(54) **Title:** PROTEASE INHIBITORS FOR TREATING TRICHOMONAS GALLINAE INFECTIONS

(57) **Abstract:** Disclosed is the use of protease inhibitors for use in the treatment of a Trichomonas gallinae infection of an animal, selected from the orders Galliformes, Passeriformes, Psittaciformes, Columbiformes and Falconiformes.

PROTEASE INHIBITORS FOR TREATING TRICHOMONAS GALLINAE INFECTIONS

The present invention relates to the use of protease inhibitors, especially cysteine protease inhibitors.

Trichomonas gallinae is a flagellated protozoan parasite which infects a variety of birds all over the world. Avian trichomonosis, caused by T. gallinae, has been reported from several continents as a major disease for numerous avian species of the orders Columbiformes, Falconiformes and Psittacifomes. They may infect other avian species as well, like Galliformes and Passeriformers. The domestic pigeon (Columba livia) is the primary host of this flagellate which has been considered re-sponsible for the worldwide spread of T. gallinae. Furthermore, serious losses among wild birds, in particular wild finches, due to T. gallinae were reported recently. T. gallinae colonizes mainly in the upper digestive tract of the birds, where, it can cause granulomatous lesions that occlude the oesophagus lumen, leading to the death of birds as a result of severe starvation. The parasite produces a variety of pathological changes depend-ing on the virulence of the strain and the species of bird in-fected. Earlier studies with T. gallinae have demonstrated a wide spectrum of virulence among different T. gallinae strains. Accordingly, some T. gallinae strains do not cause clinical signs and can induce certain immunity against a highly virulent one. Virulent strains are able to produce a systemic infection in its host and affect mainly the liver and lung beside the oro-pharynx. Pathological changes following experimental infection of a pigeon with a virulent strain of T. gallinae were described in detail in the prior art.

Applying molecular technologies genetic differences between T. gallinae strains could be demonstrated in different studies, for example it could be shown that T. gallinae clones 5895-C1/06 and 8855-C3/06 are members of a particular clade (T. gallinae like isolates) belonging to two different subgroups. In contrast clone 231-C1/07 was grouped in a different clade (Trichomonas tenax like isolates), based on phylogenetic analyses of the two ribosomal RNA internal transcribed spacers (ITS1 and ITS2) and the 5.8S rRNA gene.

In order to assess the virulence of T. gallinae in vivo a

suitable system is needed premising a simple working protocol and the protection of animals. Embryonated chicken eggs have been one of the most common substrate for isolation, propagation and characterization of different avian viruses as well as for the production of viral vaccines. Additionally, embryonated eggs have been used to investigate the virulence of various bacteria and fungi.

WO 97/05867 A1 discloses the treatment of Trichomonas infections with ditiocarb or disulfiram. WO 00/63350 A2 D2 discloses a cysteine protease and/ or a nucleic acid encoding a cysteine protease as a vaccine against neurocysticerocosis; also WO 98/44943 A1 discloses a Leishmania vaccine comprising a cysteine protease and/ or a nucleic acid encoding a cysteine protease. WO 2008/043180 A1 discloses immunostimulatory compositions comprising recombinant BCG Mycobacterium expressing a mammalian cysteine protease. WO 2010/000398 A1 discloses the use of nifurtimox for treating diseases caused by Trichomonadida. Iwalewa et al. (J. Ethnopharm. 117 (2008), 507-511) report about antiprotozoan activities of Harungana madagascariensis stem bark extract on trichomonads and malaria. Amin et al. (PLoS ONE 7 (2012), e37417) disclose that cysteine peptidases that are secreted by Trichomonas gallinae are involved in the cytopathogenic effects on a permanent chicken liver cell culture.

Recently, peptidases are described as synonyms for proteases, proteinases and peptide hydrolases which are the best known enzymes which catabolize proteins and polypeptides through cleavage of peptide bonds. Cysteine peptidases have been reported as essential factors in the biology and the pathogenesis of various parasitic diseases. In this context it was reported that cysteine peptidases are involved in degradation of extracellular matrix components such as fibronectin and laminin. Previously, it was demonstrated that liver abscesses produced by Entamoeba histolytica in severe combined immunodeficient (SCID) mice were greatly reduced by preincubation of the trophozoites with the cysteine peptidase inhibitor namely L-3-carboxyl-2,3-trans-epoxysuccinyl-leucylamido (4-guanidino) butane (E-64). Moreover, application of a specific inhibitor to major Trypanosoma cruzi cysteine peptidases (cruzain) was able to protect the infected mice from lethal infection.

Recently, cathepsin L-like cysteine peptidases were identified as secreted virulence products in a cell-free filtrate of axenic T. gallinae clonal culture. Additionally, it could also be shown that certain peptidase inhibitors are capable to reduce the cytopathogenic effects of T. gallinae in a permanent chicken liver (LMH) cell line. However, evidence that peptidase inhibitors could significantly decrease the protozoal peptidases of T. gallinae activity in vivo is still lacking.

It is therefore an object of the present invention to provide suitable means for combatting infections with T. gallinae in birds.

Therefore the present invention provides protease inhibitors for use in the treatment of a Trichomonas gallinae infection of an animal, selected from the orders Galliformes, Passeriformes, Psittaciformes, Columbiformes and Falconiformes.

With the present invention it is demonstrated that the progression of avian trichomonosis is linked with the presence of cysteine peptidases which can be neutralized by co-application of peptidase inhibitors without toxicity to the host. Such peptidase inhibitors had no direct cytotoxic effect on the viability of the trichomonads. This indicates that the reduction of pathogenicity of T. gallinae in the presence of peptidase inhibitors is due to the inhibition of cysteine peptidase activity rather than a direct effect on the trichomonad cells. The present investigations also show the importance of cysteine peptidases as drug targets as a strategy for chemotherapy.

The present invention is based on the surprising fact that the protease inhibitors are successful in preventing or reducing the pathogenic effects of T. gallinae after oral administration to the animals. Although proteases have been suggested for combatting parasitic diseases for more than 20 years (e.g. Rosenthal et al., J. Clin. Invest. 82 (1988), 1560-1566), no successful application of this concept has yet made its way to applied medicine, neither in humans nor in animals. This might also be due to the different nature and protease strategy such pathogens have with respect to their host.

The fact that the strategy of the present invention can be successfully applied for combatting T. gallinae caused pathogenicity stands in contrast to recently published results that

4

the haemolytic activity of T. gallinae does not correspond with clinical virulence (Gerhold et al., Vet. Parasitol. 160 (2009), 221-224). The successful administration of protease inhibitors according to the present invention shows that those results do not necessarily exclude combatting clinical virulence of T. gallinae, although the results according to the present invention could not have been predicted on the basis of e.g. Gerhold et al.. For example, it was also surprising that T. gallinae, in contrast to the closely related Tetratrichomonas gallinarum (Malik et al., PLoS ONE 6(2011): e20774) indeed induces distinctive cytopathogenic effects in tissue cultures.

Preferably, the protease inhibitors according to the present invention are protease inhibitors selected from cysteine protease inhibitors and serine protease inhibitors, especially cysteine protease inhibitors.

Proteases are enzymes that degrade polypeptides.

Cysteine proteases have a common catalytic mechanism that involves a nucleophilic cysteine thiol in a catalytic dyad. The first step is deprotonation of a thiol in the enzyme's active site by an adjacent amino acid with a basic side chain, usually a histidine residue. The next step is nucleophilic attack by the deprotonated cysteine's anionic sulfur on the substrate carbonyl carbon. In this step, a fragment of the substrate is released with an amine terminus, the histidine residue in the protease is restored to its deprotonated form, and a thioester intermediate linking the new carboxy-terminus of the substrate to the cysteine thiol is formed. Therefore they are also sometimes referred to as thiol proteases. The thioester bond is subsequently hydrolysed to generate a carboxylic acid moiety on the remaining substrate fragment, while regenerating the free enzyme.

Serine proteases are enzymes that cleave peptide bonds in proteins, in which serine serves as the nucleophilic amino acid at the active site. Serine proteases fall into two broad categories based on their structure: chymotrypsin-like (trypsin-like) or subtilisin-like. The main player in the catalytic mechanism in the serine proteases is the catalytic triad. The triad is located in the active site of the enzyme, where catalysis occurs, and is preserved in all serine protease enzymes. The triad is a coordinated structure consisting of three essential amino acids:

histidine, serine ("serine protease") and aspartic acid. Located very near one another near the heart of the enzyme, these three key amino acids each play an essential role in the cleaving ability of the proteases. In the event of catalysis, an ordered mechanism occurs in which several intermediates are generated. The catalysis of the peptide cleavage can be seen as a Ping-Pong catalysis, in which a substrate binds (in this case, the poly-peptide being cleaved), a product is released (the N-terminus "half" of the peptide), another substrate binds (in this case, water), and another product is released (the C-terminus "half" of the peptide).

Inhibitors to proteases are known to the person skilled in the art. In fact, numerous protease inhibitors with specificity to e.g. serine proteases or cysteine proteases are available.

The terms "serine proteases" or "cysteine proteases" and "protease inhibitors", especially "serine protease inhibitors" and "cysteine protease inhibitors" are therefore accepted prod-uct classes in the present field. The compounds showing the de-sired serine or cysteine protease functionality (or the inhibi-tion of this specific enzymatic activity) are part of the common general knowledge and this functionality is accepted in the pri-or art to have a clear technical meaning. The functionality can be verified using tests or procedures adequately specified in the prior art and widely known to the skilled person. The nature of a compound being a serine or cysteine protease inhibitor is therefore known to a person skilled in the art (see also the prior art cited herein) or can easily be defined by standard en-zymatic inhibition assays (e.g. the assays for testing cysteine or serine inhibiting function disclosed in the prior art docu-ments as cited herein). Such tests can therefore be performed as routing methods and do not require undue experimentation. As al-so disclosed herein, the present invention unambiguously shows that this functional activity is essential for the solution of the technical problem underlying the present invention, regard-less of the structure of the compounds.

It is known to a person skilled in the art that not only the term "protease inhibitors" as such is directed to a clearly defined class of compounds, this holds specifically true for "cysteine protease inhibitors" as well as for "serine protease

6

inhibitors".

According to a preferred embodiment of the present invention, the protease inhibitors are cysteine protease inhibitors selected from the group consisting of E-64 (trans-Epoxysuccinyl-L-leucylamido(4-guanidino)butane), L-trans-3-Carboxyoxiran-2-carbonyl-L-leucylagmatine, N-(trans-Epoxysuccinyl)-L-leucine 4-guanidinobutylamide), TLCK (tosyl-L-lysine chloromethyl ketone), TPCK (tosyl-L-phenylalanine chloromethyl ketone), or mixtures thereof.

A group of preferred cysteine protease inhibitors is the group of cathepsin L inhibitors, such as thiochromanone thio-semicarbazone analogs 6-bromo-TST, acetyl-leu-leu-norleucinol, NPI-8343, NPI-8344, NPI-2349, NPI-2019, NPI-3485 and NPI-3469, epoxysuccinyl acid derivatives containing aziridine-2,3-dicarboxylic acid as the electrophilic alpha-amino acid, inhibitors as disclosed in JP-00987265 A, FEBS Lett. 458 (1999), 6-10, EP 0 0611 756 A, and Antibiotics 51 (1998), 629-634; 3-(N-(1-(N-(4-aminobutyl) N-(3-aminopropyl) carbamoyl) 2-(4-hydroxyphenyl) ethyl) carbamoyl) oxirane 2-carboxylic acid, WF-14685A and WF-14865B. Examples are also given in Fig. 9.

Cysteine protease inhibitors and various suitable tests and procedures for easily observing such cysteine protease inhibiting function are also (among many other documents) disclosed in WO 99/53039 A1, WO 02/076939 A2, WO 2003/097664 A2, WO 2005/003150 A2, WO 2006/091610 A2, WO 2007/012180 A1, WO 2007/041775 A1, WO 2009/067797 A1, WO 2010/025314 A2 (especially paragraphs [0056] to [0063]), WO 2010/033658 A2 (especially paragraphs [0036] to [0045]), WO 2010/054042 A2, WO 94/06280 A1, WO 95/23229 A1 and Rosenthal et al., J.Clin. Invest 82 (1988), 1560-1566). Cysteine protease inhibitors, including their functionality and mechanistic properties are also reviewed by Otto et al. (Chem. Rev. 97 (1997), 133-171, especially 147-164), e.g. peptidyl-aldehydes, such as leupeptins, chymostatins, antipain, elastinal and β-MAPI (β-microbial alkaline protease inhibitor); peptidyl-semicarbazones, such as Z-Arg-Ile-Phe-Sc, Z-Ile-Phe-Sc, Z-Phe-Gly-Sc and Z-Gly-Phe-Gly-Sc; peptidyl-methyl ketones and -trifluormethyl ketones, such as Z-Phe-Ala-CH_3, Z-Phe-Ala-CF_3, Ac-Phe-Gly-CH_3, Z-Phe-CH_3, Bz-Phe-CHF_2, Bz-Phe-CF_3, Z-Val-Phe-CF_3 and Z-Val-D-Phe-CF_3; peptidyl-α-keto acids, -α-keto esters, -α-

7

keto amides and -diketones, such as Bz-Phe-COOMe, Z-Phe-COOEt, Z-Phe-COCH$_3$, Z-Val-Phe-COOMe, Z-Val-Phe-COCH$_3$, Z-Phe-Gly-COOH, Z-Phe-Gly-COOCH$_3$, Ac-Phe-Gly-COOCH$_3$, Ac-Phe-Gly-COCH$_3$, Z-Phe-Gly-CONHEt, Z-Phe-Gly-CO-LeuOMe, Z-Phe-Gly-COOn-But, Z-Gly-Phe-Gly-COOn-But, Z-Phe-Gly-CO$_2$CH$_2$CO$_2$Et, Z-Phe-Gly-CO$_2$(CH$_2$)$_3$CO$_2$Me, Z-Leu-Phe-CONH, Z-Leu-α-aminodimethylacetic acid-CONH$_2$, Z-Leu-Phe-CONHR, Z-Leu-α-aminodimethylacetic acid-CONHR, Z-Leu-Phe-CONEt$_2$, Z-Leu-α-aminodimethylacetic acid -CONR$_2$, Z-Leu-Phe-COOH, Z-Leu-α-aminodimethylacetic acid-COOH, Z-Leu-Phe-COOEt, Z-Leu-α-aminodimethylacetic acid-COOR, Z-Leu-nLeu-COOEt, Z-Leu-Met-COOEt, Z-Leu-α-aminodimethylacetic acid-CO-NHEt-SO$_2$Et, (CH$_3$)$_2$N-CO-Leu-α-aminodimethylacetic acid-CONHEt and morpholino-CO-Leu-α-aminodimethylacetic acid-CONHEte; peptidyl-nitriles, such as Z-NH-CH(CH$_2$OH)-CN, Ac-NH-CH$_2$-CN, Bz-NH-CH$_2$-CN, Ac-Phe-NH-CH$_2$-CN, CH$_3$-O-CO-Phe-NH-CH$_2$-CN, Ac-Phe-NH-CH(i-But)-CN, Gly-NH-CH(CH$_2$PH)-CN, Ac-NH-CH$_2$-CSNH$_2$, Bz-NH-CH2-CSNH$_2$ and CH3-O-CO-Phe-NHCH$_2$-CSNH$_2$; peptidyl-halomethyl ketones, such as TLCK, Z-Phe-Phe-CH$_2$Cl, Z-Phe-Phe-CH$_2$F, Z-Phe-Ala-CH$_2$Cl, Z-Phe-Ala-CH$_2$F, Z-Leu-Tyr-CH$_2$F, Z-Ala-Phe-CH$_2$F, Z-Leu-Leu-Tyr-CH$_2$F, Z-Tyr-Ala-CH$_2$F, Z-Leu-Leu-Phe-CH$_2$Cl, Pro-Phe-Arg-CH$_2$Cl, Leu-Leu-Phe-CH$_2$Cl, Leu-Leu-Lys-CH$_2$Cl, Ala-Phe-Lys-CH$_2$Cl, Ala-Phe-Lys-CH$_2$F, Z-Leu-Gly-CH$_2$Cl, Z-Leu-Gly-CH$_2$Br, Z-Leu-Ala-CH$_2$Cl and Z-Leu-Phe-CH$_2$Cl; peptidyl-diazomethanes, such as Z-Phe-Ala-CHN$_2$, Z-Phe-Thr(OBzl)-CHN$_2$ Z-Ala-Ala-Pro-CHN$_2$, Z-Gly-Pro-CHN$_2$, Z-Lys-CHN$_2$, Z-Ala-Phe-Ala-CHN$_2$, Z-Pyrod-Glu-CHN$_2$, Pyro-CHN$_2$, Z-Leu-Val-Gly-CHN$_2$, Z-Phe-Tyr(O-t-But)-CHN$_2$, Ser(OBzl)-CHN$_2$, Gly-Phe-CHN$_2$, Z-Leu-Leu-Tyr-CHN$_2$ and Z-Tyr(I)-Ala-CHN$_2$; peptidylacyloxymethyl ketones, such as Z-Phe-Ala-CH$_2$-O-CO-(2,6-(CF$_3$)$_2$)-Ph, Z-Phe-Ala-CH$_2$-O-CO-(2,5-(CF$_3$)$_2$)-Ph, Z-Phe-Ala-CH$_2$-O-CO-(2,6-Me$_2$-4-COOMe)-Ph, Z-Phe-Ala-CH$_2$-O-CO-4-NO$_2$-Ph, Z-Phe-Ala-CH$_2$-O-CO-(2,4,6-Me$_3$)-Ph, Z-Phe-Ala-CH$_2$-O-CO-CMe$_3$, Z-Phe-Ala-CH$_2$-O-C$_6$F$_5$, Z-Phe-Lys-CH$_2$-O-CO-(2,6-(CF$_3$)$_2$)-Ph, Z-Phe-Lys-CH$_2$-O-CO-(2,4,6-Me$_3$)-Ph, Z-Phe-Ser(OBzl)-CH$_2$-O-CO-(2,6-(CF$_3$)$_2$)-Ph, Z-Phe-Cys(SBzl)-CH$_2$-O-CO-(2,6-(CF$_3$)$_2$)-Ph, Z-Leu-Leu-CH$_2$-O-CO-(2,6-(CF$_3$)$_2$)-Ph, Z-Val-Phe-CH$_2$-O-CO-(2,6-(CF$_3$)$_2$)-Ph, Z-Leu-Leu-Phe-CH$_2$-O-CO-(2,6-(CF$_3$)$_2$)-Ph, Z-Asp-CH$_2$-O-CO-(2,6-Cl$_2$-Ph), Z-Val-Asp-CH$_2$-O-CO-(2,6-Cl$_2$-Ph), Z-Val-Ala-Asp-CH$_2$-O-CO-(2,6-Cl$_2$-Ph) and Z-Glu-CH$_2$-O-CO-(2,6-Cl$_2$-Ph); peptidyl-methlysulfonium salts, such as (CH$_3$)$_3$-S$^+$, Z-Phe-CH$_2$-S$^+$-(Me)2, Z-Phe-CH$_2$-S$^+$-(Me)(Bzl), Z-Phe-CH$_2$-S$^+$-(Et)$_2$ Z-Phe-Ala-CH$_2$-S$^+$-(Me)$_2$,

Z-Phe-Lys-CH_2-S^+-$(Me)_2$, Z-Phe-Lys-CH_2-S^+-$(Me)(Bzl)$, Z-Lys-CH_2-S^+-$(Me)_2$, Ala-Lys-Lys-CH_2-S^+-$(Me)2$, Ala-Lys-Arg-CH_2-S^+-$(Me)_2$, Ala-Arg-Lys-CH_2-S^+-$(Me)_2$, Ala-Arg-Arg-CH_2-S^+-$(Me)_2$, Bz-Phe-Arg-CH_2-S^+-$(Me)_2$ and Z-Leu-Leu-Phe-CH_2-S^+-$(Me)_2$; eposuccinyl peptides, such as E-64, E-64 D, Ep 459 (E-64a; HO-Eps-Leu-NH-$(CH_2)_4$-NH_2), Ep 460 (E-64aZ; HO-Eps-Leu-NH-$(CH_2)_4$-NH-Z), Ep 459-Ac, Ep 479 (HO-Eps-Leu-NH-$(CH_2)_7$-NH_2), Ep 174 (HO-Eps-Leu), Eps(Eps=2(S),3(S)-transepoxysuccinate)LeuOBzlb, Ep 47 LL, (E-64c) LD, DL, DD, Ep 420 (Bzl-DL-Eps-Ile-TyrOMe), Ep 429 (E-64b; HO-Eps-Leu-Leu), EpsLeuProOBzl, EtO-EpsLeuProOBzl, i-ButNH-EpsLeuProOBzl, EpsLeuPro, Et-EpsLeuPro, i-ButNH-EpsLeuPro, EpsPheOBzl, EpsArgOBzl, EpsIleOBzl, EpsPheNBzl, EpsLeuNBzl, E-64, E-64c, E-64d, CA-074 (nPr-NH-Eps-Ile-Pro), CA-028 (HO-Eps-Ile-Pro), CA-030 (Et-OEps-Ile-Pro), CA-030-OMe, EtO-Eps-Pro-Pro, EtO-Eps-Thr-Ile, EtO-Eps-Ile-Ala, EtO-Eps-Gly-Pro, HO-Eps-Leu-OEt, HO-Eps-Leu-OBzl, Bzl-O-Eps-Leu-OEt, Ho-Eps-Ile-OBzl, HO-Eps-Phe-OEt, HO-Eps-Orn(Z)-OEt, HO-Eps-Arg(NO_2)-OMe, HO-Eps-Arg-OMe, HO-Eps-Arg and HO-Eps-Lys(Z)-OBzl; unsaturated derivatives using the Michael system, such as DC-11, Fum(Fum=EtOOCCH=CH-CO trans)-Phe-OBzl Fum-Ile-NH-nBu, Fum-Ile-Pro-OBzl, N-ethylmaleimide, N-butylmaleimide, N-hexylmaleimide, N-decylmaleimide, Ac-Phe-NH-CH_2-CH=CH-COOMe, Ac-Phe-NH-CH2-CH=CH, Gly-NH-CH(Bzl)-X (X=CH=CHCOOMe trans), Gly-NH-CH(Bzl)-Y (Y= $CH=CHSO_2Me$ trans), Ac-NH-CH(Bzl)-X, Ac-NH-CH(Bzl)-Y, NH2-CH(Bzl)-X, NH2-CH(Bzl)-Y and Mu(morpholine urea)-Phe-Hphe(homophenylalanine) -VsPh(vinylsulfonyl)benzene $CH=CHSO_2Phe$); disulfides, azapeptides, azobenzenes, O-acylhydroxamates, lysosomotropic bases, such as ammonium chloride, methylamine, tributylamine, nigericin, gramicidin and chloroquine; calmodulin antagonists, aziridines and thiiranes, 1,3,2-dioxathiolane dioxide derivatives, Ac-Gly-Phe-Nle-OH, Ac-Gly-Phe-Nle-OSO_2-CH_3, aspartyl R-[(1-phenyl-3-(trifluoromethyl)pyrazol-5-yl]-oxy]methyl ketones and aspartyl R-[(diphenylphosphinyl)oxy]-methyl ketones.

A further group of preferred cysteine protease inhibitors is the group 1-Naphthalenesulfonyl-Ile-Trp-CHO (1), or [(3S)-3-[[(2S)-4-methyl-2-(10H-phenothiazine-2-carbonylamino)pen tanoyl]amino]oxolan-2-yl] (BN 82270 (and BN 82204)) (2), Z-Phe-Tyr(OtBu)-COCHO • H_2O (3), tert-butyl N-[(2S)-1-[2-[2-(2-ethylanilino)-2- oxoethyl] sulfanylcarbonylhydrazinyl]-3-(1H-

indol-3-yl)-1-oxopropan-2-yl]carbamate   and   *N*_-[(S)-2-
*tert*butoxycarbonylamino-3-(1*H*-indol-3-yl)-propionyl]-hydrazine
carboxylic acid 2-(3,4-dihydro-2*H*-quinolin-1-yl)-2-oxo-ethyl es-
ter (4), N-Acetyl-L-leucyl-L-leucyl-L-methional (5), (2S,3S)-
oxirane-2,3-dicarboxylic   acid   2-[((S)-1-benzylcarbamoyl-2-
phenyl-ethyl)-amide] 3-{[2-(4-hydroxy-phenyl)-ethyl]-amide} (6),
[N-(4-Biphenylacetyl)-S-methylcysteine-(D)-Arg-Phe-β-
phenethylamide (7), 1-Naphthalenesulfonyl-Ile-Trp-CHO (8), Z-
Phe-Tyr(t-Bu)-diazomethylketone   (9)   and   acetyl-leu-leu-
norleucinol (10). This group is characterised by a strong inhib-
itory capacity on cysteine proteases (IC_{50} = 1.9 mM (1); calpain
and cell death inhibition in C6 glial cells (IC_{50}) of 13.34 and
15.5 µM (2); K_i = 600, exhibits over 360-fold greater selectivi-
ty for cathepsin L compared to cathepsin B (Ki = 214 nM) (3);
IC_{50} of 1.0 nM and 0,4 nM (4); very potent inhibitor of cathep-
sin L (K_i = 0.6 nM) and the strongest inhibitor of cathepsin B
(K_i = 100 nM) (5); inhibits rat liver cathepsin L with IC_{50} val-
ues of 1.9 nM (6); inhibitor of human recombinant cathepsin-L
(K_i = 19 nM) (7); inhibitor of cathepsin L (IC_{50} = 1.9 nM) (8);
about 10.000-fold more effective against cathepsin L than ca-
thepsin S (9)).

Besides the inhibitors mentioned above, the present inven-
tion can also apply structural alternatives (variants) of the
substances mentioned above, e.g. derivates, conjugates and iso-
mers of such substances which preserve the general structure and
the general inhibitory capacity of the molecule, but slightly
deviates in structure. For example, (1) and suitable alterna-
tives for (1) are disclosed in WO 1996/016079 A2 and EP 0 611
756 A2 (especially the compounds of and claims 1 and 14 of the
EP 0 611 756 B1 document); (2) and suitable alternatives for (2)
(BN 82270 and BN 82204) are disclosed in Auvin et al., Bioorg.
Med. Chem. Lett. 14 (2004), 3825-3828; (3) and suitable alterna-
tives for (3) are disclosed in Lynas et al., Bioorg. Med. Chem.
Lett. 10 (2000), 1771-1773; (4) and suitable alternatives for
(4) are disclosed in WO 2009/136997 A2).

Administration amounts are typically dependent on the indi-
vidual inhibitory capacity of each inhibitor and its toxicity.
Preferred administration dosages for the inhibitors are in the
mg range, such as 1 to 1000 mg/kg bodyweight/day, preferably 5

to 500 mg/kg bodyweight/day, especially 10 to 100 mg/kg body-
weight/day.

Cysteine protease inhibitors have been specifically effec-
tive in the present invention, especially for combatting T. gal-
linae pathogenicity in preferred avian species. This has been
confirmed within the course of the present invention by using
chicken embryos, as a disease model. Accordingly, it is pre-
ferred to use the protease inhibitors according to the present
invention in the treatment of a T. gallinae infection of an an-
imal, selected from a species belonging to the orders Galli-
formes, Columbiformes, Psittaciformes or Passeriformes, espe-
cially Columba livia, Melopsittacus undulates, Serinus canaria
forma domestica, or Falco spp..

It could be shown in the course of the present invention
that the protease inhibitors according to the present invention
can be administered orally to the animal, which makes them spe-
cifically suitable for mass application. This was demonstrated
in a disease model by applying protease inhibitors via the al-
lantoic cavity to chicken embryos, which took up the parasite
and the inhibitors orally.

Preferably, the (cysteine) protease inhibitors according to
the present invention (which, as outlined above can be a single
inhibitor species or a mixture of two or more different inhibi-
tors), are for use in the oral treatment of a Trichomonas galli-
nae infection of an animal, especially wherein oral treatment
includes administration of a solution of cysteine protease in-
hibitors or cysteine protease inhibitors in tablet form.

A comfortable route of administration is via drinking water
for the birds. In this embodiment, the protease inhibitors ac-
cording to the present invention are provided in dried form,
e.g. as a powder or a lyophilisate, and reconstituted with water
or another drinkable aqueous solution before use.

Alternatively, the inhibitors are provided in administra-
tion (daily) dosage form, such as capsules, dragees, tablets,
etc.. For example, tablets (as well as all other routine dosage
formats) can be made from the inhibitors by combining those with
appropriate formulation ingredients. The tablet for use in the
present invention is composed of a mixture of the (cysteine)
protease inhibitors and excipients, usually in powder form,

pressed or compacted from a powder into a solid dose. The excip-
ients can include diluents, binders or granulating agents,
glidants (flow aids) and lubricants to ensure efficient tablet-
ting; disintegrants to promote tablet break-up in the digestive
tract. A polymer coating can be applied to make the tablet
smoother and easier to swallow, to control the release rate of
the (cysteine) protease inhibitor or to make it more resistant
to the environment (extending its shelf life). The present
invention also relates to a method for treating a Trichomonas
gallinae infection of an animal, selected from the orders Galli-
formes, Passeriformes, Psittaciformes, Columbiformes and Falcon-
iformes, wherein an effective amount of a protease inhibitor,
especially a cysteine protease inhibitor, is administered to an
animal.

According to another embodiment, the present invention con-
cerns the use of a protease inhibitor, especially a cysteine
protease inhibitor for the manufacture of a medicament for the
treatment of a Trichomonas gallinae infection of an animal, se-
lected from the orders Galliformes, Passeriformes, Psittaci-
formes, Columbiformes and Falconiformes.

Another aspect of the present invention relates to a vac-
cine against a Trichomonas gallinae infection of an animal, se-
lected from the orders Galliformes, Passeriformes, Psittaci-
formes, Columbiformes and Falconiformes, comprising a cysteine
protease and an adjuvant. Based on the results obtained with the
oral administration of the protease inhibitors according to the
present invention, also the use of cysteine proteases as active
immunogen for the prevention of infections with T. gallinae (or
the prevention of the pathogenic effects associated therewith)
is an aspect of the present invention. The vaccine containing
the cysteine protease can also be provided as a DNA-vaccine com-
prising a nucleic acid molecule, especially a DNA molecule, en-
coding the cysteine protease (i.e. containing a cysteine prote-
ase gene which can be expressed after delivery of the vaccine to
the animal and elicit an immune response to the encoded cysteine
protease after expression in the vaccinated animal). According-
ly, the cysteine proteases (or the DNA vaccine) can be combined
with any suitable adjuvant which is effective in eliciting an
immune response against the cysteine protease (either directly

or via the DNA vaccine) in the animal.

„Adjuvant" refers to a compound or mixture that enhances the immune response to an antigen. An adjuvant can serve as a tissue depot that slowly releases the antigen and also as a lymphoid system activator that non-specifically enhances the immune response. Often, a primary challenge with an antigen alone, in the absence of an adjuvant, will fail to elicit a humoral or cellular immune response. Adjuvants include, but are not limited to, complete Freund's adjuvant, incomplete Freund's adjuvant, saponin, mineral gels such as aluminum hydroxide, aluminium sulfate, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil or hydrocarbon emulsions, keyhole limpet hemocyanins, dinitrophenol, and potentially useful human adjuvants such as BCG (Bacillus Calmette-Guerin) and Corynebacterium parvum.

The present invention also relates to a method of immunizing an avian comprising administrating to the avian an effective amount of the vaccine according to the present invention. The method comprises administering to the animal an effective immunizing dose of the vaccine of the present invention. The vaccine may be administered by any of the methods well known to those skilled in the art, for example, by intramuscular, subcutaneous, intraperitoneal, intravenous, intranasally, orally, intradermal, iutrabursal, in ovo, or ocularly.

Preferred cysteine proteases are cysteine proteases from T. gallinae. These cysteine proteases from T. gallinae may either be prepared from T. gallinae or produced recombinantly (by other hosts). In the vaccine, also a mixture of different cysteine proteases may be administered, especially a mixture comprising at least two different cysteine proteases from T. gallinae. The proteases may be directly purified from T gallinae (as e.g. outlined in the example section) or produced recombinantly. In the example section, six proteolytic entities have been identified as six proteolytic spots. Provision of one or more of such spots in a vaccine is therefore a preferred embodiment of the present invention. Specifically preferred cysteine proteases are the proteases of T. gallinae. Specifically preferred proteases are: the T. gallinae protease comprising the amino acid sequence FSY-IADYPYTAR (Seq.ID.No.1) or FSYLADYPYTAR (Seq.ID.No.2); the T.

gallinae protease comprising the amino acid sequence NSWGASWGEK
(Seq.ID.No.3); the T. gallinae protease comprising the amino ac-
id sequence NYWIVR (Seq.ID.No.4) or NYWLVR (Seq.ID.No.5); and
the T. gallinae protease comprising the amino acid sequence
VNVVEGDEADLATK (Seq.ID.No.6).

The vaccine according to the present invention is prefera-
bly administered i.m. or s.c; however, any other way for suc-
cessfully eliciting an immune response to the cysteine proteases
is suitable. The compositions according to the present inven-
tion may comprise a suitable veterinarily or pharmaceutically
acceptable carrier, diluent, or excipient such as sterile water,
physiological saline, glucose or the like. The compositions can
also be lyophilized. The compositions can contain auxiliary sub-
stances, such as wetting or emulsifying agents, pH buffering
agents, adjuvants, gelling or viscosity enhancing additives,
preservatives, flavouring agents, colours, and the like, depend-
ing upon the route of administration and the preparation de-
sired. Examples for such auxiliary substances include DMSO
and/or tetradecyl maltoside (TDM). The most preferred route of
administration of the compositions according to the present in-
vention is oral administration, preferably via drinking water or
via tablets.

The present invention is further described with the follow-
ing examples and the drawing figures, yet without being limited
thereto.

Fig. 1 shows the influence of peptidase inhibitors on the
cytopathogenic effect of the cell-free filtrate. LMH monolayer
was incubated with cell-free filtrate with and without different
protease inhibitors. (A) Mean lesion scores, (B) cytotoxicity of
LMH cells, as assessed by Promega CellTiter 96® aqueous solution
at different time points. Cell-free filtrate was obtained after
24 h of incubation of 10^7 axenically grown protozoa cells ob-
tained from T. gallinae clone 5895-C1/06, passage18. Absorbance
values for Promega CellTiter 96® aqueous solution were recorded
at 490 nm using ELISA reader.

Fig. 2 shows the demonstration of the protease activity in
cell-free filtrates from T. gallinae clone 5895-C1/06 by one-
dimensional substrate SDS-PAGE. A) Substrate (0.2% gelatin) 1-D
SDSPAGE (8%) with the low passage (49x) cell-free filtrate, B)

conventional 1-D SDS PAGE (8%) with the low passage (49x) cell-
free filtrate, C) Substrate (0.2% gelatin) 1-D SDS-PAGE (8%)
with the high passage (130x) cell-free filtrate, and D) conven-
tional 1-D SDS PAGE (8%) with the high passage (130x) cell-free
filtrate.

Fig. 3 shows the time-course experiment demonstrating the
appearance of peptidases in the filtrate of the clone 5895-
C1/06, P71 (107 T. gallinae cells) following incubation in the
trichomonads growth medium (HF medium) and in the cell-culture
medium (Medium 199). A) Substrate (0.2% gelatine) 1-D SDS-PAGE
(8%).

Fig. 4 shows the two-dimensional gel electrophoresis of the
cell-free filtrate from clone 5895-C1/06, P53. A) Substrate
(0.2% gelatine) SDS-PAGE (8%) as the second dimension and B)
conventional SDS-PAGE (8%) as the second dimension.

Fig. 5 shows the gross changes in the liver of spf chicken
embryo infected with T. gallinae clone 5895-C1/06 P18, (A) yel-
low area in the liver (arrow) of embryo died at 7 dpi and (B)
dilated gizzard of embryo killed at 10 dpi.

Fig. 6 shows percentage of positive PCR signals in the liv-
er and gut (A) of embryos infected with different T. gallinae
clonal cultures with varying in vitro passages low and high pas-
sage levels and (B) of embryos infected with T. gallinae clone
5895-C1/06 P18 either alone or coincubated with cysteine pepti-
dase inhibitors (TLCK and E-64).

Fig. 7 shows histological sections of liver obtained from
(A) negative control embryos, (b-e) spf chicken embryo infected
with T. gallinae 5895-C1/06 P18 (B) congestion of hepatic blood
vessels and sinusoids, (C) perivasular leukocytic cellular in-
filtration, (D) leukocytic cellular infiltration mainly lympho-
cytes in the hepatic parenchyma, (E) necrotic area with polymor-
phonuclear leukocytes and (F) liver from an embryo infected with
5895-C1/06 P18 in the presence of cysteine peptidase inhibitor
(E-64). Haematoxylin and eosin staining.

Fig. 8 shows detection of the nucleic acid of trichomonads
by ISH in various organs of spf chicken embryos infected with T.
gallinae 5895-C1/06 P18, such as (A) the gizzard, (B) the duode-
num, (C) the caecum, (D) the liver, (E) the lung and (F) the
gizzard from embryo infected with 5895-C1/06 P18 incubated with

cysteine peptidase inhibitor (E-64).
        Fig. 9 shows cathepsin L inhibitors.

EXAMPLES

1. Cysteine peptidases, secreted by Trichomonas gallinae, are
involved in the cytopathogenic effects on a permanent chicken
liver cell culture
        This example focused on the molecular characterization of
cell-free filtrates from T. gallinae axenic cultures, which were
previously shown to possess cytopathogenic effects on permanent
chicken liver (LMH) cells. The identification of Clan CA, family
C1, cathepsin L-like cysteine peptidases in the cell-free fil-
trate and demonstration of their involvement in the cytopatho-
genic effects of the filtrate show the virulent role of these
peptidases in the pathogenesis of T. gallinae.
        The present example demonstrated that the cytopathic effect
of the cell-free filtrate on monolayers is reduced by the addi-
tion of the cysteine peptidase inhibitors TLCK and E-64. This
showed that some of the important molecules involved in the mon-
olayer destruction are cysteine peptidases. One-dimensional zy-
mograms revealed no inhibitory effect of pepstatine A and PMSF
on the proteolytic activity, demonstrating the inertness of
these two inhibitors towards peptidases present in the fil-
trates. However, TLCK and E-64 efficiently inhibited the proteo-
lytic activity of the filtrates. This generally correlated with
results obtained with the cell-culture experiments. Furthermore,
the results demonstrated that peptidases present in the fil-
trates, which were blocked by TLCK and E-64, were also partially
responsible for the cytotoxicity of the filtrates.
        The demonstration of Clan CA, family C1, cathepsin L like
cysteine peptidases in the cell-free filtrate of T. gallinae by
the 2-D substrate SDS-PAGE experiments combined with mass spec-
trometry supported the hypothesis that cysteine peptidases are
the most abundant type of peptidases in the filtrates. Seven
different proteolytic spots were detected by the 2-D substrate
SDS-PAGE experiments showing the presence of at least 7 differ-
ent peptidases in the cell-free extract of T. gallinae.

The present experiment had shown that the proteins secreted by T. gallinae possessed the proteolytic activity which contributed to the detachment of the monolayer. For the first time molecular characterization involving 1-D and 2-D zymograms in combination with mass spectrometric analysis showed that the cysteine peptidases are present in the pool of proteins secreted by T. gallinae. Finally, further demonstration that cysteine peptidases take part in the cytopathogenic effects of T. gallinae made these proteins excellent candidates for virulence factors.

1.1. Materials and Methods
1.1.1. Tested clonal culture

Cell-free filtrates of the T. gallinae axenic clonal culture named Trichomonas gallinae /Budgerigar/Austria/5895-C1/06 (shortly, clone 5895-C1/06), were investigated. The name assignment reflects the species of bird/country of origin/diagnostic number-clone number/year of isolation. In the present example a distinction between lower (P18, P49, P53) and high passages (P130) of the same clonal culture was made.

1.1.2. Cell culture

Permanent chicken liver (LMH) cells were grown in Medium 199 + Earle's salts + L-Glutamine (Invitrogen/Gibco, Paisley, U.K.). The medium was supplemented with 10% of FBS, 10% Tryptose Phosphate broth, penicillin (40,000 IU/ml) and streptomycin (40 mg/ml) (all Invitrogen/Gibco, Paisley, U.K). One milliliter of the media suspension containing 1×10^6 cells was inoculated into 75 cm² flasks with filtered caps (Sarstedt, Wiener Neudorf, Austria) containing 6 ml medium. Cells were kept in a controlled atmosphere of 5% CO_2 at 37°C and around 85-90% humidity. After 72 h of incubation, a confluent monolayer of LMH cells was obtained with an average of 7×10^6 cells per flask. Cells were passaged every three to four days depending on their density.

1.1.3. Preparation of cell-free filtrate from axenic T. gallinae culture

For the analysis on LMH monolayers cell-free filtrate was prepared in the following way: 10^7 cells of a clonal culture of T. gallinae were incubated at 37°C for 24 h in the same medium

as used for growth of LMH. Before incubation the protozoal cells were washed three times in PBS, (pH 7.2), trichomonad culture was centrifuged at 3300 x g for 5 min and then the supernatant from the culture was filtered through 0.22 μm cellulose acetate filters (Millipore, VWR).

For analysis in one- (1-D) and two-dimensional (2-D) zymogram and conventional SDS PAGE the filtrate was prepared in the following way: T. gallinae clonal culture was grown in Hollander fluid (HF) medium as described recently (Amin et al., Exp. Parasitol. 124 (2010), 202-208). After 24 h of incubation, the trichomonad culture was centrifuged at 3300 x g for 5 min; the pellet was washed three times in phosphate buffer saline (PBS, pH 7.2) and was then suspended in 1 ml HF medium without serum. Motile trichomonads were counted by a Neubauer cell counting chamber (Reichert, Buffalo, NY). The concentration of the inoculum was adjusted to 10^7 motile trophozoites. 10^7 trichomonad cells were grown for 3, 6, 16, 24 h in 10 ml HF medium without serum and the cell-free filtrate was prepared. The trichomonad culture was centrifuged at 3300 x g for 5 min, and then the supernatant from the culture was filtered through 0.22 μm cellulose acetate filters (Millipore, VWR). Afterwards, 1 mM PMSF, 5 μM of Pepstatin A, 1 mM TLCK and 270 μM E-64 were added to the cell-free filtrate and incubated for 30 min on ice before concentrating the proteins. Two types of filters were used to concentrate the proteins of the cell-free filtrate. Firstly, CENTRIPREP® Centrifugal Filter Device 3K (Millipore, Vienna, Austria) was applied to reduce the initial volume of the filtrate (15ml) to 500 μl. After that, the samples were more concentrated using Amicon® Ultra-0.5 3K centrifugal filter device (Millipore, Vienna, Austria) to obtain a final volume of 100 μl. Both types of filters were used following manufacturer´s instructions. With the samples for 2-dimensional (2-D) sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE) buffer was exchanged (three times) to 10 mM Tris-Cl pH 7.2 by using the same Amicon® Ultra-0,5 3K centrifugal filter device (Millipore,Vienna, Austria). For all samples the protein concentration was measured by Bradford Reagent (Fermentas, Thermo Scientific).

1.1.4. Assessment of LMH monolayer

Each monolayer was investigated visually by an inverted 158 light microscope to detect the effect of the cell-free filtrates on LMH monolayer. Assesment of the monolayer was performed as recently described (Amin et al., Vet.Parasitol. (2011), doi: 10.1016/j.vetpar.2011.11.037).

1.1.5. Effect of peptidase inhibitors on the cell-free filtrate

Firstly, preliminary experiments were done to establish the concentration of each peptidase inhibitor which was not toxic to the monolayer cell culture. In these initial experiments different concentrations of the following peptidase inhibitors were added to not-infected monolayer: 0.2 mM, 0.5 mM, 1 mM Phenylmethylsulfonyl fluoride (PMSF) as serine peptidase inhibitor, 1 µM, 2.5 µM, 5 µM pepstatin A as aspartic peptidase inhibitor, 135 µM, 540 µM, 1 mM N-α-p-Tosyl-L-Lysine chloromethyl ketone (TLCK) as cysteine and some serine peptidase inhibitor, and 70 µM 140 µM, 270 µM L-3-carboxyl-2,3-trans-epoxysuccinyl-leucylamido (4-guanidino) butane (E-64) as cysteine peptidase inhibitor (all purchased from Sigma-Aldrich, Steinheim, Germany).

1.1.6. Cytotoxicity assay

In order to investigate the cytotoxicity of cell-free filtrate on cell culture, CellTiter 96® aqueous one solution cell proliferation assay (Promega Corporation, Madison, USA) was used following manufacturer´s instructions. The cell-free filtrate from 10^7 trichomonad cells was prepared as described above and 270 µM of E-64 as cysteine peptidase inhibitor was added to cell-free filtrate before its incubation with the monolayer. Cytotoxicity assay was performed as described recently by Amin et al. (2011).

1.1.7. One- and two dimensional gels electrophoresis and zymographic analysis

For one dimension (1-D) zymographic analysis, 24 µg of concentrated p 183 proteins from cell free filtrates with and without peptidase inhibitors were separated by discontinuous SDS-PAGE (8%) copolymerized with 0.2% gelatin as substrate. In parallel, the same samples were investigated by conventional 1-D

SDS-PAGE (8%). After electrophoresis, the substrate gel was incubated in 2.5% Triton X-100 (Bio-Rad Laboratories GmbH, Vienna, Austria) for 1 h at room temperature to remove SDS. Subsequently the peptidases were activated by incubating the gel in zymogram development buffer (Bio-Rad Laboratories GmbH, Vienna, Austria) for 24 h at 37 °C. All gels were stained with PageBlue™ Protein Staining Solution (Fermentas, Fisher Scientific Österreich, Vienna, Austria) and clear bands in gelatin gels were indicative of proteolytic activity. All experiments were repeated twice.

For 2-D zymographic analysis of the concentrated proteins from cell-free filtrate buffer was exchanged to rehydration buffer (8M Urea (GE Healthcare, UK), 2% CHAPS (Sigma-Aldrich, Steinheim, Germany), 50 mM DTT (Sigma-Aldrich, Steinheim, Germany), 0,2% Servalyt®-Ampholytes pH 3-5 (Serva), trace bromphenol blue (Sigma-Aldrich, Steinheim, Germany) via Zeba™ Spin Desalting Columns, 7K MWCO (Pierce, Fisher Scientific Österreich, Vienna, Austria) according to manufacturer´s instructions. For first-dimension of both silver stained and PageBlue™ stained zymogram gels, 240 µg protein was applied to IPG-strip (7 cm, pH 3-6, Bio-Rad Laboratories GmbH, Vienna, Austria) by passive in-gel rehydration (16 h at room temperature). Isoelectric focusing (IEF) was carried out in a Protean IEF cell (Bio-Rad Laboratories GmbH, Vienna, Austria): voltage was increased in the slow ramp mode; 100 V in 1 h, 250 V in 1 h, 500 V in 1 hour, 1000 V in 1.5 h, 4000V in 1 h followed by focusing at 4000 V until 18 000 Vhs were reached. For both types of analyses, the IPG strips were equilibrated before the second dimensional run first in 6M Urea (GE Healthcare, UK), 2% SDS (Merck, VWR), 0.375M Tris-Cl (Merck, VWR) 20% glycerol (Sigma-Aldrich, Steinheim, Germany) pH 8.8 containing 2% DTT (Sigma- Aldrich, Steinheim, Germany) and afterwards in the same buffer supplemented with 2.5% iodoacetamide (Sigma-Aldrich) for 10 min at room temperature each. In the second-dimension proteins were resolved by SDS-PAGE (8%) and substrate (0.2% 209 gelatin) SDS-PAGE (8%) for silver-stained gels and zymograms, respectively. Silver-staining was performed according to Shevchenko et al. (Anal. Chem. 68 (1996), 850-858. Zymogram gels were treated and stained as described for 1-D zymograms gels. Clear spots were indicative for proteolytic activity. Images of all gels (1-D and 2-D) were taken with Molecular

Imager ChemiDoc™ XRS System (Bio-Rad Laboratories GmbH, Vienna, Austria) using Quantity One v4.6.3 software (Bio-Rad Laboratories GmbH, Vienna, Austria).

1.1.8. Protein digestion, peptide extraction and mass spectrometric analysis

Selected spots from 2-D gels were excised, washed, destained, reduced with DTT (Sigma-Aldrich, Steinheim, Germany) and alkylated with Iodoacetamide (Sigma- Aldrich, Steinheim, Germany). The in-gel digest was carried out with trypsin (Trypsin Gold, Mass Spectrometry Grade, Promega Corporation, Madison, USA) (Shevchenko et al., 1996) and after extraction the dried peptides were de-salted using μZip-Tips C18 (Millipore, VWR, Vienna, Austria) according to the manufacturer's instructions. Desalted peptides (0.5 μl) were spotted onto a pre-spotted Anchor-Chip MALDI (PAC target, Bruker Daltonik GmbH, Leipzig, Germany).

Data were acquired on a Matrix Assisted Laser Desorption Ionisation Tandem Time-of-Flight (MALDI-TOF/TOF) mass spectrometer (Ultraflex II, Bruker Daltonik GmbH, Leipzig, Germany) in MS and MS/MS modes. Spectra processing and peak annotation were carried out using FlexAnalysis and Biotools (Bruker Daltonik GmbH, Leipzig, Germany).

1.1.9. Data analysis and de novo peptide sequencing

Processed spectra were searched via Mascot in the Swiss-Prot database (release 56.5) or in NCBInr (20111005) using the following search parameters: taxonomy trichomonas; global modifications carbamido-methylation on cysteine; variable modifications oxidation on methionine; MS tolerance 100 ppm; MS/MS tolerance 1 Da; one missed cleavage allowed. Identifications were considered statistically significant where $p < 0.05$.

Peptide de Novo sequencing was carried out manually using FlexAnalysis. These sequences were then used for a homology search using MS Homology in ProteinProspector 5.9.0. http://prospector.ucsf.edu/prospector/cgi-bin/msform.cgi?form=mshomology. The searched database was Uni-ProtKB.2011.01.11 with following parameters: taxonomy search in Trichomonas vaginalis, 0.5k Da mass tolerance, no enzyme digest, one missed cleavage allowed, minimal match of peptides 1 and

BLOSUM62 as score matrix.

1.2. Results

1.2.1. Influence of peptidase inhibitors on the cytopathic effect of cell-free filtrate from axenically grown T. gallinae

In order to establish the maximal concentration of each peptidase inhibitor without toxicity for the monolayer, incubations with different concentrations were performed. Accordingly, the suitable concentration for each inhibitor was used in this study. The results showed that the addition of either 1 mM PMSF, 270 µM E64 or 135 µM TLCK to the cell-free filtrate of T. gallinae partially inhibited cytopathic effects induced by trichomonads (Fig. 1). The inhibition was assayed by the detachment of the monolayer in comparison to the filtrate without inhibitors (Fig. 1A). After applying peptidase inhibitors the monolayer destruction was reduced, but differences between the actions of these inhibitors were noticed. The E-64, cysteine peptidase inhibitor, produced the best inhibition causing the lowest destruction of the monolayer. The application of Pepstatin A (5 µM), aspartic peptidase inhibitor, had no inhibitory effect on the filtrate (Fig. 1A).

The effect of the E-64, cysteine peptidase inhibitor, on the cell-free filtrate was also assayed by CellTiter 96® aqueous one solution cell proliferation assay and showed the significant reduction in cytotoxicity (Fig. 1B). After 72 h of incubation the maximum cytotoxicity of T. gallinae clone 5895-C1/06, passage 18, on LMH cells, produced by the cell-free filtrate without E-64, was 70.9%. In the presence of the inhibitor the toxicity of the filtrate could be reduced to 37.1%.

1.2.2. Demonstration of peptidase activity in T. gallinae cell-free filtrates using one-dimensional substrate gel electrophoresis

In order to detect whether T. gallinae cells secreted peptidases into trichomonad-growth medium (HF medium), concentrated cell-free filtrates from clone 5895-C1/06 with and without peptidase inhibitors were separated by SDS-PAGE with gelatin copolymerized as substrate (Fig. 2). Additionally, filtrates of lower (P49) and high passages (P130) from clone 5895-C1/06 were com-

pared. The zymogram of the cell-free filtrate from P49 of clone 5895-C1/06 without peptidase inhibitors showed a proteolytic region of at least five different clear bands with molecular weights of approximately 38, 41, 50, 80 and 110 kDa (Fig. 2A). In contrary, the zymogram analysis of the cell-free filtrates from P130 of clone 5895-C1/06 demonstrated weaker proteolytic activity (Fig. 2C). The clear bands indicative for proteolytic activity were of weaker intensity and the 110 kDa band present in zymogram of cell-free filtrate from P49 was absent. No proteolytic bands were observed on both zymograms of cell-free filtrates containing peptidase inhibitors TLCK (cysteine and some serine peptidases) and E-64 (cysteine peptidases) (Fig. 2A, C). Samples treated with PMSF (serine peptidase inhibitor) and pepstatin A demonstrated the same proteolytic regions as the sample without inhibitors (Fig. 2A, C). In parallel, the same samples were investigated by conventional SDS-PAGE (figure 2B and 2D). Cell-free filtrate from clone 5895-C1/06 P49 containing TLCK and E-64 produced a pattern of sharp bands of different molecular weights (Fig 2B). In contrary, in samples without inhibitors or those with PMSF and pepstatin A, this pattern was not prominent and the remaining bands were not sharp. This additionally confirmed the presence of peptidase activity in the cell-free filtrate of P49 of clone 5895-C1/06 which could be specifically blocked by addition of TLCK and E-64. Interestingly, conventional SDS-PAGE of filtrates from P130 of clone 5895-C1/06 demonstrated sharp bands in all samples even in the ones without peptidase inhibitors (Fig. 2D).

In the time-course experiment in which passage 71 of clone 5895-C1/06 was incubated in trichomonad growth medium (HF medium) and cell-culture medium (Medium 199), peptidases were already demonstrated in the filtrate collected after 3 h of incubation of 10^7 trichomonad cells (Fig. 3). The intensity of the proteolytic activity in cell-free filtrates increased with the duration of incubation.

1.2.3. Detection of Clan CA, family C1, cathepsin L-like cysteine peptidases in T. gallinae cell-free filtrate by MS analysis of peptidase spots in 2-D gel

In order to further characterize the proteolytic activity

of the cell-free filtrate from T. gallinae, the concentrated proteins of cell free-filtrates from passage 53 of clone 5895-C1/06 were investigated by conventional and substrate 2-D gel electrophoresis (Fig. 4). Zymographic analysis identified at least seven spots with proteolytic activity between 35 and 100 kDa, which displayed pI values between 3.7 and 5.3 (calculated according to linear distribution of pH across the strip). The MS analysis of six spots of conventional 2-D gels, which corresponded to the proteolytic spots found in 2-D zymograms, identified the presence of Clan CA, family C1 and cathepsin L-like cysteine peptidases in cell-free filtrate of T. gallinae. In five proteolytic spots (6, 7, 8, 9, 10) a peptide with the same mass (m/z 1466.6) was identified. Manual peptide de Novo sequencing of this charged peptide identified the following sequence: FSY[I/L]ADYPYTAR. The MS homology search matched this peptide to different Clan CA, family C1, cathepsin L-like cysteine peptidases of T. vaginalis. In spot 8 (70 kDa, pI 5.2) two additional peptides with masses of m/z 850 and m/z 1121 could be identified. Manual sequencing of the tandem mass spectra corresponding to the single peptides identified following peptide sequences: NYW[I/L]VR (m/z 850.5) and NSWGASWGEK (m/z 1121.4). The MS homology search using all three peptides matched these peptides with highest homology to T. vaginalis Clan CA, family C1, cathepsin L-like cysteine peptidase TVAG_355480 (accession number XP_001310117).

The MS/MS spectrum of the sixth proteolytic spot, protein s 312 pot 5II (45 kDa, pI 3.7), identified two peptides with masses of m/z 850.5 and m/z 1879.8. The manual peptide de Novo sequencing identified peptides with the sequences: NYWI/LVR (m/z 850) and VNVVEGDEADLATK (m/z 1879). The MS homology search using either combination of NYWI/LVR and VNVVEGDEADLATK matched these peptides with the highest homology to T. vaginalis Clan CA, family C1, cathepsin L-like cysteine peptidase TVAG_298080 (accession number XP_001316414) and T. vaginalis CP39-Cytotoxic cysteine proteinase (accession number ABX56032).

2. Peptidase inhibitors are able to neutralize the virulence of Trichomonas gallinae in vivo

24

In this example a suitable in vivo system capable to assess the pathogenicity of T. gallinae was established in order to investigate the pathogenicity of different clonal cultures of T. gallinae in spf chicken embryos. PCR and ISH were carried out to screen the spread of trichomonads in liver and gut of embryos infected with different clonal cultures. Both techniques were found to be effective tools for demonstration of trichomonads in these organs. Most severe histological changes were detected in the liver. This may be due to the short incubation period, as the infection continued for ten days only, at which the lesions in other organs might still have been in the early stage. In addition to the oropharynx, the liver is a second predilection site for virulent T. gallinae strains in vivo. Liver lesions range from the presence of a few, yellow necrotic areas to a nearly complete caseation of the organ. In agreement with this, yellow necrotic areas on the liver surface of some embryos were noticed in the present example.

Previous reports mentioned that T. gallinae is mainly inhabiting the upper part of the digestive tract and findings in the lower parts of the proventriculus is uncommon. Contrary, in the present example, the protozoal cells were demonstrated by ISH in the gizzard and intestine of some infected embryos with absence of any lesions. This could be due to the applied in vivo system, using spf chicken embryos. The presence of protozoa in the gut of the embryos could be explained by the oral uptake of the flagellates together with the nutrients, whereas generalization and spread into other organs occurred at a second stage, following penetration of the gut epithelium and entrance into the bloodstream.

In the present experiment spf chicken embryos were established successfully as a simple model to evaluate the virulence of different clones of T. gallinae in vivo. For the first time it could be demonstrated that the progression of avian trichomonosis is linked with the presence of cysteine peptidases which can be neutralized by co-application of peptidase inhibitors without toxicity to the host. Such peptidase inhibitors had no direct cytotoxic effect on the viability of the trichomonads. This shows that the reduction of pathogenicity of T. gallinae in the presence of peptidase inhibitors is due to the inhibition of

cysteine peptidase activity rather than a direct effect on the trichomonad cells. These findings underline the importance of cysteine peptidases as drug targets for chemotherapy.

2.1. Materials and Methods
2.1.1. Clonal cultures
     Three genetically different clonal cultures of T. gallinae named Trichomonas gallinae/Budgerigar/Austria/5895-C1/06 (briefly, clone 5895-C1/06) passaged 18 (P18) and 226 (P226) times, Trichomonas gallinae/Racing Pigeon/Austria/8855-C3/06 (clone 8855-C3/06) passaged 18 (P18) and 250 (P250) times, and Trichomonas gallinae/Racing Pigeon/Austria/231-C1/07 (clone 231-C1/07) passaged 19 (P19) and 250 (P250) times were used in this experiment. The assignment reflects the species of bird/country of origin/diagnostic number-clone number/year of isolation.

2.1.2. Propagation of T. gallinae
     The axenic clonal cultures of T. gallinae were grown in Hollander fluid (HF) medium, as described above (Amin et al., 2010). At first, clonal cultures which underwent only a few passages were investigated. Higher passages were obtained by transferring 1 ml of the respective culture into 9 ml of fresh HF medium every 48 h. Assessing the virulence as well as the effect of peptidase inhibitors was performed with an equal passage number (P18). After 24 h of incubation, the cultures were centrifuged at 3300 x g for 5 min (Hettich Rotanta 460, Tuttlingen, Germany). The pellet was washed three times by addition of phosphate buffer saline (PBS, pH 7.2) followed by centrifugation. For counting cells were suspended in 1 ml PBS buffer and motile trichomonads were determined by a Neubauer cell counting chamber (Reichert, Buffalo, NY, USA). The concentration of the inoculum was adjusted to contain approximately $5x10^{6}$ actively motile trophozoites suspended in 200 µl PBS buffer. The viability of trichomonad cells was evaluated using trypan blue staining.

2.1.3. Investigated peptidase inhibitors
     In order to determine the suitable concentrations of cysteine peptidase inhibitors that have no toxic effect on protozoal cells, different concentrations (1mM, 800 µM and 675 µM) of N-α-

130 p-Tosyl-L-Lysine chloromethyl ketone (TLCK) and (270 µM, 140
µM and 70 µM) of L-3-carboxyl-2,3-trans-epoxysuccinyl-
leucylamido (4-guanidino) butane (E-64) (all purchased from Sig-
ma-Aldrich, Steinheim, Germany) were added to the axenic tricho-
monad cultures. The concentration that maintains the viability
of T. gallinae trophozoites as revealed by trypan blue staining
was used in this experiment.

2.1.4. Embryo inoculation and examination
    For each treatment, ten 9-day old embryonated spf chicken
eggs were inoculated with 200 µl of the individual sample of
protozoa suspended in sterile PBS via the allantoic cavity fol-
lowing a standard egg inoculation procedure (Senne, 1998). In
addition, ten eggs were inoculated in the same way with sterile
PBS with and without an adjusted concentration of peptidase in-
hibitors, to be used as a control. The inoculated eggs were
sealed with melted paraffin and incubated at 37°C at a relative
humidity of 55-60%. The survival of the embryos was monitored by
candling the eggs daily. At the end of the incubation period (10
days) the eggs were placed at -20°C for two hours to kill the
embryos and to avoid hatching. The allantoic fluid was harvested
and examined microscopically for the detection of flagellates,
before the embryos were necropsied. Afterwards, samples from
different organs (liver, heart, lung, proventriculus, gizzard,
kidney, spleen, and intestine) were preserved in 10% buffered
neutral formalin and later on embedded in paraffin wax for his-
tological examination. To demonstrate microscopic lesions in the
tissues haematoxylin and eosin stain was performed. For specific
150 demonstration of the protozoa or its nucleic acid in the
tissue sections in situ hybridization (ISH) was applied follow-
ing the protocol of Liebhart et al. (J. Comp. Pathol. 135
(2006), 237-242). Additionally, samples from liver and gut were
preserved at -20°C for polymerase chain reaction (PCR). DNA was
extracted from tissue samples applying the Dneasy® Tissue Kit
(Qiagen, Vienna, Austria), according to the manufacturer in-
structions. PCR was performed following the protocol of
Grabensteiner and Hess (Vet. Parasitol. 142 (2006), 223-230).
The embryos that died during the experiments were exposed to the
same examination procedures. During necropsy autoclaved scissors

and disposable tweezers were used for collecting the samples to
avoid cross contamination.

2.2. Results
2.2.1. Necropsy of embryos infected with different clonal cul-
tures of T. gallinae

    Examination of the embryos infected with T. gallinae clonal
culture 5895-C1/06 P18 revealed gross changes either in the em-
bryos which died at 7, 8 and 9 dpi or in those embryos which
were killed at the end of the incubation period (10 dpi) (Figure
5): An enlarged, severely congested liver was observed in six
out of ten infected embryos. Of these six embryos only two had
yellowish areas or tiny creamy-white foci, especially at the pe-
riphery of the hepatic lobule. In these embryos congested kid-
neys were observed. Additionally, dilated gizzards were seen in
some embryos of this group. However, no lesions were observed in
the heart, lung, proventriculus, spleen and intestine.

    In contrast, no mortalities were recorded in the group of
embryos infected with T. gallinae clone 8855-C3/06 P18. After
killing of the embryos at 10 dpi congested and enlarged livers
were observed in four embryos. In one embryo the kidney was con-
gested. Except one embryo which died on 9th day no mortalities
were observed in the group inoculated with clone 231-C1/07 P19.
The gross examination revealed a congested and slightly enlarged
liver, a finding also present in one embryo killed at the end of
the experiment. Live trichomonads were present in the allantoic
fluid investigated from those embryos which died following in-
fection. No macroscopic lesions were observed at 10 dpi in any
of the embryos inoculated with different clonal cultures of T.
gallinae after long term propagation, similar to the negative
control group.

2.2.2. Gross examination of embryos infected with a virulent T.
gallinae clone coincubated with peptidase inhibitor

    No mortalities were recorded in the group of embryos in-
fected with T. gallinae clone 5895-C1/06 P18 coincubated with
both types of peptidase inhibitors. After killing of the embryos
at 10 dpi no gross lesions in the investigated internal organs
(liver, heart, lung, proventriculus, gizzard, kidney, spleen,

and intestine) were observed in any of these embryos. Moreover, no mortalities or macroscopical changes were demonstrated in embryos inoculated with PBS alone or in the presence of the adjusted amount of peptidase inhibitors.

2.2.3. Polymerase chain reaction

Positive PCR signals were demonstrated in the liver and gut of most infected embryos with different T. gallinae clones at both low and high passage numbers (Figure 6a). No differences were noticed regardless of the passage numbers between samples processed from different clonal cultures. Moreover, positive PCR results were obtained from the liver and gut of embryos infected with clone 5895-C1/06 P18 and coincubated with TLCK or E-64 (Figure 6b). According to the obtained PCR results, there were no considerable differences comparing the infection either in the presence or absence of E-64. Additionally, TLCK had obviously a more distinct inhibitory effect on the infiltration of the flagellate to the liver than E-64.

2.2.4. Histopathology

Apparent differences were noticed between liver samples taken from control embryos (Figure 7a) and those from infected groups. The microscopical examination of livers from embryos infected with T. gallinae clone 5895-C1/06 P18 and stained by haematoxylin and eosin staining revealed severe congestion and dilatation of hepatic blood vessels and blood sinusoids (Figure 7b). Moreover, multiple haemorrhagic areas were scattered all over the hepatic parenchyma. Perivascular leukocytic cellular infiltration, mainly heterophils and lymphocytes, were seen (Figure 7c). Areas of leukocytic cellular infiltration, consisting mainly of mononuclear cells, were observed in the hepatic parenchyma (Figure 7d). Additionally, a caseated necrotic mass was noticed in the hepatic parenchyma with leukocytic cellular infiltration mainly heterophils, especially at the periphery of the lesion (Figure 7e). Most of these histological lesions were present in six of the ten embryos infected with the above mentioned clone. In addition, congestion of the renal blood vessels was observed in two embryos within this group.

Mild to severe congestion of the hepatic blood vessels and

blood sinusoids were detected in the liver of four out of ten embryos infected with the clonal culture 8855-C3/06 P18, while among the embryos infected with 231-C1/07 P19 mild congestion of the hepatic blood vessels was observed in only two embryos.

Following haematoxylin and eosin stain, no specific colour reaction of trichomonads or their organelles could be achieved. In contrast, positive signals in histological tissue sections by ISH were clearly noticed as dark blue stained cells. Protozoal cells were demonstrated adhered to the cuticle of the gizzard of embryos infected with different clonal cultures regardless of the passage number. However, for embryos infected with clone 5895-C1/06 or 8855-C3/06, both P18 the protozoa were also attached to the mucosal epithelium of the gizzard (Figure 8a). Moreover, trichomonad cells were also observed in the gastric muscular layer and in the submucosal glands of the proventriculus of these embryos. Furthermore, flagellates were noticed either in the intestinal lumen or attached to the intestinal villi in the duodenum and caecum (Figure 8b and c), in the hepatic parenchyma (Figure 8d) and in the lung where protozoon were scattered in the pulmonary tissues and adhered to the epithelium of the atria wall (Figure 8e). Although, the parasites were detected by ISH in the proventriculus, gizzard, lung, and intestine of some embryos infected with 5895-C1/06 and 8855-C3/06 both P18, no microscopical lesions were noticed in these organs. Moreover, the nucleic acid of the flagellates was demonstrated in the splenic capsule and in the renal tissue of one embryo infected with clone 8855-C3/06 P18. Overall, the microscopical examination of the heart revealed no pathological changes and no trichomonad cells were present in this organ by ISH. Positive signals were seen in the blood vessels of different organs with parasites emigrating into the tissues leading to extravascular location.

In general, no histological changes were observed in all examined organs namely liver, lung, heart, spleen, kidney, proventriculus, gizzard and intestine obtained from embryos infected with different clonal cultures which underwent a high number of in vitro passages. Tissues processed from the control group were found normal and no trichomonads could be detected by ISH.

2.2.5. Effect of peptidase inhibitors on the pathogenicity of T. gallinae

The optimum concentration that had no effect on the viability of T. gallinae trophozoites as revealed by trypan blue staining was used in this experiment. These concentrations were 675 μM for TLCK and 270 μM for E-64, respectively. Consequently, no cytotoxic effect on the embryos, no mortalities or pathomorphological changes in the investigated organs were recorded in the negative control groups. The histopathological examination of different organs of the embryos infected with T. gallinae clone 5895-C1/06 P18 in the presence of peptidase inhibitors (TLCK and E-64) revealed no histological changes as demonstrated exemplarily for one of the liver samples (Figure 7f). However, the nucleic acid of trichomonds was demonstrated by ISH in the hepatic parenchyma and in some of these embryos trophozoites were noticed attached to the cuticle of the gizzard (Figure 8f).

Preferred embodiments according to the present invention:
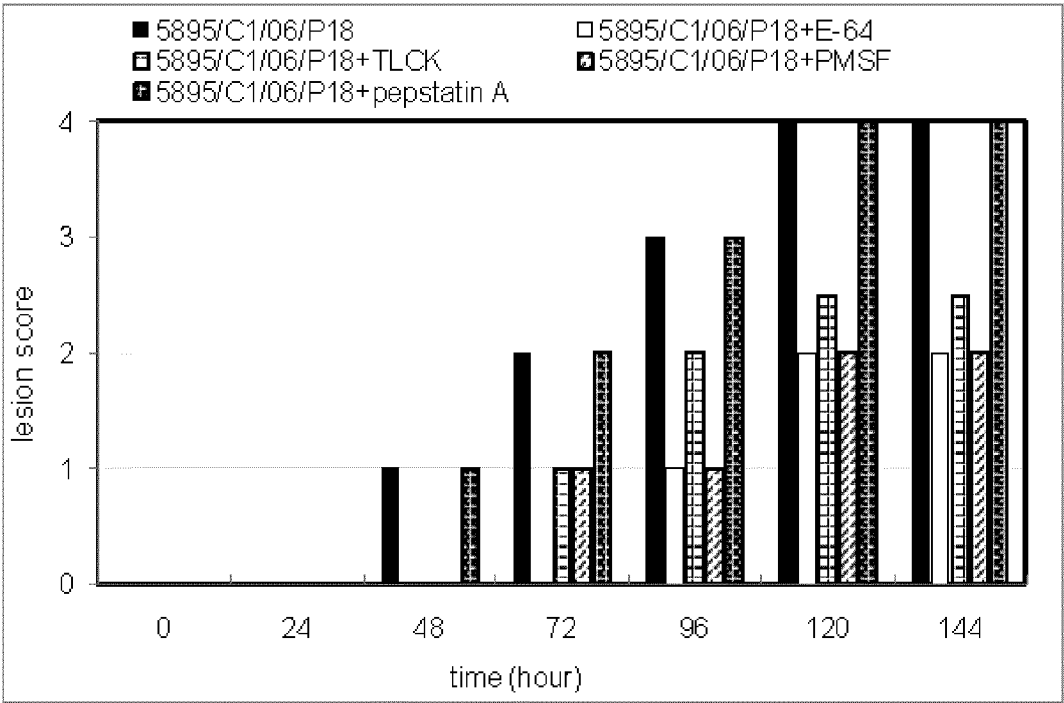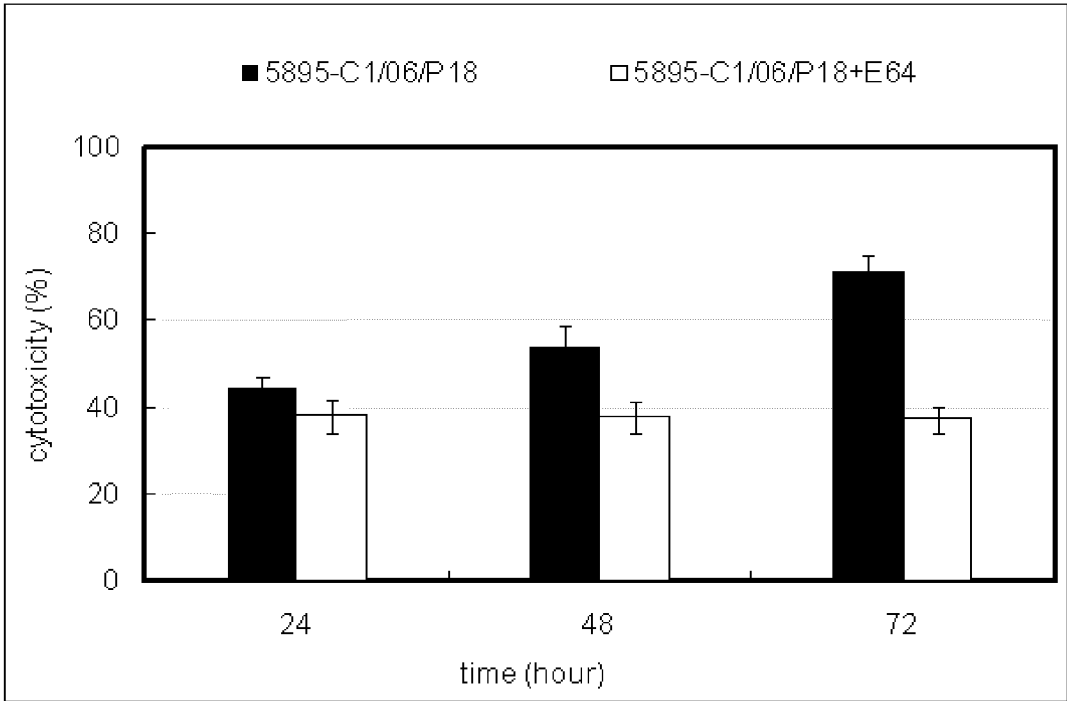1.   Protease inhibitors for use in the treatment of a Trichomonas gallinae infection of an animal, selected from the orders Galliformes, Passeriformes, Psittaciformes, Columbiformes and Falconiformes.

2.   Protease inhibitors according to embodiment 1 wherein the protease inhibitors are selected from cysteine protease inhibitors and serine protease inhibitors, especially cysteine protease inhibitors.

3.   Protease inhibitors according to embodiment 1 or 2, wherein the protease inhibitors are cysteine protease inhibitors selected from the group consisting of E-64 (trans-Epoxysuccinyl-L-leucylamido(4-guanidino)butane), L-trans-3-Carboxyoxiran-2-carbonyl-L-leucylagmatine, N-(trans-Epoxysuccinyl)-L-leucine 4-guanidinobutylamide), TLCK (tosyl-L-lysine chloromethyl ketone), TPCK (tosyl-L-phenylalanine chloromethyl ketone), or mixtures thereof.

4.   Protease inhibitors according to any one of embodiments 1 to 3 for use in the treatment of a Trichomonas gallinae infec-

tion of an animal, selected from the species Columba livia,
Melopsittacus undulates, Serinus canaria forma domestica, or
Falco spp..

5.   Protease inhibitors according to any one of embodiments 1
to 4, wherein the inhibitors are administered orally to the ani-
mal, preferably wherein oral administration includes administra-
tion of a solution of cysteine protease inhibitors or cysteine
protease inhibitors in tablet form.

6.   Method for treating a Trichomonas gallinae infection of an
animal, selected from the orders Galliformes, Passeriformes,
Psittaciformes, Columbiformes and Falconiformes, wherein an ef-
fective amount of a protease inhibitor, especially a cysteine
protease inhibitor, is administered to an animal.

7.   Use of a protease inhibitor, especially a cysteine protease
inhibitor for the manufacture of a medicament for the treatment
of a Trichomonas gallinae infection of an animal, selected from
the orders Galliformes, Passeriformes, Psittaciformes, Colum-
biformes and Falconiformes.

8.   Vaccine against a Trichomonas gallinae infection of an ani-
mal, selected from the orders Galliformes, Passeriformes, Psit-
taciformes, Columbiformes and Falconiformes, comprising a cyste-
ine protease and an adjuvant.

9.   Vaccine against a Trichomonas gallinae infection of an ani-
mal, selected from the orders Galliformes, Passeriformes, Psit-
taciformes, Columbiformes and Falconiformes, comprising a nucle-
ic acid molecule, especially a DNA molecule, encoding a cysteine
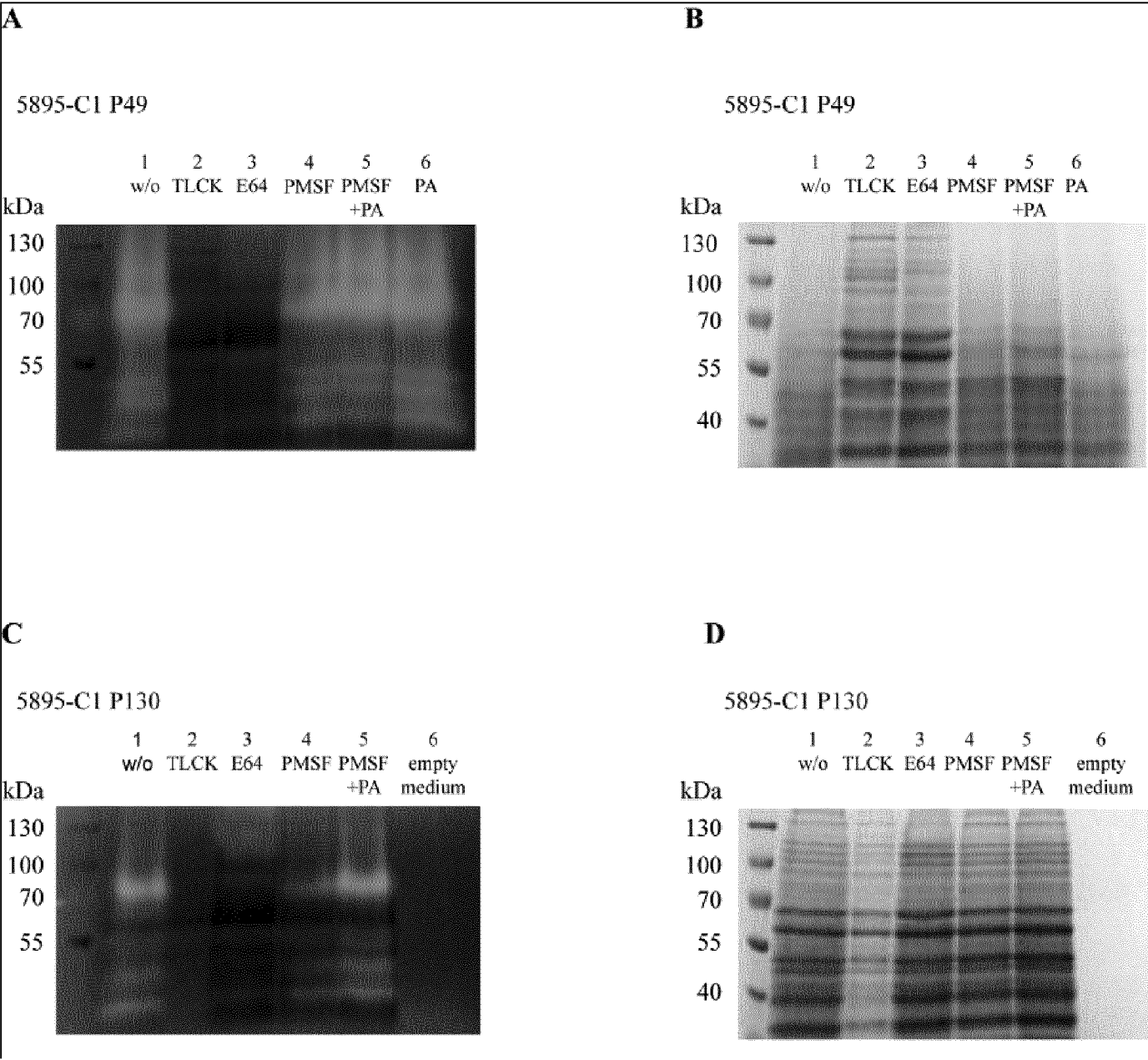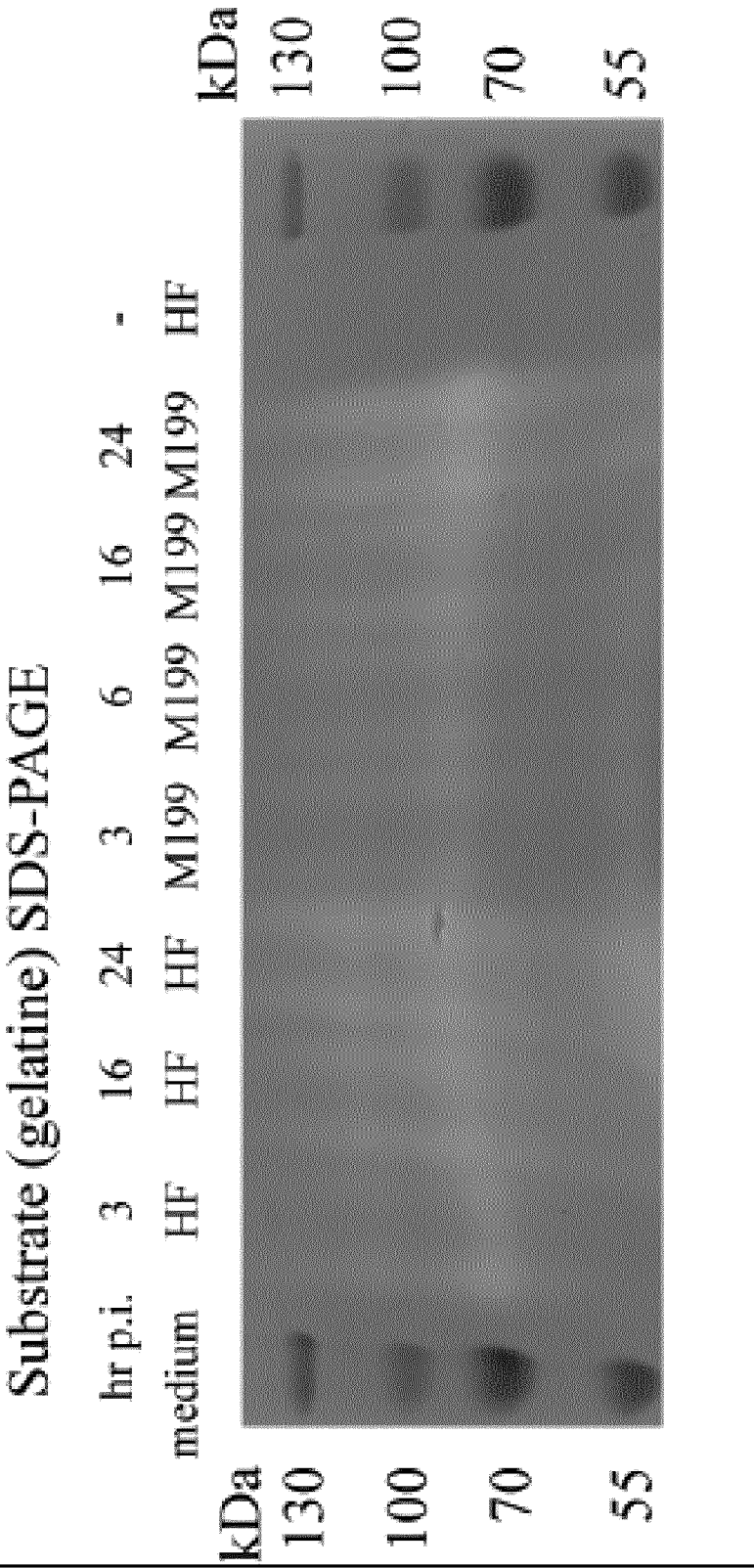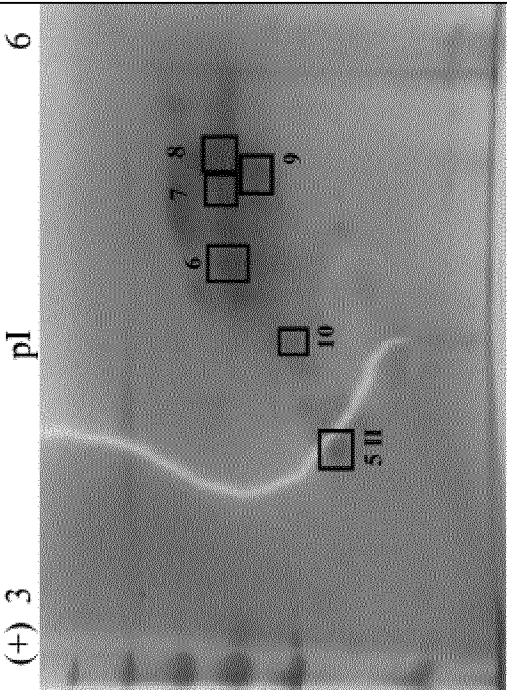protease and an adjuvant.

Claims:

1.   Cysteine protease inhibitors for use in the oral treatment
of a Trichomonas gallinae infection of an animal, selected from
the orders Galliformes, Passeriformes, Psittaciformes, Colum-
biformes and Falconiformes.

2.   Cysteine protease inhibitors according to claim 1, wherein
the protease inhibitors are cysteine protease inhibitors select-
ed from the group consisting of E-64 (trans-Epoxysuccinyl-L-
leucylamido(4-guanidino)butane,   L-trans-3-Carboxyoxiran-2-
carbonyl-L-leucylagmatine, N-(trans-Epoxysuccinyl)-L-leucine 4-
guanidinobutylamide), TLCK (tosyl-L-lysine chloromethyl ketone),
TPCK (tosyl-L-phenylalanine chloromethyl ketone), or mixtures
thereof.

3.   Cysteine protease inhibitors according to claim 1 or 2 for
use in the oral treatment of a Trichomonas gallinae infection of
an animal, selected from the species Columba livia, Melopsitta-
cus undulates, Serinus canaria forma domestica, or Falco spp..

4.   Cysteine protease inhibitors according to any one of claims
1 to 3 for use in the oral treatment of a Trichomonas gallinae
infection of an animal, wherein oral treatment includes admin-
istration of a solution of cysteine protease inhibitors or cys-
teine protease inhibitors in tablet form.

5.   Method for treating a Trichomonas gallinae infection of an
animal, selected from the orders Galliformes, Passeriformes,
Psittaciformes, Columbiformes and Falconiformes, wherein an ef-
fective amount of a cysteine protease inhibitor is administered
orally to an animal.

6.   Use of a cysteine protease inhibitor for the manufacture of
a medicament for the oral treatment of a Trichomonas gallinae
infection of an animal, selected from the orders Galliformes,
Passeriformes, Psittaciformes, Columbiformes and Falconiformes.

7.   Vaccine against a Trichomonas gallinae infection of an animal, selected from the orders Galliformes, Passeriformes, Psittaciformes, Columbiformes and Falconiformes, comprising a cysteine protease and an adjuvant.

8.   Vaccine against a Trichomonas gallinae infection of an animal, selected from the orders Galliformes, Passeriformes, Psittaciformes, Columbiformes and Falconiformes, comprising a nucleic acid molecule, especially a DNA molecule, encoding a cysteine protease and an adjuvant.

A

B

Fig. 1

Fig. 2

Fig. 3
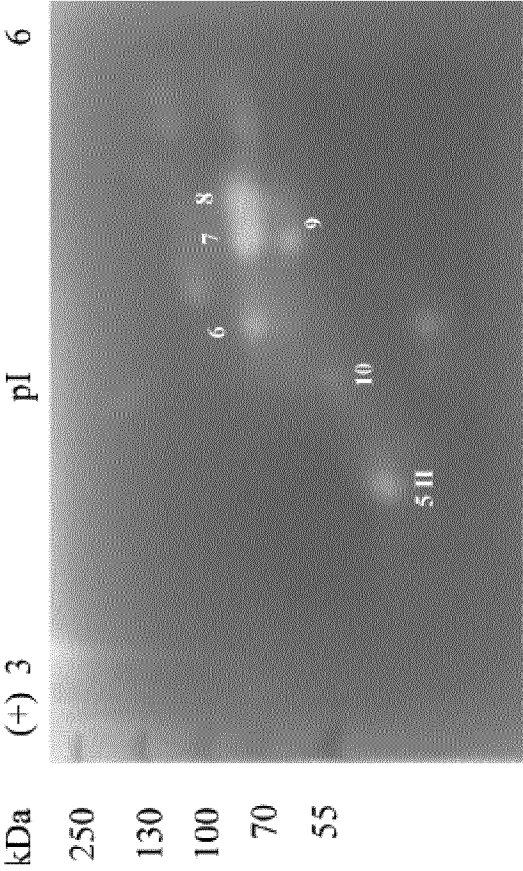
Fig. 4
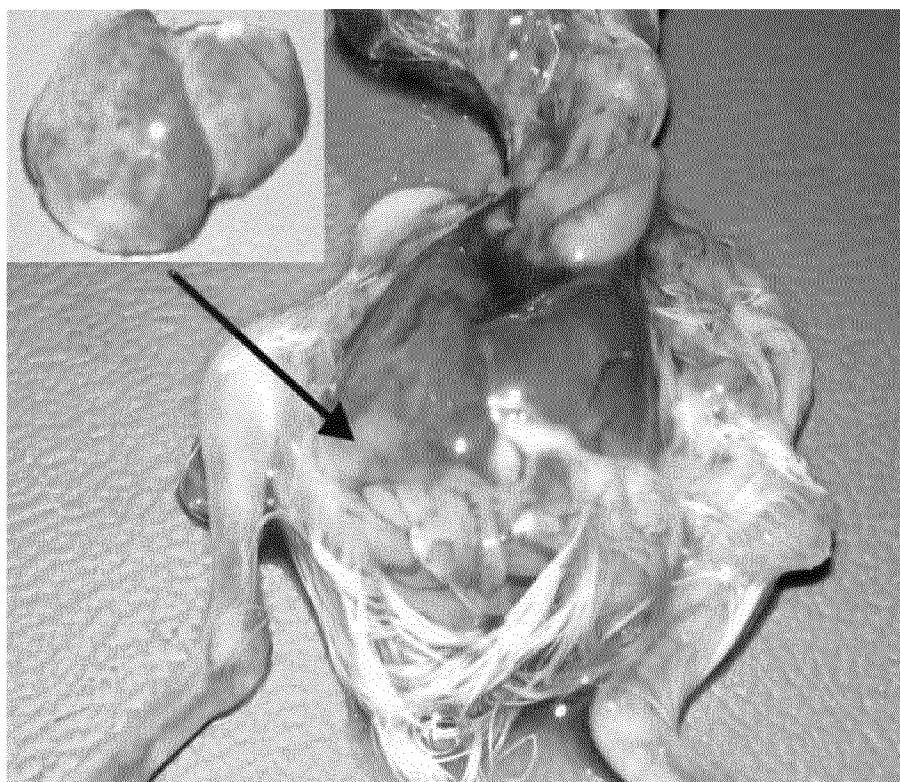
Fig. 5A
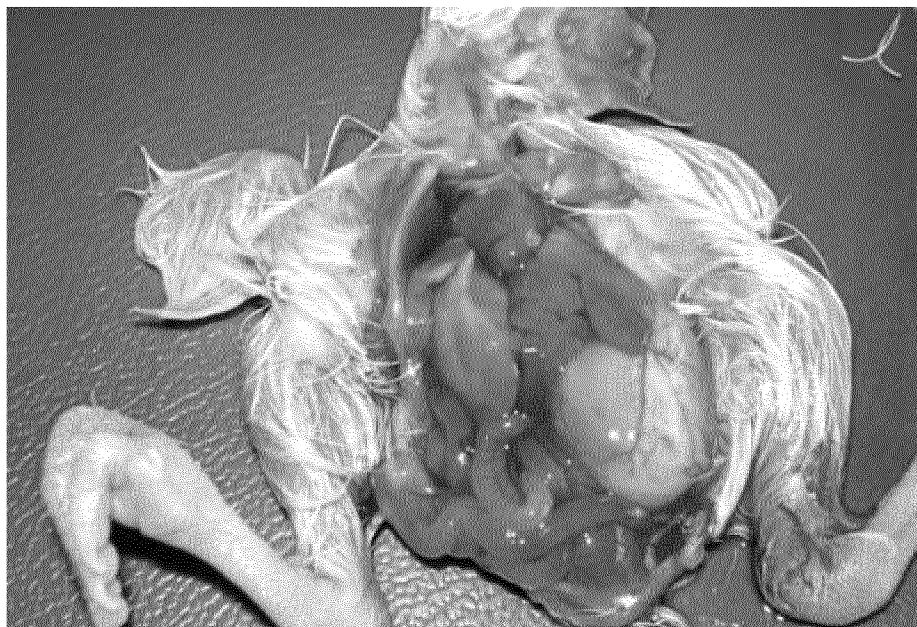
Fig. 5B

Fig. 6A
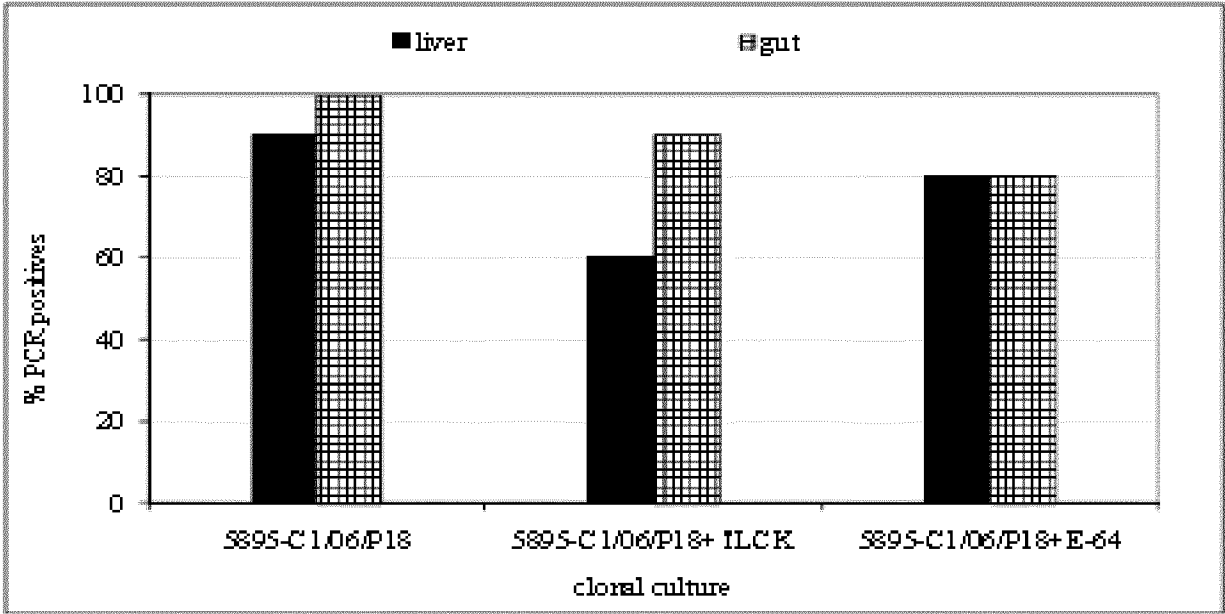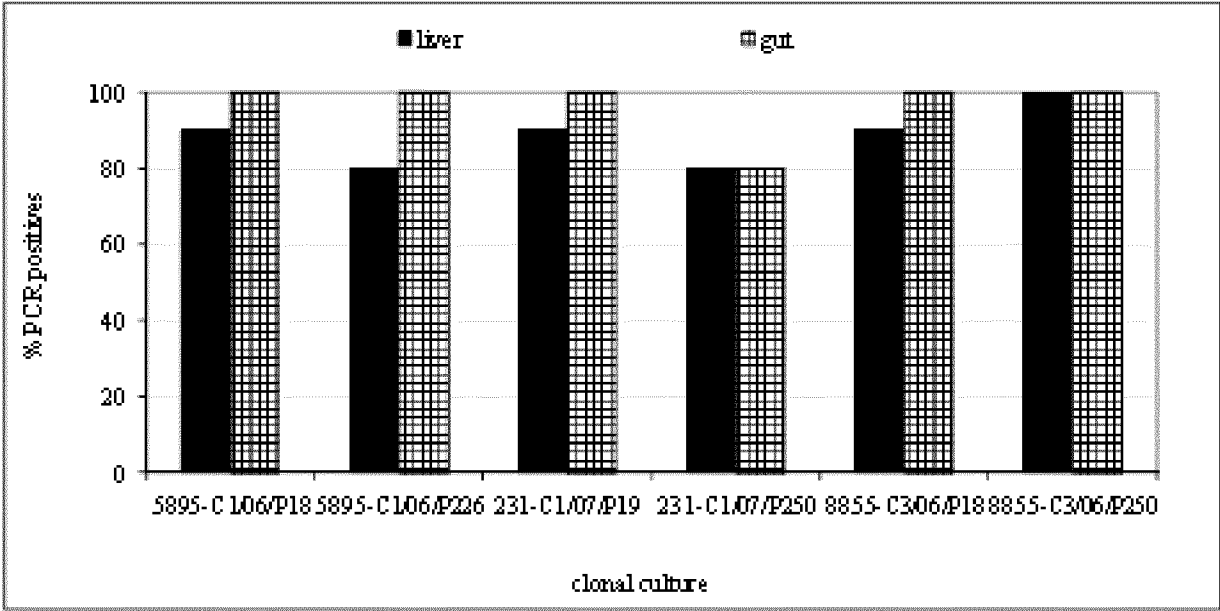
Fig. 6B

Fig. 7
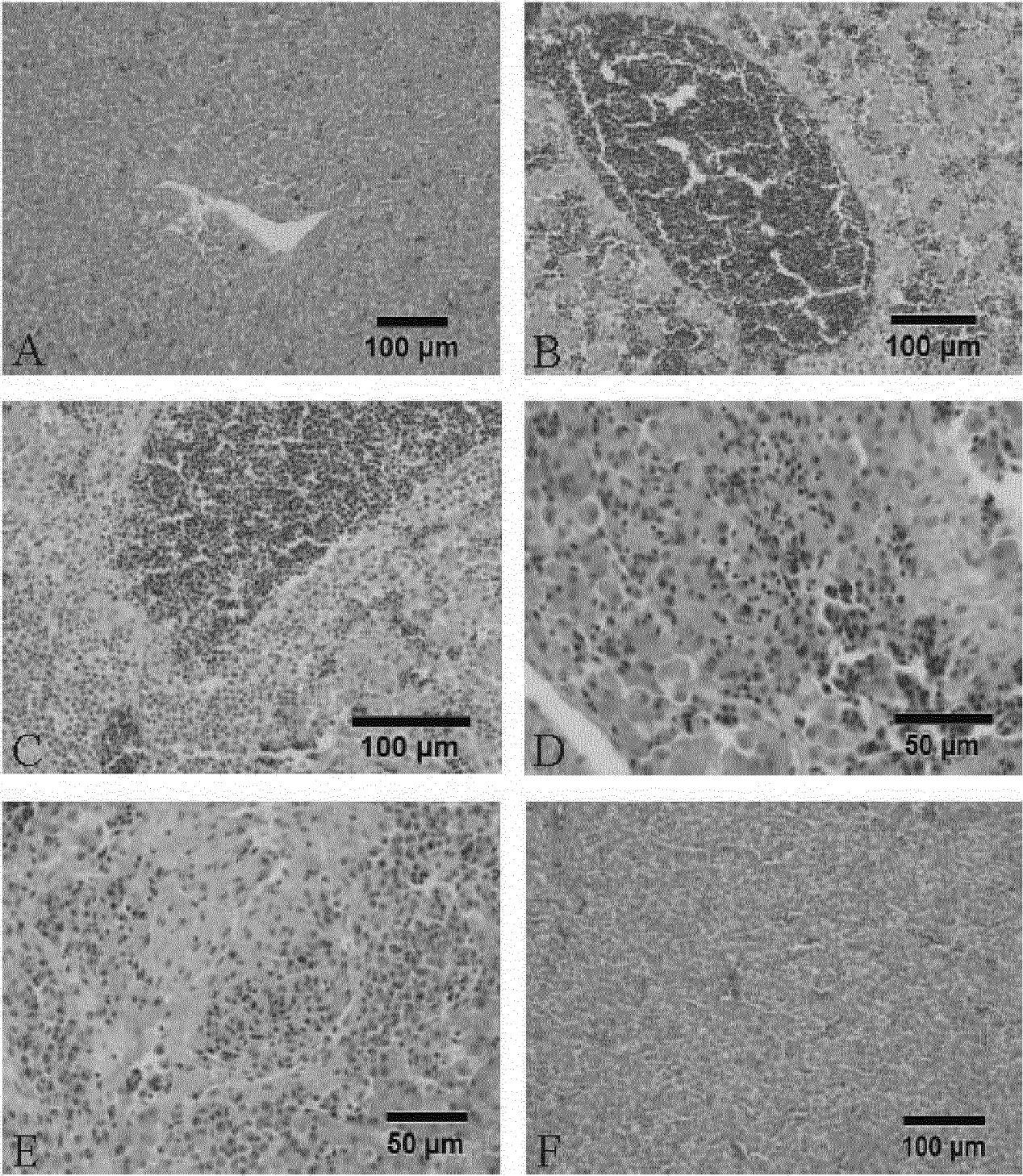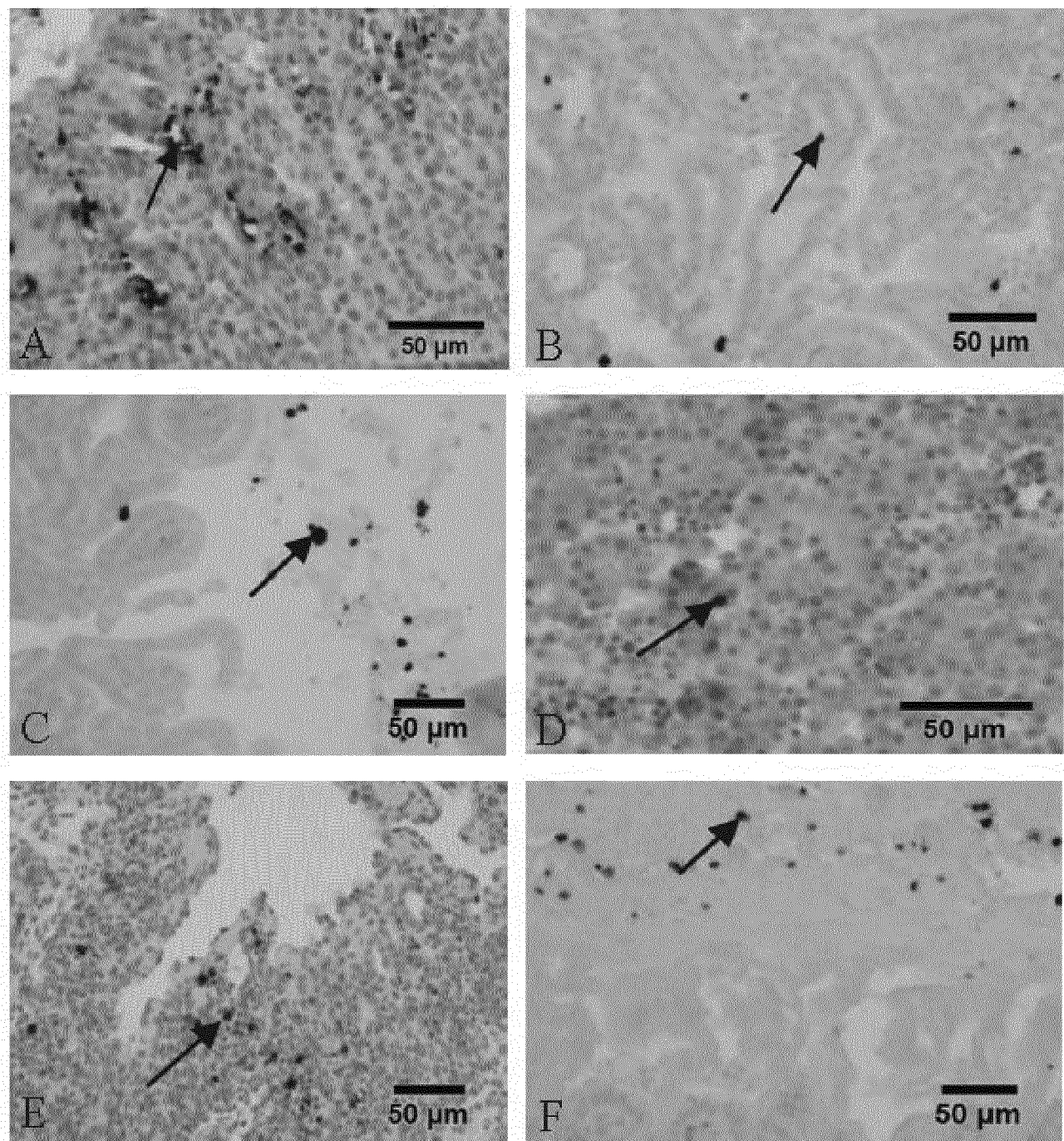
Fig. 8

Fig. 9

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

INV. A61K31/18   A61K31/336   A61K39/002   A61K39/39   A61P33/02
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, BIOSIS, CHEM ABS Data, EMBASE, SCISEARCH, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 97/05867 A1 (BRITISH TECH GROUP [GB]; ACKERS JOHN PHILIP [GB]; FAIRLAMB ALAN HUTCHI) 20 February 1997 (1997-02-20) cited in the application page 2, line 22 - page 3, line 3 page 4, lines 6-9 page 7, lines 1-12 -----	1,3-6
X	WO 00/63350 A2 (UNIV GEORGIA RES FOUND [US]; BAYLOR COLLEGE MEDICINE [US]; BAIG SALMAN) 26 October 2000 (2000-10-26) cited in the application page 20, lines 18-30 claims 4-6, 9 ----- -/--	7,8

[X] Further documents are listed in the continuation of Box C.   [X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
28 March 2013	05/04/2013

Authorized officer

Herdemann, Matthias

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C(Continuation).	DOCUMENTS CONSIDERED TO BE RELEVANT	
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 98/44943 A1 (UNIV GLASGOW [GB]; MOTTRAM JEREMY CHARLES [GB]; COOMBS GRAHAM HERBERT) 15 October 1998 (1998-10-15) cited in the application page 10, lines 23-33 claims 1-4, 11-15, 19 -----	7,8
X	WO 2008/043180 A1 (HMAMA ZAKARIA [CA]) 17 April 2008 (2008-04-17) cited in the application page 6, line 27 - page 7, line 6 claims 1-7, 11-16 -----	7,8
A	WO 2010/000398 A1 (BAYER ANIMAL HEALTH GMBH [DE]; HARDER ACHIM [DE]; GREIF GISELA [DE]; F) 7 January 2010 (2010-01-07) cited in the application page 4, lines 7-14 page 5, line 4 - page 6, line 2 page 6, lines 8-21 claims 1-6 -----	1-8
A	IWALEWA E O ET AL: "Anti-protozoan activities of Harungana madagascariensis stem bark extract on trichomonads and malaria", JOURNAL OF ETHNOPHARMACOLOGY, ELSEVIER SCIENTIFIC PUBLISHERS LTD, IE, vol. 117, no. 3, 22 May 2008 (2008-05-22), pages 507-511, XP022635286, ISSN: 0378-8741, DOI: 10.1016/J.JEP.2008.02.019 [retrieved on 2008-03-10] cited in the application abstract page 508, left-hand column, last paragraph - right-hand column, paragraph 3 table 1 -----	1-8
X,P	AZIZA AMIN ET AL: "Cysteine Peptidases, Secreted by Trichomonas gallinae, Are Involved in the Cytopathogenic Effects on a Permanent Chicken Liver Cell Culture", PLOS ONE, vol. 7, no. 5, 23 May 2012 (2012-05-23), page E37417, XP055031041, DOI: 10.1371/journal.pone.0037417 cited in the application	1-6
A,P	the whole document -----	7,8

-/--

1

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A,P	AZIZA AMIN ET AL: "Trichomonas gallinae, in comparison to Tetratrichomonas gallinarum, induces distinctive cytopathogenic effects in tissue cultures", VETERINARY PARASITOLOGY, vol. 186, no. 3-4, 1 May 2012 (2012-05-01) , pages 196-206, XP055031048, ISSN: 0304-4017, DOI: 10.1016/j.vetpar.2011.11.037 abstract page 196, right-hand column, paragraph 2 - page 197, left-hand column, paragraph 3 figure 2 -----	1-8

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INTERNATIONAL SEARCH REPORT
Information on patent family members

Patent document cited in search report		Publication date	Patent family member(s)		Publication date
WO 9705867	A1	20-02-1997	AU	6627296 A	05-03-1997
			WO	9705867 A1	20-02-1997
WO 0063350	A2	26-10-2000	AU	4650500 A	02-11-2000
			BR	0009944 A	08-01-2002
			CN	1352682 A	05-06-2002
			MX	PA01010541 A	19-08-2003
			WO	0063350 A2	26-10-2000
WO 9844943	A1	15-10-1998	BR	9807927 A	22-02-2000
			EP	0973545 A1	26-01-2000
			US	2004005326 A1	08-01-2004
			WO	9844943 A1	15-10-1998
WO 2008043180	A1	17-04-2008	NONE		
WO 2010000398	A1	07-01-2010	AR	072426 A1	25-08-2010
			AU	2009266124 A1	07-01-2010
			CA	2729727 A1	07-01-2010
			CN	102083440 A	01-06-2011
			CO	6321268 A2	20-09-2011
			DE	102008031283 A1	07-01-2010
			DO	P2010000406 A	15-01-2011
			EC	SP10010721 A	31-01-2011
			EP	2310017 A1	20-04-2011
			JP	2011526264 A	06-10-2011
			KR	20110031360 A	25-03-2011
			PE	03702011 A1	22-06-2011
			RU	2011103476 A	10-08-2012
			SV	2011003784 A	20-05-2011
			US	2011118176 A1	19-05-2011
			WO	2010000398 A1	07-01-2010