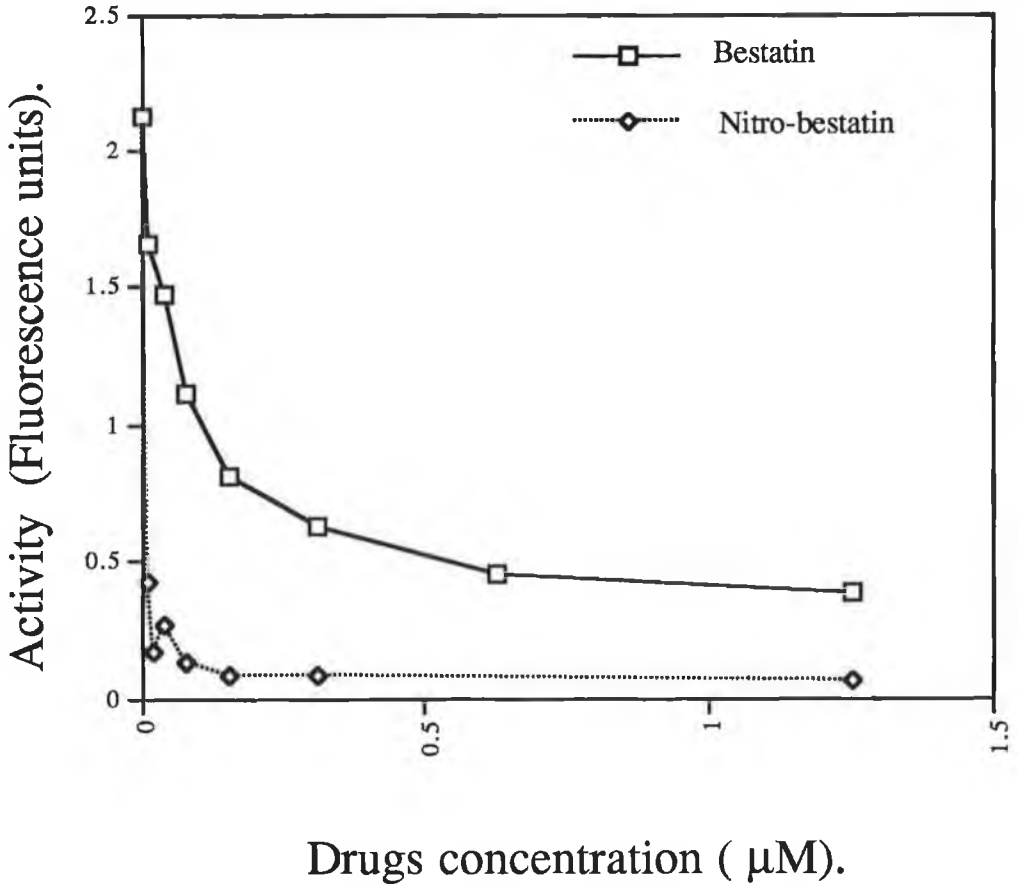


Figure 12A. Inhibition of *P.c.chabaudi* crude cell-extract aminopeptidase activity by aminopeptidase inhibitors bestatin and nitro-bestatin. Crude enzyme extract was incubated with various concentrations of aminopeptidase inhibitors bestatin or nitro-bestatin for 30 min prior to addition of L-leucine-AMC.

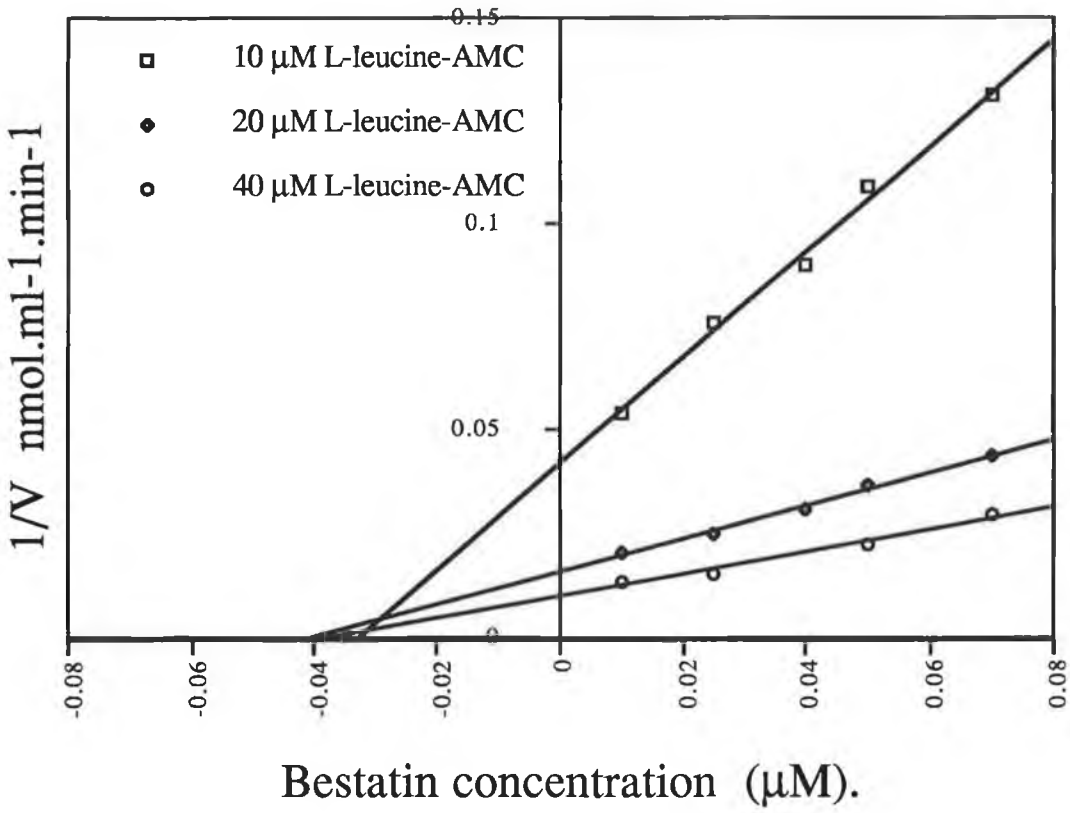


Figure 12B. Determination of the inhibitor constant (K_I) for the inhibition of crude cell-free extract of *P. c. chabaudi* aminopeptidase activity by bestatin. Dixon plot of the inhibition of crude cell-free extract *P. c. chabaudi* aminopeptidase activity by bestatin in the presence of either 10 µM, 20 µM and 40 µM of L-leucine-AMC. The intersection of the graphs represents K_I = 0.03 µM on the X axis.

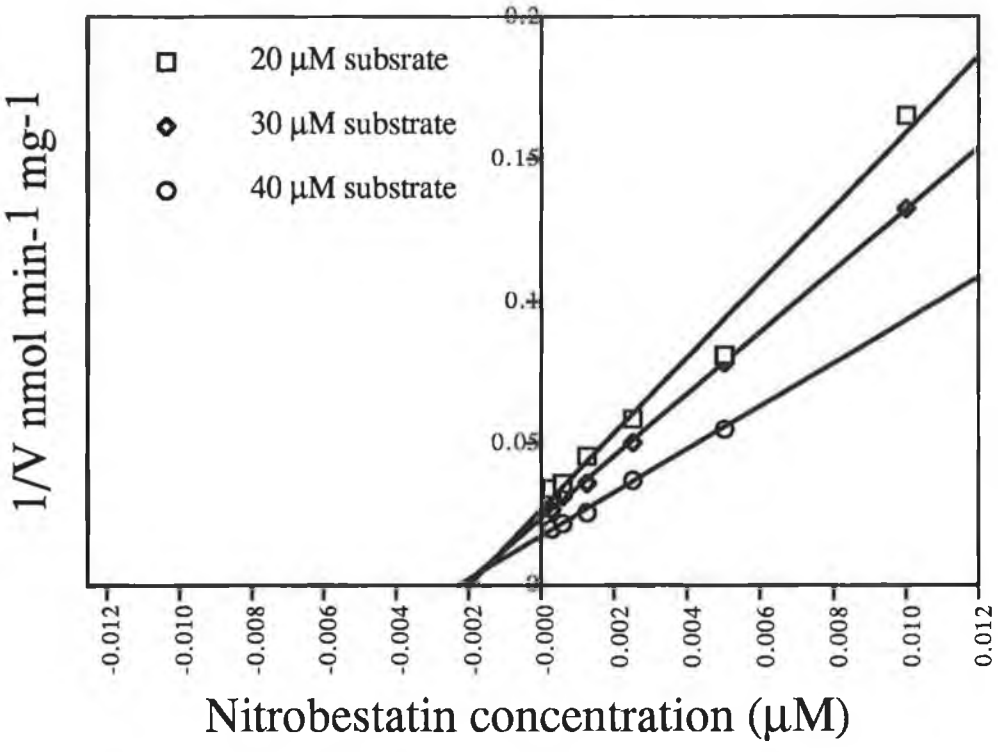


Figure 12C. Determination of the inhibitor constant (K_I) for the inhibition of crude cell-free extract of *P. c. chabaudi* aminopeptidase activity by nitro-bestatin. Dixon plot of the inhibition of crude cell-free extract *P. c. chabaudi* aminopeptidase activity by bestatin in the presence of either 20 µM, 30 µM and 40 µM of L-leucine-AMC. The intersection of the graphs represents K_I = 0.0018 µM on the X axis.

4.4. Inhibition of *P. c. chabaudi* and *P. falciparum* growth *in vitro* by bestatin and nitro-bestatin.

4.4.1. Inhibition of *P. c. chabaudi* growth *in vitro*.

The effect of bestatin and nitro-bestatin on the *in vitro* growth of *P. c. chabaudi* was investigated and compared to that of chloroquine. Ring-stage parasites were cultured for 16 hours in the presence of various concentrations of inhibitors and ^{35}S-methionine. Both bestatin and nitro-bestatin prevented the incorporation of isotope into parasite proteins. In these assays the aminopeptidase inhibitors were in the order of 2-3 times less effective than chloroquine in inhibiting parasite growth (fig. 13.A.).

Assays in which trophozoite-infected erythrocytes were incubated in the presence of ^{3}H-hypoxanthine and various concentrations of inhibitors showed that both bestatin and nitro-bestatin were effective inhibitors of *P. c. chabaudi* growth of the late stage of the asexual cycle of this malaria (fig. 13.B.). Chloroquine is most effective against trophozoites, and in our studies had an IC_{50} of 0.5 μM. Both bestatin and nitro-bestatin were approximately 100-fold less effective, having IC_{50} values of approximately 50μM. Although the inhibitory effect of nitro-bestatin on the malarial aminopeptidase is 20-fold more potent than bestatin, there was no significant difference between the two reagents' abilities to inhibit *P. c. chabaudi* growth in either assay.

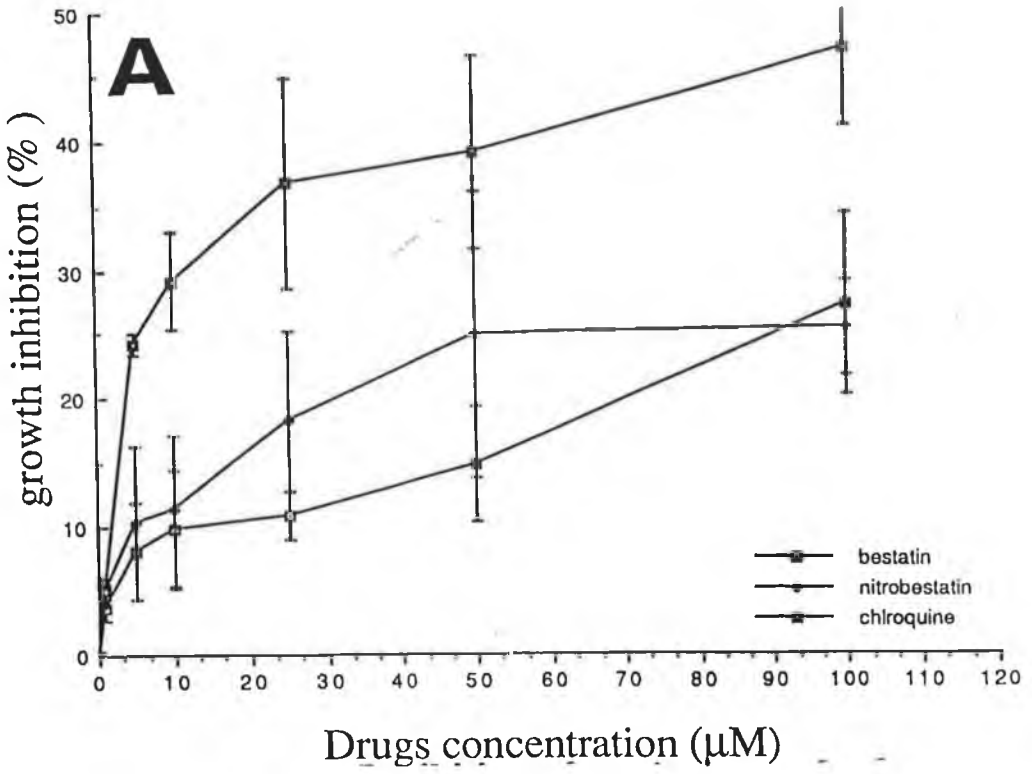


Figure 13.A. Inhibition of *in vitro* growth of *P. c. chabaudi*. Purified ring stage infected murine erythrocytes ($1x10^8$) were added to culture medium containing 2.5 µCi/ml ^{35}S-methionine and various concentrations of bestatin, nitro-bestatin and chloroquine. The amount of protein-incorporated radioisotope was estimated 16 hours later. Growth inhibition was estimated as the reduction of radioisotope incorporated into parasitised erythrocytes incubated in the presence of inhibitor relative to parasitised erythrocytes incubated in the absence of inhibitor. Non-parasitised murine erythrocytes served as background controls.

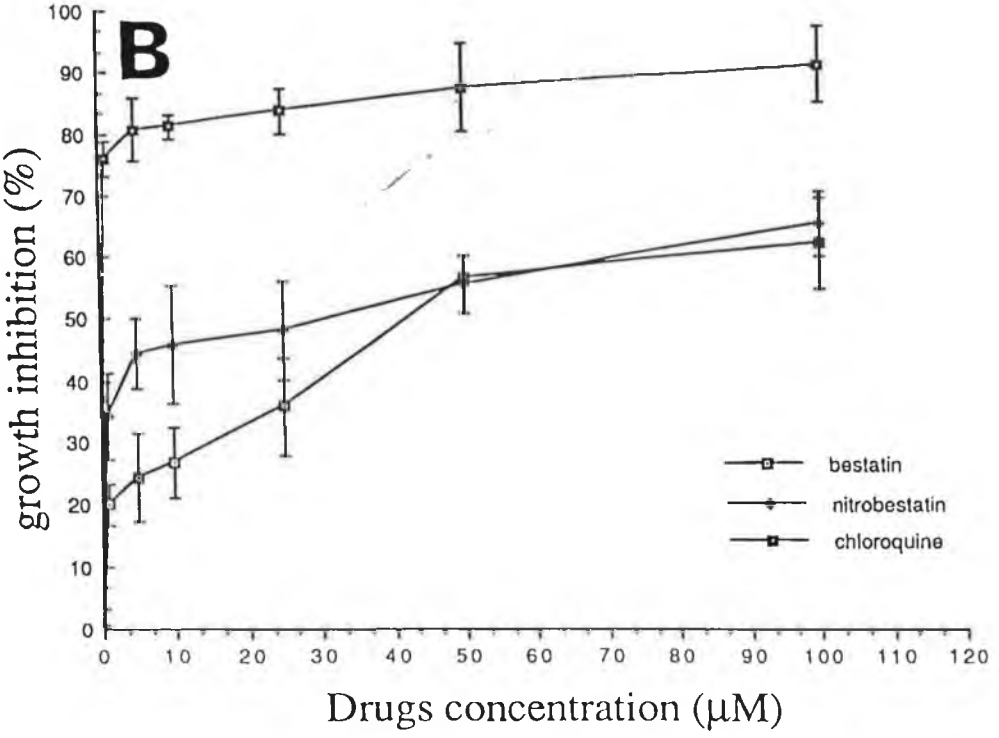


Figure 13.B. Inhibition of *in vitro* growth of P. c. chabaudi. Purified trophozoite-infected murine erythrocytes ($1x10^8$) were added to culture medium containing 1.0 μCi/ml ^{3}H-hypoxanthine and various concentrations of bestatin, nitro-bestatin and chloroquine. The amount of DNA-incorporated radioisotope was estimated 10 hours later. Growth inhibition was estimated as the reduction of radioisotope incorporated into parasitised erythrocytes incubated in the presence of inhibitor relative to parasitised erythrocytes incubated in the absence of inhibitor. Non-parasitised murine erythrocytes served as background controls.

4.4.2. Inhibition of *P. falciparum* growth *in vitro* .

Both bestatin and nitro-bestatin were potent inhibitors of the *in vitro* growth of *P. falciparum clone* FCH5.C2, with IC_{50} values of 1.7 and 0.37 μM, respectively (fig. 14.A). There was also partial sub-IC_{50} inhibition of growth by bestatin such that the potency of the two compounds between 0.02-0.1 μM was equal. In the same assay, the IC_{50} for chloroquine was 0.013 μM.

Inhibition of the growth of K1/Thailand by bestatin (IC_{50} = 5 μM) and nitro-bestatin (IC_{50} = 0.82 μM) was only slightly less than for FCH5.C2, inspite of the 10-fold higher IC_{50} of chloroquine for the former (fig. 14.B; IC_{50} = 0.11 mM). Nitro-bestatin was most potent against the early trophozoite stage (17-29 h post-invasion, data not shown).

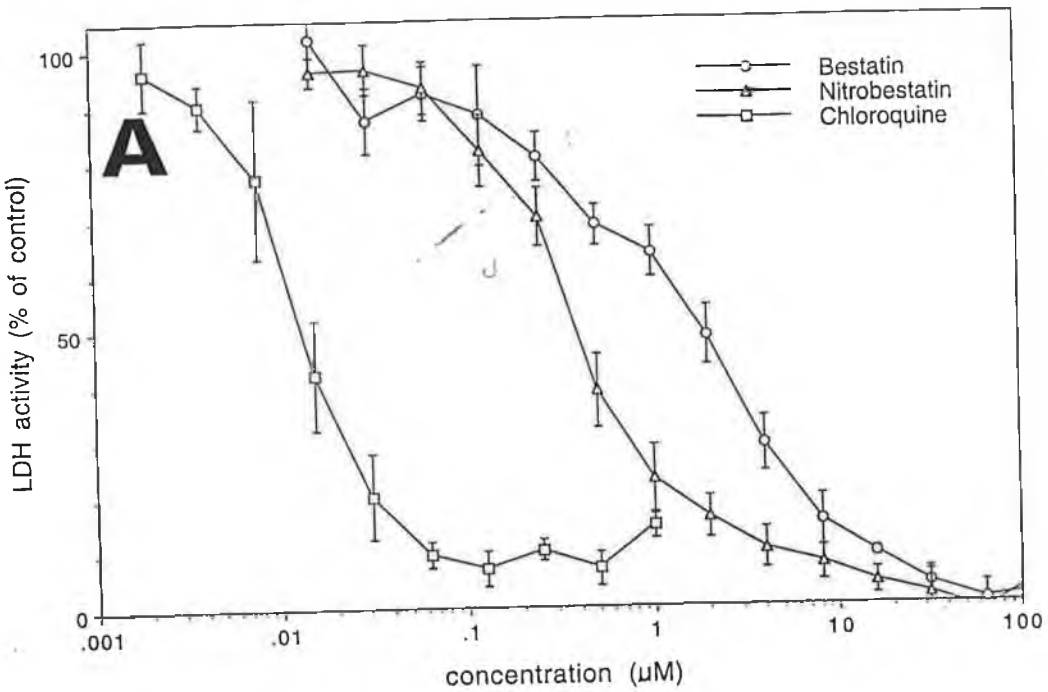


Figure 14.A. Inhibition of *in vitro* growth of *P. falciparum* by aminopeptidase inhibitors and chloroquine. *P falciparum* chloroquine sensitive clone FCH5.C2 was tested for it's inhibition by aminopeptidase inhibitors and chloroquine. Cultures of 2% haematocrit and 0.8% initial parasitaemia were incubated in various concentrations of bestatin, nitro-bestatin and chloroquine. After a 48 h incubation period LDH activity was measured as described in section 3.2.2. Growth was expressed as the LDH activity in cells incubated in the presence of inhibitor relative to cells incubated in the absence of inhibitor.

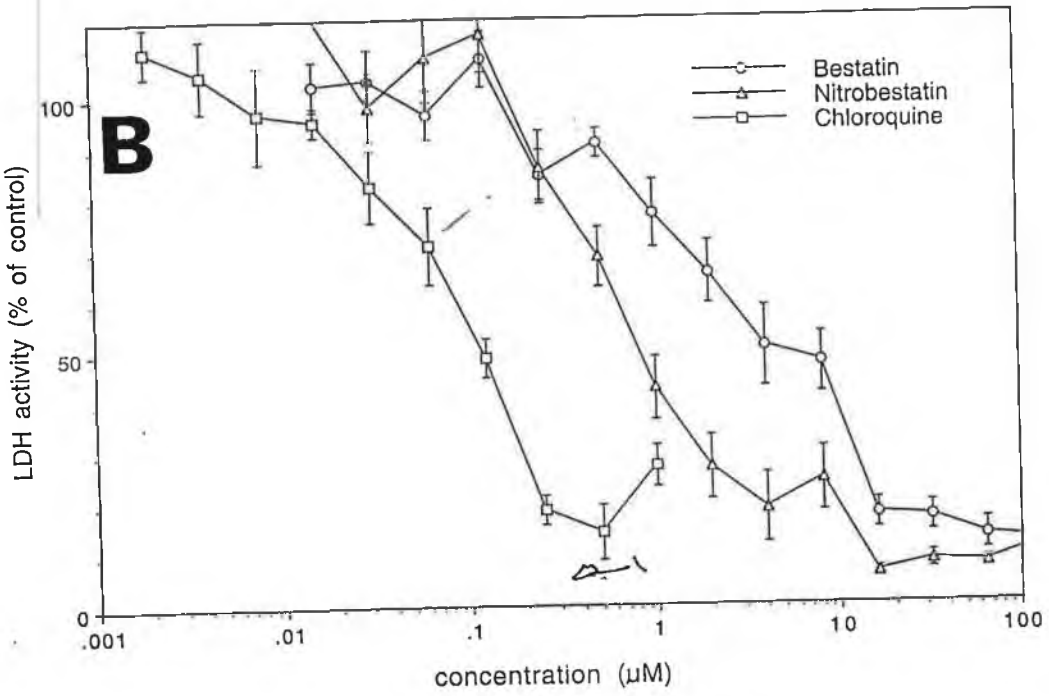


Figure 14.B. Inhibition of *in vitro* growth of *P. falciparum* by aminopeptidase inhibitors and chloroquine. *P falciparum* chloroquine resistant strain K1/Thailand was tested for it's inhibition by aminopeptidase inhibitors and chloroquine. Cultures of 2% haematocrit and 0.8% initial parasitaemia were incubated in various concentrations of bestatin, nitro-bestatin and chloroquine. After a 48 h incubation period LDH activity was measured as described in section 3.2.2. Growth was expressed as the LDH activity in cells incubated in the presence of inhibitor relative to cells incubated in the absence of inhibitor.

4 5. *In vivo* drugs tests.

4.5.1. Effects of chloroquine on *P. c. chabaudi* infection.

Female and male mice treated with chloroquine at 5% parasitaemia showed a distinctive decrease in parasitaemia after 24 hours and in 72 hours no parasites were visible in the blood stream. When parasitaemia was left to increase up to 15% before treatment with chloroquine the same results were observed indicating that chloroquine was able to clear parasitaemia in 72 hours post administration. The mice treated with chloroquine stayed alive and healthy until they were euthenased six months later (data not shown).

4.5.2. Effects of bestatin on *P. c. chabaudi* infection.

(a) Trial 1.

Female mice were subcutaneously administered with 5 or 2 mg bestatin/ injection/ mouse when parasitaemia reached 5-7% (trial 1) Parasitaemia appeared to increase earlier in mice in which bestatin had been administered subcutaneously. In addition the peak levels of parasitaemia in control mice (approx. 40%) were lower than those observed in treated mice. Parasitaemia did not exceed 40% in mice treated with 2 mg bestatin/ injection/ mouse but reached 60% in mice treated with 5 mg bestatin/injection/ mouse. The control mice appeared to clear parasitaemias slower than the treated mice (day 12-22).

However all mice showed parasite clearance from the blood stream 22 days post infection (fig. 15).

Male mice showed similar results except that the peak levels of parasitaemia in mice treated with 2 mg bestatin/ injection/ mouse were lower than the peak levels of parasitaemia in control mice. *P. c chabaudi* infection was lethal to all male mice 15 days post infection (fig. 16).

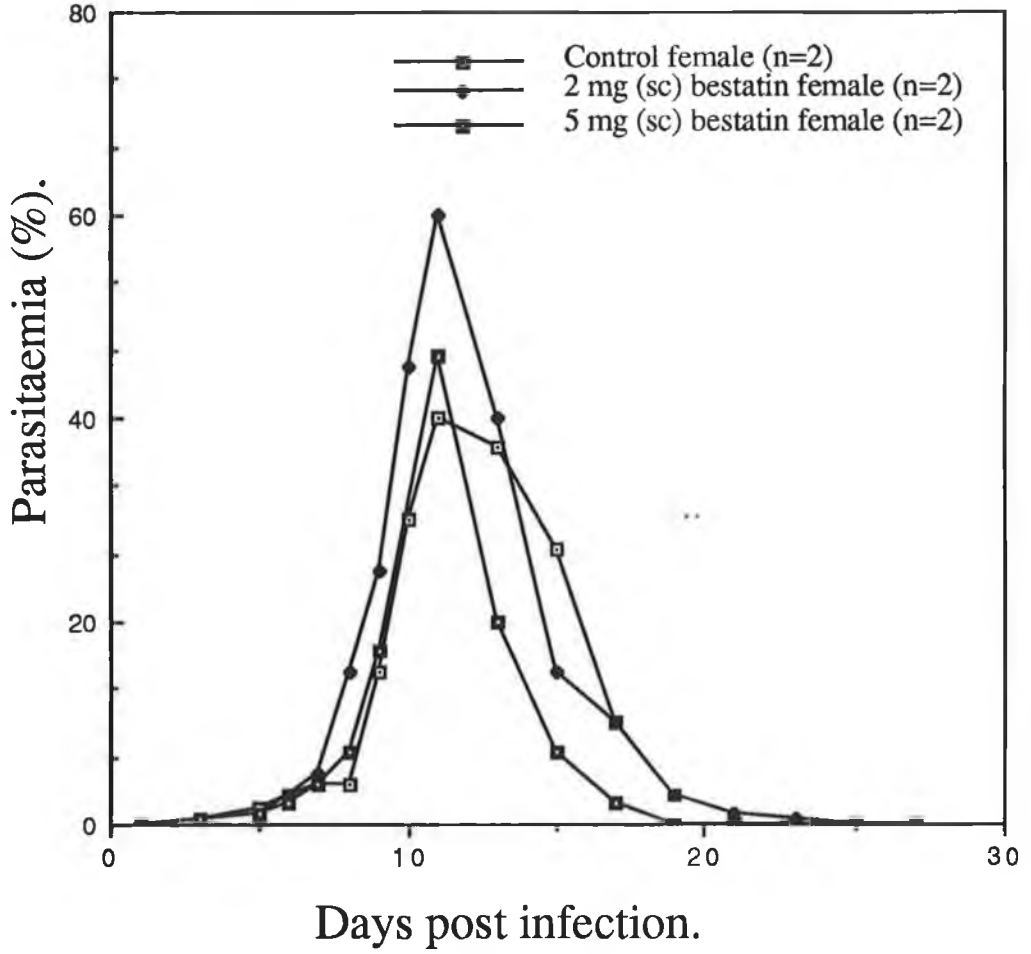


Figure 15. Drugs trial 1. Summary of the *in vivo* effects of bestatin on parasite growth (female mice). Mice were infected with ring stage *P. c. chabaudi* parasites. Subcutaneous administration of 5 or 2 mg bestatin/ injection/ mouse (PBS for control mice) was initiated when parasitaemia reached 5-7%.

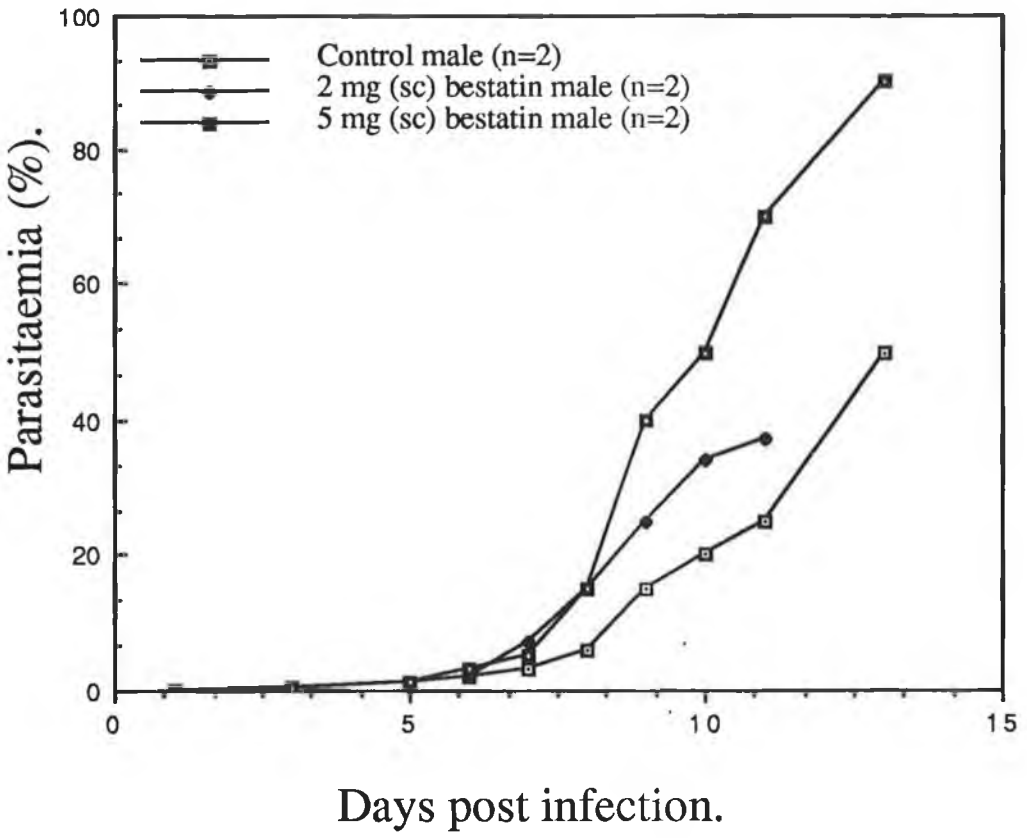


Figure 16. Drugs trial 1. Summary of the *in vivo* effects of bestatin on parasite growth (male mice). Mice were infected with ring stage *P. c. chabaudi* parasites. Subcutaneous administration of 5 or 2 mg bestatin/ injection/ mouse (PBS for control mice) was initiated when parasitaemia reached 5-7%.

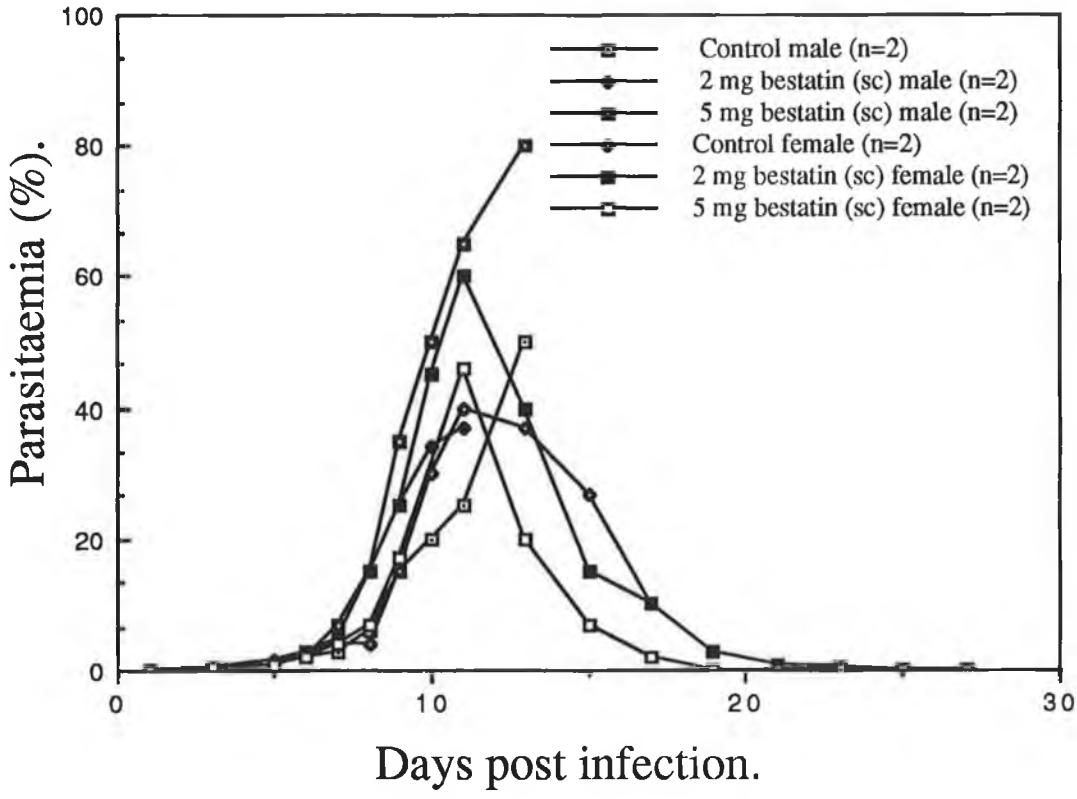


Figure 17. Drugs trial 1. Summary data of the *in vivo* effects of bestatin on parasite growth. Compilation of data shown in figures 15 & 16. Mice were infected with ring stage *P. c. chabaudi* parasites. Subcutaneous administration of 5 or 2 mg bestatin/ injection/ mouse (PBS for control mice) was initiated when parasitaemia reached 5-7%.

(b) Trial 2.

Female mice were treated subcutaneously (sc). with 2 mg bestatin/ injection/ mouse at < 0.5% parasitaemia. Parasitemia in treated mice did not exceed 40% although parasites were cleared from the blood stream in 15 days after infection as opposed to 22 days in control mice. The increase in parasitaemia appeared to be delayed in control mice. However when bestatin was administered intravenously (iv) the increase in parasitaemia was slightly delayed as opposed to female mice in which bestatin had been administered subcutaneously. Distinctively parasitaemia did not rise above 20% (fig. 18).

Male mice treated in a similar way died when parasitaemia reached > 60%. The increase in parasitaemia was delayed in control mice. Also two male mice out of three (one did not survive, data not shown) treated intravenously in a similar way reacted as the female mice in the sense that they controlled the infection and stayed alive and healthy until euthenased 6 months later (fig. 19).

This would suggest that bestatin does have an *in vivo* antimalarial effect on parasite control when administered intravenously. However as in the *in vitro* investigations, *in viv*o bestatin was not as potent an antimalarial as chloroquine.

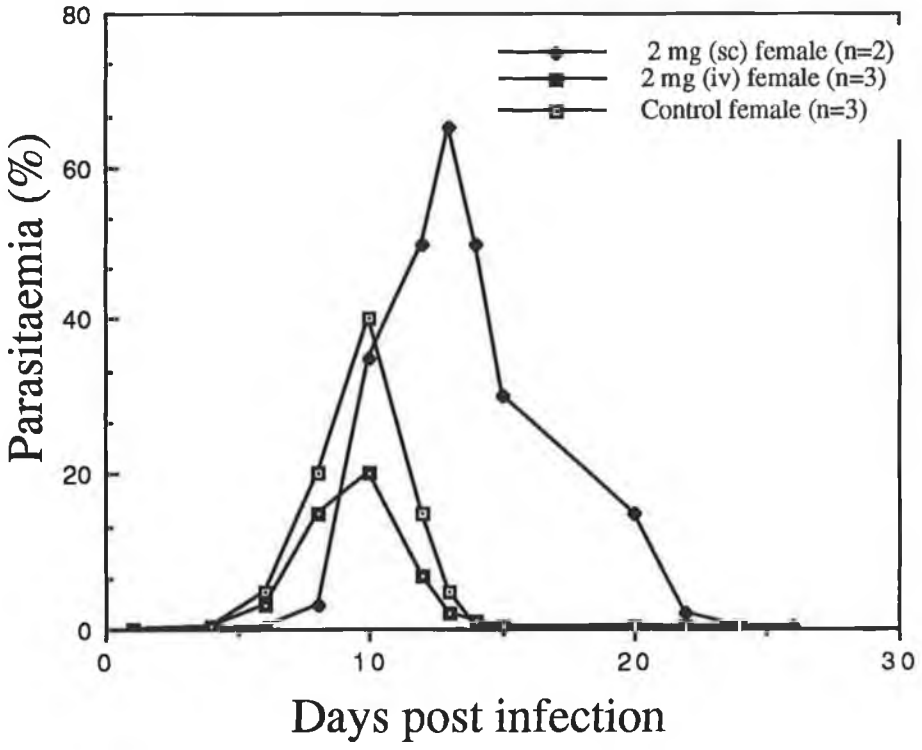


Figure 18. Drugs trial 2. Summary data of the *in vivo* effects of bestatin on parasite growth (female mice). Mice were infected with ring stage *P. c. chabaudi* parasites. Subcutaneous (sc) or intravenous (iv) administration 2 mg bestatin/ injection/ mouse (PBS for control mice) was initiated when parasitaemia reached < 0.5%.

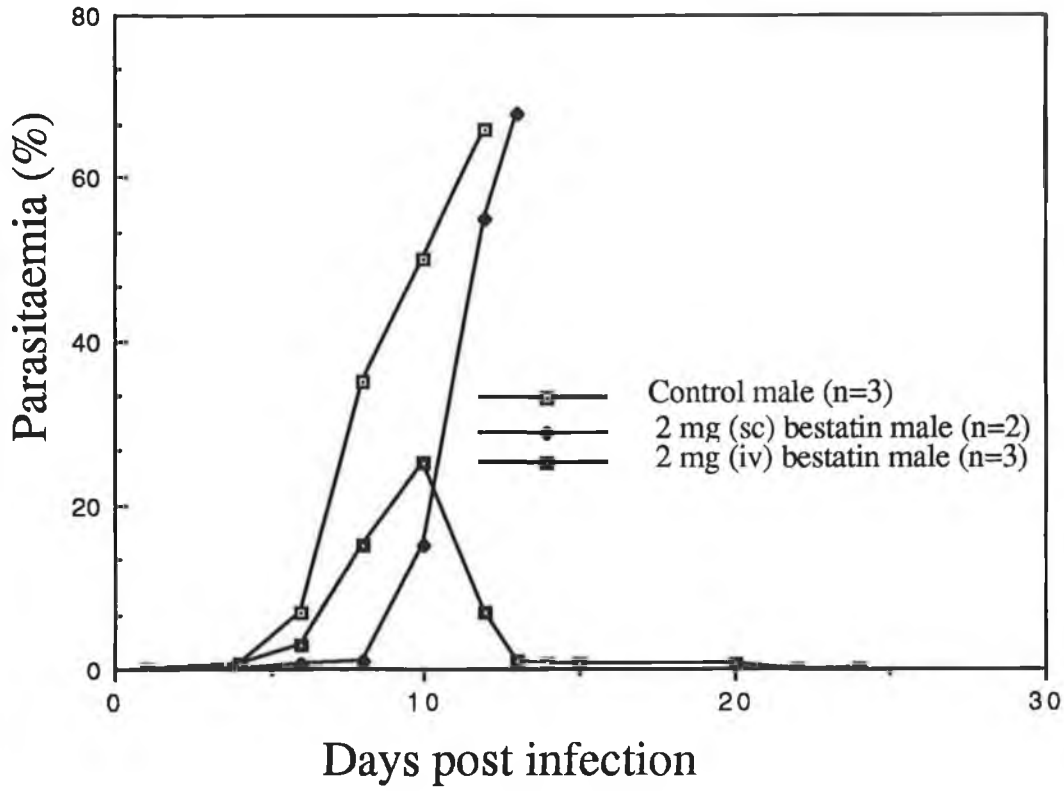


Figure 19. Drugs trial 2. Summary data of the *in vivo* effects of bestatin on parasite growth (male mice). Mice were infected with ring stage *P. c. chabaudi* parasites. Subcutaneous (sc) or intravenous (iv) administration 2 mg bestatin/ injection/ mouse (PBS for control mice) was initiated when parasitaemia reached < 0.5%.

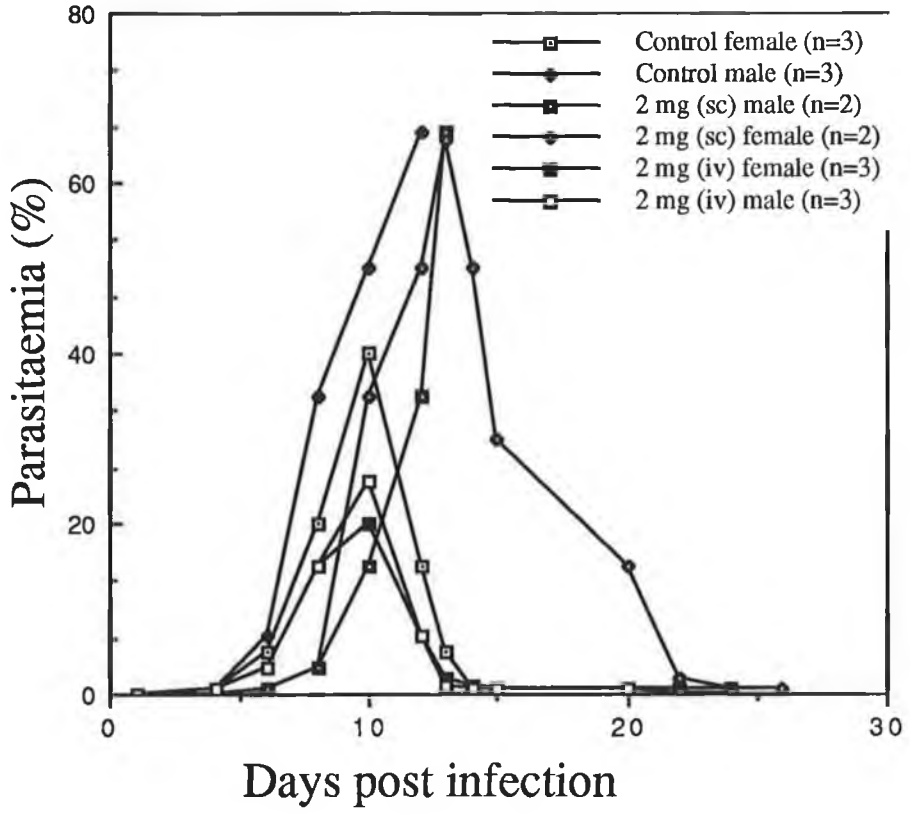


Figure 20. Drugs trial 2. Summary data of the *in vivo* effects of bestatin on parasite growth. Compilation of data shown in figures 18 & 19. Mice were infected with ring stage *P. c. chabaudi* parasites. Subcutaneous (sc) or intravenous (iv) administration of 2 mg bestatin/ injection/ mouse (PBS for control mice) was initiated when parasitaemia reached < 0.5 %.

4.6. Purification and characterisation of *P. c. chabaudi* aminopeptidase.

4.6.1. Purification of aminopeptidase.

Purification of *P. c. chabaudi* aminopeptidase was first attempted using a combination of size-exclusion HPLC and ion-exchange. Crude soluble *P. c. chabaudi* extract was separated on an anion-exchange column (QAE-Sepharose) The aminopeptidase activity bound to this column was eluted from the column with 0.1 M NaCl (fig. 21A). This would indicate that the *P. c. chabaudi* aminopeptidase has a moderate negative charge.

After this purification step an increase in total aminopeptidase activity was observed (yield 155%) and a purification. factor of 3.9 was obtained (Table 1.). The sample (I.1) was analysed by direct fluorogenic analysis in a 10% non-reducing, non-denaturing polyacrylamide gel. In this gel the proteins are electrophoretically separated according to their overall electrical charge, molecular weight size and conformation. A fluorescent band localising aminopeptidase activity was observed (fig. 23; lane 3). Sample I.1 was subsequently subjected to size-exclusion HPLC. The proteins resolved in one sharp peak and the aminopeptidase activity eluted as a symmetrical peak at approximately 60-70 kDa (Sample IH.2, fig. 21B). The overall purification factor was approximately 124 fold (Table 1). Forty to fifty percent of initial activity was lost during these two purification steps.

When the purification was performed in the reverse order i.e., by size-exclusion HPLC followed by ion-exchange chromatography, an

increase in total aminopeptidase activity was observed after the size-exclusion step indicating the presence of an aminopeptidase inhibitor in the crude cell-free extract (corroborating observations made by ion-exchange and HPLC purifications). Greater aminopeptidase activity was observed in diluted crude cell-free extract than the undiluted crude cell-free extract emphasising the presence of an endogenous aminopeptidase inhibitor. A similar overall purification factor was obtained.

Another strategy to purify the aminopeptidase to a high degree of purification was performed by exploiting the biological properties of this aminopeptidase to bind specifically and non-covalently to bestatin by affinity chromatography. Crude cell-free extract separated on a size-exclusion HPLC column resolves as two broad protein peaks. The aminopeptidase activity eluted as a symmetrical peak at 80 kDa (Sample (H.1) in the first of the protein peaks (fig 22A). A purification factor of 5-6 fold was obtained at this step (Table 2). In addition, an increase of total aminopeptidase activity was observed (yield 225%) again confirming the presence of an aminopeptidase inhibitor in the crude extracts. Sample H.1 was applied to the bestatin affinity column. The aminopeptidase binds to the bestatin-affinity column and is eluted with 0.2-0.3 M NaCl (sample HB.1, fig.22B). This step increased the purification factor by approximately 15-fold providing an overall purification factor of > 100 fold (Table 2).

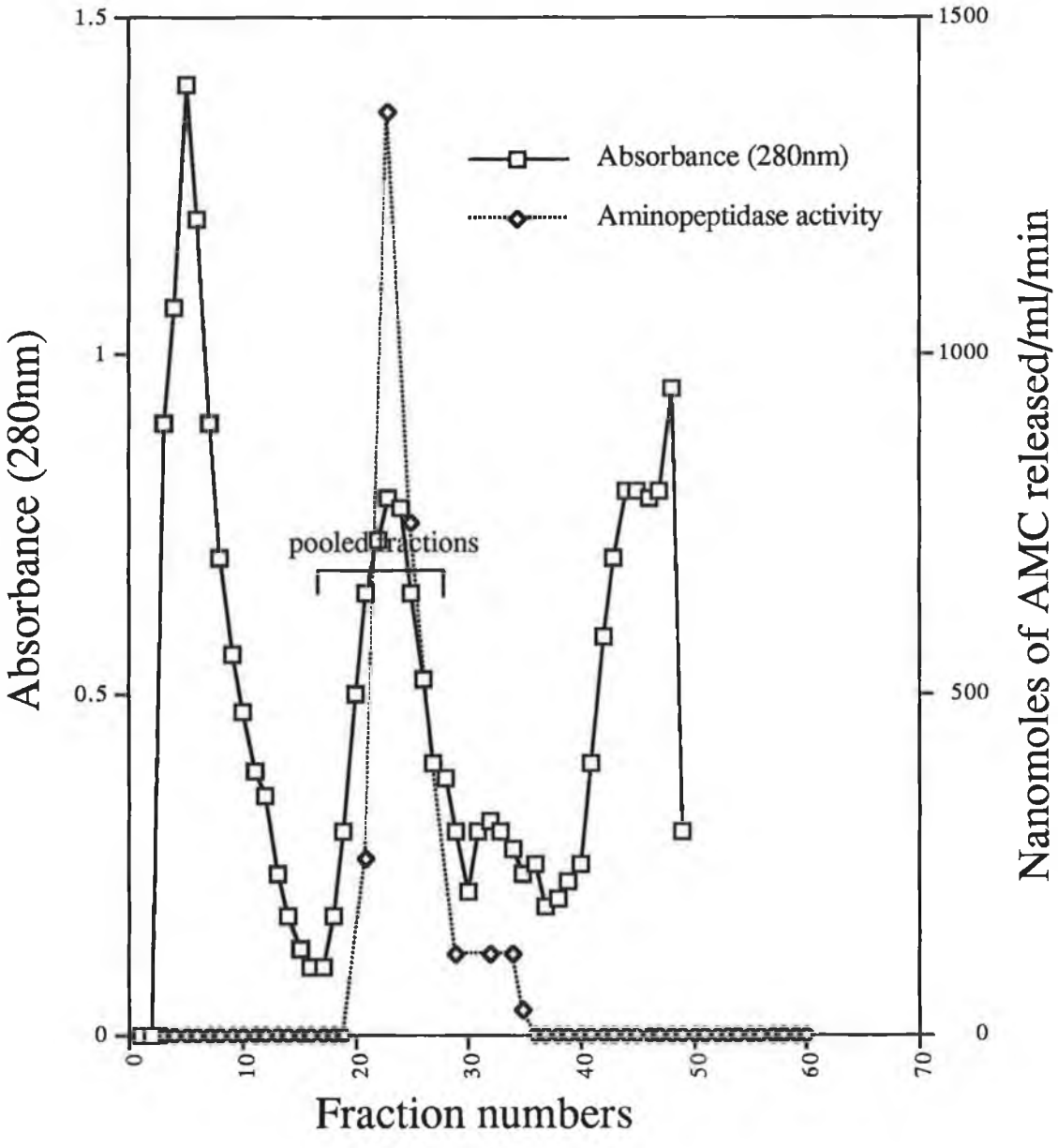


Figure 21A. Purification of *P. c. chabaudi* aminopeptidase. Crude cell-free extract of aminopeptidase (approx. 1 mg), was separated on an anion-exchange chromatography column equilibrated in 0.1 M Tris-HCl, pH 8.0. Bound aminopeptidase activity eluted with 0.1M NaCl using a step-wise gradient and was detected using the fluorogenic substrate L-leucine -AMC.

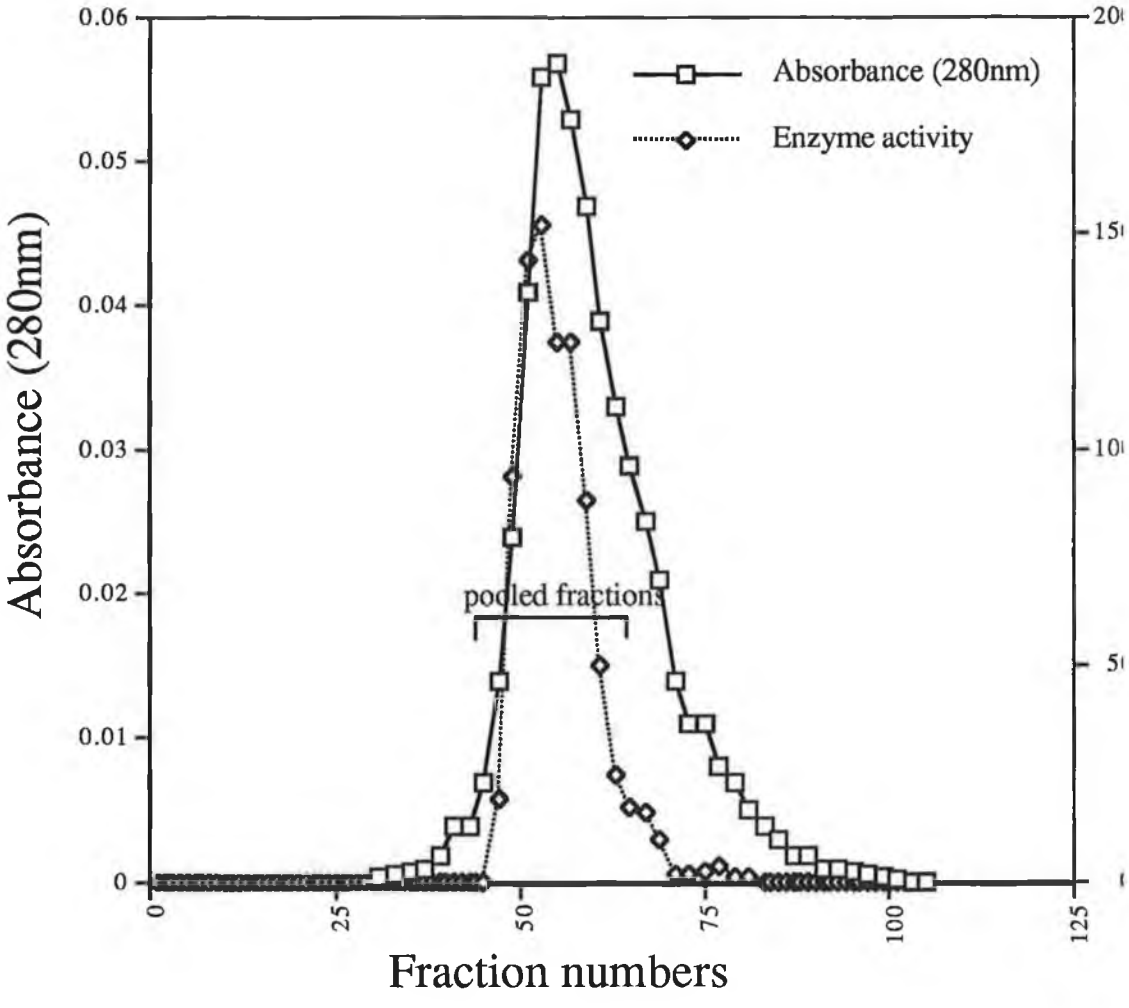


Figure 21B. Purification of *P. c. chabaudi* aminopeptidase. Crude cell-free extract of aminopeptidase (approx. 1 mg), was separated on an anion-exchange chromatography column, followed by a size-exclusion HPLC gel filtration column equilibrated in 0.1 M KH_2PO_4, pH 6.8. Aminopeptidase activity was detected using the fluorogenic substrate L-leucine -AMC.

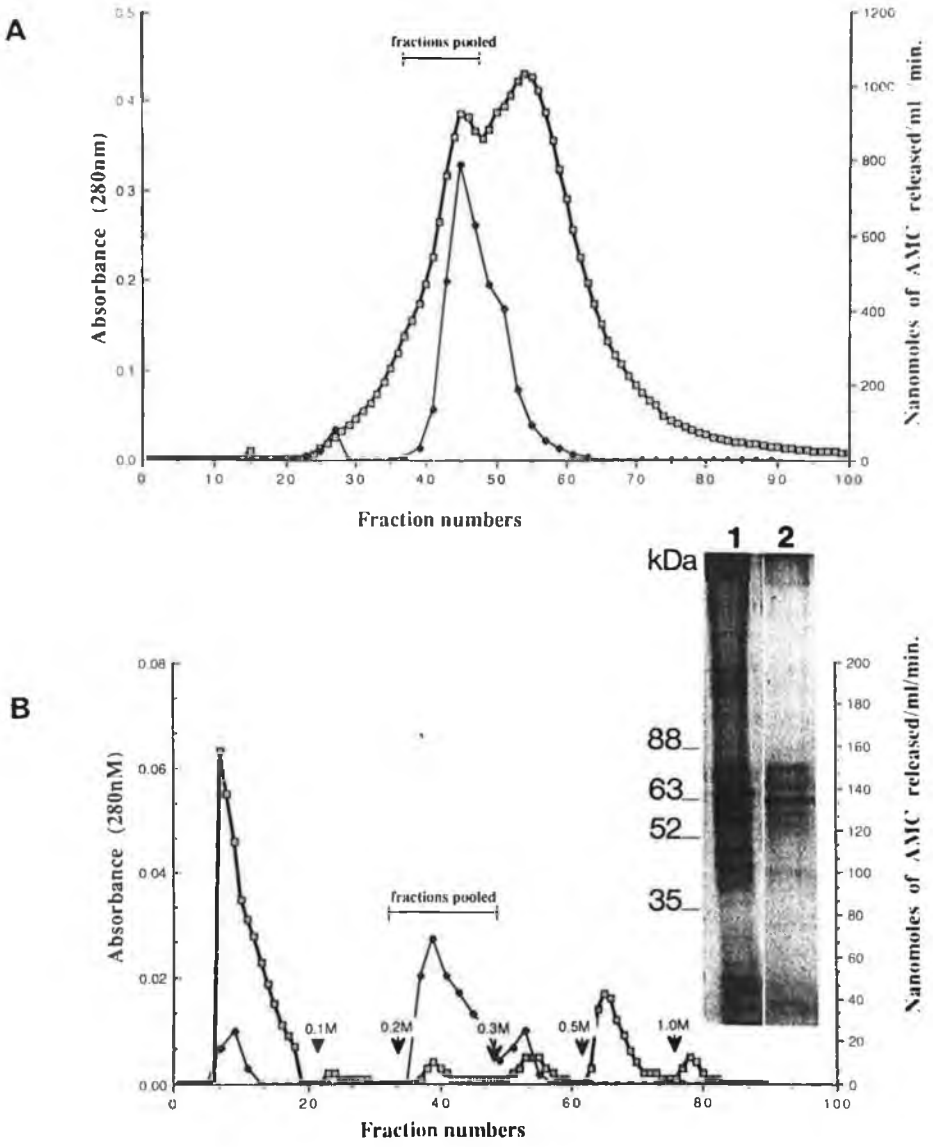


Figure 22. Purification of *P. c. chabaudi* *aminopeptidase*. (A) Crude cell extract of *P. c. chabaudi* was separated on a HPLC gel filtration column equilibrated in 0.1 M KH_2PO_4, pH 6.8. Eluted protein was measured by absorbance at 280 (▫) nm and aminopeptidase activity was detected using the fluorogenic substrate L-leucine-AMC (◆). (B) Aminopeptidase activity eluted from the gel filtration column (sample H.1) was pooled and applied to a bestatin EAH-Sepharose 4B affinity column equilibrated in 0.05M Tris-HCl, pH 8.0. Bound aminopeptidase activity was eluted between 0.2-0.3 M NaCl using a step-wise gradient. ***Inset***: 12 % SDS-PAGE analysis of crude cell-free extract (lane 1) and purified fraction HB (lane 2)

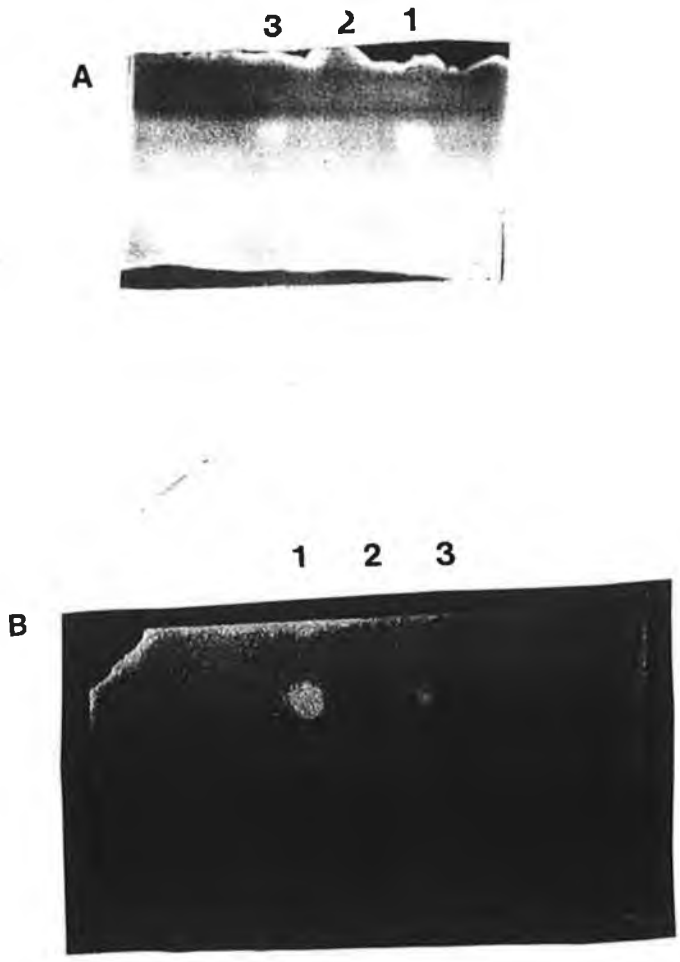


Figure 23. Direct fluorogenic substrate analysis of aminopeptidase activity in polyacrylamide gels. (A) Crude cell-free of aminopeptidase was purified by a combination of ion-exchange and size-exclusion HPLC. Fractions from each purification step were analysed on a non-denaturing, non-reducing 10% polyacrylamide gel. Lane 1: crude extract, lane 2: fraction IH.1., from ion-exchange chromatography followed by size-exclusion HPLC, and lane 3: fraction I.1., from ion-exchange chromatography.(B) Crude cell-free extract of aminopeptidase was purified by a combination of size-exclusion HPLC gel filtration and bestatin-EAH-Sepharose 4B affinity chromatography. Fractions from each purification step were analysed on a non-denaturing, non-reducing 10% polyacrylamide gel. Lane 1: crude extract, lane 2: fraction H.1., from size-exclusion HPLC gel filtration, and lane 3: fraction HB.1., from size-exclusion HPLC gel filtration followed by bestatin-affinity chromatography.

Table 1. Purification table of *P. c. chabaudi* aminopeptidase. Crude cell-free extract of *P. c. chabaudi* aminopeptidase (100 µl) was purified by a combination of anion-exchange chromatography and size-exclusion HPLC gel fitration.

Step	Protein (mg)	Total activity (units)	Specific activity (Units/mg)	Yield (%)	Purification factor
Crude extract	0.106	0.312	2.95		1
Ion-exchange	0.040	0.48	12	153.8	3.9
Size-exclusion HPLC	0.0009	0.33	366.66	105.8	124.3

Table 2. Purification table of *P. c. chabaudi* aminopeptidase. Crude cell-free extract of *P. c. chabaudi* aminopeptidase (600 µl) was purified by a combination of size-exclusion-HPLC gelfiltration and bestatin-affinity chromatography.

Step	Protein (mg)	Total activity (units)	Specific activity (Units/mg)	Yield (%)	Purification factor
crude extract	0.6	3.2	5.3		1
size-exclusion HPLC	0.234	7.2	30.7	225	5.79
Bestatin-affinity chromatography	0.0097	5.2	536	162	101.13

4.6 2. Characterisation. of purified *P. c. chabaudi* aminopeptidase.

(a) SDS-PAGE analysis

One hundred ng of concentrated samples from each purification step was mixed with reducing and denaturing sample buffer (see section 2.1.4a). SDS is an ionic detergent which denatures the proteins by breaking all non covalent bonds thus unfolding the proteins, in doing so, SDS also provides a uniformed negative charge to the denatured proteins this ensures that all the proteins will migrate in the same direction when submitted to an electric field, β-mercaptoethanol is a reducing agent which reduces RS-SR covalent bonds to 2RSH thus further unfolding the proteins, should there be any covalent disulphide bridges. When submitted to electrophoresis the proteins migrate and are separated solely according to their molecular weight. The samples including prestained molecular weight markers were then boiled for 2 min to dissolve any aggregated proteins. The samples were loaded on a 12% gel and submitted to an electric field using SDS-PAGE running buffer (see section 2.1.4a) at 30 mA.

Silver staining of these gels revealed a concentration of bands in the region of 60-70 kDa compared to the crude extract. Sample HB was very interesting as one of the bands at approximately 63 k Da has the same intensity as the co-migrating band in the crude extract (fig 22, inset., lane 2).

(b) Direct fuorogenic localisation of aminopeptidase activity in polyacrylamide gels.

The pooled samples from each purification step were submitted to non-reducing and non-denaturing PAGE on a 10% gel (native gels) using PAGE running buffer (section 2.1.4) at 30 mA. In this type of gels the proteins migrate and are separated according to their overall electrical charge , molecular weight and conformation when submitted to an electric field. These gels were analysed by direct fluorogenic localisation . Samples H.1., I.1 and HB.1., revealed intense fluorescent bands thus localising the presence of aminopeptidase activity in native gels (fig. 23 A & B lanes 1 & 3). Samples H.1 and IH.1 revealed very weak bands.

(c) Quantitative analysis of polyclonal antisera.

Antiserum was obtained from immunised rabbits as described (see section 3.6.2b). 500 ng of samples H.1 and HB.1., and the crude cell extract of aminopeptidases were coated onto ELISA plates and analysed with 1:100 and 1:1000 increasing dilutions up to 1:10.000 of rabbit anti- sera. *p*-nitrophenyl phosphate was used as a substrate for alkaline phosphatase. When pNpp is hydrolysed by the alkaline phosphatase the soluble end product is yellow in colour. The intensity of the yellow colour is directly proportional to the amount of antibodies bound to the coated protein extracts This amount of bound antibodies was determined by spectrophotometry at 405 nm. The quantity of antibodies bound were inversely proportional to the anti-sera dilutions between 1:100 and 1:4000. Beyond this value the readings were very

fluctuant and would indicate non specific or background readings. Sample HB.1 showed the highest values of bound antibodies. Indicating that the anti-sera was reacting specifically to the sample against which it was produced (fig. 24). These results were used to determine the best dilutions to use in any further work which necessitated the use of this antisera. ELISA analysis of rat antisera showed similar results (data not shown)

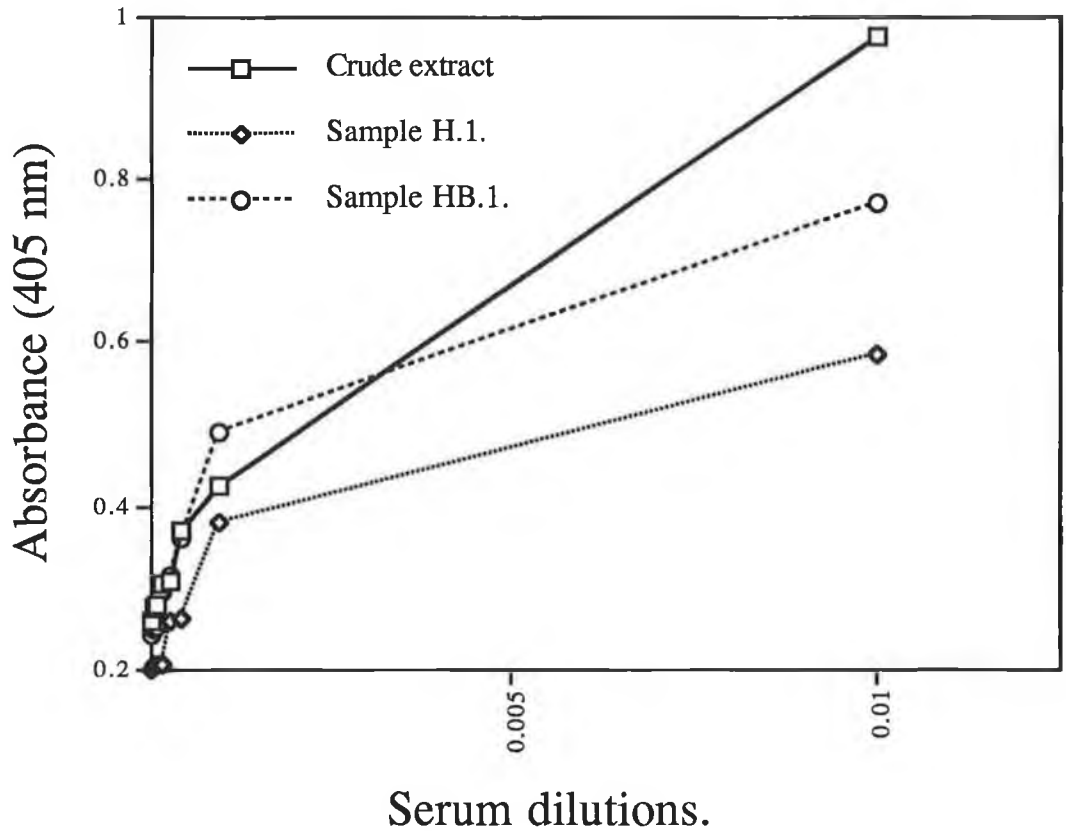


Figure 24. Quantitative analysis of rabbit anti-sera raised against purified *P. c. chabaudi* aminopeptidase. ELISA analysis of Rabbit anti-sera. Rabbits were immunised with sample HB.1. Bound antibodies were detected with the colorigenic substrate p-Nitro phenyl phosphate at 405 nm.

(d) Qualitative analysis of polyclonal antisera.

The quality of the polyclonal antisera was analysed by Western blotting. The pooled fractions from each purification step were concentrated and freeze dried before resuspension in 50 µl PBS. 10 µl of each sample were subsequently submitted to reducing-SDS-PAGE or non-reducing PAGE. The gels were transfered to nitrocellulose filters which were probed with 1:50, 1:100 and 1:500 dilutions of rat anti-sera raised against sample IH.1., or rabbit anti-sera raised against fractions HB.1.,. Samples I.1., IH.1., and crude extract probed with rat anti-sera revealed four distinctive protein bands with molecular weights in the region of 60-70 kDa (fig. 25). The samples H.1., HB.1., and crude extract probed with rabbit anti-sera revealed a smear in the same region (fig. 26 A.). Probing of non-reduced, non-denatured samples H.1., HB.1., with rabbit anti-sera revealed a smear with the crude extract only (fig. 26 B.) no protein bands were detected with native samples probed with rat anti-sera.

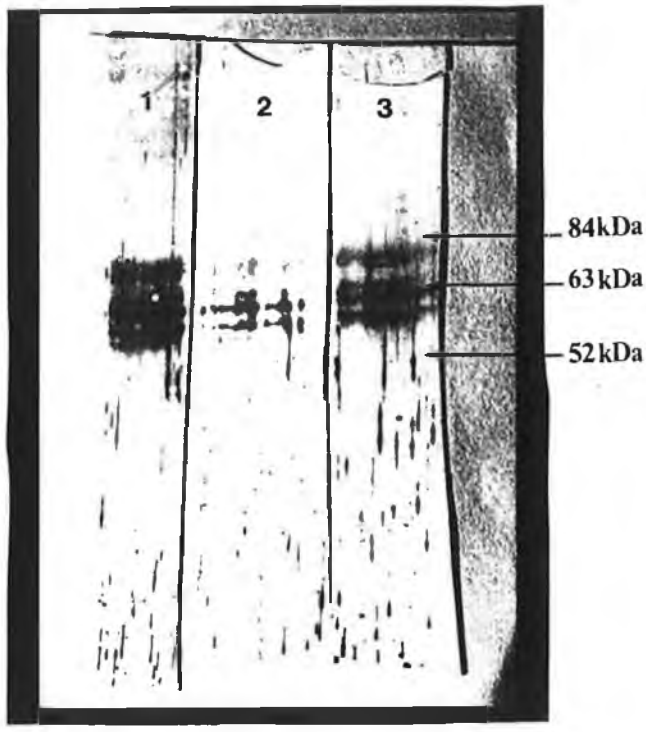


Figure 25. Qualitative analysis of polyclonal rat anti-sera. Samples from each purification step were submitted to reducing SDS-PAGE and transferred to nitrocellulose filters. The filters containing crude cell extract (lane 1), fraction IH.1., (lane 2) and fraction I.1., (lane 3) were probed with rat anti-sera raised against *P. c. chabaudi* aminopeptidase, fraction IH.1.

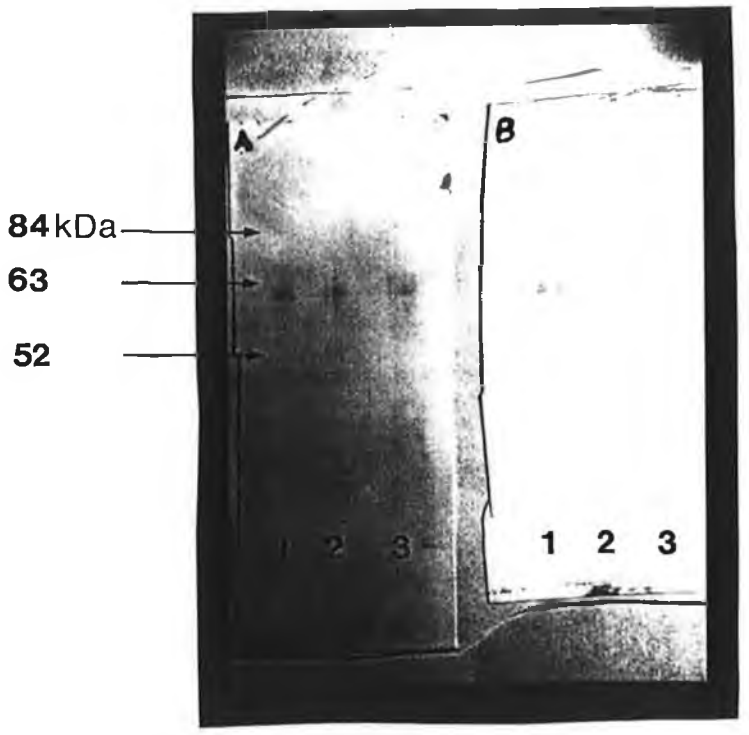


Figure 26. Qualitative analysis of polyclonal rabbit anti-sera. Samples from each purification step were submitted to (A) reducing SDS-PAGE and transferred to nitrocellulose filters. The filters containing crude cell extract (lane 1), fraction H.1., (lane 2) and fraction HB.1., (lane 3) were probed with rabbit anti-sera raised against *P. c. chabaudi* aminopeptidase fraction HB.1. (B) The samples were submitted to non-denaturing, non-reducing PAGE before being transferred to nitrocellulose filters . Lane 1, contains crude cell extract, lane 2, fraction H.1 and lane 3. fraction HB.1.

4.7. Enzyme kinetics and inhibition studies of the purified *P. c. chabaudi* aminopeptidase.

4.7.1. Effect of substrate concentration on the rate of the aminopeptidase catalysed reaction.

The effect of the concentration of L-leucine-AMC on the rate of the aminopeptidase catalysed reaction was investigated. Plots of enzyme activity (v_o) versus substrate concentration showed that at a low substrate concentration, the initial reaction velocity, vo, was nearly proportional to the substrate concentration, and the reaction was thus approximately first order with respect to the substrate. However, as the substrate concentration was increased, the initial rate increased less, so that it was no longer nearly proportional to the substrate concentration; in this zone, the reaction was mixed order. As the concentration was further increased, the reaction rate became essentially independent of substrate concentration and asymptotically approached a constant rate. In this range of substrate concentration the reaction was essentially zero order therefore the enzyme was saturated with respect to the substrate. These observations are in accordance with simple Michaelis-Menten kinetics (data not shown). The Michaelis-Menten theory assumes that this reaction is reversible and that the enzyme first combines with the substrate to form the enzyme-substrate complex ES; the latter then breaks down in a second step to form free enzyme and the product. Based on this theory we can therefore write-

$$[E] + [S] \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} [ES] \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} [E] + [P]$$

With [E] = Aminopeptidase concentration

[S] = L-leucine-AMC concentration

[ES] = Aminopeptidase:L-leucine-AMC complex concentration

[P] = (AMC + L-leucine) concentration

We can also write the Michaelis-Menten equation (Briggs & Halden) v_O=Vmax [S] /K_M+[S].

Where vo is defined as the initial reaction velocity, Vmax as the maximum initial reaction velocity, [S] as the concentration of substrate and K_M as the Michaelis-Menten constant the substrate concentration at which the initial reaction velocity is half maximal.

This equation can be transformed into the Lineweaver -Burk equation

1/vo = K_M/Vmax X.1/[S] + 1/Vmax

Plots of 1/vo versus 1/[S] determined that K_M = 20 mM and Vmax = 5.6 mmol AMC released/min/mg protein for the *P. c. chabaudi* aminopeptidase with respect to L-leucine-AMC

4 7.2. Inhibition kinetics of malaria aminopeptidase by bestatin.

Purified aminopeptidase was pre-incubated with various concentrations of bestatin or nitro-bestatin for 45 min at room temperature prior to adding the substrate and measuring aminopeptidase activity. Plots of activity versus the concentration of bestatin or nitrobestatin showed a decreasing hyperbole which asymptotically approaches a constant rate. This would suggest that bestatin and nitro-bestatin inhibit aminopeptidase activity in a Michaelis-Menten manner.

Lineweaver-Burk plots of $1/v_O$ versus 1/[S] for each inhibitor concentration revealed a family of straight lines intersecting at a common intercept on the $1/v_O$ axis (fig 27.A). This graph is characteristic of competitive inhibition (Lehninger, 1978). The presence of bestatin increased the apparent K_M of the aminopeptidase for L-leucine-AMC, but did not affect the Vmax. We can therefore assume that bestatin and nitrobestatin can combine with the free aminopeptidase in such a way that it competes with L-leucine-AMC for binding at the active site. This theory also assumes that a competitive inhibitor binds reversibly with the enzyme to form an enzyme-inhibitor complex [EI] analogous to the enzyme-substrate complex. The inhibitor molecule is not chemically changed by the enzyme.

The reversibility of inhibition of the aminopeptidase by bestatin was confirmed as the malarial aminopeptidase incubated with 2 μM of bestatin regained total activity upon a 50 fold dilution (fig. 28.B). This concentration of bestatin inhibits the enzyme more than 95%. This reactivation occurred immediately, and no difference in activity was

observed between samples assayed at time 0 and after 3 hours, indicating that bestatin dissociates rapidly from the malarial enzyme.

Based on the Michaelis-Menten formalism we can write:

$$[E] + [I] \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} [EI]$$

$$K_I = \frac{[E][I]}{[EI]}$$

[I]= bestatin concentration

[EI]=bestatin:aminopetidase complex concentration

The inhibitor constant K_I can be defined as the dissociation constant of the enzyme-inhibitor complex:

From the plots of $1/v_O$ versus 1/[S] we know that the slope of the uninhibited reaction is K_M/Vmax and the slope for the inhibited reaction is K_M/Vmax(1+ [I]/K_I). The inhibiting constant K_I can be calculated from this relation however, using the method of Dixon which consists of plotting $1/v_O$ versus [I] (Dixon, M. *et al.*, 1953), values for the inhibitor constants were determined for bestatin (fig 27.B; K_I = 50.7nM) and nitro-bestatin (K_I = 2.51 nM). These constants are similar to those reported for the "slow-binding, tight-binding" bestatin-like inhibitors of a variety of mammalian and plant aminopeptidases (Taylor, 1993).

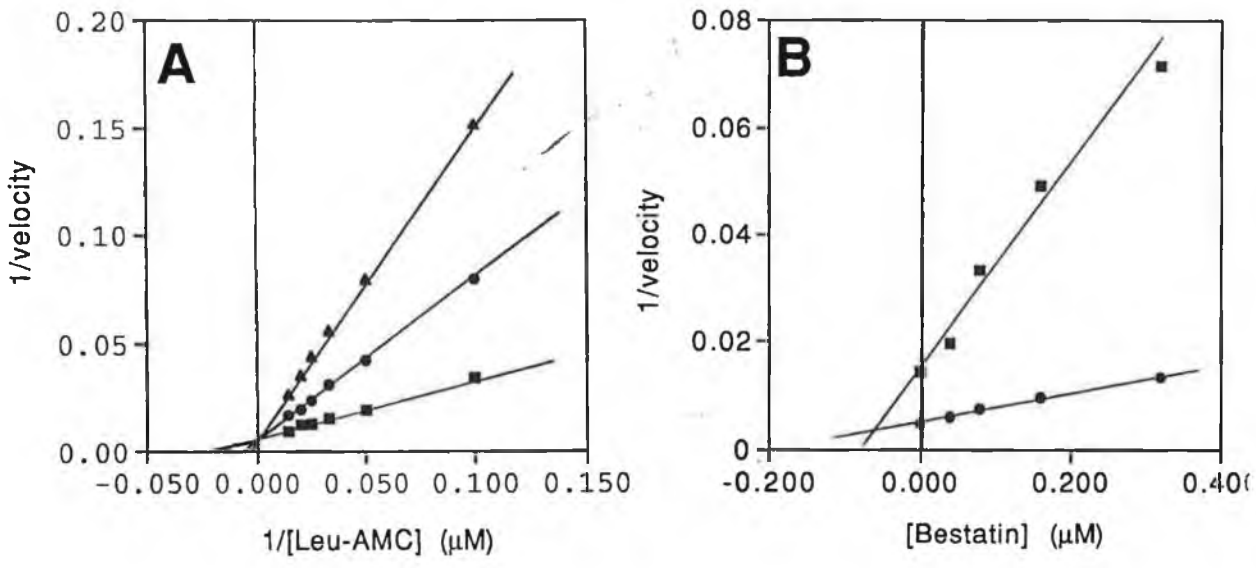


Figure 27. Determination of the inhibitor constant (K_I) of purified *P. c. chabaudi* aminopeptidase by bestatin. (A) Lineweaver-Burk plot of activity (arbitrary units) of *P. c. chabaudi* aminopeptidase incubated with varying concentrations of L-leucine-AMC in the presence of 0 µM (■), 0.1 µM (●) and 0.3 µM bestatin (▲). (B) Dixon plot of the inhibition of *P. c. chabaudi* aminopeptidase by bestatin. The enzyme was incubated with varying concentrations of bestatin, in the presence of either 15 µM (■) and 60 µM (●) L-leucine-AMC.

4.7.3. Comparison of malarial aminopeptidase with porcine kidney aminopeptidase.

In contrast to the time-dependent inhibition observed for porcine kidney aminopeptidase, the plot of activity versus time reveals that the binding of bestatin to the malarial aminopeptidase is rapid (fig. 28.A). When the reversibility of bestatin inhibition was investigated the malarial aminopeptidase regained total activity upon dilution (fig. 28.B). This reactivation occurred immediately, and no difference in activity was observed between samples assayed at time 0 and after 3 hours, indicating that bestatin dissociates rapidly from the malarial enzyme. In contrast similar experiments with the porcine kidney aminopeptidase reveal that its activity increases with the length of the post-incubation period (fig 28.B).

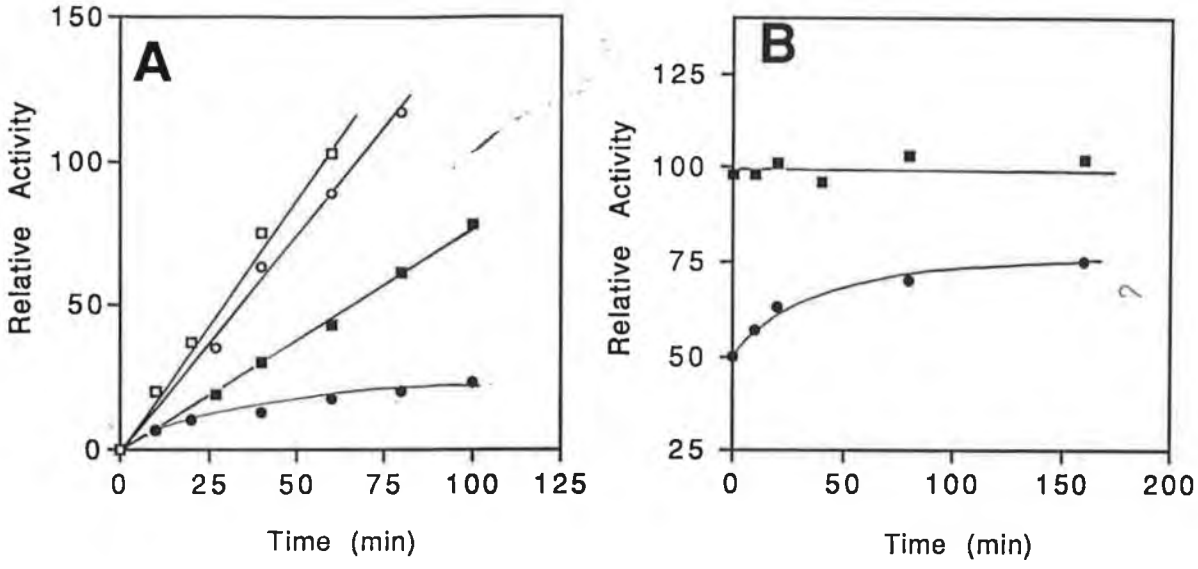


Figure 28. Inhibition kinetics of aminopeptidase by bestatin. (A) *P. c. chabaudi* (■) and porcine kidney aminopeptidases (●) were incubated with 50 μM L-leucine-AMC in the presence of 0.5 μM and 2.0 μM bestatin, respectively. Control reaction with no inhibitor for *P. c .chabaudi* (□) and porcine aminopeptidase (○) are also shown. (B) *P. c. chabaudi* (■) and porcine kidney aminopeptidases (●) were incubated for 1 hour with 2 μM bestatin, diluted 50-fold and incubated for various time periods prior to addition of L-leucine-AMC. The measured activities are given as a percentage of the activities determined for the enzymes incubated in the absence of inhibitor.

4.8. Effects of novel bestatin analogues on *P. c. chabaudi* aminopeptidase activity.

4.8.1. Structure and nomenclature of synthesised bestatin analogues.

1.A. 1,5-diphenyl-4-amino-3-hydroxy-pent-1-ene.

2.B. *N*-Benzoyl-1,4-diphenyl-4-amino-3-hydroxy-but-1-ene

3.C. 1-phenyl-3-hydroxy-4-amino-but-1-ene

4.D. 1,4-diphenyl-4-amino-3-hydroxy-but-1-ene

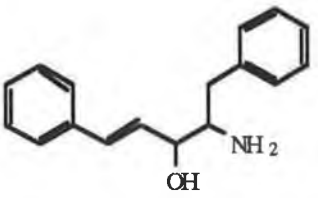
5.E. 4-phenyl-4-amino-3-hydroxy-but-1-ene

6.F. 6-phenyl-5-amino-4-hydroxy-hex-2-ene

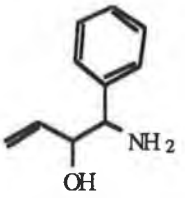
7.G. 5-amino-4-hydroxy-hex-2-ene

8.H. 5-phenyl-5-amino-4-hydroxy-pent-2-ene

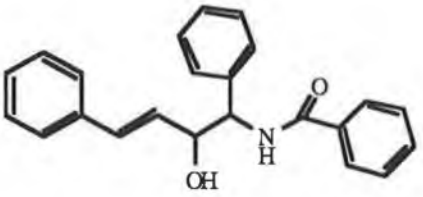
9. [(2S,3R)-3-amino-2-hydroxy-4-phenylbutanoyl]-L-leucine or Bestatin



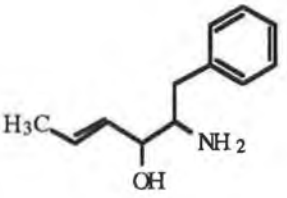
A. 1,5-diphenyl-4-amino-3-hydroxy-pent-1-ene



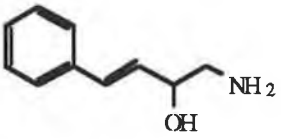
E. 4-phenyl-4-amino-3-hydroxy-but-1-ene



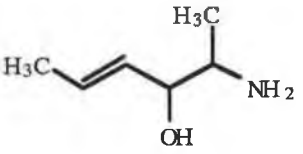
B. *N*-benzoyl-1,4-di-phenyl-4-amino-3-hydroxy-but-1-ene



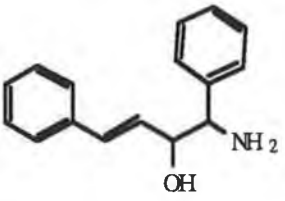
F. 6-phenyl-5-amino-4-hydroxy-hex-2-ene



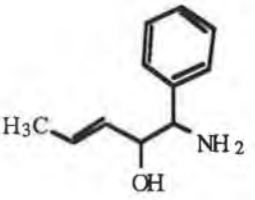
C. 1-phenyl-3-hydroxy-4-amino-but-1-ene



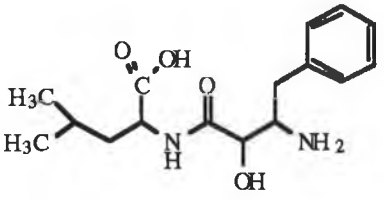
G. 5-amino-4-hydroxy-hex-2-ene



D. 1,4-diphenyl-4-amino-3-hydroxy-but-1-ene



H. 5-phenyl-5-amino-4-hydroxy-pent-2-ene



[(2S,3R)-3-amino-2-hydroxy-4-phenylbutanoy]-L-leucine or Bestatin

4.8.2. Effects of novel bestatin analogues on aminopeptidase activity

Most of these compounds were insoluble in PBS but they were all soluble in DMSO. A control assay was performed with aminopeptidase in PBS and DMSO, pH 7.4 without bestatin and in the presence of bestatin. Comparison of aminopeptidase activity in DMSO and PBS (with and without bestatin) showed that DMSO did not affect aminopeptidase activity in any way. All the activity assays were therefore performed in DMSO, pH 7.4. Each set of compounds consisted of a mixture of the four isomers of the molecule. Activity assays were performed three times in duplicate with molecules A-C. The results obtained with compounds A-C can therefore be considered reproducible, however activity assays with compounds D-H were only performed once in duplicate. It can therefore be justifiably argued that the results obtained with compounds D-H are not necessarily reproducible as they are. For this reason the discussion on this section will be mostly based on compounds A-C although the other molecules will also be referred to.

At concentrations of 2-10 μM bestatin analogues D, A, B and C inhibited aminopeptidase activity, with compound D showing the highest level of inhibition. (fig. 29; Table 3). Compounds E, G and F enhanced aminopeptidase activity (fig. 30; Table 3). at higher concentrations (10-100 μM) of bestatin analogous compounds, only compounds A, B and C continued to inhibit aminopeptidase activity (fig. 29; Table 4), compounds D and F on the other hand enhanced aminopeptidase activity at these concentrations. Compounds E, G and H continued to enhance aminopeptidase activity. (fig. 30; Table 4).

Molecules D and F behave like "allosteric effectors" of aminopeptidase activity (fig. 31).

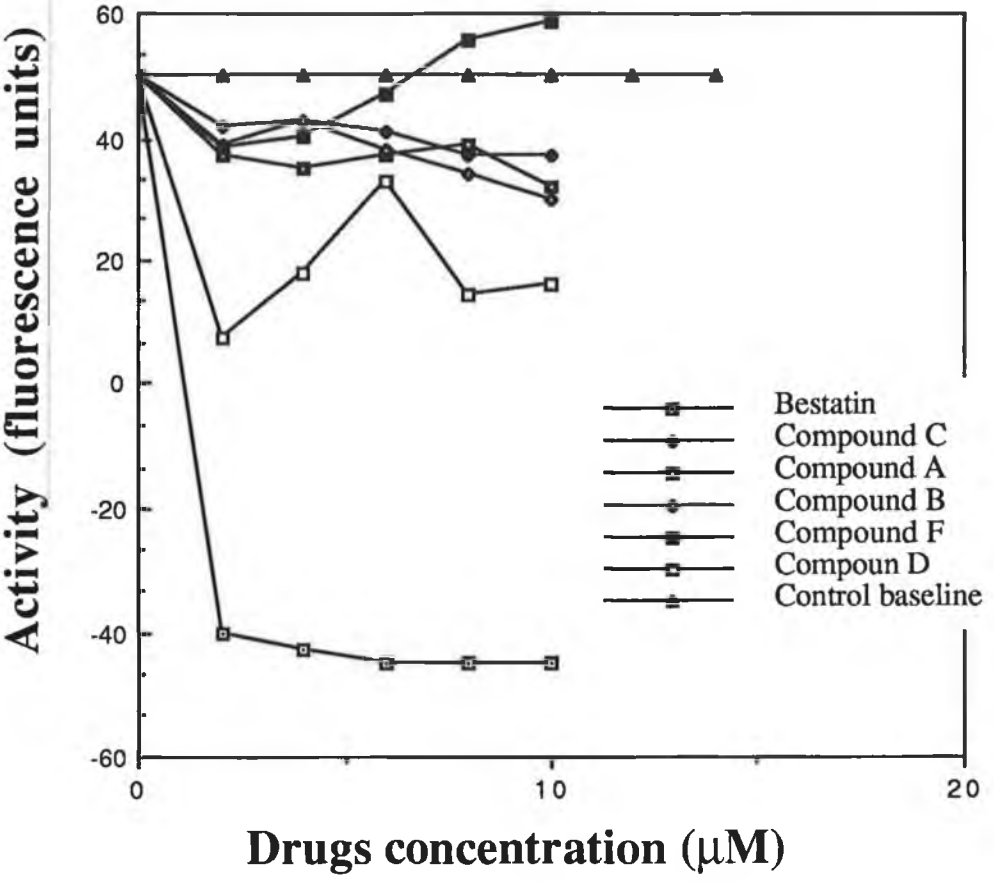


Figure 29. Effects of bestatin analogues on *P. c. chabaudi* aminopeptidase activity. Inhibition of aminopeptidase activity by synthetic bestatin analogous compound. *P. c. chabaudi* purified aminopeptidase was incubated with various concentrations of bestatin analogues. Aminopeptidase activity was detected using the fluorogenic substrate L-leucine-AMC.

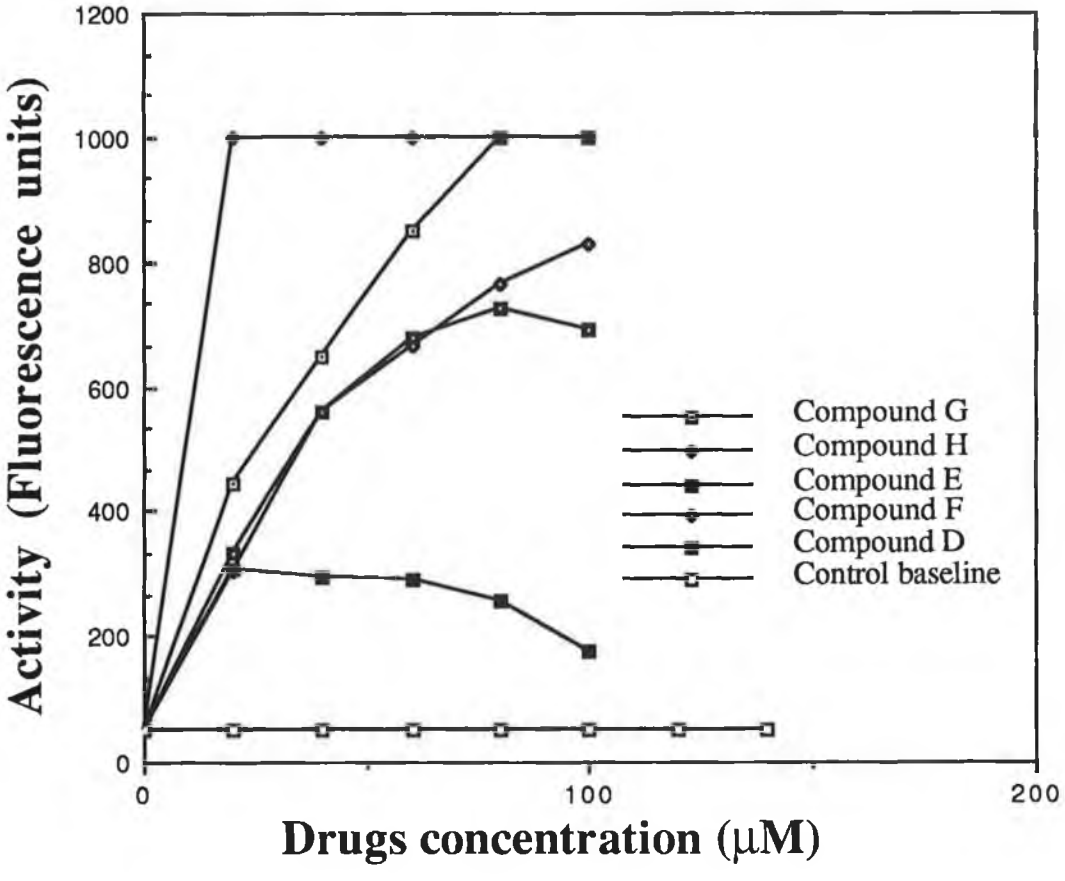


Figure 30. Effects of bestatin analogues on *P. c. chabaudi* aminopeptidase activity. Activation of aminopeptidase activity by synthetic bestatin analogous compound. *P. c. chabaudi* purified aminopeptidase was incubated with various concentrations of bestatin analogues. Aminopeptidase activity was detected using the fluorogenic substrate L-leucine-AMC.

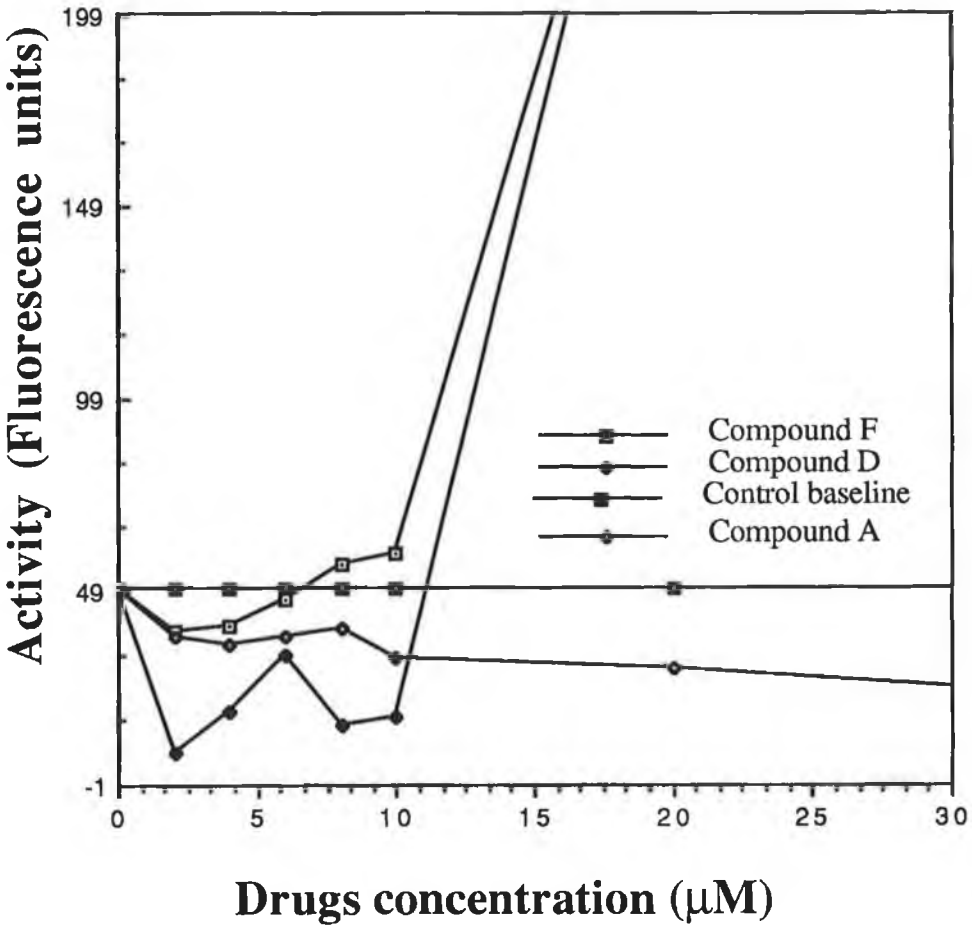


Figure 31. Effects of bestatin analogues on *P. c. chabaudi* aminopeptidase activity. Note-these analogues inhibit aminopeptidase activity at low concentrations but enhance aminopeptidase activity at higher concentrations.

Table 3. Inhibition and activation of aminopeptidase activity by bestatin and bestatin analogues at 2-10 μM

Compounds	**Residual activity (%)**	**Inhibition (%)**	**Activation (%)**
Bestatin	-96	196	
Molecule D	14.6	85.4	
Molecule A	60	40	
Molecule B	64	36	
Molecule C.	74	26	
Molecule F	76	24	
Molecule E	200		100
Molecule G	366		266
Molecule H	808		708

Residual activity when aminopeptidase was preincubated with 2-10 μM bestatin and bestatin analogous molecules.

Table 4. Inhibition and activation of aminopeptidase activity by bestatin and bestatin analogues at 10-100 μM.

Compounds	Residual activity (%)	Inhibition (%)	Activation (%)
Bestatin	-96	196	
Molecule A.	38	62	
Molecule B.	56	44	
Molecule C.	68	32	
Molecule D.	616		516
Molecule E.	1452		1352
Molecule F.	1660		1560
Molecule G.	>2000		>1900
Molecule H.	>2000		>1900

Residual activity when aminopeptidase was preincubated with 10-100 μM bestatin bestatin and bestatin analogous molecules

4.9. Cloning of *P. c. chabaudi* aminopeptidase.

4.9.1. Immunoscreening of *P. c. chabaudi* cDNA expression library.

The titre of the library was calculated as 2.95 x 10^6 pfu/ml for library A and 3.68 x 10^7 pfu/ml for library B

Twenty plates with ~ 2000 pfu were used in the primary screening with 1/100 and 1/1000 dilutions of preabsorbed anti *P. c. chabaudi* aminopeptidase extract. After four rounds of primary screening from each library a total of seven clones expressed proteins specific to the antisera (primary positive clones). These clones were picked and left to diffuse in λ diluent buffer for secondary screening in order to increase the immunoscreening specificity.

4.9.2. PCR screening of *P. c. chabaudi* cDNA expression library

(a) Primers.

The internal primer BB10 (5'-GGWACWTTRACWGGTGCT-3') was designed as a consensus sequence from the bestatin binding site of *E. coli* and Bovine lens leucine aminopeptidases. LF (5'-GGTGGCGACGACTCCTGGAGCCCG-3') and LR (5'-TTGACACCAGACCAACTGGTAATG-3') are universal 1 primers situated on either side of the unique λgt11 EcoR1 cloning site .The

annealing temperature for the universal primers is 45°C-55°C. However the annealing temperature for the internal primer is 40°C-60°C due to the presence of the wobble sequences.

(b) Amplification of DNA fragments by PCR.

Attempts to amplify DNA fragments directly from the cDNA library DNA preparations, using BB10 and λF or λR primers produced amplified fragments in the region of 70 bp. This fragment was obtained only when large quantities of DNA preparations were used (3-6 µl). When smaller quantities were used no amplification was observed. However a DNA preparation from which fragments of 500 and 200 bp had been amplified were supplied by Dr. P. Curley.

In order to minimise non-specific binding of this primer the PCRs were performed with various concentrations of $MgCl_2$. Amplification of the DNA preparations with 1.5, 3 mM $MgCl_2$ and Touch up or Touch down thermocycling programmes, revealed the presence of 5 fluorescent bands of 1000 bp, 500bp, 450bp 200bp, and 170 bp (fig. 32 A & B). BB10 and LF primers were used for this amplification. At different concentrations of magnesium different size fragments were amplified. The bands obtained with 1.5 mM $MgCl_2$ were of very weak intensity for the larger fragments and the fragment of 1000 bp is not visible or absent. PCR performed with 3 mM $MgCl_2$ however revealed high intensity fragments regardless of the size.

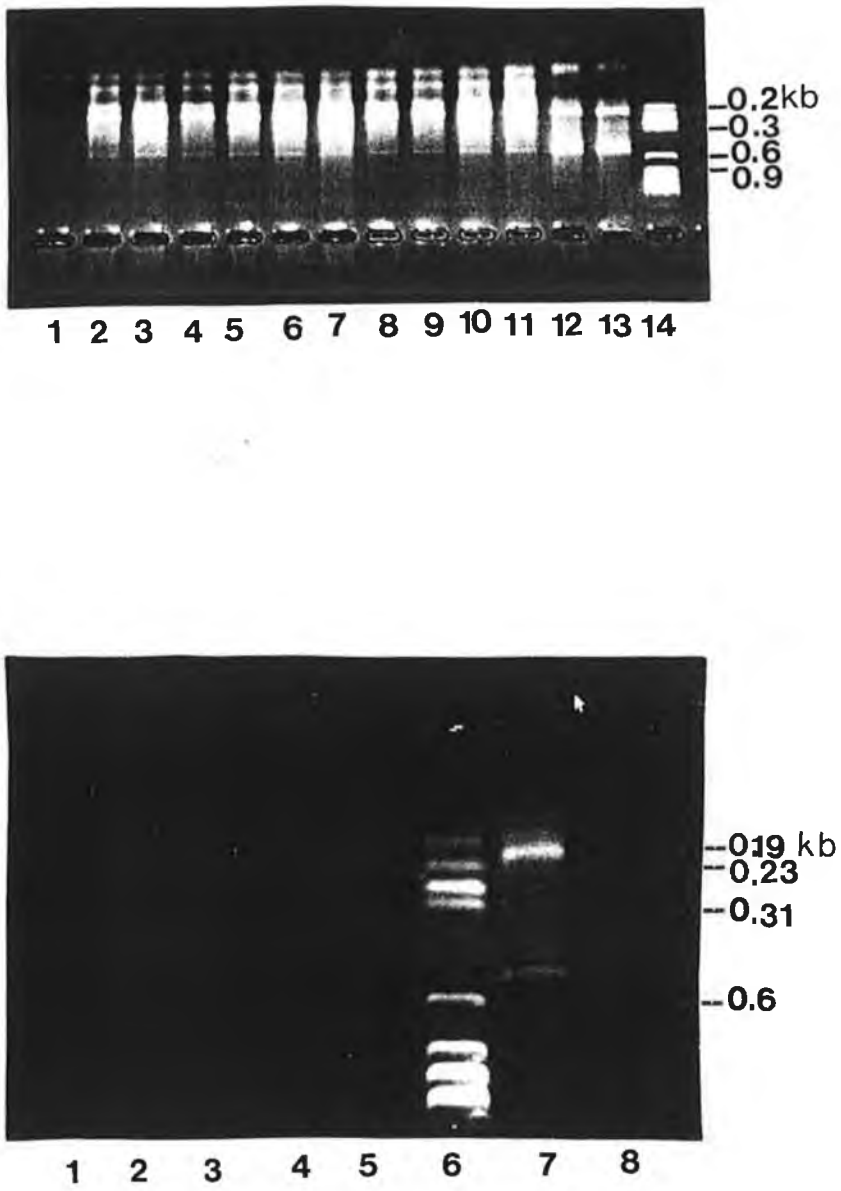


Figure 32. Screening of *P. c. chabaudi* expression library by PCR. (A) PCR was performed in the presence of 1.5 mM (lanes 2-11) and 3 mM (lane 12 & 13) $MgCl_2$ and various increasing amounts of template DNA using touch up thermocycling. Lane 1 is a negative control without template DNA. (B) Fragments were amplified by PCR in the presence of 1.5 mM $MgCl_2$ by touch up (lane 7) and touch down (lanes 2-5) thermocycling. Lanes 1 & 8 are negative controls without template DNA.

(c) Sequencing of amplified DNA fragments.

The amplified DNA fragments were sequenced as described in section 3.8.6 and the Blast Network Service was used to screen the gene bank for homologous sequences. The most homologous proteins and nucleic acid sequences retrieved from the Blast service were *E. coli* proteins or Lambda cloning. The presence of Lambda homologous sequences suggests that the amplified fragments were from *E. coli* competent cells used to amplify the cDNA library. or contamination.

In light of these results the Gene bank was screened for leucine aminopeptidases. These aminopeptidases were aligned in order to detect a consensus aminoacid and nucleotide sequence. Indeed the metal ion (Burley *et al.*, 1992; Wood *et al., 1993*) binding site turns out to be quite conserved and a consensus nucleotide and aminoacid sequence was revealed (see appendix).

Region 1.

R. prowazekii 5'GTT.GGT.AAA.GGA.GTT.ATT.TTT.GAC

L. esculentum 5'GTT.GGA.AAG.GGA.TTA.ACT.TTT.GAC

Cattle kidney 5'GTT.GGG.AAG.GGG.ATT.ACC.TTT.GAC

A. thaliana 5'GTT.GGA.AAA.GGA.TTG.ACG.TTT.GAC

S. tuberosum- 5'GTT.GGA.AAG.GGA.TTA.ACT.TTT.GAC

Consensus- 5'GTT.GGa. AAg. GGA. tTw. ACt.TTT. GAC

R. prowazekii	V	G	**K**	G	V	I	F	D-
L. esculentum	V	G	**K**	G	L	T	F	D-
Cattle kidney	V	G	**K**	G	I	T	F	D
A. thaliana	V	G	**K**	G	L	T	F	D
S. tuberosum	V	G	**K**	G	L	T	F	D
E. coli	V	G	**K**	G	L	T	F	D
Bovine lens	V	G	**K**	G	I	T	F	D-
P. crispum	V	G	**K**	G	L	T	F	D
C. elegans	V	G	**K**	G	L	T	F	D

Potential primers from region 1

Nan.1-(5'GTT GGA AAG GGA TTT ACT TTT GAC-3') Most conserved nucleotide sequence

Nan 1a (5'GTT GGA AAG GGA TTA ACT TTT GAC-3')

Nan 1b-(5'GTT GGA AAG GGA TTG ACT TTT GAC-3')

The Tm for the most conserved nucleotide sequence is 66°C ± 10°C.

These aminoacids in bold have been shown to be involved in metal interaction when bestatin is bound to Bovine lens leucine aminopeptidase (Burley *et al.*, 1992)

Region 2.

R. prowazekii- 5'AAT.ACC.GAT.GCT.GAA.GGT.CGT.TTA

L. esculentum- 5'AAC.ACG.GAT.GCT.GAG.GGT.AGA.CTC

Cattle kidney- 5'AAC.ACC.GAT.GCT.GAG.GGG.AGA.CTT

A. thaliana-----5'AAC.ACA.GAT.GCT.GAA.GGT.CGT.CTA

S. tuberosum- 5'AAT.ACT.GAT.GCT.GAG.GGT.AGG.CTC

Consensus- 5'AAc. ACc. GAT.GCT.GAg. GGT.aGw. CTm

R. prowazekii	N	T	**D**	A	**E**	G	**R**	L
L. esculentum	N	T	**D**	A	**E**	G	**R**	L
Cattle kidney	N	T	**D**	A	**E**	G	**R**	L
A. thaliana	N	T	**D**	A	**E**	G	**R**	L
S. tuberosum	N	T	**D**	A	**E**	G	**R**	L
E. coli	N	T	**D**	A	**E**	G	**R**	L
Bovine lens	N	T	**D**	A	**E**	G	**R**	L
P. crispum	N	T	**D**	A	**E**	G	**R**	L
C. elegans	N	T	**D**	A	**E**	G	**R**	L

primers region 2

Nan.2-(5'AAC ACC GAT GCT GAG GGT-3') most conserved nucleotide sequence.

Nan 2a-(5'AAC ACC GAT GCT GAA GGT-3')

The Tm for the most conserved nucleotide sequence is 56°C ± 10°C.

These aminoacids in bold have been shown to be involved in metal interaction when bestatin is bound to Bovine lens leucine aminopeptidase (Burley *et al.*, 1992)

Region 3.

R. prowazekii---GCC.ACT.CTA.ACA.GGA.GCA-3'

L. esculentum---GCA.ACA.TTA.ACT.GGT.GCT-3'

Cattle kidney----GCC.ACC.CTG.ACA.GGT.GCC-3'

A. thaliana-------GCT.ACT.TTG.ACC.GGG.GCC-3'

S. tuberosum----GCA.ACA. TTA.ACT.GGT.GCT-3'

Consensus---------GCm.ACm.tTa. ACm. GGt.GCm-3'

R.prowazekii -----A T L T G A

L. esculentum ----A T L T G A

Cattle kidney -----A T L T G A

A. thaliana -------A T L T G A

S. tuberosum ----A T L T G A

E. coli ----------A T L T G A

Bovine lens ------A T L T G A

P. crispum -------A T L T G A

C. elegans -------A T L T G A

Primer region 3

5'GC(A/C) AC(A/T) TTA AC(A/T) GGT GC(C/T) which is infact BB10. designed from this region contains mostly C, A or T for every 3' position of the aminoacids. This region is less ideal for primer design than regions 1 and 2

Chapter 5
Discussion

5.1. *Plasmodium chabaudi chabaudi* in CD1 and Balb/C mice

5.1.1. *P. c. chabaudi* infection in mice.

P. c. chabaudi AS strain was lethal to male CD1 and Balb/c mice whereas female CD1 and Balb/c mice infected with the same strain of rodent malaria in the same conditions survived *P. c. chabaudi* infection (fig. 11, section 4.1.2). These observations suggest that survival/death after *P. c. chabaudi* infection is under hormonal control regardless of the strain of infected mice. The female mice are able to develop protective immune mechanisms. The hypothesis that the survival/death after *P. c. chabaudi* infection was under hormonal control and independent of the strain of mice used was similar to studies carried out by Wunderlich and co-workers (Wunderlich *et al.*, 1988). Their studies suggest that this self-healing capacity of female mice against *P. c. chabaudi* AS strain infection is due to the low circulating levels of circulating testosterone. Male mice can exert the same self-healing behaviour after castration. The protective mechanisms exherted by female mice towards *P. c. chabaudi* are not only under the control of testerone but other gonadal factors are thought to be implicated (Schmitt *et al.*, 1990). These protective mechanisms are mediated by splenic non-T cells involving secreting B-cells (Wunderlich *et al.*, 1988). Susceptible mice also show deficiency in oxygen metabolism and Ia expression which may contribute to the suceptibility of these hosts to this intraerythrocytic parasites (Stevenson *et al.*, 1992)

Innate resistance to *P. falciparum* has only been observed in individuals possesing genetically modified haemoglobin, deficiency in G-6-PD (Friedmann *et al.*, 1979; Nagel, 1990; Beutler, 1994), and, individuals who belong to the Duffy negative blood group type (Boyd & Stratman-Thomas).

5.1.2. Observations on the immunological response to *P. c. chabaudi* infection.

A few days after infection circulating immune cells were visible on Giemsa's stained blood smears from both male and female mice, although no parasitised erythrocytes were visible, indicating an immunological response to an infection as most of the immune cells observed at this stage are known to be phagocytes and antigen presenting cells when stimulated by lymphokines. This first immunological response observed a few days after infection could be explained by the inflammatory system being triggered by the inoculation of parasites.

The number of circulating immune cells (phagocytes) decreased as parasitised erythrocytes begun to appear in the circulation in both male and female mice, indicating an immuno-supression reaction. This first immunological response is not sufficient to block parasite devellopement in the host as parasitaemia continued. A second wave of circulating phagocytes appear only in female mice when parasitaemia aproached lethal doses indicating immuno-stimulation. This second wave of immuno-stimulation must be specific to the parasitised erythrocytes as within a week of this reaction there is total clearance of parasitised erythrocytes from the blood stream. The lysed erythrocytes are simultaneously replaced by new red blood cells indicating

activation of hemopoiesis. In male mice parasitaemia augemented until death occured without any detactable rise in circulating immune cells nor stimulation of hemopoiesis.

5.1.3. Transfer of protective immune cells.

Adoptive transfer experiments showed that male mice were able to control *P. c. chabaudi* infection when the infections were performed with infected blood from female mice. This indicates that protective immune cells are transferable from female to male mice. These protective immune cells maintain their protective capacities when transfered to male mice which do not have the natural capacity to induce protective immunity against *P. c. chabaudi*.

5.2. Anti-malarial effects of aminopeptidase inhibitors bestatin and nitro-bestatin.

5.2.1. Inhibition of *P. c. chabaudi* and *P. falciparum* growth *in vitro* by bestatin and nitro-bestatin.

Bestatin and nitro-bestatin both exhibited anti-malarial effects when they were added at various concentrations to *P. c. chabaudi* and *P. falciparum* cultures although they were not as efficient as chloroquine (fig. 13 A & B; section 4.4.1). The implication is that these reagents inhibit growth by blocking malarial aminopeptidase activity, although it is possible that they may interfere with other enzymes. The aminopeptidase inhibitors could inhibit growth of *P. c. chabaudi*

cultures initiated at ring or trophozoites stages. In addition the aminopeptidase inhibitors were most effective against the early trophozoite stage of *P. falciparum*. These inhibitors appear to have a greater anti-malarial effect on the trophozoites. The very stage when most cell metabolism such as haemoglobine degradation, nucleic division, and protein synthesis is taking place (slomianny *et al.*, 1983, Goldberg *et al.*, 1991; fig. 13 B; section 4.4.1).

The IC_{50} obtained for bestatin (1.7 μM) and nitro-bestatin (0.37 μM) in the assay revealed that in comparison to chloroquine these inhibitors were 131 to 28-fold less effective, respectively. However the aminopeptidase inhibitors were equally effective against a chloroquine sensitive clone FCH5.C2 and resistant strain K1/Thailand (fig. 14 A&B, section 4.4.2). It is important to note that the K_I values for bestatin and nitro-bestatin were in the order of 30-fold to 150-fold less than their respective IC_{50} values, which may indicate that these polar molecules do not readily penetrate the erythrocyte membrane and probably the parasite plasma membranes.

Aminopeptidase from *P. c .chabaudi* has been localised in the cytoplasm, associated to ribosomes where it is synthesised and then migrates to associate to pynocytotic vesicles as soon as these are formed (Slomianny *et al.*, 1983) These vesicles empty their contents into the food vacuole where haemoglobin degradation is initiated (Goldberg *et al.*, 1991). The existence of a transport system for haemoglobin derived peptides to be vehicled outside the acid food vacuole is a possibility These observations indicate that bestatin and nitro-bestatin could block aminopeptidase activity either in the cytoplasm where it's optimal activity can be exherted, or/and in the pinocytotic vesicles where maximal activity can not be exherted due to the acidic pH of these vesicles. However enzymes have been reported to be localised and

be physiologically active in cellular environments that did not correspond to their *in vitro* determined pH optimum. Either way these reagents need to cross the erythrocyte, parasite and/or pinocytotic vesicles and digestive vacuole membranes before reaching their site of action.

It follows, therefore, that modifications of these molecules to make them more hydrophobic, lipophilic and osmiophilic may increase their anti-malarial activity as such analogues would cross membrane barriers with greater ease regardless of the localisation of the aminopeptidase. The hydrophobicity of the molecule should be such that the molecule is still soluble in physiological and intracellular fluids. The modifications should preferably improve malarial aminopeptidase inhibition while diminishing mammalian aminopeptidase interaction.

5.2.3. Effects of bestatin on *P. c. chabaudi in vivo.*

(a) Subcutaneous administration of bestatin to P. c. chabaudi infection in mice.

Plots of parasitaemia versus days post infection indicated a delay in increase of parasitaemia in both male and female mice treated subcutaneously with bestatin (fig. 15, section 4.5.2). The higher level of parasitaemia in treated female mice compared to female control mice, suggests an antagonistic effect of bestatin on the female mice's ability to control parasitaemia. The amount of bestatin administered and the frequency of administration does not appear to modify these observations. Subcutaneous administration of 2 mg or 5 mg of bestatin does not improve the overall health of the mice even when the

frequency of admistration was modified inspite of the delay in infection.

This lack of therapeutic effects could be due to 1) The dosage used in these studies was not sufficient to induce therapy. Bestatin being a peptidomimetic is prone to proteolysis by endogenous proteases hence short bioavailability. These reason imply that bestatin would have a high renal clearance rate. Increasing the dosage and the frequency of administration would increase the plasma concentration of bestatin in the renal artery hence decreasing the renal clearance of bestatin and bioavailability. 2) Bestatin circulation may be limited by it's polarity when admistered subcutaneously. Intravenous admistration would deliver the drug closer to it's target. At the same dosage bestatin could show therapeutic effects as expected from the *in vitro* studies. 3) A combination of hypothesis 1 and 2, i.e., increase the dosage and frequency of admistration. Administer the drug closer to it's target therefore intravenously. I decided to maintain the dosage and frequency but administer the drug intravenously.

(b) Intravenous administration of bestatin to P. c. chabaudi infected mice.

When bestatin was administered intravenously at the same dosage. A small delay in infection was observed confirming that the delay observed in s.c treated mice was indeed engendered by the aminopeptidase inhibitor bestatin. Parasitaemia did not exceed 20% in self-healing treated female mice as opposed to 60% (fig. 18-20) when bestatin was administered s.c., indicating a possible increase in bioavailabilty, Most striking was the results obtained with i.v treated male mice, parasitaemia did not exceed 25% unlike the male control

mice and the s.c treated male mice, these male mice were able to control *P. c. chabaudi* infection just like the female mice. These were healthy non-castrated male mice.

(c) Bestatin as an immuno-modulator and a biological modifier.

In both i.v treated female and male mice a decrease in parasitaemia was evident on Giemsa's stained slides and parasitaemia did not exceed 25%. Intravenous administration of bestatin also appeared to induce self-healing in male mice. This would suggest that bestatin treatment helps male mice to override the immunosuppressive effects engendered by the high ciculating levels of testosterone or/and other gonadal factor(s). However, bestatin is not as effective as chloroquine *in vivo* . Chloroquine treatment clearered *P. c. chabaudi* infected erythocytes from the blood stream in 72 hours when administered subcutaneously. Bestatin was only effective as an anti-malarial within the dosage range used in these studies, when administered i.v. This would support the suggestion that the antimalarial effects of bestatin are limited by the dificulty encountered by this compound when crossing membrane barriers . No toxicity was observed with bestatin whether bestatin was administered subcutaneously or intravenously.

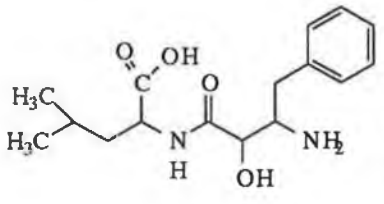
The dosage used in these *in vivo* trials would suggest that the therapeutic effect exherted by (iv) bestatin treatment would be more likely due to the immunomodulatory effects of bestatin which do not require high doses of bestatin rather than a direct antimalarial activity of bestatin. This suggestion would explain the observed diminution of parasitaemia accompanied by a large increase in circulating immune cells when parasitaemia reached approx. 15% in intravenously treated

male mice. Although the same phenomenon of a sudden apparition of lymphocytes is visible in un treated self-healing female mice and is immediately followed by a decrease in parasitaemia. This same phenomenon of a sudden invasion of circulating lymphocytes is not observed in control male mice to whom *P. c. chabaudi* infection is lethal. Therefore male mice would be better models for an intensive bestatin trial for *in vivo* anti-malarial effects. First, because they are incapable of self-healing this malaria unlike female mice, therefore any diminution in parasitaemia will be the consequence of bestatin treatment. Second, although there is a slight apparition of lymphocytes a few days after infection no immune cells increase is observed on Giemsa's stained smears when parasitaemia reaches near the lethal dose of parasitised erythrocytes in control male mice. Therefore any sudden outburst of circulating lymphocytes observed after treatment can be attributed to bestatin treatment as this was indeed the case with male mice after intravenous administration of bestatin.

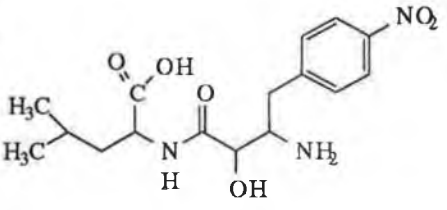
These *in vivo* bestatin trials have shown the immunomodulatory effects of bestatin and chloroquine. Macrophage cytotoxicity was observed as parasites were cleared from the male mice who received intravenous administration of bestatin. The same results were obtained with subcutaneous administration of chloroquine in 72 hours. This would indicate that the half life time of chloroquine is far greater than that of bestatin and that chloroquine has a much easier access to it's site of action than bestatin. Host aminopeptidases may also have been affected by the bestatin treatment. A good antimalarial should be able to target parasite enzymes easily with minimal effect on host enzymes.

5.2.4 Effect of some bestatin analogues on aminopeptidase activity.

Nitro-bestatin is a commercial synthetic analogue of bestatin. These two molecules differ by a single addition of a nitro group on parabenzyl of bestatin. This single addition increases the inhibiting activity of nitro-bestatin by at least five times (fig. 9, Nishizawa *et al.*, 1977) showing that a single chemical modification of natural aminopeptidase inhibitors such as bestatin and amastatin can affect aminopeptidase activity and hence anti-malarial activity.



Bestatin



Nitro-bestatin

There are three functional groups in bestatin, COOH, NH_2, OH and the phenyl group for steric purposes. The carboxyl group was eliminated from all the analogues.

The bestatin analogues synthesised and assayed for aminopeptidase activity showed that although the leucine moiety of bestatin was important for aminopeptidase activity inhibition, aminopeptidase inhibition was still possible (fig. 29, section 4.8.2). In comparison to bestatin, the lower levels of inhibition could be attributed to the elimination of the COOH group in all the analogues analysed. Modification of the amino group by addition of *N*-Boc also lowered the level of inhibition (Compound B, section 4.8.1). The best level of inhibition was obtained with compound A, 1,5-di-phenyl-4-amino-3-hydroxy-pent-1-ene in which these functional groups were maintained with the exception of the carboxyl group. However Compound D showed the highest levels of inhibition at lower concentration (fig. 31). Substitution of the phenyl group by a less bulky alkyl group showed activation of aminopeptidase activity (fig. 30 section). This indicates that the steric effects of the phenyl group are implicated in aminopeptidase inhibition and so is the carboxylic group (Nishizawa *et al.*,1977, Taylor *et al*, 1993) . Substitution of the Leucine in bestatin by methyl showed a dramatic increase in aminopeptidase activity (Compound H). This increase in aminopeptidase activity is still an unclear phenomenon frequently observed with some bestatin analogues (Taylor *et al.*, 1993). All these molecules are in actual fact analogues of 3-amino-2 hydroxy-4-phenyl-butanoic acid (AHPBA)

The concentration of the compounds also appeared to modify the direction of aminopeptidase activity. Two of the compounds investigated for aminopeptidase activity appeared to have allosteric effects on the aminopeptidase (fig. 31, section 4.8.2). At low concentrations they behaved as inhibitors whereas at high concentrations they behaved as activators (Tables 3 & 4).

The results observed can be biased in the sense that the activity assays were carried out with a mixture of the four isomers of each compound. These activity assays should be repeated with the single isomers of each compound. In addition to the polar functional groups the design of bestatin analogues will have to include biosteric requirements to improve aminopeptidase inhibition.

However some inhibitors with low K_I constants on aminopeptidases are strong anti-malarial. Chloroquine showed a K_I = 0.41 mM (Vander jagt *et al.*, 1989) It would be of great scientific and therapeutic interest to investigate the anti-malarial effects of these bestatin analogues.

5.3. Purification and characterization of *P. c. chabaudi* aminopeptidase.

5.3.1. Purification of aminopeptidase from *P.c.chabaudi* cell-free extracts.

The *P. c. chabaudi* aminopeptidase interacted with the anion exchange column QAE-Sepharose equilibrated in 0.1 M Tris-HCl, pH 8.0. This interaction would suggest that this *P. c. chabaudi* aminopeptidase is negatively charged at pH 8.0. This observation is in agreement with the pI detected in *P.c.chabaudi* extracts described by charet *et al.*, (1980). Indeed four pIs were described of 5,85 to 5,5 (Table 5) indicating that at pH 8.0 the aminopeptidase would be charged negatively. The bound fraction containing aminopeptidase activity was eluted with a low ionic

strength of sodium chloride (0.1 M NaCl) suggesting that this *P. c .chabaudi* aminopeptidase is only slightly negatively charged at pH 8.0.

Whether the first purification step was ion-exchange , size-exclusion or affinity chromatography an increase in aminopeptidase activity was observed. This increase in activity after every first purification step could indicate the presence of an endogenous reversible aminopeptidase inhibitor in the crude extract. These purification steps were performed in non-reducing conditions. Therefore the endogenous aminopeptidase inhibitor would not be bound to the aminopeptidase via disulphide bonding. A reversible inhibitor that combines directly to the enzyme can be removed by dialysis or simple dilution to restore full enzymatic activity. This was verified by diluting the crude cell-free extract and comparing the activity with the same volume of undiluted crude cell-free extract. These observations are in agreement with an association-dissociation equilibrium between the enzyme and the inhibitor, where by the dissociation constant is increased upon dilution and during purification. Therefore in the crude extract only part of the enzymatic activity of the aminopeptidase is measured. During the first step of purification the endogenous aminopeptidase inhibitor dissociates from the aminopeptidase thus revealing the full enzymatic activity of the aminopeptidase. However this inhibitor would not be present at a saturating concentration to totally inhibit the aminopeptidase in the crude cell-free extract.

Reducing SDS-PAGE analysis followed by silver staining revealed the presence of several bands with prominent bands in the region of 60-70 k Da. This analysis appears to suggest that 1) Other proteins other than the aminopeptidase bind to the bestatin affinity column. No other proteins other than aminopeptidases have been

purified using bestatin affinity columns as one of the purification steps in my knowledge. In addition no aminopeptidase has been purified in one step by using bestatin affinity columns (Masatoshi *et al.*, 1994 Inversen *et al.*, 1992). 2) The presence of more than one aminopeptidase. The suggestion of four different aminopeptidases in *P. c. chabaudi* with the same apparent molecular mass has previously been reported (*Charet et al.*, 1980). Western blot analysis using polyclonal antisera raised against the ion-exchange purified aminopeptidase (fig., 25, section 4.6.2) revealed four bands in the region of 60-70 k Da. Charet and co. workers describe *P. c. chabaudi* aminopeptidase activity detected by non reducing gel filtration at approximately 90,000 Da. However analysis on an electrofocalising column, revealed four different pIs (5.85, 5.7, 5.6, 5.5) indicating a possibility of four different aminopeptidases with a very close apparent molecular weight of 90,000 Da (Charet *et al* 1980). This molecular weight is also consistent with the 80 ± 10 kDa determined by non-reducing HPLC for the *P. c. chabaudi* aminopeptidase by Curley *et al* (1994). 3). That the aminopeptidase is not a monomeric enzyme. All the proteins revealed by western blot analysis with both antisera (section 4.6.2) appear to suggest that the aminopeptidase(s) is/are (a) monomeric enzyme(s) as no bands are detected below or above the 60-90 kDa region (fig. 25 & 26, section 4.6.2). Direct substrate analysis also localises all aminopeptidase activity in the same region (fig. 23A, section 4.6.1)

SDS-PAGE analysis of the bestatin affinity purified aminopeptidase shows that a band with the same intensity as the co-migrating band in the crude extract is more prominent than the other bands in the same region. This prominent band is situated at approximately 63 kDa (fig. 22B, section 4.6.1). Western blot analysis using polyclonal antisera raised with this same fraction reveals a smear

band in the same region (fig. 26, section 4.6.2). The appearance of a smear rather than distinct bands also indicates the presence of more than one protein interacting with the polyclonal antibodies. Although these aminopeptidases have been classified as leucine aminopeptidases highest enzymatic activity was observed with alanyl- substrates (Charet *et al.*, 1980; Vander jagt et al., 1984). In cannot be excluded that the four aminopeptidases could be a mixture of leucine and alanine aminopeptidases which are genetically different (Sanderink *et al.*, 1988) thus different epitopes could be produced and recognised by different antibodies. However alanine aminopeptidases are generally membrane bound (Sanderink *et al.*, 1988) whereas all these aminopeptidases were cytosolic. To date no plasmodial aminopeptidase sequence is available. A summary of aminopeptidases from *P. c. chabaudi* and *P. falciparum* is presented in tables 5 & 6.

Table 5 . Aminopeptidases from rodent malaria.

Author	Source	App. mw. (kDa.)	pH optimum	pI	Temp. optimum (°C)	Inhibiting ions	Activating ions	Effect of antimalarials
Charet *et al.*, 1980	*P. c. chabaudi*	90	7.2	5.85 5.7 5.6 5.5	52	Co^{2+} Zn^{2+} Ca^{2+} Mn^{2+}	Ca^{2+} Mg^{2+}	Chloroquine ++++ Quinacrine + Primaquine ++ Quinine -
Gyang *et al.*, 1982	*P. yoelii nigeriensis*	90	7.2	5.3	42	$\pm Hg^{2+}$ $\pm Zn^{2+}$ $\pm Mn^{2+}$	Ca^{2+} Mg^{2+}	
Curley *et al.*, 1994	*P. c. chabaudi*	80 ± 10	7.2	ND	50	Mn^{2+} Hg^{2+} Cd^{2+} $\pm Zn^{2+}$		
Curley *et al.*, 1994	*P. berghei*	80 ± 10	7.2	ND	42	Mn^{2+} Hg^{2+} Cd^{2+} $\pm Zn^{2+}$	Ca^{2+}. Mg^{2+} Mn^{2+}	
This work	*P. c. chabaudi*	63±10		ND				Chloroquine ++++ Nitro-bestatin +++ Bestatin ++

App. mw. (apparent molecular weight); Temp (temperature).

Table 6 . Aminopeptidases from human malaria.

Author	Source	App.mw. (kDa.)	pI	pH opt.	Temp. opt. (°C)	Inhibiting ions	Act. ions	Effect of antimalarials
Gyang *et al.*, 1982	*P. falciparum*	186	6.05	7.5		Hg^{2+}, Zn^{2+}, Cd^{2+}	Ca^{2+}	Amodiaquin ++++ Chloroquine +++ Primaquine -
Vander jagt *et al.*, 1984	*P. falciparum*	100	6.8	7.2		Hg^{2+}, Zn^{2+}		Chloroquine ++++ Mefloquine ++ Quinacrine + Quinine - Primaquine -
vander jagt *et al.*, 1987	*P. falciparum*							Chloroquine ++++
	Chloroquine S (HB3)	63		7.5				
	Chloroquine R (NC1)	63		7.5				
Curley *et al.*, 1994	*P. falciparum*	> 80	N.D	7.2	50-55	Mn^{2+}, Hg^{2+}, Cd^{2+}		
This work								Chloroquine ++++ Nitro-bestatin +++ Bestatin ++

App. mw (apparent molecular weight); opt. (optimum); Temp (temperature); Act. (activating) S. chloroquine (sensitive), R. (resistant)

5.4. Kinetic characterization of purified *P. c. chabaudi* aminopeptidase.

The kinetic studies of this aminopeptidase show that the malarial aminopeptidase extracted from *P .c. chabaudi* parasites as described earlier is a metallo-aminopeptidase although the metal co-factor has not yet been determined, moderately thermophilic with a temperature optimum of 35-45°C and has a near neutral pH optimum of 7.2 (Curley *et al.*, 1994). The malarial aminopeptidase showed simple Michaelis-Menten kinetics with respect to the substrate L-leucine-AMC, Lineweaver-burk plots were used to determine a K_M = 20 µM and a Vmax=5.6 µM AMC released/ml/min/mg protein (fig. 27B, section 4.7.2). The stoichiometry of the catalysed reaction has not yet been determined experimentally. These results confirm the results obtained with crude cell extract *P. c. chabaudi* aminopeptidase (section 4.3).

5.4.1. Inhibition kinetics of *P. c. chabaudi* aminopeptidase by bestatin and nitro-bestatin.

The natural aminopeptidase inhibitor bestatin (Umezawa *et al.*, 1976) and it's derivative nitro-bestatin were both potent competitive inhibitors of the purified aminopeptidase. Dixon plots were used to determine a K_I = 50.7 nM for bestatin and K_I = 2.51 nM for nitro-bestatin. Nitro-bestatin was 5-10 times more potent an inhibitor than bestatin.

The binding of bestatin to purified malarial aminopeptidase and porcine kidney aminopeptidase was shown to be fully reversible. However, the binding and dissociation of bestatin to the purified malarial aminopeptidase appeared to be rapid as the malarial aminopeptidase regained activity immediately upon dilution and the degree of inhibition was totally independent of the time of incubation, in accordance (fig. 28, section 4.7.3) with the Michaelis-Menten theory. This observation would exclude bestatin from the "slow-binding, tight-binding inhibitors" category in which bestatin is suggested to belong. In contrast, the binding and dissociation of bestatin to porcine kidney aminopeptidase is time-dependent as the degree of inhibition appears to increase with time (fig. 28, section 4.7.3.). This time-dependence is superficially similar to that observed with an enzyme undergoing irreversible inhibition. The same superficial similarity to irreversible inhibition was observed with other plant and mammalian aminopeptidases (Wilkes *et al.*, 1985, Burley *et al.*, 1991). However the inhibition of porcine kidney and other plant and mammalian aminopeptidases by bestatin has been shown to be fully but slowly reversible by greatly diluting the enzyme:inhibitor complex, and then incubating for periods of 3-48 hours prior to assay (Wilkes *et al.*, 1985). This observation would maintain bestatin in the "slow-binding, tight binding inhibitor" category. These molecules usually resemble the transition state for the substrate-bound enzyme (Burley *et al.*, 1991) which accounts both for their very low inhibitor constants (K_I for bestatin is typically in the range of 10^{-8}-10^{-9}M) and their slow binding/dissociation kinetics (Wilkes *et al.*, 1985).

This difference in binding and dissociation of bestatin to malarial and mammalian aminopeptidases could indicate that the bestatin binding

site of this malarial aminopeptidase differs from that of the mammalian aminopeptidases.

5.5. Cloning of *P. c. chabaudi* aminopeptidase.

Screening of *P. c. chabaudi* cDNA expression library.

The amplification was performed on cDNA templates which are complementary to the mRNA transcripts. The non specificity of the amplified DNA fragments can be explained by the presence of wobble sequences in the internal primer BB10. The use of touch up and touch down thermocycling increases non-specific annealing of the primers The wobble sequences in the primers also indicate that the region of the bestatin binding site from which this primer was designed is not a very "conserved" region in terms of the nucleotide sequence. When thermocycling is performed by the touch up or touch down programme the annealing temperature is gradually increased or decreased. At some of these temperatures the non specific primer anneals to the cDNA to prime cDNA amplification. This annealing can happen at different sites. In one reaction five fragments of different sizes ranging from 1000 bp-170 bp (fig. 32A, section 1.9.1). The weaker fragments correspond to the fragments annealed and polymerised at the temperatures at the end of the thermocycling programme as they are submitted to less polymerising cycles. The stronger fragments correspond to those submitted to the highest number of polymerising cycles and hence to fragments annealed at temperatures close to the beginning of the thermocycling programme.

The fragments at 1000 and 500 bp are annealed at low temperatures as they are only visible with the touch up thermocycling programme (fig. 32A, section 4.9.1.).

Regions 1 and 2 contain aminoacids residues that have been shown to be involved in the interaction with the metal ions (commonly Zn^{2+}. Mg^{2+} or Mn^{2+}) described from the three dimensional structure of bovine lens LAP complexed with bestatin. Hence this site is also the active site (Burley *et al., 1992*). Regions 1, 2 and 3 are well conserved in all the leucine aminopeptidase proteins available in the Blast Network Service except *Aeromonus proteolytica* (Guenet *et al.*, 1992) leucine aminopeptidase which does not align with these conserved regions (data not shown). The aminoacids which interact with bestatin are also well conserved and are located in these 3 regions (Burley *et al., 1992*; Wood *et al.*, 1993). From the computed consensus nucleotide sequence corresponding to the most conserved nucleotide sequence, more specific primers have been designed. BB10., was designed from region 3 using Bovine lens and *E. coli* leucine aminopeptidase only, however inclusion of other nucleotide sequences does not increase the specificity of BB10., design in this region. Region 3 is not an ideal region for primer designing.

5.6. Summary and suggestions.

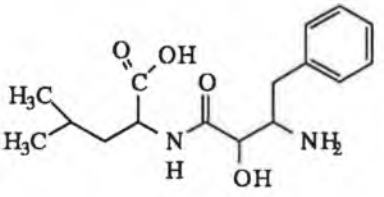
Two methods have been described to obtain highly purified aminopeptidase (>100-fold) in two steps. The *P. c. chabaudi* aminopeptidase activity is probably composed of four different leucine aminopeptidase with molecular weights ranging from 60-90 kDa. The enzyme is a metallo-protease although Zn^{2+} does not appear to be the metal co-factor for enzymatic activity. The enzyme is moderately thermophilic and shows simple Michaelis-Menten kinetics with respect to L-leucine-AMC. It is inhibited in a competitive manner by bestatin and nitro-bestatin. However the bestatin binding site of this enzyme appears to differ from the mammalian bestatin binding site. Aminopeptidase inhibitor bestatin shows potential anti-malarial activity both in vivo and in vitro. Primers Nan1 and Nan2 were designed from the most conserved regions with respect to the nucleotide sequence and aminoacid sequence. These primers can be used to screen the cDNA library as labelled nucleotide probes or as PCR primers. They are specific without wobble sequences.

Drug designing and delivery.

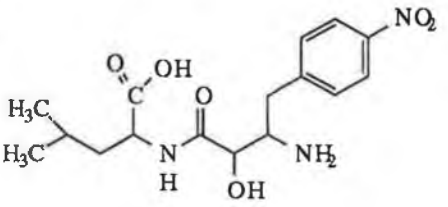
For a drug to be effective it must be effectively delivered primarily to the infected erythrocytes and minimally to other parts of the body. Use of carrier molecules such as glycoproteins or antibodies with recognition sites on the surface of the malarial parasites cell surface have shown increased therapeutic effects (Singh *et al.*, 1993). The

bestatin binding studies revealed possible differences between mammalian and malarial aminopeptidases at the active site. Attaching bestatin or an effective bestatin analogue to this antibody can be used for site specific drug targeting. However the malarial aminopeptidase has not yet been localised. Only one description of *P. c. chabaudi* aminopeptidase localisation has been reported so far to my knowledge (Slomianny *et al.*, 1983). To confirm this report localisation studies could be attempted by using the fluorogenic substrate leucine AMC. The idea being that when the fluorogenic substrate is added to malarial parasites it will bind to the aminopeptidase and the site of binding could be visualised by fluorescent microscopy. Monoclonal antibodies against the malarial aminopeptidase if they are found to be strictly selective for malarial aminopeptidase would be even better candidates to localise the aminopeptidase.

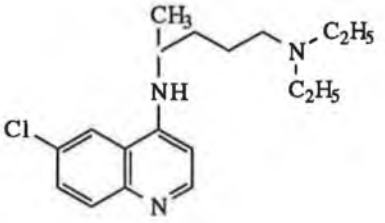
The *in vivo* and *in vitro* antimalarial studies suggested that the polarity of bestatin and nitro bestatin was interfering the anti-malarial effects of these aminopeptidase inhibitors. However, activity assays have shown that the addition of a polar group NO_2 on the phenyl ring of bestatin to produce nitro-bestatin created a more effective aminopeptidase inhibitor and this higher efficiency was observed when *P. falciparum* was cultured *in vivo* in the presence of nitro-bestatin Elimination or modification of any functional polar groups reduces the inhibiting effects of some bestatin analogues (Table 3 & 4 section 4.8.2). These polar groups and the bulky phenyl ring appear to be essential for aminopeptidase activity inhibition. However polar molecules are not the best candidates to cross membrane barriers but transport systems for this type of molecules do exist in the membranes (glucose transporter for example, (Wingard *et al.*, 1991).



Bestatin



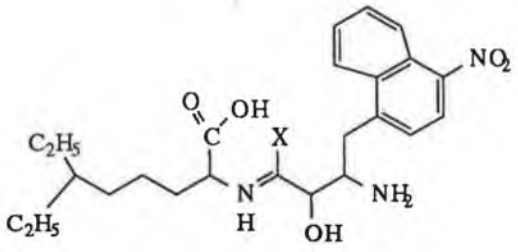
Nitro-bestatin



Chloroquine

Bestatin could be rendered less prone to proteolysis by blocking the peptide bond hence making bestatin or nitro-bestatin a non-hyrolysable peptidomimetic. This may decrease the renal clearance of bestatin and therefore the required dose to obtain a therapeutic effect. The alkyl chain of leucine could be increased as this would increase liposolubility of bestatin. Addition of an extra phenyl would make the molecule almost analogous to chloroquine. The resulting molecule would resemble chloroquine yet maintain the functional groups of bestatin, hence maybe the mechanism by which chloroquine is delivered to it's site of action could be appropriate for this molecule.

This suggested molecule would look like this:



Chloroquine/Bestatin analogue

X should be an electronegative atom like Cl or F.

5.7. Conclusion.

Malarial leucine aminopeptidase activity from *P. c. chabaudi* is probably composed of four monomeric enzymes with a very close apparent molecular weight of 63 ± 10 kDa. An N-terminal sequence on the four bands revealed by Western blotting needs to be performed in order to confirm this suggestion. It is a moderately thermophilic enzyme with a near neutral pH optimum of 7.2 and displays optimum activity at 50-55°C. This aminopeptidase could be implicated in the degradation of haemoglobin derived peptides, into free aminoacids, used by the parasite for nutrition and growth. Due to the acidic pH of the food vacuole in which haemoglobin degradation is initiated, a transport system (Gero & Kirk, 1994) of haemoglobin derived peptides from the food vacuole towards a near neutral cellular environment would be essential for the aminopeptidase to display optimum activity. However it is possible that in physiological conditions this enzyme could degrade haemoglobin derived peptidases in the food vacuole, as

enzymes have been shown to have physiological activity in cellular environments with different pHs as those observed *in vitro.*

The aminopeptidase inhibitor bestatin and it's synthetic analogue nitro-bestatin have been shown to have antimalarial effects on *P. c. chabaudi* and *P. falciparum* chloroquine resistant and sensitive strains. In addition the binding kinetics of bestatin to the malarial aminopeptidase have been shown to differ from those observed with mammalian aminopeptidases. These findings indicate a possible difference in the active site of the malarial and mammalian aminopeptidase that could be exploited for selective antimalarial drugs design.

Bestatin is currently being used as a therapeutic agent for various diseases due to it's effects as a biological modifier and an analgesic agent. For the first time it is shown in this thesis that aminopeptidase inhibitor bestatin or it's analogues are also potential antimalarial agents. However a more intensive study on the *in vivo* antimalarial effects of bestatin is necessary.

Primers / nucleotide probes have been designed from a conserved region of leucine aminopeptidases in terms of aminoacid and nucleotide sequence. These primers can be used for future cloning of plasmodial leucine aminopeptidases.

Chapter 6
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6.0. References.

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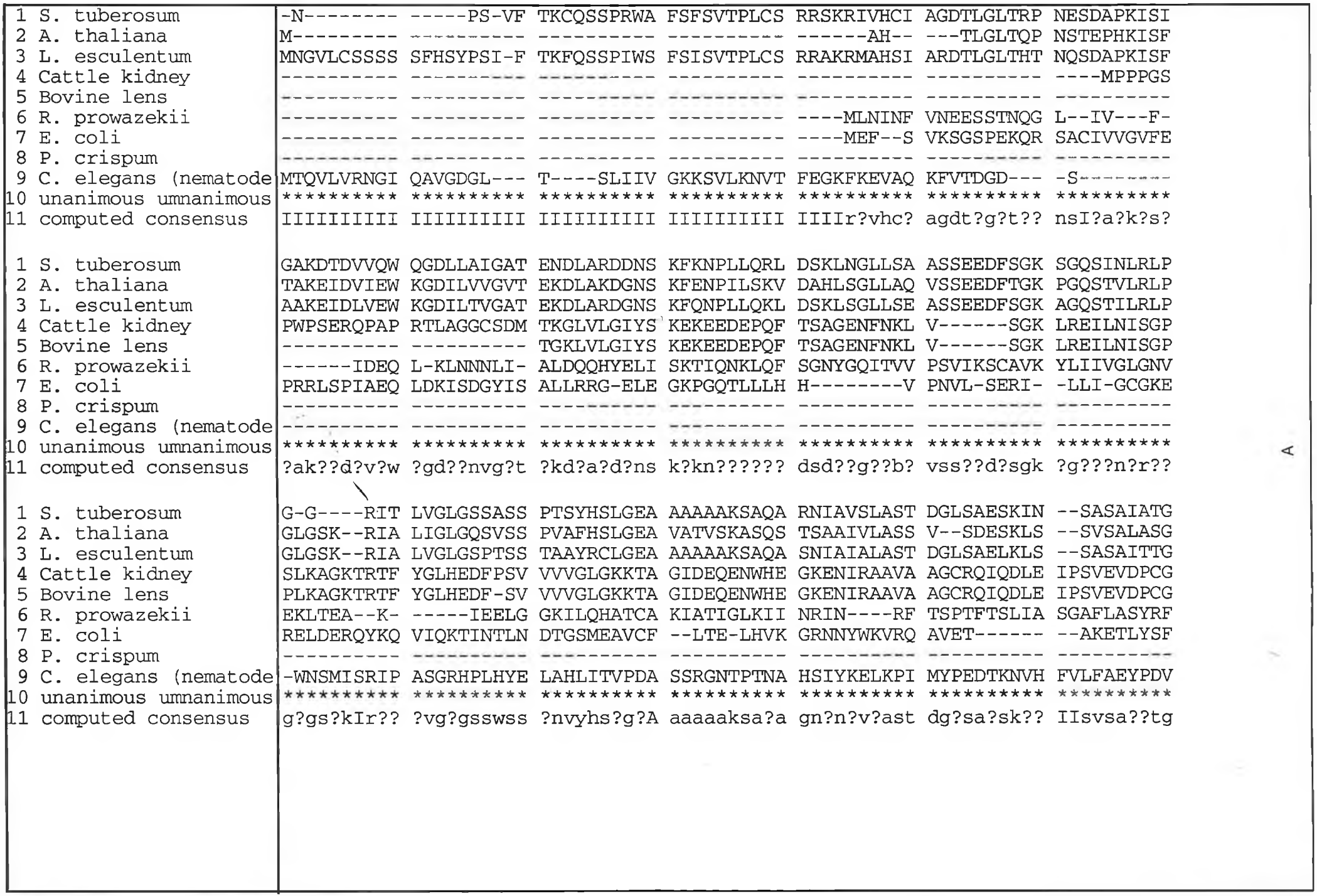
APPENDIX

Alignment of Leucine aminopeptidases.

Leucine aminopeptidase sequences were retrieved from the Blast Network Service and aligned using the SeqEd multiple alignment programme. Regions 1, 2, and 3 are highlighted in the computed consensus and contain aminoacids involved in bestatin and metal ion interactions. (Burley *et al.*, 1992).


1 S. tuberosum -N-------- -----PS-VF TKCQSSPRWA FSFSVTPLCS RRSKRIVHCI AGDTLGLTRP NESDAPKISI
2 A. thaliana M--------- ---------- ---------- ---------- ------AH-- ---TLGLTQP NSTEPHKISF
3 L. esculentum MNGVLCSSSS SFHSYPSI-F TKFQSSPIWS FSISVTPLCS RRAKRMAHSI ARDTLGLTHT NQSDAPKISF
4 Cattle kidney ---------- ---------- ---------- ---------- ---------- ---------- ----MPPPGS
5 Bovine lens ---------- ---------- ---------- ---------- ---------- ---------- ----------
6 R. prowazekii ---------- ---------- ---------- ---------- ----MLNINF VNEESSTNQG L--IV---F-
7 E. coli ---------- ---------- ---------- ---------- ----MEF--S VKSGSPEKQR SACIVVGVFE
8 P. crispum ---------- ---------- ---------- ---------- ---------- ---------- ----------
9 C. elegans (nematode MTQVLVRNGI QAVGDGL--- T----SLIIV GKKSVLKNVT FEGKFKEVAQ KFVTDGD--- -S--------
10 unanimous umnanimous ********** ********** ********** ********** ********** ********** **********
11 computed consensus IIIIIIIIII IIIIIIIIII IIIIIIIIII IIIIIIIIII IIIIr?vhc? agdt?g?t?? nsI?a?k?s?
1 S. tuberosum GAKDTDVVQW QGDLLAIGAT ENDLARDDNS KFKNPLLQRL DSKLNGLLSA ASSEEDFSGK SGQSINLRLP
2 A. thaliana TAKEIDVIEW KGDILVVGVT EKDLAKDGNS KFENPILSKV DAHLSGLLAQ VSSEEDFTGK PGQSTVLRLP
3 L. esculentum AAKEIDLVEW KGDILTVGAT EKDLARDGNS KFQNPLLQKL DSKLSGLLSE ASSEEDFSGK AGQSTILRLP
4 Cattle kidney PWPSERQPAP RTLAGGCSDM TKGLVLGIYS KEKEEDEPQF TSAGENFNKL V------SGK LREILNISGP
5 Bovine lens ---------- ---------- TGKLVLGIYS KEKEEDEPQF TSAGENFNKL V------SGK LREILNISGP
6 R. prowazekii ------IDEQ L-KLNNNLI- ALDQQHYELI SKTIQNKLQF SGNYGQITVV PSVIKSCAVK YLIIVGLGNV
7 E. coli PRRLSPIAEQ LDKISDGYIS ALLRRG-ELE GKPGQTLLLH H--------V PNVL-SERI- -LLI-GCGKE
8 P. crispum ---------- ---------- ---------- ---------- ---------- ---------- ----------
9 C. elegans (nematode ---------- ---------- ---------- ---------- ---------- ---------- ----------
10 unanimous umnanimous ********** ********** ********** ********** ********** ********** **********
11 computed consensus ?ak??d?v?w ?gd??nvg?t ?kd?a?d?ns k?kn?????? dsd??g??b? vss??d?sgk ?g???n?r??
1 S. tuberosum G-G----RIT LVGLGSSASS PTSYHSLGEA AAAAAKSAQA RNIAVSLAST DGLSAESKIN --SASAIATG
2 A. thaliana GLGSK--RIA LIGLGQSVSS PVAFHSLGEA VATVSKASQS TSAAIVLASS V--SDESKLS --SVSALASG
3 L. esculentum GLGSK--RIA LVGLGSPTSS TAAYRCLGEA AAAAAKSAQA SNIAIALAST DGLSAELKLS --SASAITTG
4 Cattle kidney SLKAGKTRTF YGLHEDFPSV VVVGLGKKTA GIDEQENWHE GKENIRAAVA AGCRQIQDLE IPSVEVDPCG
5 Bovine lens PLKAGKTRTF YGLHEDF-SV VVVGLGKKTA GIDEQENWHE GKENIRAAVA AGCRQIQDLE IPSVEVDPCG
6 R. prowazekii EKLTEA--K- -----IEELG GKILQHATCA KIATIGLKII NRIN----RF TSPTFTSLIA SGAFLASYRF
7 E. coli RELDERQYKQ VIQKTINTLN DTGSMEAVCF --LTE-LHVK GRNNYWKVRQ AVET------ --AKETLYSF
8 P. crispum ---------- ---------- ---------- ---------- ---------- ---------- ----------
9 C. elegans (nematode -WNSMISRIP ASGRHPLHYE LAHLITVPDA SSRGNTPTNA HSIYKELKPI MYPEDTKNVH FVLFAEYPDV
10 unanimous umnanimous ********** ********** ********** ********** ********** ********** **********
11 computed consensus g?gs?kIr?? ?vg?gsswss ?nvyhs?g?A aaaaaksa?a gn?n?v?ast dg?sa?sk?? IIsvsa??tg

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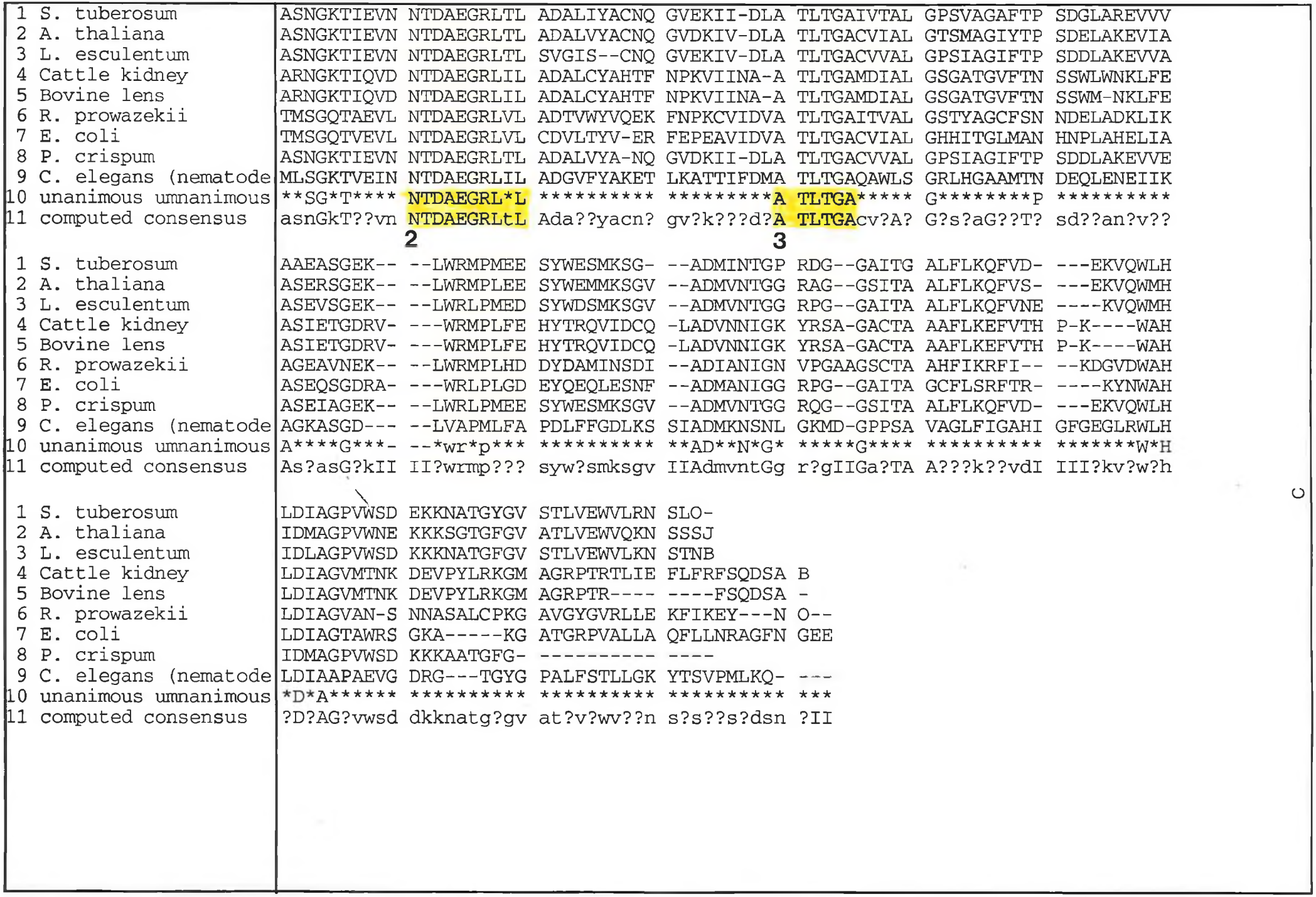
 1 S. tuberosum          VMLGIFEDNR FRSESKTPAL ESLDILGLGT GPEIESKIKY AEHVCAGVIL GRELVNA--P ANIVTPGALA
 2 A. thaliana           IVLGLFEDGR YKSESKKPSL KAVDIIGFGT GAEVEKKLKY AEDVSYGVIF GRELINS--P ANVLTPAVLA
 3 L. esculentum         AVLGTFEDNR FKSESKKPTL KSLDILGLGT GPEIEKKIKY AADVCAGVIL GRELVNA--P ANVLTPAVLA
 4 Cattle kidney         DAQAAAEGAV LGLYEYDDLK QKRKVVVSAK LHGSEDQEAW QRGVLFASGQ NLARRLMETP ANEMTPTKFA
 5 Bovine lens           DAQAAAEGAV LGLYEYDDLK QKRKVVVSAK LHGSEDQEAW QRGVLFASGQ NLARRLMETP ANEMTPTKFA
 6 R. prowazekii         HKYKTTLKEV E----K--FA VESIEILTDN NSEAMKLFEV KKL-IAEAVF FTRDISNE-P SNIKTPPSLC
 7 E. coli               DQLKTNKSEP RRPLRKMVFN VPTRRELTSG ER-A-----I QHGLAIAAGI KAAKDLGNMP PNICNAAYLA
 8 P. crispum            ---------- ---------- ---------- ---------- ---------- ---------- ----------
 9 C. elegans (nematode  LSHVAAIART FCKFSMKTSG IRELNVNIDV VCDKLTNEDA VFLTDLSESV RETARLIDTP ANILTTDALV
10 unanimous umnanimous  ********** ********** ********** ********** ********** ********** *_********
11 computed consensus    dv?gw??dnr ?rs?skk??? ?s?d??g?gt g????kk?ky a?gvcagv?? gr??v?a?I? an??T?aa?a

 1 S. tuberosum          EEAKKIASTY SDVITVNILD AEQCKELK-M GAYLGVAAAA TENPPYFIHL CFKTNSRERK TKIALVGKGL
 2 A. thaliana           EEAAKVASTY SDVFTANILN EEQCKELK-M GSYLAVAAAS A-NPPHFIHL VYKPPNGSVK TKLALVGKGL
 3 L. esculentum         EEAKKIASTY SDVFSANILD VEQCKELK-M GSYLRVAAAS A-NPAHFIHL CYKPSSGEIK KKIALVGKGL
 4 Cattle kidney         EIVEENLKSA SIKTDVFIR- PKSWIEEQEM G-SFLSVAKG SEEPPVFLEI HYKGSPNASE PPLVFVGKGI
 5 Bovine lens           EIVEENLKSA SIKTDVFIRR PKSWIEEQEM GESFLSVAKG SEEPPVFLEI HYKGSPNASE PPLVFVGKGI
 6 R. prowazekii         RKNSJDTJTA WSKYJCYRJT JYON---LGM GALL-GVGQG SQNESKLVVM EYKGG-SRDD STLALVGKGV
 7 E. coli               SQARQLADSY SKNVITRVIG EQQMKELGMH SYLAVG00QG SQNESLMSVI EYKGNASEDA RPIVLVGKGL
 8 P. crispum            ----KIASLY SDVISATILD VEQCKELKM- GSYLGVAAAA SENPAHFIHL CYKPPSGTVK SKLALVGKGL
 9 C. elegans (nematode  DEAVKVGNAT GS--KITVIR GEELLKAGFG GIYHVGK-AG PTPPA-FVVL --SHEVPGST EHIALVGKGV
10 unanimous umnanimous  ********** ********** ********** ********** ********** ********** *****VGKG*
11 computed consensus    ??akk?asty sdv?tvn??d ???ck??k?m Gvy?gvaaag s?n??h??h? cykgnsg?sk tk?a?VGKG?
                                                                                                1

 1 S. tuberosum          TFDSGGYNLK TGAGSKIELM KNDMGGAAAV LGAAKALGEI KPRGVEVHFI VAACENMISG AGMRPGDIVT
 2 A. thaliana           TFDSGGYNIK TGPGCSIELM KFDMGGSAAV LGAAKAIGEI KPPGVEVHFI VAACENMISG TGMRPGDVIT
 3 L. esculentum         TFDSGGYNIK TGPGCSIELM KFDMGGAAAV LGAAKALGQI KPAGVEVHFI VAACENMISG TGMRPGDIIT
 4 Cattle kidney         TFDSGGISIK AAANMD--LM RADMGGAATI CSAIVSAAKL DLPINIVGLA PL-CENMPSG KANKPGDVVR
 5 Bovine lens           TFDSGGISIK AAANMD--LM RADMGGAATI CSAIVSAAKL DLPINIVGLA PL-CENMPSG KANKPGDVVR
 6 R. prowazekii         IFDTGGISLK PSSNMH--LM RYDMAGSAAV VGTIIALASQ KVPVNVVGVV GLV-ENMQSG NAQRPGDVVV
 7 E. coli               TFDSGGISIK PSEGMD-E-M KYDMCGAAAV YGVMRMVAEL QLPINVIGVL AG-CENPGGG RAYRPGDVLT
 8 P. crispum            TFDSGGYNIK TGPGCSIELM KFDMGGSAAV LGAAKAI--I KPAGVEVHFI VAACENMISG AGMRPGDILT
 9 C. elegans (nematode  VYDTGGLQIK TKTGMPN--M KRDMGGAAGM LEAYSALVKH GFSQTLHACL CIV-ENNVSP IANKPDDIIK
10 unanimous umnanimous  **D*GG***K ***G*****M **DM*G*A** ********** ********** ****EN**** ****P*D***
11 computed consensus    T?DsGGyn?K tgagmn???m k?dmGGaAav ?gAaka?r?? k??gv?vh?? vaaCENm?sG armrPGd?vt
```

B


1 S. tuberosum ASNGKTIEVN NTDAEGRLTL ADALIYACNQ GVEKII-DLA TLTGAIVTAL GPSVAGAFTP SDGLAREVVV
2 A. thaliana ASNGKTIEVN NTDAEGRLTL ADALVYACNQ GVDKIV-DLA TLTGACVIAL GTSMAGIYTP SDELAKEVIA
3 L. esculentum ASNGKTIEVN NTDAEGRLTL SVGIS--CNQ GVEKIV-DLA TLTGACVVAL GPSIAGIFTP SDDLAKEVVA
4 Cattle kidney ARNGKTIQVD NTDAEGRLIL ADALCYAHTF NPKVIINA-A TLTGAMDIAL GSGATGVFTN SSWLWNKLFE
5 Bovine lens ARNGKTIQVD NTDAEGRLIL ADALCYAHTF NPKVIINA-A TLTGAMDIAL GSGATGVFTN SSWM-NKLFE
6 R. prowazekii TMSGQTAEVL NTDAEGRLVL ADTVWYVQEK FNPKCVIDVA TLTGAITVAL GSTYAGCFSN NDELADKLIK
7 E. coli TMSGQTVEVL NTDAEGRLVL CDVLTYV-ER FEPEAVIDVA TLTGACVIAL GHHITGLMAN HNPLAHELIA
8 P. crispum ASNGKTIEVN NTDAEGRLTL ADALVYA-NQ GVDKII-DLA TLTGACVVAL GPSIAGIFTP SDDLAKEVVE
9 C. elegans (nematode MLSGKTVEIN NTDAEGRLIL ADGVFYAKET LKATTIFDMA TLTGAQAWLS GRLHGAAMTN DEQLENEIIK
10 unanimous umnanimous **SG*T**** NTDAEGRL*L ********** *********A TLTGA***** G********P **********
11 computed consensus asnGkT??vn NTDAEGRLtL Ada??yacn? gv?k???d?A TLTGAcv?A? G?s?aG??T? sd??an?v??
2
3
1 S. tuberosum AAEASGEK-- --LWRMPMEE SYWESMKSG- --ADMINTGP RDG--GAITG ALFLKQFVD- ---EKVQWLH
2 A. thaliana ASERSGEK-- --LWRMPLEE SYWEMMKSGV --ADMVNTGG RAG--GSITA ALFLKQFVS- ---EKVQWMH
3 L. esculentum ASEVSGEK-- --LWRLPMED SYWDSMKSGV --ADMVNTGG RPG--GAITA ALFLKQFVNE ----KVQWMH
4 Cattle kidney ASIETGDRV- ---WRMPLFE HYTRQVIDCQ -LADVNNIGK YRSA-GACTA AAFLKEFVTH P-K----WAH
5 Bovine lens ASIETGDRV- ---WRMPLFE HYTRQVIDCQ -LADVNNIGK YRSA-GACTA AAFLKEFVTH P-K----WAH
6 R. prowazekii AGEAVNEK-- --LWRMPLHD DYDAMINSDI --ADIANIGN VPGAAGSCTA AHFIKRFI-- --KDGVDWAH
7 E. coli ASEQSGDRA- ---WRLPLGD EYQEQLESNF --ADMANIGG RPG--GAITA GCFLSRFTR- ----KYNWAH
8 P. crispum ASEIAGEK-- --LWRLPMEE SYWESMKSGV --ADMVNTGG RQG--GSITA ALFLKQFVD- ---EKVQWLH
9 C. elegans (nematode AGKASGD--- --LVAPMLFA PDLFFGDLKS SIADMKNSNL GKMD-GPPSA VAGLFIGAHI GFGEGLRWLH
10 unanimous umnanimous A****G***- --*wr*p*** ********** **AD**N*G* *****G**** ********** *******W*H
11 computed consensus As?asG?kII II?wrmp??? syw?smksgv IIAdmvntGg r?gIIGa?TA A???k??vdI III?kv?w?h
1 S. tuberosum LDIAGPVWSD EKKNATGYGV STLVEWVLRN SLO-
2 A. thaliana IDMAGPVWNE KKKSGTGFGV ATLVEWVQKN SSSJ
3 L. esculentum IDLAGPVWSD KKKNATGFGV STLVEWVLKN STNB
4 Cattle kidney LDIAGVMTNK DEVPYLRKGM AGRPTRTLIE FLFRFSQDSA B
5 Bovine lens LDIAGVMTNK DEVPYLRKGM AGRPTR---- ----FSQDSA -
6 R. prowazekii LDIAGVAN-S NNASALCPKG AVGYGVRLLE KFIKEY---N O--
7 E. coli LDIAGTAWRS GKA-----KG ATGRPVALLA QFLLNRAGFN GEE
8 P. crispum IDMAGPVWSD KKKAATGFG- ---------- ----
9 C. elegans (nematode LDIAAPAEVG DRG---TGYG PALFSTLLGK YTSVPMLKQ- ---
10 unanimous umnanimous *D*A****** ********** ********** ********** ***
11 computed consensus ?D?AG?vwsd dkknatg?gv at?v?wv??n s?s??s?dsn ?II