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Antifungal and antitumor models of bioactive protective peptides

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ABSTRACT

Peptides are remarkably reactive molecules produced by a great variety of species and able to display a number functions in uni- and multicellular organisms as mediators, agonists and regulating substances. Some of them ex cytotoxic effects on cells other than those that produced them, and may have a role in controlling subpopulatio and protecting certain species or cell types. Presently, we focus on antifungal and antitumor peptides and discu a few models in which specific sequences and structures exerted direct inhibitory effects or stimulated a protecti immune response. The killer peptide, deduced from an antiidiotypic antibody, with several antimicrobial activities a other Ig-derived peptides with cytotoxic activities including antitumor effects, are models studied *in vitro* and *in vi* Peptide 10 from gp43 of *P. brasiliensis* (P10) and the vaccine perspective against paracoccidioidomycosis is anoth topic illustrating the protective effect *in vivo* against a pathogenic fungus. The cationic antimicrobial peptides wi antitumor activities are mostly reviewed here. Local treatment of murine melanoma by the peptide gomesin is anoth model studied at the Experimental Oncology Unit of UNIFESP.

Key words: bioactive peptides, *Paracoccidioides brasiliensis*, tumor cells, killer peptide, melanoma, apoptosis.

INTRODUCTION

Bioactive peptides arise from proteins by the action of peptidases or are chemically synthesized based on certain templates of natural sequences that have been selected by a variety of screening methods. Peptides can be designed aiming at enhanced functional activity by using amino acid substitutions and chemical modification. Owing to their great diversity of binding properties, peptides can play roles of biochemical reagents, pharmacological drugs, hormones, antibiotics, vaccines and mediators of neural and immunological signaling. Peptides interact with membrane structures, are specifically recognized by cell surface receptors or act as liga teracting with intracellular compounds and subc structures. Peptides can include epitopes recogn antibodies and TCRs, and those called protecti topes elicit a protective immune response. On fo the actual fungal and tumor models, peptides that direct cytotoxicity on target cells or elicit a pro immune response in animals experimentally infe challenged with tumor cells have been investigat

ANTIFUNGAL PEPTIDES

During the past decades, an increase in the incid fungal diseases has been recognized mainly ca

In commemoration of the 75th anniversary.

the use of antimycotics is the only option for the treatment of fungal infections. Currently used antimycotics, however, frequently have a limited activity spectrum, are available only in intravenous formulations, favor resistance development, and cause serious side-effects (reviewed in François et al. 2005). Thus, the search for new antifungal therapies is strongly stimulated, and the use of antifungal peptides is a promising alternative.

Antifungal properties of peptides have been reviewed by De Lucca and Walsh (2000). There are 405 peptides with antifungal properties described, comprising linear or cyclic, hydrophobic or amphipathic structures (http://aps.unmc.edu/AP/main.php).

Their cytotoxicity may involve binding to and disruption of the membrane (Shai 1995), membrane penetration and interaction with the mitochondria (Helmerhorst et al. 1999) or pore formation (Bechinger 1997). Antifungal peptides have been studied in bacteria, fungi, plants, insects, amphibians and mammals. Relevant examples are given next.

Syringomycins, syringostatins and syringotoxins from *Pseudomonas syringae* are lipodepsipeptides highly lethal to *Candida albicans*, *Aspergillus* and *Fusarium* species (De Lucca and Walsh 2000, De Lucca et al. 1999, Sorensen et al. 1996). Glycopeptide cepacidines from *Burkholderia cepacia* are active against *Candida sp.*, *Aspergillus niger*, *Fusarium oxysporum* and *Cryptococcus neoformans* (Lee et al. 1994, Lim et al. 1994). Antifungal peptidylnucleoside nikkomycins are produced by *Streptomyces tendae*, act by inhibiting chitin biosynthesis and were effective in murine infections by *Coccidioides immitis* and *Blastomyces dermatitidis* (Hector et al. 1990). Zeamatin, the 22 kilodalton (kDa) peptide produced by *Zea mays*, permeabilizes the fungal membrane and kills *C. albicans* with a minimal inhibitory concentration (MIC) of 0.5 μg/ml (Roberts and Selitrennikoff 1991). Cecropins from the silk moth *Hyalopora cecropia* are linear, lytic peptides effective against germinating conidia of *F. oxysporum* and *A. fumigatus* (De Lucca et al. 1998). Both the L- and D-isomeric forms of cecropin B were fungicidal (De Lucca et al. 2000). Drosomycin is a 44 amino acids (aa) do not coevolve with pathogens (Jiggins and Kim 2005). In contrast, antimicrobial peptides (AMPs) appear to undergo a rapid adaptive evolution in vertebrates. In frogs, each species produces 10-20 AMPs that differ in size, sequence and specificity, and this rapid diversification is driven by evolutionary selection (Duda et al. 2002). Dermaseptins, produced by *Phyllomedusa sauvagii*, a South American frog, are lysine-rich linear peptides fungicidal for *A. flavus*, *A. fumigatus* and *F. oxysporum* (Mor et al. 1994). Magainins are antifungal peptides produced by the African frog *Xenopus laevis* (De Lucca and Walsh 2000). They are not hemolytic and inhibit *Candida albicans* (Zasloff 1987). Plant [DmAMP1 from dahlia (*Dahlia merckii*), RsAFP2 from radish (*Raphanus sativus*), HsAFP1 from coral bells (*Heuchera sanguinea*), Psd1 from pea (*Pisum sativum*), MsDef1 from alfalfa (*Medicago sativa*) and MtDef2 from barrel medic (*Medicago truncatula*)], insects (Termicin from the termite *Pseudacanthotermes spiniger*, Drosomycin from the fruitfly *Drosophila melanogaster*, Heliomicin from the tobacco budworm *Heliothis virescens*) and human [β-defensin 1 (HBD1), β-defensin 2 (HBD2), β-defensin 3 (HBD3)] defensins showed antifungal properties (reviewed in Aerts et al. 2008). Although there are no clear similarities in the mode of action of these defensins, the presence of sphingolipid glucosyl ceramide (GlcCer) in fungal membranes seems to play a central role in the action of some defensins (Thevissen et al. 2004). Only Psd1 was internalized in the fungal cell, affecting the normal progression of the cell cycle (Lobo et al. 2007), and it is possible that the other defensins stay outside the cell inducing fungal cell death after interaction with their target (e.g. sphingolipids) and modulation of intracellular signaling cascades (Aerts et al. 2008). RsAFP2 was also effective in an *in vivo* prophylactic model of murine candidiasis (Tavares et al. 2008).

β-Defensins include porcine cationic, cysteine-rich protegrins which inhibited *C. albicans* (Cho et al. 1998). Gomesin, a cationic AMP isolated from the hemocytes of the unchallenged Brazilian spider *Acanthoscurria gomesiana* (Silva et al. 2000), is structurally related to

the presence of the peptide, induced a decrease in capsule expression, rendering cells more susceptible to brain phagocytes and, in association with fluconazole, in concentrations with low antimicrobial activity (0.1–1μM), inhibited fungal growth and enhanced the antimicrobial activity of brain phagocytes (Barbosa et al. 2007). One of the models described in the present review is that of gomesin cytotoxicity in murine and human tumor cells (Rodrigues et al. 2008).

Among the antifungal peptides produced by fungi, the echinocandins interfere with the cell wall biosynthesis (Denning 1997) and the pneumocandins, aculeacins, WF11899, and mulundocandins have a modified echinocandin B peptide core (Debono and Gordee 1994, Kurtz and Douglas 1997). Echinocandins are produced by *Aspergillus nidulans* and *A. rugulosus* and are effective against *Candida* (MIC = 0.6μg/ml for echinocandin B and *C. albicans*) (reviewed in De Lucca and Walsh 2000). Clinical trials have started with molecules of the echinocandin group, VER-002, FK463 and caspofungin (MK-0991) modified for increased solubility and active against *Candida* spp. and *Aspergillus* spp. V-echinocandin and FK463 were effective in the treatment of esophageal candidiasis, the latter in AIDs patients (reviewed in De Lucca and Walsh 2000). Clinical trials with caspofungin (derived from pneumocandin), a drug that inhibits β-1,3 D-glucan synthase, have shown excellent results in the treatment of *Candida* infections and invasive aspergillosis refractory to other antifungal agents (i.e., conventional or lipid formulations of amphotericin B and/or itraconazole). Aureobasidins are produced by *Aureobasidium pullulans*, interfere with sphingolipid synthesis and are effective against murine candidiasis (Nageic et al. 1997, Takesako et al. 1993).

KILLER TOXINS AND KILLER PEPTIDES

Killer yeasts secrete killer toxins that target susceptible cells in a two-step receptor-mediated manner. They bind to cell wall receptors and translocate to the plasma membrane. They can then interact with secondary receptors or enter susceptible cells to exert a cytocidal effect (Magliani et al. 1997, Schmitt and Breinig

susceptible cells by various mechanisms, includ
induction of cation-selective ion channels in the
membrane, interference in the cell cycle (G1, G
arrest), chromosomal DNA synthesis and anticod
clease (Schmitt and Breinig 2006, Santos and Ma
2004, Jablonowski and Schaffrath 2007, Klasse
2004). Killer toxins can induce apoptosis media
yeast caspase Yca1p, characterized by DNA frag
tion, and phosphatidylserine external membrane
sion. This could be a general cell death mechani
der natural environmental conditions (Paluszynsk
2007, Schmitt and Reiter 2008).

The direct use of killer toxins in antifunga
apy was discouraged owing to some of their
ties. They are generally heat-labile, protease-se
and act within a narrow pH and temperature range
are antigenic and toxic, as shown for *Pichia a*
killer toxin (Pettoello-Mantovani et al. 1995). T
come these pitfalls of a potential therapeutic age
munological derivatives were generated on the b
the idiotypic network that mimicked the toxic e
P. anomala killer toxin (Polonelli et al. 1991).
antibodies with the internal image of the active
a killer toxin, which acted as antibiotics, were th
tained. They exerted significant therapeutic eff
experimental models of candidiasis, aspergillos
pneumocystosis.

Toxic effects were also obtained with singl
variable fragment (scFv) preparations and they w
ther examined by synthesizing overlapping decap
which correspond to the light chain of antibodie
and heavy chain of antibodies (V_H) regions. Th
gions include the complementary determining
(CDRs) that were tested *in vitro* against *C. al*
Several peptides were active and one of them,
ponding to the framework sequence with the fina
amino acids belonging to V_L CDR1, was selec
was very cytotoxic and the substitution of the
minal glutamic acid by alanine generated a pepti
the AKVTMTCSAS sequence that was severa
more active and was called killer peptide (KP). T
interacted with β-glucan and this binding was in

dition to *C. albicans*, and was effective even in normal and immunocompromised animals against vaginal and systemic candidiasis (Polonelli et al. 2003), disseminated cryptococcosis (Cenci et al. 2004) and paracoccidioidomycosis (Travassos et al. 2004a). The KP is very stable forming dimers in non-reducing conditions without loss of activity (Magliani et al. 2004a, b).

The remarkable cytotoxicity of KP was also examined by electron microscopy. *C. albicans* cells treated with KP showed important internal alterations, including cell wall swelling with middle electron-dense region, collapse of the plasma membrane, condensation and fragmentation of nuclear material, and alteration of mitochondria structure (Fig. 1A). In a dividing cell with a big vacuole and chromatin condensation and fragmentation, cellular alterations were seen beyond the septum separating both cells, with the daughter cell already affected by the KP showing an altered cell wall (Fig. 1B).

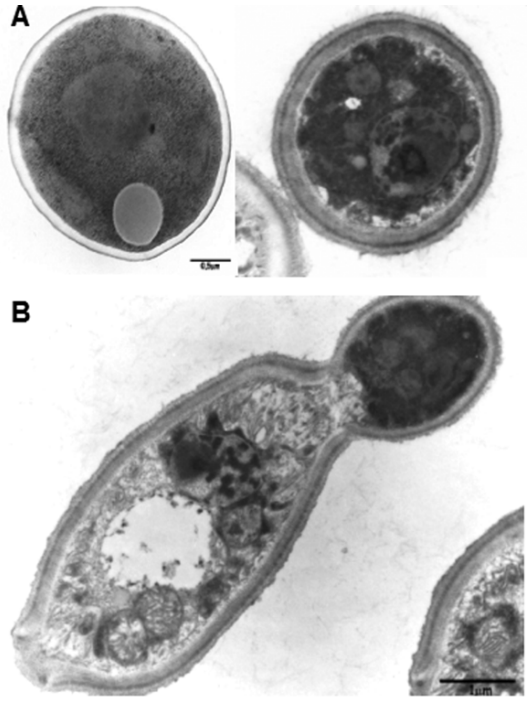

Fig. 1 – Electron micrographs showing the cytotoxic effects of the killer peptide (KP) on *Candida albicans.* (**A**) Normal untreated or treated with the inactive scrambled peptide *C. albicans* yeast cell (left) as compared with the KP-treated yeast cell (right). Major alterations can be seen as the swelling of the cell wall, plasma membrane collapse, chromatin condensation and nuclear fragmentation. (**B**) An elongated *C. albicans* cell with a budding cell, both affected by KP treatment. The same alterations as in (A) are seen with nuclear fragmentation and cytoplasmic blebs invading the daughter cells beyond the septum.

A MODEL OF DIRECT ANTIFUNGAL EFFECT OF A PEPTIDE

Glucans, chitin and mannoproteins, in addition to plasma membrane sterols, are natural targets of antifungal drugs. Additional targets are ceramide monohexosides, ubiquitously present on the fungal cell wall and displaying several roles in fungal cells (Nimrichter et al. 2008). In *C. neoformans* (Rodrigues et al. 2000), *C. albicans* and *Pseudallescheria boydii*, these glycolipids were identified as targets of human antibodies that inhibited fungal growth. Other targets are melanin, adhesion factors, and cell wall enzymes. The killer decapeptide (KP) described above was synthesized and engineered demonstrating a strong candidacidal activity *in vitro* and curing rat vaginal infections caused by fluconazole-susceptible and -resistant *C. albicans* strains (Polonelli et al. 2003). The fungicidal activity of KP *in vitro* against *P. brasiliensis* and its therapeutic activity *in vivo* have been reported (Travassos et al. 2004a).

Paracoccidioidomycosis (PCM) is the prevalent systemic mycosis in South America with most reported cases in Brazil. It is a major cause of disability and death among young adult rural workers. Sequels are frequent and therapy with itraconazole, amphotericin or sulfamethoxazole/trimethoprim are used in clinical practice, relapses are a significant unsolved problem (Travassos et al. 2008b). Vaccination against PCM is now a prospective goal after P10, and four other peptides derived from the major diagnostic antigen gp43 were found to be promiscuously presented by several human leukocyte antigens DR, MHC class II molecules (HLA-DR) (Iwai et al. 2003). Such a vaccine could function as an adjuvant to chemotherapy significantly reducing the time of treatment (Travassos et al. 2008a, b).

Wide-spectrum antimicrobial peptides, such as KP

tion in cases of anergy and drug resistance. Multiply-budding yeast cells of *P. brasiliensis* had their viability hampered at 39 ng of KP/yeast in distilled water. The D-isomeric form of KP was also active. Further, the decapeptide was therapeutic in B10A mice infected intravenously with 3×10^6 cells of *P. brasiliensis* Pb18 isolate administered intraperitoneally at 3.3 μg/g of body weight, 1 h after infection and 1 and 2 days later. With this protocol, no colony forming units (CFUs) were obtained from lung, spleen and liver after 8 days of fungal challenge in the KP treated animals. In these animals compared to those injected with the scrambled peptide, the liver granulomas were smaller and fewer with no visible fungi. The lungs were less infiltrated with extensive areas of normal alveoli and no visible fungi. Spleens also were little affected, with no detectable fungi.

It was clear therefore that KP was an effective inhibitor of *P. brasiliensis in vitro* and *in vivo* (Travassos et al. 2004a).

It is still not clear whether α-1,3 glucan, the predominant polysaccharide of yeast forms of *P. brasiliensis*, is a target of KP. There is, however, evidence that yeast forms may have β-glucans at the cell surface. Macrophages from pentraxin 3 transgenic (PTX3 Tg) mice showed improved opsonin-independent phagocytosis of zymosan particles and yeast forms of *P. brasiliensis.* In the case of *P. brasiliensis*, an enhanced microbicidal activity accompanied by high production of nitric oxide was observed in macrophages from transgenic mice. Blockade of dectin-1 receptor for β-1,3 glucan inhibited the phagocytosis of zymosan particles by PTX3 Tg macrophages, pointing out the relevant role of dectin-1 as the main receptor involved in zymosan and possibly also of *P. brasiliensis* uptake (Diniz et al. 2004).

BIOACTIVE PEPTIDES EXPRESSED AS IMMUNOGLOBULIN ISOLATED CDRs

The discovery by Polonelli et al. (Polonelli et al. 2003, Magliani et al. 2004a, b) that internal sequences of immunoglobulin variable regions may display antibiotic properties prompted us to investigate the activity of monoclonal antibody (mAb) CDRs tested as synthetic pep-

to the hypervariable domains called complemer
determining regions (CDRs). There are 6 CDRs
variable regions of light (V_L) and heavy chain
with background variability on each side of the
The CDRs are named H1, H2, H3 and L1, L2
heavy and light chains, respectively. The fran
sequences between CDRs can be similar or id
Although all CDRs are expected to contribute t
gen binding with variable affinity, only the CDR
V_H when tested as an isolated linear or cyclic
was found to have the same specificity of the c
antibody, sharing some of its biological properti
CDR3 (H3) peptides with such properties have th
called micro (mini) antibodies (Levi et al. 1993
geois et al. 1998). They can even compete with
tibody for binding to a certain antigen. The other
generally do not show a similar reactivity when
as isolated peptides.

Recently we showed, in collaboration with
nelli's and Ponton's groups from Parma and Bilb
spectively, that, independently of the specificity
native Ab, CDRs other than H3 may display, wi
frequency, antimicrobial, antiviral and antitumo
ities in a way reminiscent of molecules of early
immunity (Litman et al. 2005). The following
were studied as sources of the CDRs: Ab (mA
raised against a *C. albicans* antigen; mouse mAb
sharing H1 and H2 with mAb C7; and human
HuA, sharing no CDR either with mAb C7 or mA
with specificity for difucosylated blood group
mAbs generated CDRs that, represented by sy
peptides, showed *in vitro*, *ex vivo* and/or *in vivo* d
tial antimicrobial (*C. albicans*), antiviral (HIV-1)
antitumor activities (Polonelli et al. 2008).

CDRs C7/pc42 H2 and HuA L1 were directl
toxic for melanoma and HL-60 (human leukemi
causing caspase-dependent apoptosis. H2 pept
tivity was receptor-mediated in melanoma cells
C7 H2 and HuA L1 peptides in the C-terminal an
form were active against lung colonization by me
cells by intravenous injection (i.v.). Peptides w
ministered by intraperitonial injection (i.p.) (2

ules in the lungs of peptide treated animals were very few. Presumably, even better results could have been obtained by optimization of the peptide administration protocol (Polonelli et al. 2008). C7 H3 but not C7/pc42 H2 competed with mAb C7 for binding to phosphatidylcholine, the probable ligand of polyreactive C7 (IgM) on melanoma cells. This CDR (C7 H3) together with the H3 CDRs of two anti-melanoma mAbs (A4 and A4M), that competed with the antibodies for binding to melanoma cells, were three examples of micro (mini) antibodies shown in our laboratory (unpublished results).

A PEPTIDE VACCINE AGAINST PARACOCCIDIOIDOMYCOSIS

The main diagnostic antigen of *P. brasiliensis* was identified in our laboratory in 1986 (Puccia et al. 1986; reviewed Travassos et al. 2004b). Glycoprotein gp43 reacts with 100% sera of patients with paracoccidioidomycosis from a vast region of South America, with the possible exception of sera from certain Western areas. It elicits an immune response that protects against the intratracheal challenge by virulent *P. brasiliensis* yeast cells. This molecule has been cloned and sequenced (Cisalpino et al. 1996). Apart from B cell epitopes, which are beginning to be identified, the gp43 carries an immunodominant epitope that elicits a predominant IFN-γ-mediated Th-1 response. It is responsible for delayed type sensitive (DTH) reactions in infected animals (Rodrigues and Travassos 1994). The T-CD4+ cell epitope was mapped to a peptide called P10 with the QTLIAIHTLAIRYAN sequence, the HTLAIR hexapeptide core being essential for priming the immune response (Taborda et al. 1998). P10 was as protective as the gp43 in intratracheal injection (i.t.) challenged mice, being administered i.p with complete Freund's adjuvant (CFA). The nucleotide sequence encoding P10 was conserved in a number of isolates (Travassos et al. 2004b).

The T cell epitope in peptide P10 is presented by major histocompatibility complex (MHC) class II molecules from three different mouse haplotypes (Taborda et al. 1998). Promiscuity of P10 was also observed with Caucasian HLA-DR molecules (Iwai et al. 2003). Additional gp43 peptides were also identified using the TEPITOPE algorithm, which bound promiscuously to several HLA-DR molecules. As pointed out before (Travassos et al. 2008a, b) this is an essential property of a vaccine peptide candidate considering the genetic diversity of the target immunizable population.

In 29 patients with PCM and submitted to chemotherapy, 79% of them recognized one peptide selected by the TEPITOPE algorithm. By pooling peptides $gp43^{45\text{-}59}$, $gp43^{106\text{-}120}$, $gp43^{181\text{-}195}$ or P10, and $gp43^{283\text{-}298}$, the recognition frequency increased to 86% (Iwai et al. 2007). Overall for 25 Caucasian HLA-DRs, P10 and neighboring peptides were predicted to bind (TEPITOPE) to 90% or more of these molecules. Very few healthy individuals had peripheral blood mononuclear cells (PBMC) proliferating with gp43 and even fewer with gp43 derived peptides. They may have been exposed to *P. brasiliensis* on a trip to reserve areas of the fungus or cross-reacted with related fungal antigens, possibly also exo-β-1,3-D-glucanases. Site homologous but unidentical sequences, in comparison with P10, were found in β-1,3-glucanases from *Aspergillus nidulans*, *Histoplasma capsulatum*, *Blastomyces dermatitidis* and *Lacazia loboi* (a gp43-like protein).

The rationale for a peptide vaccine based on P10 has been discussed recently (Travassos et al. 2008a). Basically: "Stimulation of an effective IFN-γ-producing T-helper response can simultaneously trigger the production of potentially protective antibodies and the activation of $CD8^+$ T cells in addition to activation of phagocytic cells. In the presence of several immunogenic molecules of the fungal agent, stimulation of one arm of the immune system may alter a state of early or installed immunosuppression". Since treatment of fungal infections and particularly of PCM involves chemotherapeutic drugs, a peptide vaccine could work as an adjuvant to reduce the treatment period, which is usually long, avoid relapses and reverse the potentially lethal anergic cases. It also could help to treat those cases of fungal drug resistance.

To tackle the above issues while using experimental

with P10 and/or a chemotherapeutic drug starting after 48h of infection. In the second protocol, P10 and/or drug treatment was started after 30 days of infection. It aimed at reproducing a condition of established infection as in patients with PCM. The treatment was held for 30 days, during which groups of mice received i.p. doses of itraconazole, fluconazole, ketoconazole, sulfamethoxazole or trimethoprim-sulfamethoxazole at every 24 h. Amphotericin B was given at every 48 h. P10 was administered weekly for 4 weeks, initially in CFA and three times in incomplete Freund's adjuvant (Marques et al. 2006).

In all cases, there was an additive protective effect with the combination of P10 immunization and chemotherapy. Animals treated with sulfamethoxazole showed early protection followed by relapse. Significantly, the association of sulfamethoxazole and P10 successfully controlled the infection. In the second protocol, the fungal burden was examined after 60 and 120 days of infection. An additive protective effect of P10 immunization and drug treatment was also observed, with 60 to 80% reduction in lung CFUs. Chemotherapy alone induced a predominant Th-2 response with increased production of IL-4 and IL-10 detected in lung homogenates, whereas P10 vaccination stimulated a Th1 response, rich in IFN-γ and IL-12 without suppressing the Th-2 response (Marques et al. 2006). These are encouraging results in short term experiments. It is probable that an increased protective effect will be obtained in long term trials in which the animals will have time to completely recover of the fungal infection.

The condition of anergy was addressed as follows. Balb/c mice were treated with dexamethasone-21 phosphate added to drinking water. Negative DTH with *P. brasiliensis* antigen was obtained after 30 days. Immunosuppressed mice (n=10), infected with virulent *P. brasiliensis*, began to die 10 days after infection, and all animals were dead after 70 days. Chemotherapy and/or P10 immunization of immunosuppressed animals was started 15 days after i.t. infection and all treated animals survived thereafter. Chemotherapy and P10 immunization conferred additive protection. A significant increase

results suggest that P10 immunization can be pro
in anergic patients.

Delivery of peptides for an efficient immun
has always been a concern of our group because p
experiments have always used CFA as an adjuva
following alternatives therefore have been investi

Early studies have shown that immuniza
Balb/c mice with a mammalian expression vecto
gp43) carrying the full gene of gp43 with Cyton
virus (CMV) promoter induced B and T cell-m
immune responses which were protective against
challenge by virulent *P. brasiliensis* yeast forms
et al. 2000). The cellular immune response in m
munized with VR-gp43 was kept for at least 6 m
after immunization. A similar construction wi
was made several years later. Immunization w
P10 minigene in plasmid DNA alone or associate
a plasmid carrying mIL-12 insert was tested in
mice i.t. infected with a virulent isolate (Pb18
brasiliensis. A significant reduction of fungal bu
lung, spleen and liver was obtained with produc
IL-12 and IFN-γ and reduction of IL-4 levels
homogenates (G. Rittner et al., unpublished resul

The construction of MAP (multiple antige
tide) was also tried to deliver a tetravalent antige
taining P10 sequence. MAP-10, or M10, had fou
LIAIHTLAIRYAN (QT-less P10) chains synthes
a branched lysine core. Lymph node cell prolif
from P10 or M10-sensitized mice was identical
vitro stimulation with either P10 or M10. Immun
with single dose of M10 without adjuvant was
tive with few lung, spleen and liver CFUs and
no yeasts in lung histopathological sections (Tab
al. 2006).

In Balb/c mice infected i.t. for 30 days, th
tective effect of P10 was tested alone or mixe
adjuvants: alum, monophosphoryl lipid A or co
Freund's adjuvant (Travassos et al. 2008a, b). Un
edly, P10 administered in phosphate-buffered sali
most effective with a significant reduction in lung
with no fungi detected in spleens and livers.

The protective effect of P10 has also been

therapy, both gp43 and gp70 are markers for monitoring successive treatment and cure through their decreased antigenemia and specific antibody response (Marques da Silva et al. 2004, Silva et al. 2004). In the experimental Balb/c model of PCM infection, anti-gp43 mAb 3E effectively reduced the fungal burden and promoted phagocytosis *in vitro* (Buissa-Filho et al. 2008). The recognized epitope in the gp43 was mapped to the sequence NHVRIPIGYWAV shared with *Aspergillus fumigatus*, *A. oryzae* and *B. graminis* internal sequences of β-1,3-glucanases. This peptide could increase the protective effect of P10 in a possible peptide vaccine against PCM.

Again, as stressed, we quote our own thought expressed before (Travassos et al. 2008a): "Short term protocols (30 to 45 days) have the advantage of allowing repeated experiments to define a certain response. However, longer periods of treatment and observation may lead to even more effective results, aiming at sterilization in experimental models with massive infection loads".

ANTITUMOR PEPTIDES

Cancer remains as a major source of mortality and morbidity around the world, despite numerous recent advances in treatment alternatives. Chemotherapy and, more recently, biochemotherapy, is still the choice treatment for advanced and metastatic disease (Espinosa et al. 2003). It is, though, often associated with deleterious side effects caused by drug-induced damage to healthy cells and tissues (Buzaid and Atkins 2001). Quiescent or slowly proliferating cancer cells are refractory to the cytotoxic effect of drugs interfering with DNA synthesis (Naumov et al. 2003) and, frequently, cellular changes affected sensitivity to chemotherapeutic drugs by increased expression of drug-detoxifying enzymes and/or drug transporters, altered interactions between the drug and its target, increased ability to repair DNA damage and defects in the apoptotic pathway (Gatti and Zunino 2005). Development of a new class of anticancer drugs that lack toxicity to healthy cells and are tides (CAPs), are promising candidates for antitumor treatment.

CAPs have been found in all species that have been tested so far, including bacteria, fungi, plants and animals, and they probably represent one of the first evolved forms of defense of eukaryotic cells against pathogens (Zasloff 2002). An updated list of CAPs can be found in http://aps.unmc.edu/AP/main.php, with 1,393 entries. Most CAPs have a broad spectrum of antimicrobial activities; only 82 of the listed CAPs were active, however, against tumor cells.

Despite their diverse origins, antimicrobial peptides have common biophysical parameters, including small size, positive charge, and amphipathicity, that are likely important for peptide activity. These molecules are grouped according to structural characteristics, and are usually separated in three classes: (1) linear, often forming alpha-helical structures; (2) cysteine stabilized, beta-sheet structures; and (3) peptides with one or more predominant amino acid residues, but variable in structure (Yount et al. 2006). As stated before, not all CAPs are able to kill cancer cells, and to date, it has not been possible to predict an antitumor activity based on the peptide structure.

The short length and cationic/amphipathic properties of these molecules enable CAPs to interact and disrupt lipid membranes. Positively charged amino acid residues, such as lysine and arginine, and hydrophobic residues are frequently found in large numbers in CAPs (Hoskin and Ramamoorthy 2008). The high expression of anionic molecules, such as phosphatidylserine in the outer membrane leaflet of human tumor cells (Utsugi et al. 1991, Dobrzynska et al. 2005), as well as O-glycosylated mucins (Yoon et al. 1996) on cancer cell membranes, account for the net negative charge of these cells and their electrostatic interactions with cationic CAPs. In the case of magainin peptides, the cytotoxic activity for tumor cells was abolished by eliminating the electrical gradient across the plasma membrane. Apparently, the cellular potential is critical for peptide channel formation in tumor cell membranes and could determine the selective killing of tumor cells by

major membrane components, such as sphingomyelin, phosphatidylethanolamine and phosphatidylcholine (Zachowski 1993).

CAPs interaction with cancer cell membranes is not mediated by receptors, since D-amino acid peptide analogues displayed an activity similar to the all-L-amino acid peptide (Rodrigues et al. 2008, Hetru et al. 2000).

Another mechanism for cancer cell killing by CAPs is the induction of apoptosis by permeation of mitochondrial membrane after internalization, release of cytochrome c, leadind to caspase 9 and 3 activation (Pardo et al. 2001). Both cationic and hydrophobic amino acids play a role in the peptide permeation of mitochondrial membranes (Horton et al. 2008). Alternatively, apoptosis may be induced by CAPs interaction with cell death receptors, such as Fas ligand, leading to caspase 8 activation. Interestingly, arginine, glycine and asparagine, integrin homing domain (RGD)-conjugated tachyplesin induced both pathways, suggesting that some CAPs may have more than one effect on cancer cells (Chen et al. 2001).

Protein glycosylation may alter the secondary structure of a membrane-associated protein or peptide, and altered glycosylation of membrane proteins is frequently found in malignant cells. Moreover, differential branching and sialic acid content of N-linked glycans are associated with an increase in the net negative charge in the membrane of many cancer cells. Interestingly, peptide-glycosylation was associated with increased potency of drosocin *in vitro* (McManus et al. 1999). It is therefore likely that glycosylation of CAPs and/or cancer cell membrane proteins may influence the binding affinity of some CAPs for the cancer cell.

CAPs may be used in combination with conventional chemotherapeutic antitumor drugs in order to reduce effective doses, and thereby reduce harmful side-effects frequently observed in treated patients. Cecropin A, in combination with 5-fluorouracil and cytarabine, showed a synergistic cytotoxic effect on human leukemia cells (Hui et al. 2002).

Representative naturally occurring CAPs with antitumor activities are depicted on Table I.

tides can exhibit direct tumor cell cytotoxicity, immunomodulators or as antiangiogenic factors. review on these peptides, see Daffre et al. (2008)

A MODEL OF ANTITUMOR EFFECT OF A PEPT

Gomesin is a CAP isolated from hemocytes of challenged Brazilian spider *Acanthoscurria go na*. It is a hairpin-like two-stranded antiparallel structure formed by 18 amino acid residues and tw translational modifications, the N-terminal pyrogl acid (Z) and the C-terminal amidated arginine (Silva et al. 2000, Mandard et al. 2002; Tabl rigid conformation is maintained by two interna fide bridges formed by four cysteine residues, and $Cys^{6\text{-}11}$, together with six hydrogen bonds central part of the molecule, as well as at each the β-sheet (Mandard et al. 2002). The peptide phypathic, with a hydrophobic face (residues Leu Val^{12} and Tyr^{14}) and three hydrophilic regions c ing positively charged and polar amino acids at terminus (Arg^3 and Arg^4), at the C-terminus (Ar Arg^{18}) and within the canonical β-turn (Lys^8, G Arg^{10}) (Fazio et al. 2006). A representation of g is depicted on Figure 2.

As stated before, gomesin has a broad and microbicidal activity. The peptide is active against positive and Gram-negative bacteria, filamentous yeast (Silva et al. 2000), *Cryptococcus neoforman* bosa et al. 2007) and parasites, such as *Plasmodi ciparum* and *Plasmodium berghei* (Moreira et al.

The antitumor activity of gomesin was te *vitro* and *in vivo* (Rodrigues et al. 2008). Gome erted direct cytotoxic effects on murine and hu mor cells *in vitro*. The estimated IC_{50} for the melanoma cell line B16F10-Nex2 was 3.58 μ was below 10 μM for human tumor cell lines (Human endothelial cells were also sensitive to g *in vitro*, with an IC_{50} of 5.30 μM. The cytotoxi was time- and dose-dependent, and was not reve ter peptide removal. The β-hairpin structure and phipathicity of the peptide are important for an activity, since substitution of cysteine residues by

TABLE I
Naturally occurring CAPs with antitumor activity.

Peptides	AA sequence*	Source	Antitumor Activity	Refs.
α-helical				
BMP27, BMP28	GRFKRFRKKFKKLFKKLSPVIPLLHL, GGLRSLGRKILRAWKKYGPIIVPIIRI	Bovine Cathelicidin-derived	*In vitro*	Risso et al. 1998, Risso et al. 2002
Cecropin A, Cecropin B	KWKLFKKIEKVGQNIRDGIIKAG-PAVAVVGQATQIAKY KWKVFKKIEKMGRNIRNGIVKAG-PAIAVLGEAKAL	Insects and mammals	*In vitro*, Xenogeneic model *in vivo*	Moore et al. 1994, Chan et al. 1998, Winder et al. 1998, Hui et al. 2002, Ye et al. 2004, Suttman et al. 2008
LL-37/hCAP-18	LLGDFFRKSKEKIGKEFKRIVQRIK-DFLRNLVPRTES	Human	*In vitro*	Okumura et al. 2004, Li et al. 2006
Magainins and analogues	GIGKFLHSAKKFGKAFVGEIMNS (magainin 2)	Frog skin	*In vitro*, Xenogeneic model *in vivo* (local therapy)	Cruciani et al. 1991, Soballe et al. 1995, Takeshima et al. 2003, Cruz-Chamoro et al. 2006, Lehman et al. 2006
Gaegurin 5, Gaegurin 6	FLGALFKVASKVLPSVKCAITKKC FLPLLAGLAANFLPTIICFISYKC	Frog skin	*In vitro*	Kim et al. 2003, Won et al. 2006
Aurein 1.2	GLFDIIKKIAESF	Frog skin	*In vitro*	Rozek et al. 2000
Citropin 1.1	GLFDVIKKVASVIGGL	Frog skin	*In vitro*	Doyle et al. 2003
Melittin	GIGAVLKVLTTGLPALISWIKRKRQQ	Insect venom	*In vitro, In vivo* (melittin-avidin conjugate)	Tosteson and Tosteson 1981, Killion and Dunn 1986, Saini et al. 1999, Holle et al. 2003
Epinicidin-1	GFIFHIIKGLFHAGKMIHGLV	Fish	*In vitro*	Lin et al. 2009
Polybia-MP1	I D W K K L L D A A K Q I L	Wasp venom	*In vitro*	Wang et al. 2008
β-sheet				
Defensins HNP-1 HNP-2 HNP-3	A**C**Y**C**RIPA**C**IAGERRYGT**C**IYQGRLWAF**CC** **C**Y**C**RIPA**C**IAGERRYGT**C**IYQGRLWAF**CC** D**C**Y**C**RIPA**C**IAGERRYGT**C**IYQGRLWAF**CC**	Human	*In vitro, In vivo* xenogeneic model (HNP-1)	Lichtenstein et al. 1986, Müller et al. 2002, McKeown et al. 2006, Xu et al. 2008
Bovine Lactoferricin	FK**C**RRWQWRMKKLGAPSIT**C**VRRAF	milk	*In vitro*, *In vivo* Xenogeneic model, antiangiogenic	Yoo et al. 1997a, b, Eliassen et al. 2002, Mader et al. 2005, Eliassen et al. 2006
Tachyplesin I	KW**C**FRV**C**YRGI**C**YRR**C**R	Crustacean hemocytes	*In vitro*, *In vivo* (RGD-tachyplesin)	Li et al. 2000, Chen et al. 2001, Ouyang et al. 2002, Chen et al. 2005, Shi et al. 2006
Gomesin	Z**C**RRL**C**YKQR**C**VTY**C**RGR	Insect	*In vitro, In vivo* (local therapy)	Rodrigues et al. 2008
Linear, with predominant AA				
PR-39, Proline arginine-rich porcine	RRRPRPPYLPRPRPPPFFPPRLPPRIPP-GFPPRFPPRFP	Porcine cathelicidin-derived	*In vitro*	Ohtake et al. 1999

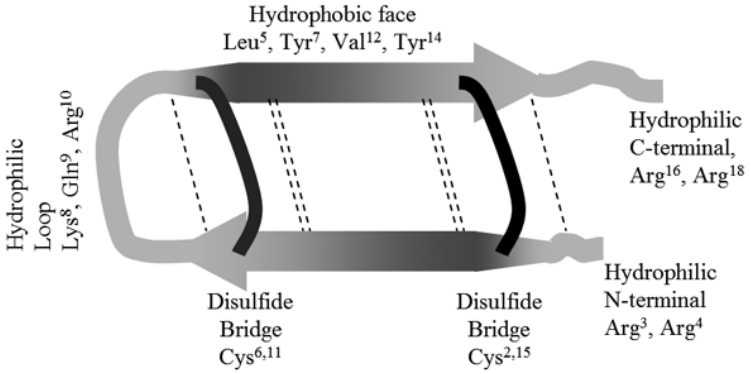

Fig. 2 – Schematic representation of gomesin. The molecule is formed by two antiparallel β-strands stabilized by 2 disulphide bridges (black lines) and 6 hydrogen bounds (hatched lines). Gomesin contains a hydrophobic face and three hydrophilic regions.

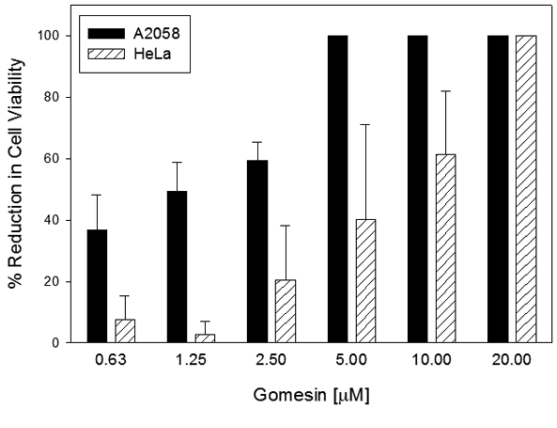

Fig. 3 – Gomesin cytotoxicity *in vitro* against human tumor cells. Human melanoma (A2058) and cervical cancer (HeLa) cells were treated *in vitro* with different concentrations of gomesin for 12 hours, and viable cells were counted in presence of Trypan Blue. The percentage of reduction of cell viability in relation to untreated cells is shown. The melanoma A2058 was the most sensitive and HeLa the most resistant cell line amongst all lineages studied (Rodrigues et al. 2008).

the cytotoxic effect. The enantiomer D-gomesin, synthesized employing D-amino acids and containing both disulfide bridges, was equally cytotoxic for tumor cells, suggesting that chiral recognition is not required for the antitumor effect (Rodrigues et al. 2008).

The peptide concentrates at the tumor cell mem-

tion, caused (1) early morphological alteration increased granularity and loss of cytoplasmic c (2) release of lactate dehydrogenase (LDH) in a dependent way; (3) partial inhibition of the r tion-dependent proton gradient; (4) internaliza immunoglobulins that reacted with tubulin fil and with nuclear histone H1 (monoclonal A4M peptide did not induce apoptosis of tumor cel drigues et al. 2008).

Interestingly, the monoclonal antibody (mAb is an IgM that recognizes nuclear histone H1 in B Nex2 murine melanoma cells, but is not cytotoxi intact tumor cell (A.S. Dobroff et al., unpublis sults). After treatment with low doses of gomesi ever, the mAb A4M was internalized in B16F1 cells and showed additive cytotoxic activity *in* Therefore, gomesin at low concentrations could tate the penetration of drugs inside tumor cells, tially reducing toxic doses and allowing penetra molecules that are not directly cytotoxic to cel intact membranes.

More importantly, topical *in vivo* treatmen gomesin significantly delayed subcutaneous melanoma development and significantly increa survival of animals with tumors below the allowe imal size limit. Male mice with established su

oil-in-water cream. This effect can be explained by the direct effect of gomesin on tumor cells, but also by an effect on tumor neoangiogenesis, since endothelial cells were sensitive to low concentrations of the peptide. Repeated topical applications of gomesin did not affect the peripheral healthy skin of treated mice (Rodrigues et al. 2008).

Some patients may develop extensive, confluent regional metastases near the primary nodular melanoma. In these cases, surgical excision or radiotherapy are unsuitable, and topical treatment is a preferred alternative. Some topical treatments have indeed being used tentatively, but only partial responses were obtained with 5-aminolevulinic acid photodynamic therapy (Wolf et al. 1993), imiquimod (Steinmann et al. 2000, Hesling et al. 2004), dinitrochlorobenzene (Malek-Mansour 1973, Illig et al. 1984, von Nida and Quirk 2003), and diphencyprone (Damian and Thompson 2007). Gomesin could be an alternative for treatment of these patients and eventually also patients with other skin cancers.

PERSPECTIVES

Peptides used in protective protocols against pro- and eukaryotic cells, including fungi and tumor cells, can act directly on target cells or will elicit an immune response that may be effective to control infections and tumor development. Peptides allow structural changes to incorporate protective substitutions, chiral derivatives, non-natural amino acids and other modifications aiming at increased stability, efficiency and resistance to proteolysis. In this sense, they are much more drug-like than recombinant proteins. A great number of peptide sequences with biological activity is now recognized, and the finding that fragments of immunoglobulin variable chains have increased frequency of bioactivity opens a broad field of investigation. Peptide-based vaccines are now in development for various pathologies including cancer (Purcell et al. 2007). The possibility of chemical synthesis of a limitless variety of peptide sequences and derivatives poses the question of how many more reagents can be produced compared to our capacity to test them in different biological systems. The use of vaccine candidate being presented by most Caucasian HLA-DR molecules, and being able to protect against massive *P. brasiliensis* infection in normal and immunosuppressed mice. The combination of chemotherapy and P10 vaccination is therefore a very promising strategy to treat human PCM. Antitumor peptides for systemic and topical treatment are additional tools that can be largely developed as adjuvants of conventional treatment.

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RESUMO

Peptídeos são moléculas particularmente reativas produzidas por uma grande variedade de espécies, aptos a exercer um número de funções em organismos uni- e multicelulares como mediadores, agonistas e substâncias regulatórias. Alguns deles exercem efeitos citotóxicos em células outras das que os produzem, e podem ter um papel controlando subpopulações e protegendo certas espécies ou tipos celulares. No presente, focalizamos peptídeos antifúngicos e antitumorais e discutimos alguns modelos nos quais seqüências específicas e estruturas exercem efeitos inibitórios diretos ou estimulam uma resposta imune protetora. O peptídeo letal ("killer"), deduzido de um anticorpo anti-idiotípico, com várias atividades antimicrobianas bem como outros peptídeos derivados de imunoglobulinas com atividades citotóxicas incluindo efeitos antitumorais são modelos estudados *in vitro* e *in vivo*. O peptídeo P10 da gp43 de *P. brasiliensis* e a perspectiva de vacina contra a paracoccidioidomicose é outro tópico ilustrando o efeito protetor *in vivo* contra um fungo patogênico. Peptídeos antimicrobianos catiônicos com atividades antitumorais são os principais revistos aqui. O tratamento local do melanoma murino com o peptídeo gomesina é outro modelo estudado na Unidade de

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