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RENATA MARIA SOARES TERRA

ANÁLISE CONFORMACIONAL DA MELITINA POR DINÂMICA MOLECULAR E CARACTERIZAÇÃO DOS EFEITOS DO PEPTÍDEO NA FUNÇÃO PLAQUETÁRIA

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Dissertação submetida ao programa de pós-graduação em Biologia Celular e Molecular, Centro de Biotecnologia, da Universidade Federal do Rio Grande do Sul como requisito parcial para obtenção do título de Mestre.

Orientador Prof. Dr. Jorge Almeida Guimarães

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Aos meus pais e irmão,

meus grandes motivadores e amigos

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“*Pode-se viver no mundo uma vida magnífica, quando se sabe trabalhar e amar: trabalhar pelo que se ama e amar aquilo em que se trabalha*.”

Liev Tólstoi

Resumo

Acidentes envolvendo abelhas africanizadas são freqüentemente relatados, particularmente na América do Sul. As picadas de abelhas causam reações localizadas e sistêmicas e os sintomas do envenenamento incluem náuseas, vômitos, hemólise, falência renal e coagulação intravascular disseminada. Durante muito tempo todas as reações tóxicas eram atribuídas à presença de uma fosfolipase A2, mesmo sendo o veneno uma mistura complexa de substâncias. A melitina, o componente mais abundante e tóxico do veneno de abelha, é um peptídeo de 26 aminoácidos com a habilidade de interagir e danificar membranas celulares. A melitina é também capaz de modular muitas proteínas, aumentando a diversidade de atividades biológicas do peptídeo. Até recentemente, acreditava-se que a estrutura tri-dimensional biologicamente ativa do peptídeo, que possui conformação em hélice, era um tetrâmero.

Neste trabalho avaliamos a conformação da melitina e seus estados oligoméricos em solução por dinâmica molecular e a interferência da melitina na função plaquetária. Aqui está demonstrado que a melitina possui uma conformação randômica em condições fisiológicas e que sua estrutura tri-dimensional sofre alterações de acordo com as condições ambientais. Ainda, foi demonstrada uma nova atividade biológica do peptídeo melitina. O peptídeo é capaz de induzir a

agregação plaquetária de forma dose-dependente e de interagir diretamente com a superfície de plaquetas. A correlação entre a conformação da melitina e suas atividades biológicas é discutida.

Os resultados aqui apresentados podem ser valiosos no entendimento do papel da melitina nas coagulopatias induzidas por veneno de abelha. O estudo estrutural mostrado aqui pode ser aplicado para explicar as diferentes atividades do peptídeo.

Abstract

Accidents involving africanized bees are frequently reported, particularly in South America. Bee stings cause localized and systemic reactions and the symptoms of envenomation include nausea, vomiting, hemolysis, kidney failure and disseminated intravascular coagulation. For a long time, all toxic reactions were ascribe to the presence of a phospholipase A2, despite of being the venom a complex mixture of substances. Melittin, the most abundant and the major toxic component of bee venom, is a 26 amino acid peptide with the ability to interact and disrupt cell membranes. Melittin is also able to modulate many proteins, enhancing the wide range of the peptide biological activities. The biologically active tridimensional structure of the peptide, which has a helical conformation, has been described until now as a tetramer.

In this work we evaluated the conformation of melittin and its oligomeric states in solution by molecular dynamics simulations and performed studies of melittin effect on platelet function. Here we demonstrate that melittin has a random conformation under physiological conditions and its tridimensional structure changes under different environmental conditions. Moreover, here we describe a new biological activity of melittin. The peptide is able to induce platelet aggregation in a dose-

dependent manner and can interact directly with the platelet surface. The correlation between melittin conformation and biological activity is discussed.

Our results might contribute to elucidate the role of melittin in bee venom induced coagulopathies. The structural data gathered in this work may explain the different activities of the peptide.

Sumário

Introdução

Abelhas e sua importância médica

As abelhas melíferas (*Apis mellifera mellifera*, *Apis mellifera lingustica*, *Apis mellifera carnica* e *Apis mellifera iberica*) foram trazidas para as Américas por imigrantes europeus. Porém, o fato de abelhas africanas (*Apis mellifera scutellata*) produzirem maior quantidade de mel despertou o desejo de se introduzir esta nova espécie no continente americano. Como espécie isolada, estas abelhas não conseguiram manter-se na região. Entretanto, em 1957, no Brasil, durante um experimento com abelhas africanas, alguns indivíduos fugiram, hibridizando-se com abelhas européias e dando origem ao que hoje conhecemos como abelhas africanizadas (Sherman, 1995). Estima-se que este processo de africanização tenha se espalhado cerca de 320 km por ano, norte e sul, atingindo o estado americano do Texas em 1990 e espalhando-se pela costa oeste até o estado da Califórnia, Estados Unidos (Sherman, 1995). Apesar da miscigenação, as abelhas mantiveram muitas das características das abelhas africanas, especialmente o caráter migratório,

a agitabilidade e a agressividade, fato que lhes rendeu o apelido de “abelhas assassinas” (do inglês, *killer bees*) (Sherman, 1995). Pouco se conhece a respeito da dinâmica da hibridização, contudo uma análise de DNA mitocondrial sugere que o processo tenha ocorrido principalmente pela migração das fêmeas (Sherman, 1995).

A característica comportamental destas abelhas faz com que elas estejam freqüentemente envolvidas em ataques massivos a seres humanos e animais, tornando esse tipo de envenenamento um problema de saúde pública. Os ataques ocorrem em resposta a um estímulo ameaçador e são fatais para as abelhas envolvidas, porém bastante efetivos para a colônia (Sherman, 1995; Vetter *et al.*, 1999). Pequenos acidentes com abelhas da espécie *Apis mellifera* usualmente causam dor intensa localizada, pequeno edema e eritema imediato (Ewan, 1998). No entanto, reações alérgicas são bastante comuns, podendo o paciente ser sensibilizado após poucas picadas ou mesmo em um único evento (Ewan, 1998). As reações alérgicas podem ser classificadas como imediatas ou tardias e locais ou sistêmicas, subdividindo-se as últimas em leves, moderadas ou graves (Ewan, 1998). As reações localizadas causam prurido intenso e edema em algumas horas e eventualmente são seguidos de infecção secundária (Ewan, 1998; Sherman, 1995; Steen *et al.*, 2005). As reações sistêmicas, subdivididas em três graus de severidade, têm seus sintomas descritos na Tabela 1, assim como seu tratamento e uma possível indicação de terapia de dessensibilização. Reações sistêmicas são capazes de ocasionar o óbito do paciente atingido, principalmente se o mesmo não receber tratamento adequado na primeira hora após o acidente (Sherman, 1995).

A imunoterapia, ou terapia de dessensibilização, é indicada para pacientes que sofreram reação alérgica sistêmica severa (Ewan, 1998). A imunoterapia convencional deve ser realizada em centros especializados onde um aparato para

ressuscitação deve estar disponível, uma vez que é elevado o risco de choque anafilático (Ewan, 1998; Sherman, 1995; Steen *et al.*, 2005). Os pacientes recebem pequenas doses de veneno semanalmente durante três meses até uma dose máxima de 100 µg de veneno (equivalente a 2 picadas). A manutenção é realizada com injeções mensais da dose máxima por um período de até três anos (Ewan, 1998). A despeito de se acreditar que um paciente com histórico de reação alérgica sistêmica tenha o processo exacerbado em um novo contato, não existem métodos capazes de prever o grau da resposta em novos eventos (Ewan, 1998). Paradoxalmente, um paciente com reação sistêmica pode sofrer apenas de reações locais em um novo contato com o veneno, tornando o emprego da terapia uma decisão a ser cuidadosamente julgada.

Tabela 1

Classificação de reações alérgicas sistêmicas a picadas de abelha, sintomatologia e conduta clinica desejável.

Tipo de reação	Símbolo	Sintomas	Tratamento	IgE específica	Imunoterapia
Leve	+	Eritema, prurido, urticária, angio-edema, rinite e náusea	Anti-histamínico (oral ou intramuscular)	Positivo	Não
Moderada	++	Náusea, angio-edema, asma leve e dor abdominal	Anti-histamínico (intramuscular), hidrocortisona (intramuscular), agonista β2 inalatório (se asma), epinefrina inalatória (se edema de laringe)	Positivo	Alguns casos, usualmente não
Severa	+++	Dificuldade respiratória (edema de laringe), hipotensão severa, perda de consciência e colapso	Epinefrina (intramuscular), hidrocortisona (intramuscular ou endovenosa), clorfeniramina (intramuscular ou endovenosa)	Positivo	Sim

Adaptado de Ewan, PW (1998)

Além da anafilaxia, o veneno de abelhas pode ocasionar uma série de outras reações sistêmicas imediatas ou tardias. Ataques massivos - de dezenas a centenas de picadas, e em alguns casos, milhares - têm elevada toxicidade, podendo ocasionar a falência de diversos órgãos (Franca *et al.*, 1994; Grisotto *et al.*, 2006; Kolecki, 1999; Vetter *et al.*, 1999). Reações tóxicas incluem hemólise com conseqüente hematúria, rabdomiólise, trombocitopenia, coagulação intravascular disseminada, dano cardíaco, hepático e renal, hipercalemia, hiperglicemia e hipertensão (Franca *et al.*, 1994; Gawlik *et al.*, 2004; Grisotto *et al.*, 2006; Kolecki, 1999; Sherman, 1995; Steen *et al.*, 2005; Vetter *et al.*, 1999). Achados bioquímicos demonstram um aumento nos níveis das enzimas creatina quinase e lactato desidrogenase assim como de aminotransferases hepáticas em pacientes envenenados, demonstrando importante lesão tecidual (Grisotto *et al.*, 2006; Kolecki, 1999; Vetter *et al.*, 1999). Casos graves podem ocasionar morte do paciente em um período de 4 horas a 12 dias e a mesma ocorre usualmente em decorrência de falência renal por subprodutos de hemólise e miólise ou parada cardíaca pela toxicidade do veneno (Vetter *et al.*, 1999).

Componentes do veneno da abelha Apis mellifera

O veneno de abelhas, assim como de outros animais, é uma complexa mistura de substâncias bioativas. A composição dos venenos de diferentes espécies de *Apis* parece não variar, entretanto pequenas diferenças quantitativas de seus componentes são observadas (Vetter *et al.*, 1999). Dentre os componentes do veneno, duas proteínas têm maior relevância: o peptídeo hemolítico melitina,

compondo cerca de 50% do peso seco do veneno e a enzima fosfolipase A2, representando 10-12% da massa do veneno bruto (Habermann, 1972; Peiren *et al.*, 2005; Vetter *et al.*, 1999). Aminas biogênicas como histamina, dopamina e norepinefrina, assim como peptídeos ativos – apamina e o peptídeo degranulador de mastócitos (MCD) – e componentes enzimáticos – hialuronidase, serino-protease com domínio CUB e uma fosfatase ácida, também compõem esta complexa mistura (Habermann, 1972; Peiren *et al.*, 2005; Vetter *et al.*, 1999). Em recente abordagem proteômica, três novas proteínas foram encontradas no veneno de *Apis mellifera carnica*, sendo que para duas delas a identificação foi possível (Peiren *et al.*, 2005). A primeira proteína foi identificada como contendo domínio PDGF (fator de crescimento derivado de plaquetas)/VEGF (fator de crescimento endotelial vascular) e a segunda mostrou homologia com uma proteína hipotética similar a MRJP (*major royal jelly protein*) (Peiren *et al.*, 2005). Estas proteínas são de baixa abundância no veneno e sua participação na cinética do envenenamento é desconhecida (Peiren *et al.*, 2005).

Aminas Biogênicas

O papel das aminas biogênicas no veneno e nos quadros clínicos observados após o envenenamento ainda é bastante obscuro. Estas substâncias parecem ter seus efeitos mascarados e até mesmo estimulados por outros componentes protéicos (Grisotto *et al.*, 2006; Habermann, 1972). A dopamina e a norepinefrina foram descritas *in vivo* nos reservatórios de veneno de abelhas, porém sua presença no veneno secretado e liofilizado não foi observada (Habermann, 1972). Entretanto, acredita-se que estas catecolaminas tenham um efeito importante nas alterações

hemodinâmicas renais (Grisotto *et al.*, 2006). Dentre as aminas, a histamina é a substância de maior relevância. Representando de 0,1-1,5% do peso seco do veneno, estima-se que uma única picada possa injetar cerca de 2 µg de histamina, representando 14 µg de histamina exógena /Kg em um caso de envenenamento massivo (quando estima-se 500 picadas em um indivíduo de 70 Kg) (Grisotto *et al.*, 2006). Alterações cardiovasculares podem ser observadas em humanos saudáveis na dose de 1 µg de histamina /Kg, atribuindo-se a ela um papel fundamental na hipotensão observada pós-envenenamento (Grisotto *et al.*, 2006). Além disso, existe a presença de peptídeo degranulador de mastócitos (MCD) que é capaz de liberar histamina endógena (Grisotto *et al.*, 2006).

Componentes enzimáticos

A fosfolipase A2, dentre as enzimas que compõem o veneno de abelha, é a proteína mais estudada e talvez a enzima de maior importância. A identificação da atividade fosfolipásica fez com que durante muitos anos se creditassem todos os efeitos biológicos do veneno a esta enzima (Habermann, 1972). A fosfolipase é a proteína com maior potencial imunogênico e, como tal, vem sendo usada em estratégias para imunização e dessensibilização (Jones *et al.*, 1999; Vetter *et al.*, 1999). A atividade catalítica da enzima é potencializada pelo peptídeo melitina, outro componente importante do veneno (Mingarro *et al.*, 1995). Assim como outras fosfolipases de venenos animais, a enzima possui atividade neurotóxica através de ligação receptor-específica (Nicolas *et al.*, 1997). Necrose muscular pode ser observada após a injeção da enzima pura, evidenciando sua atividade miotóxica, demonstrada como sendo diretamente relacionada à atividade catalítica (Ownby *et*

al., 1997). A ocorrência de rabdomiólise em pacientes que sofreram ataques massivos por abelhas parece estar relacionada à fosfolipase e sua capacidade de alterar membranas celulares (Grisotto *et al.*, 2006). A modulação da função plaquetária pela fosfolipase parece ter efeito bimodal e dose-dependente (Ouyang e Huang, 1984). Distúrbios na coagulação foram observados *in vitro*; a fosfolipase A2 de veneno de abelha é capaz de alterar todas as fases da coagulação (via intrínseca, extrínsica e comum) (Petroianu *et al.*, 2000). Demonstrou-se que a enzima é capaz de reduzir a atividade dos fatores da coagulação, trombina, fator V e fator VIII, e esta capacidade pode servir de parâmetro para o monitoramento do envenenamento (Petroianu *et al.*, 2000).

Outras três enzimas parecem ser componentes importantes dos venenos de abelha, apesar da literatura a respeito das mesmas ser bastante escassa. Duas dessas proteínas são importantes alergenos, sendo a hialuronidase a enzima com segundo maior potencial imunológico do veneno de abelha. Verificou-se que 78% dos pacientes alérgicos apresentam IgE específica para fosfolipase e 71% para hialuronidase (Markovic-Housley *et al.*, 2000). A terceira enzima é uma serino-protease que apresenta sítio catalítico tipo tripsina e a presença de um domínio CUB (Garcia, 2006; Winningham *et al.*, 2004). Este domínio lhe confere a capacidade de fazer interações proteína-proteína, sendo sua ligação à IgE recentemente comprovada (Winningham *et al.*, 2004).

Peptídeos bioativos

A apamina é um peptídeo neurotóxico de apenas 18 aminoácidos diferenciando-se de neurotoxinas clássicas de serpentes e escorpiões que possuem

cerca de 60 resíduos (Habermann, 1972). A apamina tem dose letal (DL_{50}) de 4 mg/Kg em camundongos e doses altas (1 mg/Kg) são capazes de produzir movimentos descoordenados e involuntários após 15 minutos, culminando em convulsões (Habermann, 1972). O peptídeo é um bloqueador específico de um subtipo de canal de potássio ativado por cálcio (SK_{Ca}) presente em neurônios e, em baixas doses, parece aumentar a resposta cognitiva, o aprendizado e ter uma atividade anti-depressiva (van der Staay *et al.*, 1999).

Outra importante proteína do veneno é o denominado peptídeo degranulador de mastócitos (MCD). O MCD é o principal componente do veneno capaz de induzir a liberação de histamina (Habermann, 1972). O peptídeo é praticamente atóxico, com uma dose letal (DL_{50}) >40 mg/Kg em camundongos, todavia é capaz de produzir cianose em doses acima de 0,5 mg/Kg (Habermann, 1972). O MCD tem uma atividade dupla na inflamação, apresentando atividades pró- e anti-inflamatórias dependendo da dose e tem importante potencial imunogênico (Buku, 1999). Recentemente mostrou-se que na degranulação de mastócitos ocorre a liberação de carboxipeptidase A e outras proteases que oferecem um efeito protetor contra o envenenamento por abelhas e serpentes (Metz *et al.*, 2006; Rivera, 2006).

Peptídeo melitina

A melitina é o componente majoritário do veneno da abelha *Apis mellifera*, representando cerca de 50% do peso seco do mesmo (Habermann, 1972). É um peptídeo anfipático, em hélice, composto de 2.9 KDa, com a seqüência GIGAVLKVLTTGLPALISWIKRKRQQ e uma carga global de +4, concentrada na porção carboxi-terminal. Sua estrutura tridimensional tem sido estudada por diversas

técnicas como cristalografia de raios X, ressonância magnética nuclear (RMN), dicroísmo circular e dinâmica molecular (Bello *et al.*, 1982; Glattli *et al.*, 2006; Lam *et al.*, 2001; Lin e Baumgaertner, 2000; Terwilliger e Eisenberg, 1982; Wang e Polavarapu, 2003). A estrutura terciária do peptídeo altera-se em função do meio, formando uma hélice perfeita quando em contato com membranas (Glattli *et al.*, 2006; Qiu *et al.*, 2005; Sengupta *et al.*, 2005; Yang *et al.*, 2001).

A atividade hemolítica da melitina é seu efeito biológico mais característico, sendo usado para a identificação do peptídeo em frações de veneno (Tosteson *et al.*, 1985). Esta atividade é modulada pela presença de colesterol em eritrócitos, sendo a capacidade hemolítica do peptídeo aumentada em células depletadas de colesterol (Raghuraman e Chattopadhyay, 2005). O peptídeo ainda é capaz de interagir e extravasar o conteúdo de leucócitos e mastócitos (Habermann, 1972). Além disso, a capacidade lítica da melitina não se restringe às células humanas e animais uma vez que o peptídeo apresenta uma capacidade antibacteriana e antifúngica (Asthana *et al.*, 2004; Habermann, 1972). As características líticas da melitina podem ser explicadas pela sua elevada capacidade de interagir e desestabilizar membranas naturais ou artificiais (Dempsey, 1990; Habermann, 1972). A inserção em membranas provoca a formação de poros e a permeabilização das mesmas, causando rompimento celular (Dempsey, 1990; Yang *et al.*, 2001).

A melitina apresenta atividade tóxica frente a diversos tipos de células. O peptídeo é uma conhecida cardiotoxina (Okamoto *et al.*, 1995), efeito este relacionado a um aumento no influxo de cálcio. O fluxo de íons pode ser modulado através da interação da melitina com canais iônicos, efeito observado também em uma diversidade de outros tecidos (Baker *et al.*, 1995; Shorina *et al.*, 2004; Voss *et al.*, 1995). Igualmente, a melitina é capaz de modular proteínas G (Fukushima *et al.*,

1998), podendo afetar uma série de receptores acoplados a estas proteínas em maneira similar ao peptídeo do veneno de vespas – mastoparan (Higashijima *et al.*, 1990; Higashijima *et al.*, 1988).

A modulação de proteínas pela melitina confere a ela uma série de outras atividades biológicas interessantes. Fosfolipases são moduladas pelo peptídeo que aumenta a atividade catalítica da fosfolipase A2 do veneno de abelha assim como de uma fosfolipase D humana e ainda tem efeito bimodal sobre uma fosfolipase A2 humana (Koumanov *et al.*, 2003; Mingarro *et al.*, 1995; Saini *et al.*, 1999). A proteína ligadora de cálcio calmodulina também tem sua atividade regulada por melitina e a estrutura tridimensional do complexo vem sendo estudada por diferentes abordagens (Hait *et al.*, 1985; Kaetzel e Dedman, 1987; Kataoka *et al.*, 1989; Scaloni *et al.*, 1998). A geração de ácido araquidônico e seus metabólitos pelo peptídeo é explicada através da modulação de cicloxigenases e lipoxigenases (Salari *et al.*, 1985). Outra característica interessante é a capacidade do peptídeo de simular superfícies ou fosfolipídios de membrana. Acredita-se que a melitina é capaz de suportar a atividade do fator IX da coagulação na ativação do fator X fornecendo a superfície necessária para uma eficiente atividade proteolítica (Blostein *et al.*, 2000).

Objetivos

A presente dissertação teve como objetivo geral a caracterização estrutural e biológica da melitina. Especificamente, os objetivos deste trabalho são:

1. Caracterização conformacional da melitina por dinâmica molecular.
    - Avaliação dos estados de oligomerização do peptídeo em solução
    - Avaliação do efeito do pH sobre a estrutura tridimensional (3D) do peptídeo
    - Avaliação do efeito da força iônica sobre a estrutura 3D do peptídeo
    - Avaliação do efeito do solvente sobre a estrutura 3D do peptídeo
2. Identificação dos efeitos da melitina sobre a função plaquetária
    - Avaliação da capacidade pró-agregante do peptídeo
    - Avaliação da secreção de adenosina tri-fosfato como efeito do peptídeo
    - Avaliação das características morfológicas das plaquetas tratadas com o peptídeo
    - Avaliação dos efeitos do peptídeo sobre a adesão plaquetária

Resultados

1. Análise conformacional da melitina por dinâmica molecular

1.1 Dinâmica molecular no estudo de estrutura e conformação de proteínas

Há quase trinta anos, a primeira simulação de dinâmica molecular foi publicada utilizando o inibidor pancreático da tripsina bovina como alvo de estudo (McCammon *et al.*, 1977). O emprego desta técnica, apesar de bastante limitada na época, ajudou na desmistificação de que proteínas eram estruturas estáticas (Karplus, 2002). O entendimento de que as estruturas protéicas são dinâmicas vem auxiliando o estudo de sistemas biológicos complexos e a caracterização molecular das proteínas é valiosa. As simulações de dinâmica molecular de proteínas, assim como de outros sistemas, podem ajudar a descrever detalhadamente a mobilidade e flexibilidade das moléculas em função do tempo (Karplus, 2002; Karplus e

McCammon, 2002). A validação dos modelos obtidos deve ser verificada por comparação a resultados experimentais, porém, a técnica vem sendo também amplamente utilizada para o refinamento de dados obtidos por outras metodologias como cristalografia de raios-x e ressonância magnética nuclear (Karplus e McCammon, 2002).

Nos últimos anos, as simulações de dinâmica molecular foram popularizadas e reconhecidas pela comunidade científica e os avanços na capacidade computacional permitem hoje uma descrição complexa da dinâmica das moléculas. Em consulta ao banco de dados do portal ISI WEB of Knowledge (http://portal.isiknowledge.com/), quando os termos *"molecular dynamics"* e *"proteins"* são consultados, cerca de 7600 artigos são encontrados, sendo 743 trabalhos publicados no ano de 2006.

1.2 Artigo 1: Terra, RMS; Guimarães, JA; Verli, H. Structural and functional behavior of biologically active monomeric melittin. J. Mol. Graph. Model. (2006)

O trabalho aqui apresentado é uma análise detalhada da conformação do peptídeo do veneno de abelha melitina por simulações de dinâmica molecular. Aspectos conformacionais são avaliados em função dos graus de oligomerização do peptídeo, assim como seu comportamento em solução sob diferentes condições físico-químicas. Os resultados obtidos neste trabalho permitiram um entendimento global do comportamento da melitina em solução e ajudaram em uma melhor compreensão da diversidade de suas funções biológicas. O artigo foi submetido ao

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Structural and functional behavior of biologically active monomeric melittin

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Abstract

Melittin is a well-known water-soluble toxic peptide present in bee venom of *Apis mellifera*, capable of interacting with and disrupting cell membranes thus producing many effects on living cells. Additionally, melittin induces activation of phospholipases and calmodulin upon interaction with cellular membranes. The conformation and aggregation state adopted by melittin in solution depends on several factors including the peptide concentration, ionic strength, pH and the nature of the ions in the aqueous medium. Such conformational dependence on the peptide environment gives new insights over the currently available 3D structures of melittin and, ultimately, over its biologically functional unit. Based on crystallographic data, the melittin tetramer has been proposed as its bioactive form. Contrarily to such data, we show in this work the results obtained from molecular dynamics simulations, which clearly indicate that the tetrameric organization of melittin is not stable under biological conditions dissociating after 2.5 ns through a 10 ns trajectory. We found that the tetrameric form of melittin is stable only in conditions of high pH and high peptide concentration in the molecular dynamics simulations. Moreover, when in plasma melittin appears to be a random coil monomer, folding only upon interaction with biological membranes. In summary, these findings elucidate several properties of melittin structure and dynamics, projecting significant implications in the study of its biological function.

Keywords: Melittin; Molecular dynamics; Bee venom; *Apis mellifera*; Peptide folding; Melittin biological unit

1. Introduction

Melittin is a well-known water-soluble toxic peptide present in bee venom of *Apis mellifera*, comprising about 50% of its dry weight. This peptide is able to disrupt membranes, producing many effects on living cells [1,2]. Like other amphiphilic α-helical peptides, melittin has an antibacterial activity, induces voltage-gated channel formation and can also produce micellization of phospholipids bilayers due to its membrane-interacting effect [1,3,4]. However, the major effect of melittin on erythrocytes is to cause lyses, as its binding to cell membranes results in the release of hemoglobin to the extracellular medium [3]. The molecular mechanism underlying melittin interaction with biological membranes and lipid bilayers is not well understood. Moreover, it seems that different molecular mechanisms could generate different actions of the peptide [3]. Interestingly, melittin interaction with certain proteins in the cell, such as phospholipases and calmodulin, thus inducing their functional modulation, has been also described [5–7].

This helical amphipatic peptide consists of 26 amino acid residues, comprising the sequence: GIGAVLKVLTTGLPALIS-WIKRKRQQ, and a total net charge of +5, four of which (KRKR) are in the C-terminal portion. Its conformation and structure has been studied by different approaches, including X-ray crystallography, nuclear magnetic resonance (NMR) spectroscopy, circular dichroism (CD), and molecular dynamics (MD) simulations [8–16]. Melittin is soluble in both water and methanol, and its monomeric structure is described as shaping like a bent rod due to the presence of a proline residue in position 14 [9]. The peptide's crystallographic structure was deposited in the Protein Data Bank (PDB) under accession number 2MLT [10]. According to this data melittin crystallizes as a dimer,

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and the proposed biologically active unit has been described as a tetramer [10,11]. Contrarily to crystallographic data, biochemical studies show that the helical content and aggregation state adopted by the peptide depends, in a complex way, on several factors including the peptide concentration, ionic strength, pH and the nature of the ions in the aqueous medium [2,8]. Such apparent contradiction between structural and biochemical data has not been explained or correlated to conformational and functional data by any molecular model so far.

A recent publication [17] presented a molecular dynamics simulation of the melittin tetramer as the bioactive form of this peptide. The authors describe the tetrameric form as a 'channel', defined between two melittin dimers, which collapse during a water drying transition. Surprisingly, using the same methodology we were unable to reproduce these computational results. We believe that this is due to the fact that computational models should be built in a way that the system factors that are essential to a precise quantification of the property of interest must be both adequately considered and described, as well as sufficiently sampled [18]. The correct representation of these factors can be inferred by how well a molecular dynamics simulation reproduces known quantities [19]. Regarding melittin description by molecular dynamics, an adequate model should necessarily be capable of reproducing its conformational dependence of pH, salt and peptide concentrations.

In this work we used the crystallographic structure (PDB 2MLT) as the starting point for molecular dynamics simulations of melittin. We investigate the conformation of the peptide on both monomeric and oligomeric forms under different solvents, peptide concentrations, ionic strength and pH conditions. We describe here the conformational equilibrium assumed by the peptide in solution, a result that reproduces and correlates, for the first time, the peptide' structural properties with its biochemical data so far reported.

2. Methods

2.1. Software

Energy minimization calculations, molecular dynamics simulations and trajectory analysis were done using the GROMACS simulation suite [20]. Molecular visualization was done in Swiss PBD viewer (SPDBV) environment [21] and the secondary structure content analyses were performed with PROCHECK [22].

2.2. Molecular dynamics simulations

Melittin in its tetrameric form was retrieved from Protein Data Bank under code 2MLT. This oligomerization state was kept for simulation of the tetramer, the chains AB were isolated for simulation of the dimer, and the chain A was isolated for simulation of the monomeric form of the peptide. The monomeric, dimeric, and tetrameric forms of melittin were further solvated in a cubic box using periodic boundary conditions and using single point charge (SPC) water model [23] or Gromos96 methanol model [24] for water and methanol modeling, respectively. The ionic strength was adjusted by addition of chloride or sulfate ions, while the peptide concentration was obtained by varying the box size. Different protonation states of melittin under pH 7.0 and 11.0 were adjusted manually according to the standard pK_a values of the amino acids side chains. Consequently, at pH 11.0 lysine side chains were deprotonated. The final systems composed by peptide, water and ions comprise up to 28,000 atoms. The Lincs and Settle methods [25,26] were applied to constrain covalent bonds lengths, allowing an integration step of 2 fs after an initial energy minimization using the Steepest Descents algorithm. Electrostatic interactions were calculated with the generalized reaction-field method [27], with Coulomb and Lennard-Jones cut-off adjusted at 16 Å. The simulations were performed under constant-pressure (1 atm) and constant-temperature (310 K), using Gromos96 or OPLSAA force fields. The dielectric constant was treated as $\varepsilon = 1$. The monomeric, dimeric and tetrameric systems were heated slowly, from 50 to 310 K, in steps of 5 ps. At each step the reference temperature was increased by 50 K, allowing a progressive thermalization of the molecular systems. The simulation was then extended up to 10 ns. As several simulations were performed, the total conformational sampling of the peptide was about 0.1 μs.

3. Results and discussion

3.1. Simulations of oligomeric melittin

In order to elucidate the oligomeric organization of melittin in biological solutions we performed unrestrained molecular dynamics simulations of a melittin tetramer in three different conditions: (1) 23.5 mM melittin, 120 mM chloride ions, pH 7.0; (2) 54.6 mM melittin, 273 mM chloride ions, pH 7.0; (3) 23.5 mM melittin, 120 mM chloride ions, pH 11.0. Observing

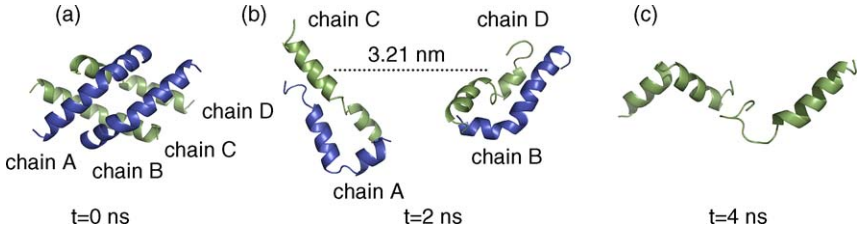

Fig. 1. Molecular dynamics simulation of oligomeric melittin. (a) Melittin crystallographic conformation (PDB 2MLT). (b) Melittin tetrameric conformation after 2 ns of molecular dynamics simulation in 120 mM chloride ions and pH 7.0; the distance in nm between dimers AC and BD is shown in dotted lines. (c) Snapshot of melittin dimeric conformation after 4 ns of molecular dynamics simulation.

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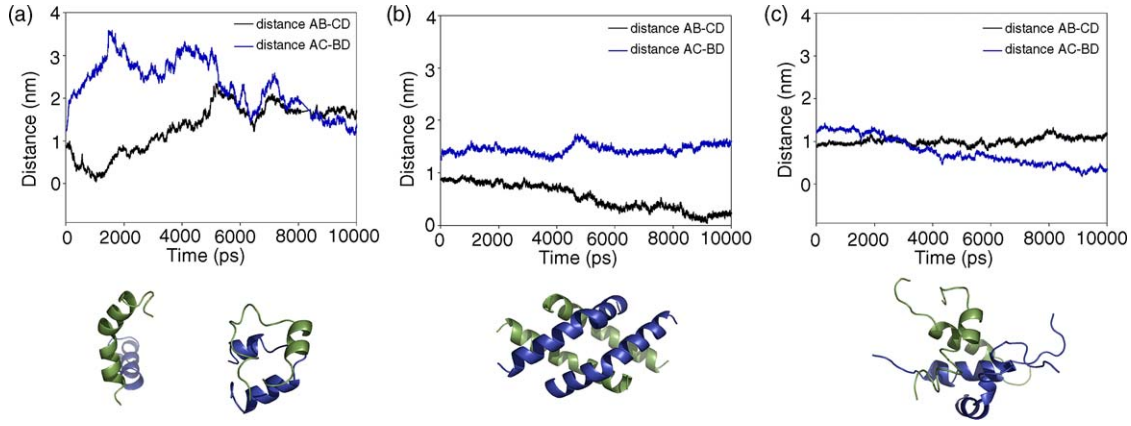

Fig. 2. Plot of the distances between dimers of melittin against molecular dynamics simulation time (upper) and snapshots of tetrameric conformation after 4 ns of molecular dynamics simulations (lower). (a) Condition 1, 23.5 mM melittin, 120 mM Cl^-, pH 7.0, Gromos96 force field. (b) Condition 2, 56.6 mM melittin, 273 mM Cl^-, pH 7.0, Gromos96 force field. (c) Condition 3, 56.6 mM melittin, 273 mM Cl^-, pH 7.0, OPLSAA force field.

the simulation condition closest to the plasmatic medium (condition 1), the monomers that compose the tetrameric structure present a progressive re-orientation, irreversibly splitting into two dimers within 2.5 ns of the trajectory (see Supplementary data). The initial conformation of tetrameric melittin can be observed in Fig. 1a and the resulting tetrameric form unfolded into two dimers (AC and BD) is seen in Fig. 1b. The distances between dimers increases drastically, as shown in Fig. 2a, becoming larger than 3.5 nm after the first 1.5 ns. Such behavior allowed us to conclude that the formation of the 'channel' as proposed in a previous work [17] on this system, seems to be an artifact.

In fact, the stability of the tetrameric form of melittin (and of its 'channel') in unrestrained simulations was obtained only through the modulation of the medium physical–chemical conditions. Simulation under condition 2 presents a 2.3-fold increase in both melittin and salt concentration, while condition 3 presents an increase in pH from 7.0 to 11.0. Under conditions 2 and 3 the initial tetrameric conformation (Fig. 1a) could not be set apart (Fig. 2b) during the trajectories indicating a stable tetrameric form. It must be emphasized that even the lowest melittin concentration (23.5 mM) tested (simulation conditions 1 and 3) is at least eight-fold higher than its concentration in crude bee venom. This observation is of extreme importance regarding any mechanism proposed for explaining the peptide's biological activity, which should take into consideration both the conformation and oligomerization status of the peptide when acting in a physiological medium. As clearly indicated here, a lower plasmatic concentration would induce an even faster unfolding of the melittin tetramer.

Since the previous work on melittin simulation used the OPLSAA force field to describe the tetrameric form as a 'channel' [17] and in order to avoid any force field dependent results, we also simulated the melittin tetramer using such force field under both low (23.5 mM) and high (56.6 mM) peptide concentrations. The conformational behavior of melittin tetramer was very similar in both Gromos96 and OPLSAA force fields, being stable only at high peptide concentrations. However, under OPLSAA a partial unfolding of its constituent peptide units was observed (Fig. 2c). Our data demonstrate that the 'channel' between the dimers in the crystallographic structure remains stable only under extreme and non-physiological conditions (Fig. 2b), being thus inappropriate to attribute a biological role for such melittin structure. In agreement with these observations, a recent work [29] reported that aggregation of melittin in a tetrameric form was only observed in high salt aqueous solutions.

Considering the fact that the melittin tetramer disassembles into two dimers under conditions close to that of a physiological medium, we also performed molecular dynamics simulations of the crystallographic dimeric form of the peptide. The parameters of pH, melittin and salt concentration chosen for such molecular dynamics simulation were the same as the simulation condition 1. Doing so, we observed that the dimeric form of melittin also dissociates in solution, with the distance between monomers changing from 1.3 to 4.5 nm in the first 2.5 ns of the trajectory (Fig. 1c). This result led us to the conclusion that, in plasma, melittin should not be in a tetrameric nor a dimeric oligomerization state, but is probably present as a monomeric peptide.

3.2. Simulations of monomeric melittin

The data obtained by molecular simulations of oligomeric melittin indicate that the aggregation state of the peptide is highly dependent on the environment conditions, i.e. peptide concentration and/or pH. The effect of physical–chemical parameters of the medium on the structure of melittin can be easily noticed in the monomeric form of the peptide. For example, the helical content of melittin increased from 12% to 65% in response to a salt concentration changing from near zero to 1 M NaCl, as reported by others [8,16]. Thus, in addition to the oligomerization state, the peptide secondary structure is also dependent on solution conditions.

Considering the lack of stability of the peptide oligomeric states under 'physiological' conditions and aiming to correlate these data to the peptide's helical content, we investigated the behavior of monomeric melittin under a number of different

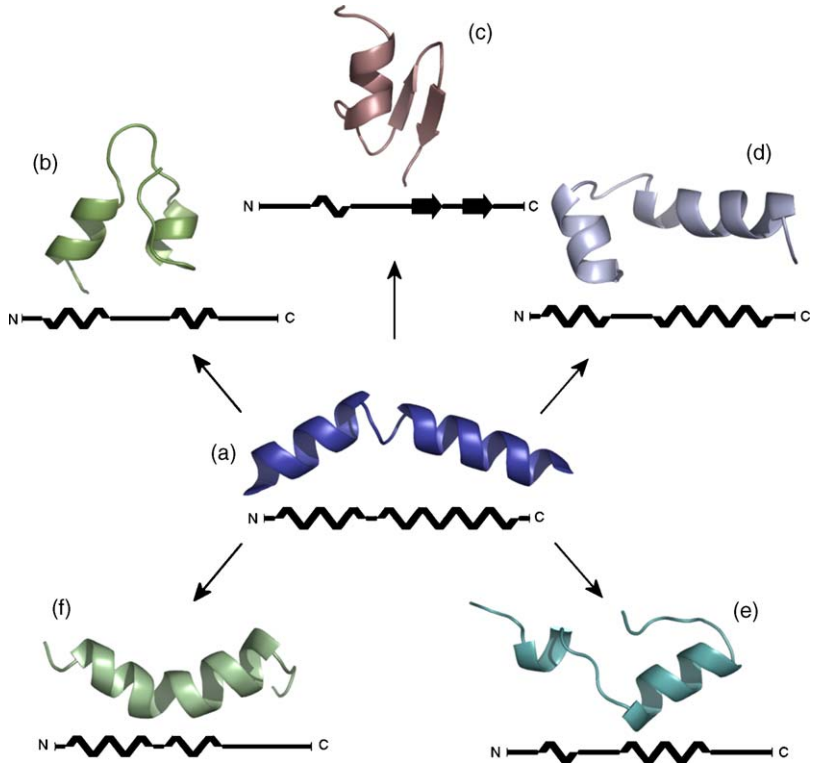

Fig. 3. Monomeric melittin conformations after 10 ns of molecular dynamics simulations. (a) Melittin crystallographic structure (PDB 2MLT). (b) Melittin in water pH 7.0, Cl^- 50 mM. (c) Melittin in water pH 7.0, SO_4^{2-} 50 mM. (d) Melittin in water pH 7.0, Cl^- 1.2 M. (e) Melittin in water pH 7.0, SO_4^{2-} 1.2 M. (f) Melittin in water pH 11.0, SO_4^{2-} 50 mM. Secondary structure prediction by PROCHECK [23] is presented in black.

conditions. An overview of melittin conformations after 10 ns of molecular dynamics simulations under different simulation parameters, is presented in Fig. 3.

In agreement with biochemical observations, the simulations of a melittin monomer in presence of 50 mM chloride ions indicated a significant decrease in its helical character (Fig. 3b), a behavior also observed under an equivalent concentration of sulfate ions (Fig. 3c). On the other hand, the increase in ionic strength, using either chloride or sulfate ions, enhance the helix content of melittin in an ion-nature independent manner (Fig. 3d and e). In addition, a curious behavior is observed at 50 mM SO_4^{2-}, in which the helical character is partially transformed in a β-sheet structure. Such conformational modification is reproducible and can be reverted under higher ionic strength.

The pH has a critical effect on the stabilization of the helical structure of melittin, as reported by other authors [8]. Again, molecular dynamics simulations correctly reproduce experimental data: loss of the helical character is observed when the peptide is exposed to pH 7.0 (Fig. 3c), being the secondary structure almost completely recovered upon increasing the pH medium to 11.0 (Fig. 3f). These data are illustrated in Fig. 4 by

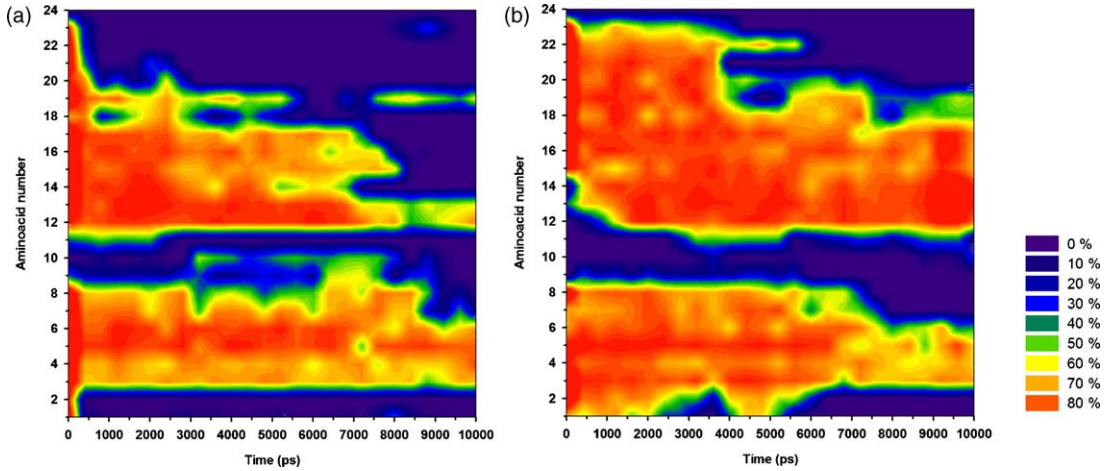

Fig. 4. Ellipticity maps [27] of monomeric melittin as a function of time and aminoacid number. (a) 26.5 mM melittin, 50 mM Cl^-, pH 7.0. (b) 26.5 mM melittin, 1.2 M Cl^-, pH 7.0.

means of the ellipticity parameter [28] as a function of both time and amino acid sequence.

Besides the critical parameters of ionic strength and pH, some solvents, especially methanol, have been considered in the study of melittin structure and conformation. Methanol is an interesting solvent for investigation, since it was the solvent used to obtain the NMR structure of melittin [9], which shows the peptide as a completely folded helix. However, contrarily to this a 10 ns molecular dynamic simulation in presence of methanol presented an unfolded structure on the peptide N-terminal portion (data not shown). Conversely, the C-terminal helix remained stable over the entire simulation. Similar results were obtained in previous work, in which a completely unfolded melittin was seen after a 120 ps molecular dynamics simulation in methanol [15]. Regarding the molecular basis for the difference between NMR and molecular dynamics findings, a possible reason are the conditions in which the peptide structure is simulated. As shown in Fig. 3, the peptide conformation and dynamics is highly determined by the surrounding environment, which is influenced by the procedures used for the peptide purification. For example, residual ions in the purified sample could be the key factor for inducing the differences between folded and unfolded helix, as shown in Fig. 3d being the increase in helical character due to an increase in chloride ions concentration.

The simulations of the monomeric form of the peptide, in a total sampling of about 0.1 μs in this study, suggest a rather simple explanation for the discrepancy so far existing in the literature about the peptide structure and its biochemical properties in solution. As shown here the conformation of melittin can be highly influenced by physical–chemical conditions (Fig. 3), inducing the peptide to adopt a diversity of forms under different media. On the other hand, both crystallographic and NMR structures are usually obtained under artificial aqueous conditions (i.e. different from plasma composition), as has been the case for melittin. As a consequence, the helical structure observed in crystallographic and NMR data for this peptide should be interpreted as a consequence of both high salt and melittin concentrations. In this context, our calculations suggest that in physiological medium such as human plasma, melittin should assume a monomeric form, mostly unfolded.

3.3. Implications to current understanding of melittin biological properties

The melittin conformation responsible for its pore formation capabilities has been a matter of controversy [3,30–32], especially because some authors assume that melittin approaches and binds to membranes in a defined conformation and orientation. In fact, the orientation of the peptide over lipid bilayers has been proposed as a function of the peptide concentration, e.g. at low concentration melittin binds parallel to the membrane surface and as the peptide concentration increases in the medium the binding turns to a perpendicular orientation [32]. The pore model has also been assumed as a toroidal model by neutron diffraction [32], and a previous molecular dynamics simulation study has proposed that even when inserted into lipid bilayers the position of the melittin monomers is not stabilized as a perpendicular tetramer [14]. Besides, the orientation of melittin chains in a pore is enormously different [32] to that proposed by X-ray crystallography [10,11]. Thus, considering the whole of experimental and theoretical data available for melittin structure and dynamics, the peptide appears to fold into a helix conformation only when interacting with highly negative biological surfaces, as membranes of activated platelets or to membrane proteins, as phospholipases. Such charged surfaces would perform a role equivalent to high salt conditions in the peptide folding. Actually it has been experimentally shown [33] that melittin binds to a lipid vesicle as a disordered monomeric peptide form, inserts itself into the lipid membrane, and its concerted folding as a helix is promoted upon the peptide penetration. The characterization of this process at the atomic level was recently shown, with the observation that DMPC bilayers are capable to stabilize the secondary structure of melittin related to the unfolded peptide in aqueous solution [34].

4. Conclusion

In this work we performed molecular dynamics simulation of melittin in three different oligomeric states, i.e. monomeric, dimeric, and tetrameric forms, in order to have a complete overview of the peptide structure, conformation and dynamics according to environmental conditions. Our results show that the tetrameric organization of melittin is not possible in plasma, but only in extreme physical–chemical conditions. We have seen that the crystallographically observed inner channel does not remain stable in solution under physiological conditions and cannot be responsible for the biological properties of melittin.

Additionally, we observed that the peptide is not a perfect helix, but a random structure at pH 7.0 in physiological ionic strength. Our findings are important not only for the understanding of the melittin mechanism of action concerning its lytic activity, but are also relevant for the study of its ability to modulate phospholipases and others physiologically important proteins.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jmgm.2006.06.006.

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2. Caracterização dos efeitos da melitina na função plaquetária

2.1 Função e avaliação de função plaquetária

As plaquetas têm um papel fundamental na hemostasia. Durante um dano vascular o subendotélio exposto provoca uma reação rápida de adesão plaquetária ao tecido, seguida da ativação das plaquetas, promovendo a formação de um tampão e impedindo a hemorragia (Rand *et al.*, 2003; Willoughby *et al.*, 2002). A adesão das plaquetas às proteínas dos sítios de dano vascular, principalmente fator de von Willebrand, é mediada pelo receptor glicoprotéico (GP)Ib-V-IX, exclusivamente expresso em plaquetas e megacariócitos (Nieswandt *et al.*, 2005). Este processo ainda é auxiliado pela presença de receptores específicos para outras proteínas de membrana basal, como a integrina $\alpha_2\beta_1$ ligadora de colágenos (Nieswandt *et al.*, 2005). O processo de adesão é seguido da ativação das plaquetas e conseqüente recrutamento de outras plaquetas circulantes.

As plaquetas circulantes são discóides e possuem uma meia-vida aproximada de 8-10 dias (Willoughby *et al.*, 2002). O processo de ativação ocorre na presença

de agonistas sendo mediado por receptores na superfície das plaquetas. A ativação desencadeia quatro distintos processos: (a) alteração morfológica (*shape change*), (b) agregação, (c) secreção e (d) produção de ácido araquidônico (Chow e Kini, 2001; Smith e Brinkhous, 1991). A indução deste processo pode ser realizada por uma série de substâncias endógenas - como adenosina difosfato (ADP), colágeno, tromboxana e trombina – ou substâncias exógenas – como ionóforos e análogos de endoperóxidos cíclicos (Willoughby *et al.*, 2002). Existe ainda uma grande quantidade de toxinas de venenos animais que também possuem a capacidade de interagir e alterar a função plaquetária (Chow e Kini, 2001; Smith e Brinkhous, 1991). A maioria dos agonistas utiliza receptores acoplados a proteína G, sendo a proteína $\alpha_{IIb}\beta_3$ essencial na ativação plaquetária. Quando ativada, a $\alpha_{IIb}\beta_3$ provoca a ativação de fosfolipases, aumento do cálcio intracelular, supressão da produção de AMPc e reorganização do citoesqueleto (Nieswandt *et al.*, 2005).

Uma vez ativada, as plaquetas sofrem uma mudança de forma (*shape change*); a mobilização de actina, miosina, tropomiosina e outras proteínas do citoesqueleto resulta na emissão de pseudópodos, a exposição de diversos receptores na superfície da célula e a sua mudança para uma forma globular (Zucker e Nachmias, 1985). Na Figura 1 pode ser observada a diferença morfológica das plaquetas por microscopia eletrônica. Após o processo de *shape change*, as células passam então a formar agregados. A secreção dos conteúdos de grânulos intracelulares, principalmente ADP, e a liberação de ácido araquidônico provoca um aumento no sinal de ativação e o recrutamento de outras plaquetas, promovendo a formação de grandes agregados, tampão vascular, impedindo a hemorragia (Willoughby *et al.*, 2002).

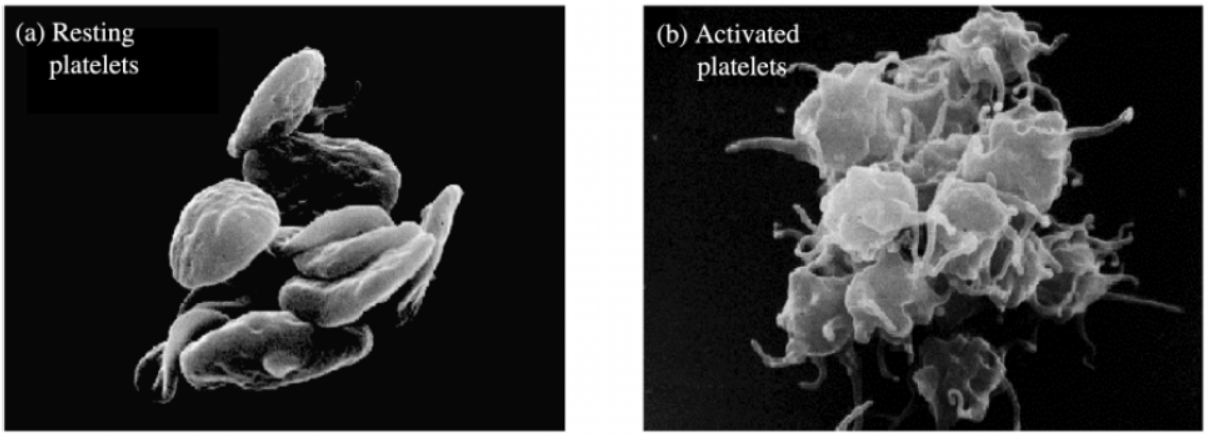

FIGURA 1 – Ativação e agregação plaquetária. Diferenças morfológicas evidenciadas por microscopia eletrônica de varredura. Em (a) plaquetas circulantes e em (b) plaquetas ativadas. Retirado de Willoughby, S. et al (2002)

Diversas técnicas são utilizadas para avaliar a função plaquetária. O primeiro ensaio descrito para esta finalidade foi o tempo de sangramento, porém sua falta de sensisibilidade e especificidade provocaram o desenvolvimento de novas técnicas (Rand *et al.*, 2003). O ensaio mais utilizado atualmente para avaliar a função plaquetária é a medida de agregação plaquetária em plasma rico em plaquetas (PRP) (Rand *et al.*, 2003). A técnica espectrofotométrica baseia-se no princípio de que, na presença de um agonista, as plaquetas sofrem ativação provocando sua alteração morfológica (*shape change*) com conseqüente e transitória diminuição na transmitância do meio medida a 650 nm. Em seguida, as plaquetas agregam resultando em um aumento na transmitância do meio. A quantificação da resposta de agregação é realizada através da intensidade com que ocorre esta diferença na passagem da luz (Rand *et al.*, 2003).

2.2 Artigo 2: Terra, RMS; Pinto AFM; Berger, M; de Oliveira, SK; Juliano, MA; Juliano, L; Guimarães, JA. Melittin-induced platelet signaling and aggregation. Manuscrito em preparação.

O presente trabalho é uma avaliação da influência do peptídeo melitina na função plaquetária. Para tal, foi realizada a análise da sua atividade pró-agregante assim como de sua capacidade secretora. Durante o desenvolvimento deste estudo foi possível ainda identificar a seqüência de aminoácidos relacionada a esta atividade biológica através da avaliação de peptídeos sintéticos derivados de melitina. Além disso, uma demonstração clara da interação direta peptídeo-plaqueta está aqui apresentada. O trabalho ainda indica uma ativação pouco específica, envolvendo várias possíveis rotas de sinalização.

Os resultados apresentados aqui estão escritos na forma de manuscrito que será submetido para publicação no periódico Biochemical and Biophysics Research Communications.

Melittin-Induced Platelet Signaling and Aggregation

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ABSTRACT

Envenomation involving africanized bees results in many biochemical alterations, including coagulation disorders. The main toxic substance of *Apis mellifera* venom is melittin, a peptide with the ability to interact with cell membranes. In this work we evaluate melittin and five melittin-derived peptides (Mel-1 to Mel-5) in their ability to affect platelet function. Here we describe the capability of melittin to promote human platelet aggregation in dose-dependent manner and to secrete ATP from dense granules. The peptides Mel-1 and Mel-2, which correspond to the amino-terminal portion of melittin, are related to the pro-aggregating activity as well as the adhesion of the peptide to platelet surfaces. Additionally, we demonstrate that melittin induced platelet aggregation is inhibited by indomethacin, esculetin, verapamil, naloxone and cilostazol. Altogether these findings contribute to the actual knowledge of the biological activities of melittin and give new insights to the better understanding of the haemostatic abnormalities during bee envenomations.

Keywords: Melittin, platelet aggregation, platelet adhesion, bee venom, *Apis mellifera*, indomethacin, esculetin, cilostazol, naloxone, verapamil

INTRODUCTION

Accidents involving africanized bees are frequently reported and massive envenomations results in nausea, hemolytic and other haemostatic abnormalities, shock, disseminated intravascular coagulation, kidney failure and coma and, in some cases, a delayed multi-organ-failure [1, 2]. Altogether these clinical symptoms points to profound blood coagulation disorder appearing in patients after bee sting. For a long time, practically all biological effects of bee venoms were ascribed to its phospholipase activity, specially regarding the haemostatic abnormalities [3-5]. In fact, the effect of bee venom phospholipase A2 upon platelet reactivity has been already described [6-8]. However bee venom is a complex mixture of pharmacologically and toxicologically active proteins and peptides that could interfere in platelet function. Recently, the publication of the bee genome gave rise to the interest to investigate others platelet active proteins such as desintegrins [9].

Melittin, a 2.8 kDa peptide that accounts for about 50% of the venom dry weight [3], is the most important toxic compound present in *Apis mellifera* venom with respect to weight and activity. The cationic 26 amino acid long peptide (GIGAVLKVLTTGLPALISWIKRKRQQ) is composed by a hydrophobic amino-terminal portion and a carboxy-terminal end rich in hydrophilic amino acids. As we have previously demonstrated [10], in solution melittin is found in a random conformation, a fact that could explain its wide range of biological activities [11, 12].

The hemolytic action of melittin as well as its ability to interact and disrupt natural and artificial membranes is well described [3, 13, 14]. The peptide is also able to modulate phospholipases [15-18] and calmodulin being then an important inhibitor of cell growth [19]. The stimulation of arachidonic acid-derived lipoxygenase

metabolites induced by melittin in leukocytes and platelets [20] denotes its interesting potential as a platelet aggregating agent [6, 8, 21]. However, such aspect has been neglected or poorly explored so far.

Considering the variability of actions of melittin upon many target proteins and cells, the importance of a better understanding of both the pharmacological and toxicological effects produced by bee venoms and furthermore, its impact on the clinical management of bee envenomation, this work aimed to investigate the effects of melittin upon platelet morphology and function.

MATERIALS AND METHODS

Materials

Melittin was either obtained from commercial source (Sigma Chemical Co., St. Louis, MO, USA) or synthesized. Luciferin-luciferase (Chrono-lume) was obtained from Chrono-log (Havertown, PA, USA); glutaraldehyde 25% EM grade was purchased from Electron Microscopy Sciences (Hatfield, PA, USA) and von Willebrand factor (vWF) was a commercial preparation (vWF/fVIII) from Octapharma (Langfeld, Germany). Verapamil (Sandoz pharmaceuticals, Brazil) and cilostazol (Libbs pharmaceuticals, Brazil) were obtained as commercial drugs. Indomethacin, esculetin, naloxone and apyrase grade VII as well as all other chemicals used were purchased from Sigma (St. Louis, MO, USA).

Peptides synthesis and purification

Peptides were synthesized by the Fmoc [N-(9-fluorenyl)methoxycarbonyl] methodology as previously described [22] using an automated benchtop simultaneous multiple solid-phase peptide synthesizer (PSSM 8 system from Shimadzu, Tokyo, Japan) and were purified to homogeneity by high-pressure liquid chromatography on a Vydac C18 analytical column. The molecular mass and sequence were checked by amino acid analysis, MALDI-TOF (TOFSpec E instrument from Micromass, Manchester, U.K.), and Edman degradation (PPSQ 23 system from Shimadzu).

Blood collection and platelet preparation

Human venous forearm blood was collected from five healthy volunteers in anticoagulant ACD (2:10 v/v) or 3.2 % sodium citrate (1:10 v/v). Platelet rich plasma (PRP) was prepared from blood anticoagulated with sodium citrate, centrifuged at 200 X g thrice for 5 minutes. Washed platelets were obtained from blood collected on ACD, centrifuged as above and re-centrifuged at 620 X g for 10 min. The platelet pellet was re-suspended in 1 mL ACD and washed through a modified protocol from Timmons and Hawinger [23] by gel filtration in a Sepharose 2B column equilibrated and eluted with Albumin-Tyrode's buffer, pH 7.4. The final platelet suspension O.D. (650nm) was adjusted to 0.15 O.D.

Platelet aggregation and ATP secretion

Platelet aggregation (PRP and washed platelets) was measured turbidimetrically as described [24] using either a Lumi-aggregometer (Chrono-log, USA) and/or a SpectraMax microplate reader (Molecular Devices, USA). Briefly, platelets were incubated for 2 minutes at 37 °C under stirring and aggregation was induced by melittin and melittin-derived peptides. ADP (10 μM) and collagen (2 μg/mL) were used as control inducers.

Adenosine triphosphate (ATP) secretion was determined as described [24], using a Luciferin-luciferase reagent (Chrono-lume). Luminescence gain was monitored at 37 °C for 5 minutes in the dark.

Scanning electron microscopy (SEM)

SEM sample preparation procedure was modified from Gear [25]. Briefly, PRP samples were pre-warmed at 37 °C and exposed to ADP, saline or melittin under low stirring. Platelets were fixed overnight in 2.5% glutaraldehyde in 0.1 M cacodilyc buffer, pH 7.2. The samples were washed twice for 30 minutes in 0.1 M cacodilyc buffer and filtered in a 0.4 μm polycarbonate membranes (Millipore, USA). The fixed cells were sequentially dehydrated for 5 minutes in 30, 50, 70 and 90% (v/v) acetone and finally treated twice for 10 minutes in 100% acetone. Critical-point drying and gold coating treatments were performed at the University's Center of Electron Microscopy (CEM-UFRGS, Brazil). Specimens were visualized in a JEOL-JSM 6060 scanning electron microscope with automated image digitization and archiving.

Platelet aggregation inhibition

Platelet aggregation inhibition assays were performed in PRP as already described [26]. Indomethacin (100 μM), esculetin (10 μM), verapamil (50 μM), naloxone (0.5 mM), cilostazol (50 μM) and apyrase grade VII (5 units/mL) were used as inhibitors of platelet aggregation. ADP (5 μM) was used as experiments control. The inhibitors were incubated with pre-warmed (37 °C) PRP for 5 minutes under low stirring. Platelet aggregation was induced upon the addition of melittin or ADP.

Platelet Adhesion

A modified platelet adhesion procedure described by Bellavite et al. [27] was used. Melittin was assayed either as an adhesion protein (Direct Adhesion) or as a competitive agonist when other proteins were added as an adhesive substrate (Competitive Adhesion). In the direct adhesion experiment, 96 wells microtiter plates (Flacon, USA) were coated overnight at 4 °C and then for 1 hour at 37 °C with 10 μg of melittin or melittin-derived peptides (100 μL aliquots). Immediately before use, plates were manually washed twice with 0.9 % NaCl solution. Aliquots (100 μL each) of washed platelets preparation (2.5 X 10^6 cells) were added to the wells and incubated at 37 °C for 30 minutes. At the end of incubation the wells were washed twice with 0.9 % NaCl and each one immediately supplemented with 100 μL of 0.1 M citrated buffer, pH 5.4, containing 5 mM ρ-nitrophenyl phosphate and 0.1% Triton X-100 in order to measure platelets acid phosphatase activity. The plate was incubated for one hour at room temperature and the reaction was stopped with 50 μL of 2M NaOH. The amount of ρ-nitrophenol produced by the reaction was measured at 405

nm against a platelet-free blank by using a SpectraMax microplate reader (Molecular Devices, USA). The number of adherent platelets was calculated on the basis of a standard curve obtained with known number of lysed platelets. For the competitive adhesion experiments the plates were coated with 100 µL of diluted human plasma (1:2 in saline), bovine fibrinogen (2mg/mL), collagen types I and III (2 mg/mL) and human von Willebrand Factor (20 µg/mL). The experiment then followed the protocol described above. In these experiments melittin was assayed as a competitive agonist of known pro-aggregating inducers. In these cases, platelets were treated with 10 µg of melittin for 10 minutes or immediately before their addition to the plates.

Statistical analysis

Results were analyzed by ANOVA followed by the Bonferroni test using Instat Graph Pad software in order to estimate difference between groups. Values of *$p<0.05$, **$p<0.01$ and ***$p<0.001$ were considered statistically significant.

RESULTS

Melittin capability to induce platelet aggregation was tested in both platelet rich plasma and washed human platelets. As shown in Figure 1A, the aggregation effect in PRP was produced in a dose-dependent manner where the platelets shape change response could be clearly visualized as an initial decrease in the medium transmittance. Melittin was also able to induce aggregation in washed platelets (not shown) indicating that its pro-aggregating activity is not dependent or mediated by

plasma proteins. ATP secretion was demonstrated by luminescence gain in platelet rich plasma. The secretion of ATP is related to dense granules content release and was comparable to the secretion obtained by known platelet-aggregation agonists, such as ADP and collagen type I (Figure 1B). Electron microscopy techniques showed (Figure 1C) that the platelet aggregates produced by melittin presents a morphological pattern similar to the one induced by ADP. It also confirms that at these concentrations, melittin does not produce lyses of human platelets.

With the aim of identify a possible amino acid sequence responsible for melittin effects regarding both platelet activation and aggregation, five peptides (Mel-1 to Mel-5) containing from 5 to 9 amino acid residues and covering the entire melittin sequence were designed and synthesized (Table I). Platelet pro-aggregating activity was found only in peptides Mel-1 and Mel-2 (Figure 2A). Even presenting such activity at a much higher concentration (ca 0.8 mM or about 40-fold) than the full-length peptide, these results are indicative of a possible role of peptides in melittin-induced platelet aggregation.

Inhibitory studies of platelet aggregation induced by melittin were performed upon pre-incubation of PRP with indomethacin, esculetin, verapamil, naloxone, cilostazol and apyrase. As shown in Figure 3, indomethacin (100 μM) and esculetin (10 μM) produced a discrete inhibitory effect of melittin-induced platelet aggregation (ca 18 and 15% inhibition, respectively) while a more expressive effect (ca 70% inhibition) was produced by verapamil (50 μM). Naloxone was able to produce a 30% inhibition which was comparable to the effect of the phosphodiesterase inhibitor (cilostazol, ca 25%). On the other hand, apyrase (5 units/mL) was unable to block the pro-aggregating effect of melittin (Figure 3) although, in this concentration, the enzyme was able to reduce 75% of 5 μM ADP induced aggregation.

In order to evaluate the interaction of melittin and its platelet active derived-peptides Mel-1 and Mel-2 the direct adhesion experiment was conducted. Peptides to be tested were immobilized in a 96-well plate (100 µg per well) and then incubated with washed platelets for 30 minutes at 37 °C. The adherent platelets were lysed and acid phosphatase activity measured in a colorimetric assay. Figure 2B shows that melittin as well as its sequence related peptides Mel-1 and Mel-2 were able to induce direct platelet adhesion, being however, the full-length peptide more effective to adhere to the platelets surface. When testing melittin interference (competitive adhesion experiment) in platelet adhesion to known adhesive proteins, such as collagen, fibrinogen and von Willebrand factor, we found that the peptide inhibited platelet interaction with all these adhesion proteins (Figure 4). There were no statistical differences between pre-incubation (10 minutes, 37 °C) and no incubation of melittin with washed platelets before their addition to coated microplates.

DISCUSSION

Accidents involving bee stings can cause several hematological manifestations that are still not fully understood [1, 2, 5]. In this work we have investigated the platelet-aggregating activity of melittin, the main toxic component of honey bee venom. Using a series of different though complementary methodologies we were able to demonstrate that melittin interacts with human platelets leading to its activation and subsequent aggregation. This pro-aggregating effect is coherent with the platelet-aggregating activity of the whole venom, which is also a platelet-aggregating agent (data not shown), even thought the bee phospholipase posses an inhibitory activity in the tested concentration range [7]. Thus, it should be concluded that during

envenomation the predominant platelet effect of bee venom components can be attributed to melittin rather than to phospholipase A_2.

The platelet-aggregating activity of bee venom components has been poorly studied. In the case of its component melittin a single and preliminary report [21] just mention such activity but this action of melittin has never been included in any inventory of venoms components that cause platelet aggregation [6, 8]. Here we demonstrated that the aggregation induced by melittin is dose-dependent and is followed by the platelets shape change as observed by the slight decrease in light transmission seen in Figure 1A and also demonstrated by electron microscopy (Figure 1B). Moreover, the activation of blood platelets by the peptide was followed by dense granules release with ATP secretion (Figure 1B) and possibly other granules components such as ADP, an important platelet-aggregating agonist.

The known amino acid composition of melittin allowed us to design and synthesize partial-sequence melittin peptides in order to evaluate the possible involvement of structural motifs of melittin molecule responsible for its unique platelet pro-aggregating activity. Five peptides were tested and among then only Mel-1 (GIGAVLKV) and Mel-2 (AVLKVLTTG) promoted platelet aggregation (Table I), although much less potent than the full peptide (Figure 2A). The two designed peptides with an overlap in the sequence AVLKV, represent the N-terminal portion of melittin and furthermore, they constitute the first structural helix before the proline kink in the folded conformation of the entire peptide [10]. Interestingly, this region is also responsible for the known hemolytic activity of melittin [28]. However, such lytic effect of melittin upon human platelets was not detected neither using electron microscopy (Figure 1C) nor in the release of lactic acid dehydrogenase experiments (data not shown). Moreover, large platelets aggregates could be easily visualized in

a naked eye inspection when platelets were stimulated with melittin, thus confirming the absence of a lytic action of the peptide in this case.

Considering the pro-aggregating effect and the release of dense granules content, an inhibitory assay was designed in order to elucidate the mechanism by which melittin activates blood platelets. It was already reported that melittin has a modulatory effect over phospholipases and lipoxygenases that are essential to arachidonic acid pathway, a key signaling route in platelets [20]. Intracellular phospholipases A_2 and C catalyze the production of arachidonic acid from platelet membrane phospholipids [29]. Arachidonic acid is a substrate for lipoxygenases and cyclooxygenases and their activity, together with other intracellular enzymes, produces thromboxane A_2 (TXA_2) [29]. This pathway enhances the aggregating signal since TXA_2 is also an agonist that can liberate intracellular calcium thus amplifying platelet aggregation [29]. It seems that melittin is able to modulate more than one enzyme in this cascade. This let us to use several platelet inhibitors in order to investigate melittin's pro-aggregating effects: indomethacin (a cyclooxygenase inhibitor), esculetin (a lipoxygenase inhibitor), verapamil (a voltage-dependent calcium-gated channel blocker) and naloxone (an intracellular calcium mobilization inhibitor) to investigate the role of the peptide in this signaling pathway. All four platelet-aggregation inhibitors were effective in blocking melittin signaling although calcium intracellular mobilization and influx seems to be pivotal in this process (Figure 3). The role of phosphodiesterase in melittin-induced aggregation was also evaluated by the incubation of platelets with cilostazol and it seems that melittin could also activate this enzyme during the signaling route. However, despite melittin's ability to induce cellular secretion of dense granules contents, apyrase (an ADP hydrolase), by itself was ineffective in inhibiting platelet aggregation induced by

the peptide (Figure 3). These data could be understood as ATP and ADP release being not the most essential event in platelet activation produced by melittin.

Moreover, melittin as well as mastoparan – a toxic peptide from wasp venom - was already described as an important G-protein modulator [30, 31]. This remarkable activity is of special concern once many platelet receptors are G-coupled proteins. ATP $P2Y_1$ and ADP $P2Y_{12}$ receptors are both G-coupled-proteins [32]. Then is plausible to speculate that melittin could interact directly with platelets receptors generating a pro-aggregating effect or even mimic the G-coupled receptors promoting the cell signaling response like mastoparan seems to do [33].

The direct interaction of melittin with human platelets was demonstrated in an adhesion assay. The adhesion occurs directly to melittin as well as to the platelet-active melittin-fragments Mel-1 and Mel-2, reflecting the affinity of the peptides for the platelets surface (Figure 2B). Our data also reveled that both melittin (Figure 4) and Mel-1 and Mel-2 (data not shown) could inhibit the adhesion of platelets to surfaces coated with plasma, fibrinogen, vWF and collagens I and III. Altogether, these findings indicate a direct interaction of melittin to platelet surface, an action that corroborate to our hypothesis of an unspecific association of melittin to platelet surface proteins and receptors.

In conclusion, our results show that melittin is able to aggregate human platelets in a dose-dependent manner, releasing dense granules contents. Moreover, we were able to identify the amino terminal portion of the peptide as partially responsible for the observed activity. In addition we demonstrated a direct adhesion of the peptide to the cell surfaces. Here we could also explore some of the platelet signaling pathways involved in melittin platelet activation such as the arachidonic acid activating cascade. This study contributes to the actual knowledge of the biological activities of

melittin and could give insights to the better understanding of the haemostatic abnormalities during bee envenomations and its clinical management.

ACKNOWLEDGEMENTS

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FIGURE LEGENDS

Table I – Peptides based on the melittin sequence. The full sequence of melittin is shown together with the designed peptides (Mel-1 to Mel-5) derived from its full length sequence.

Figure 1 – Melittin induced platelet aggregation and ATP secretion. **A.** Dose-response curve of melittin induced aggregation of human platelets. Results (mean ± SD) are shown as percentage of maximal aggregation (considering ADP 10 μM as 100%) for four replicates measured in a microplate reader. Shape change and profile of the aggregation registered in an aggregometer are shown in the inset. **B.** Melittin induced ATP secretion was detected by an increase in luminescence. PRP (270 μL) was incubated with 30 μL of luciferin-luciferase reagent (Chrono-log) for 2 minutes at 37 °C in the dark. Platelet aggregation was induced by 23 μM melittin or ADP (10 μM) and collagen type I (2 μg/mL) as control. Luminescence was monitored for 5 minutes. **C.** Surface morphology of human platelets by scanning electron microscopy (SEM). Control (saline), 10 μM ADP and 23 μM melittin stimulated platelets are shown in panels, white bar represents 2 μm.

Figure 2 – Effects of melittin and melittin-derived peptides upon platelet aggregation and adhesion. **A.** Platelet aggregation induced by melittin, Mel-1 (GIGAVLKV) and Mel-2 (AVLKVLTTG) peptides was recorded in a microplate reader. Results (mean ± SD) are expressed in percentage of aggregation, considering 10 μM ADP as 100% of aggregation, for four replicates. **B.** Direct adhesion was measure as number of platelets adhered to immobilized 10 μg melittin, 10 μg Mel-1 or 10 μg Mel-2 by an acid phosphatase colorimetric assay. Control is represented as the number of

platelet adhered to 20 µg bovine albumin. Results are shown as mean ± SD for triplicates, values of *p<0.05 and **p<0.01 were considered statistically significant.

Figure 3 – Effect of platelet aggregation inhibitors. PRP was incubated with inhibitors for 5 minutes under low stirring and platelet aggregation was stimulated with 23 µM melittin (dark grey) or 5 µM ADP (light grey - as a standard platelet-aggregating agent). Results (mean ± SD) are expressed as percentage of aggregation (5 µM ADP and 23 µM melittin as 100% of aggregation) for four replicates. Values of *p<0.05, **p<0.01 and ***p<0.001 were considered statistically significant.

Figure 4 - Competitive adhesion assay. Interference of melittin in platelet adhesion to immobilized adhesive proteins. Microplates were coated with 100 µL of human plasma (1:2 in saline), collagens I and III (20 mg/mL), bovine fibrinogen (2 mg/mL) and von Willebrand factor (vWF) (20 µg/mL). Immediately before the addition of human washed platelets to the microplates, 10 µg of melittin was added. After 30 minutes at 37 °C, platelets were lysed and the adhesion was measure by a colorimetric assay of acid phosphatase activity. Results (mean± SD) are expressed in number of platelets for triplicates, values of *p<0.05, **p<0.01 and ***p<0.001 were considered statistically significant. Control (ADP 10 µM) is represented in light grey bars and melittin treatment is represented in dark grey bars.

Table I

Peptide	Amino acid sequence
Melittin	GIGAVLKVLTTGLPALISWIKRKRQQ
Mel-1	GIGAVLKV
Mel-2	AVLKVLTTG
Mel-3	LPALISWI
Mel-4	ISWIKRKR
Mel-5	KRKRQQ

Figure 1

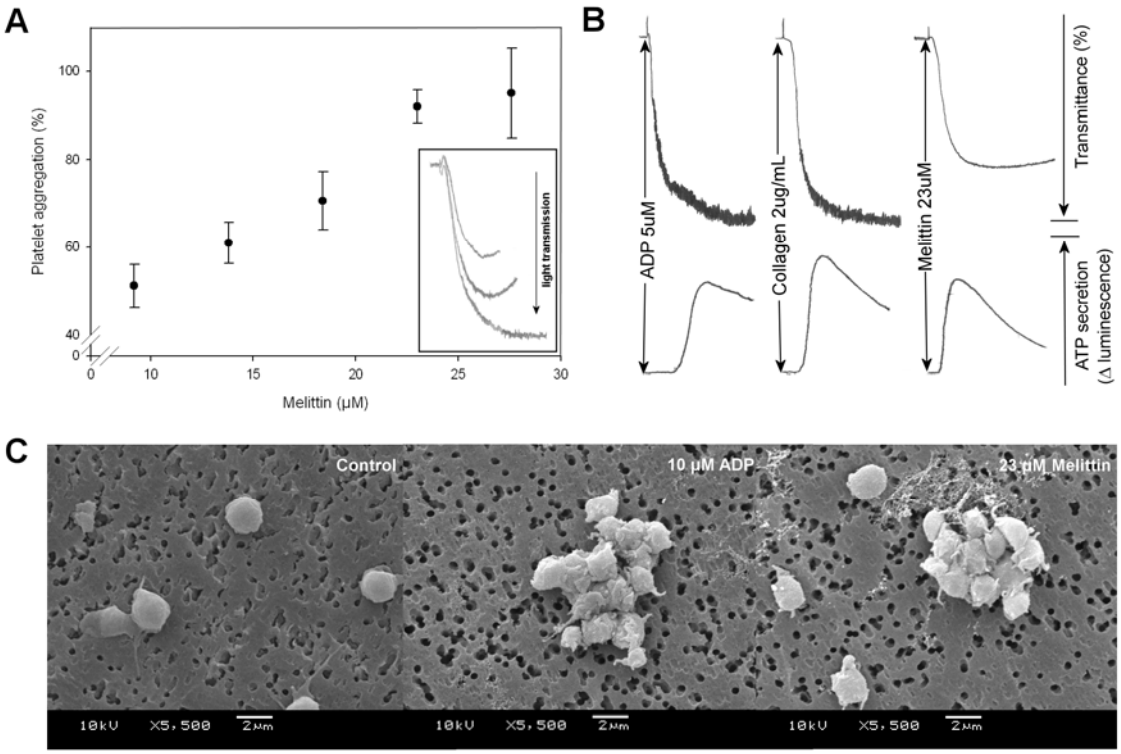

A
Platelet aggregation (%)
Melittin (μM)
light transmission
B
ADP 5uM
Collagen 2ug/mL
Melittin 23uM
Transmittance (%)
ATP secretion
(Δ luminescence)
C
Control
10 μM ADP
23 μM Melittin
10kV X5,500 2μm
10kV X5,500 2μm
10kV X5,500 2μm

Figure 2

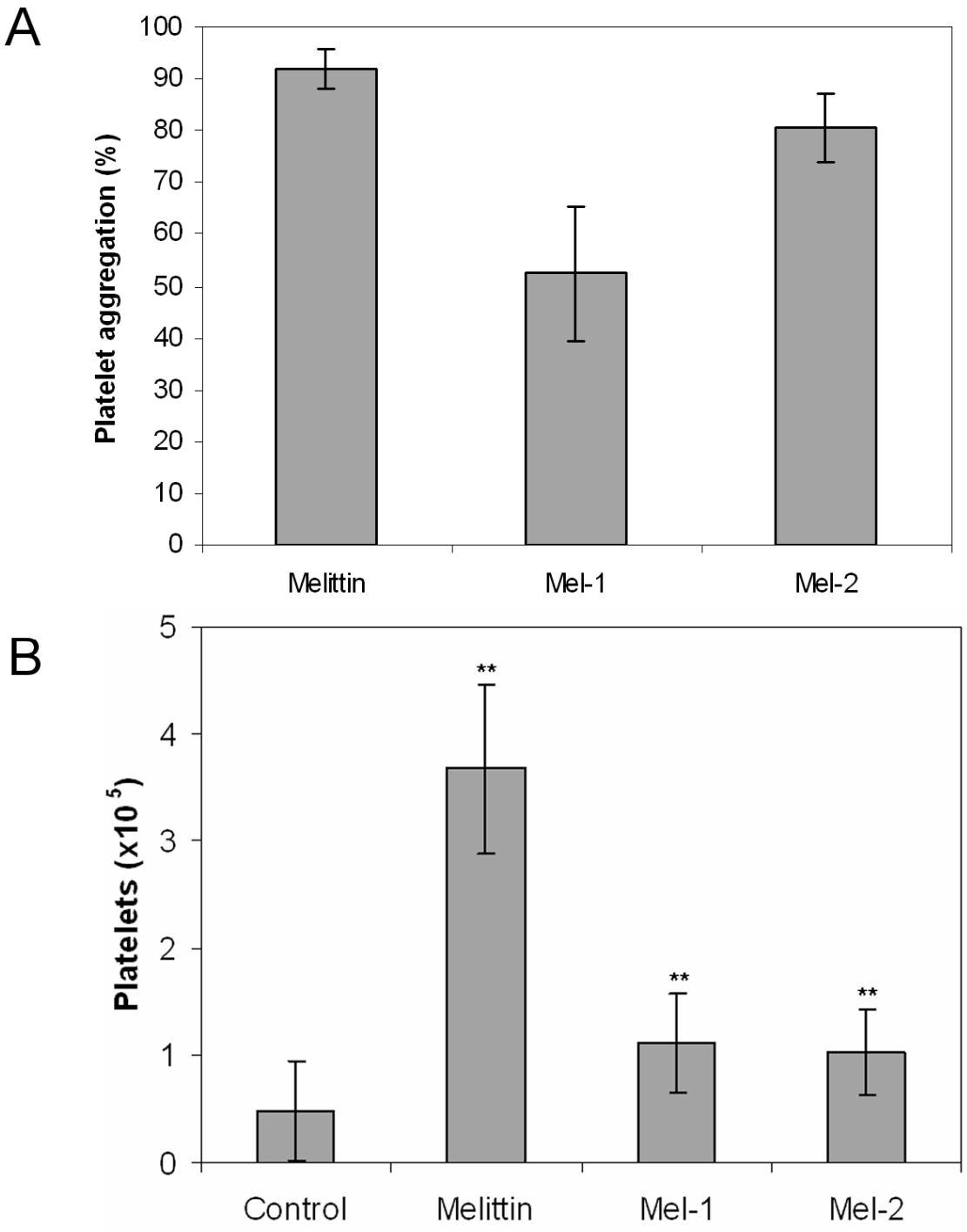

A
Platelet aggregation (%)
100
90
80
70
60
50
40
30
20
10
0
Melittin
Mel-1
Mel-2
B
Platelets (x10 5)
5
4
3
2
1
0
**
**
**
Control
Melittin
Mel-1
Mel-2

Figure 3

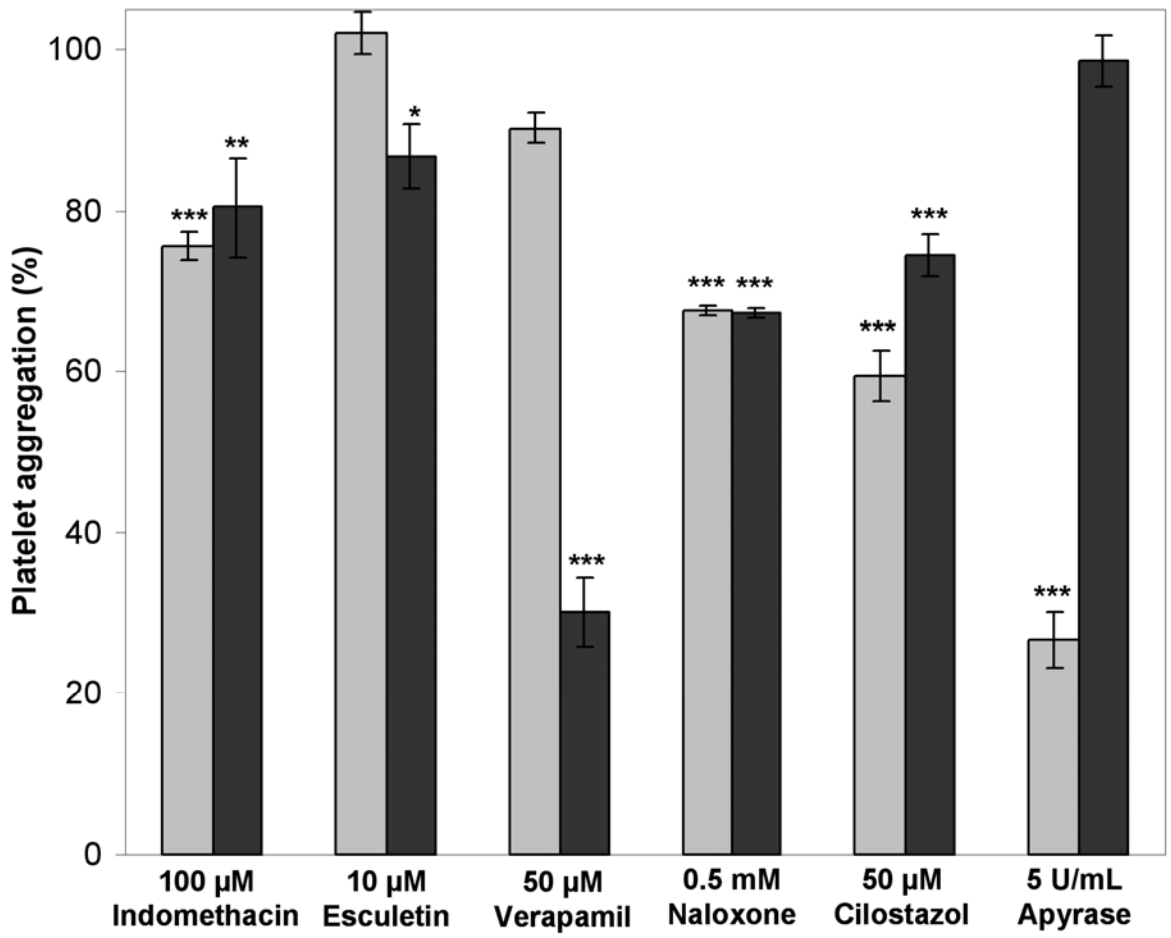
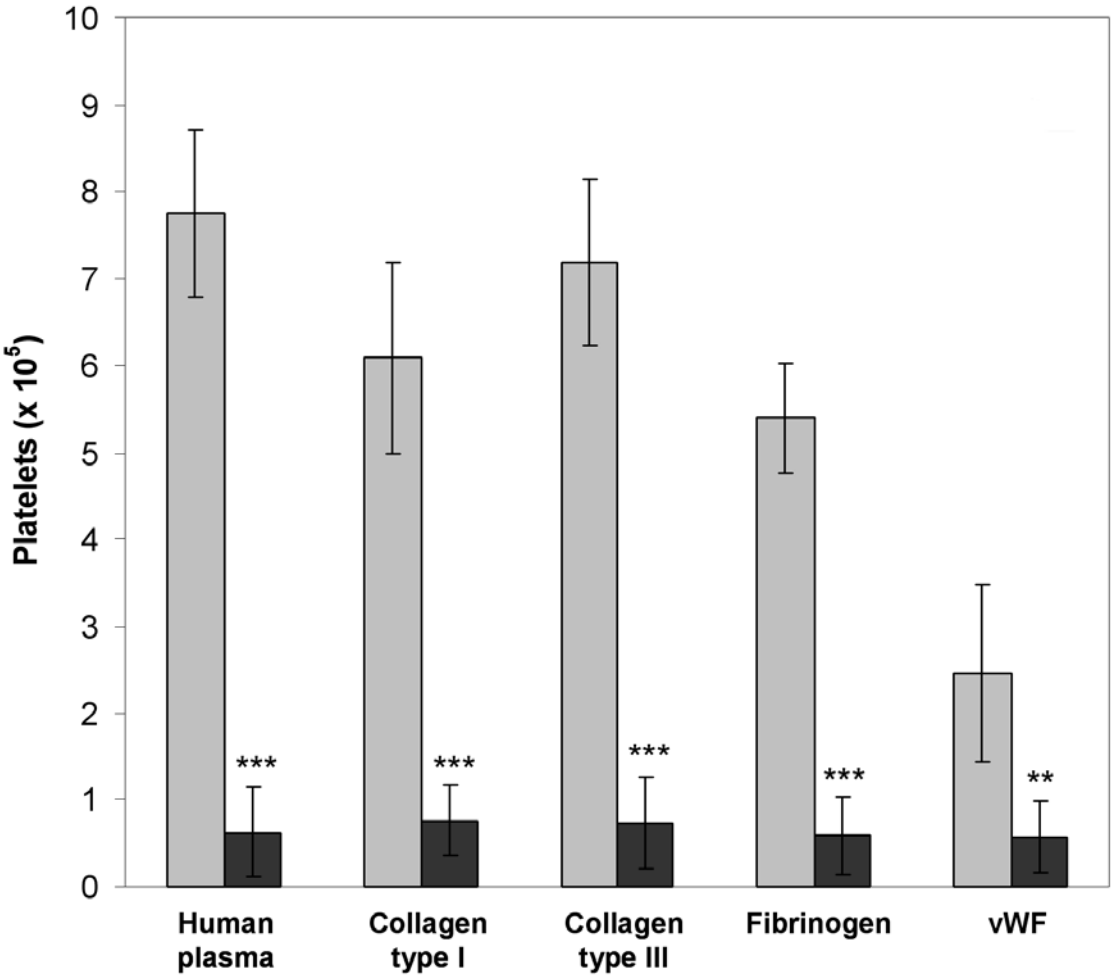

Platelet aggregation (%)
100
80
60
40
20
0
***
**
*
***
***
***
***
***
***
100 µM Indomethacin
10 µM Esculetin
50 µM Verapamil
0.5 mM Naloxone
50 µM Cilostazol
5 U/mL Apyrase

Figure 4


10
9
8
7
6
5
4
3
2
1
0
Platelets (x 10^5)
***
***
***
***
**
Human plasma
Collagen type I
Collagen type III
Fibrinogen
vWF

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Conclusões

Os resultados obtidos no desenvolvimento deste trabalho e aqui apresentados demonstram a complexidade conformacional e funcional do peptídeo melitina do veneno da abelha *Apis mellifera*. Os dados aqui descritos evidenciam que:

1. A estrutura terciária do peptídeo é altamente influenciada pelo meio;
2. A melitina em condições fisiológicas de pH e força iônica apresenta-se monomérica uma vez que as estruturas oligoméricas só são mantidas em elevado pH;
3. A presença de solvente orgânico (metanol) é capaz de desestabilizar a estrutura em hélice da melitina;
4. A conformação cristalográfica, em hélice, pode ser mantida apenas em condições elevadas de pH e força iônica, não sendo influenciada pelo caráter do íon;
5. A estrutura cristalográfica (PDB 2MLT), antes proposta como biologicamente ativa, não tem relevância biológica, uma vez demonstrado aqui que o peptídeo é randômico em condições fisiológicas de pH e íons;
6. O enovelamento da proteína é dependente de condições ambientais específicas;

7. A melitina é capaz de provocar a agregação plaquetária sem lise celular;
8. O peptídeo provoca secreção de grânulos densos das plaquetas, com liberação de ATP;
9. A porção amino-terminal da melitina (GIGAVLKVLTTG) é a responsável pela atividade biológica aqui estudada;
10. A melitina é capaz de interagir diretamente com a superfície das plaquetas assim como seus peptídeos relacionados (Mel-1 e Mel-2) aqui avaliados;
11. Ocorre inibição da adesão das plaquetas a algumas proteínas, indicando uma interação inespecífica com a superfície plaquetária;
12. A modulação de diversas proteínas intracelulares, assim como a interação inespecífica parecem ser responsáveis pela ativação plaquetária.

Os resultados aqui apresentados mostram o quão importante é o estudo conjunto de estrutura e função.

A falta de estrutura definida da melitina em condições fisiológicas e seu enovelamento sob diferentes condições ambientais ajudam-nos a entender a diversidade de atividades biológicas. Este entendimento é importante na avaliação e conduta clínica em casos de envenenamento. Os acidentes causados por ataques de abelhas hoje são considerados um problema de saúde pública a ser manejado e a urbanização das abelhas africanizadas tem aumentado o número de acidentes. A melitina, sendo a principal toxina do veneno, tem um papel essencial em diversas manifestações bioquímicas e patológicas. Neste trabalho foi possível descrever seus efeitos sobre a função plaquetária e contribuir para o conhecimento das coagulopatias causadas pelo envenenamento. Porém, há ainda muito a ser entendido a respeito da fisiopatologia do envenenamento e do impacto de cada um de seus componentes na evolução clínica.

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Publicações em Periódicos

1 TERRA, Renata Maria Soares; GUIMARÃES, Jorge Almeida; VERLI, Hugo. Structural and functional behavior of biologically active monomeric melittin. Journal of molecular graphics & modelling, *in press*, 2006

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4 BIZZANI, Delmar; MOTTA, Amanda S; MORRISSY, Juliana Alonso Campos; TERRA, Renata Maria Soares; SOUTO, André Arigony; BRANDELLI, Adriano. Antibacterial activity of cerein 8A, a bacteriocin-like peptide produced by Bacillus cereus. International microbiology, v. 8, p. 125-131, 2005.

Trabalhos apresentados em Congressos e Simpósios

1 RECK JR., José; BERGER, Markus; TERRA, Renata Maria Soares Terra; MARKS, Fernanda; DA SILVA VAZ JR., Itabajara; GUIMARÃES, Jorge Almeida; TERMIGNONI, Carlos.Avaliação de parâmetros Hemostáticos de bovinos infestados pelo carrapato Rhipicephalus (Boophilus) Microplus. In: CONGRESSO BRASILEIRO DE PARASITOLOGIA VETERINÁRIA, 2006, Ribeirão Preto. 2006

2 TERRA, Renata Maria Soares; KOBE, Simone de Oliveira; GUIMARÃES, Jorge Almeida. Evaluation of melittin effect over hemostasis. In: REUNIÃO ANUAL DA SOCIEDADE BRASILEIRA DE BIOQUÍMICA, 2006, Águas de Lindóia. 2006.

3 PINTO, Antônio Frederico Michel; TERRA, Renata Maria Soares; NAGASE, Hideaki; SERRANO, Solange Maria de Toledo; GUIMARÃES, Jorge Almeida; FOX, Jay William. Insight into the molecular interaction of snake venom metalloproteinase, atrolysin C, and tissue inhibitors of metalloproteinases. In: REUNIÃO ANUAL DA SOCIEDADE BRASILEIRA DE BIOQUÍMICA,2006, Águas de Lindóia. 2006.

4 TERRA, Renata Maria Soares; VERLI, Hugo; GUIMARÃES, Jorge Almeida. Evaluation of Melittin Structure, Conformation and Oligomerization in Solution Using Molecular Dynamics Simulation. In: REUNIÃO ANUAL DA SOCIEDADE BRASILEIRA DE BIOQUÍMICA, 2005, Águas de Lindóia. 2005.

5 JAGER, Alessandro; TERRA, Renata Maria Soares; SOUTO, André Arigony. Altas concentrações de trans-resveratrol em sucos de uva ecológicos. In: 26ª REUNIÃO ANUAL DA SOCIEDADE BRASILEIRA DE QUÍMICA, 2003, Poços de Caldas. 2003.

Participação em Congressos e Reuniões

1 Reunião Anual da Sociedade Brasileira de Bioquímica e Biologia Molecular (SBBq). 2006. (Participação em eventos/Congresso).

2 Reunião Anual da Sociedade Brasileira de Bioquímica e Biologia Molecular (SBBq). 2005. (Participação em eventos/Congresso).

3 III Jornada de Terapia Nutricional do HCPA. 2004. (Participação em eventos/Outra).

4 The 2nd Brazilian Symposium on Medicinal Chemistry: Current Trends in Drug Discovery and Development. 2004. (Participação em eventos/Simpósio).

5 X Escola de Verão em Química Farmacêutica e Química Medicinal. 2004. (Participação em eventos/Outra).

6 2ª Semana de Antibióticos do Hospital de Clínicas de Porto Alegre. 2003. (Participação em eventos/Seminário).

7 26ª Reunião Anual da Sociedade Brasileira de Química. 2003. (Participação em eventos/Congresso).

8 Encontro de Farmácia Hospitalar AFARGS/SBRAFH-RS. 2003. (Participação em eventos/Encontro).

9 6º Congresso de Produtos Farmacêuticos e Cosméticos do Rio Grande do Sul. 2002. (Participação em eventos/Congresso).

Participação em outros eventos

Cursos de extensão

2004 - 2004 História da Descoberta de Fármacos. (Carga horária: 12h) Universidade Federal do Rio de Janeiro, UFRJ, Rio de Janeiro, Brasil.

2004 - 2004 Relações Quantitativas 3D Estrutura-Atividade. (Carga horária: 12h) Universidade Federal do Rio de Janeiro, UFRJ, Rio de Janeiro, Brasil.

2004 - 2004 Síntese em Fase Sólida Aplicada ao Desenho de Fármacos. (Carga horária: 12h) Universidade Federal do Rio de Janeiro, UFRJ, Rio de Janeiro, Brasil.

2004 - 2004 Gestão em Projetos de Pesquisa, Desenv. e Inovação. (Carga horária: 12h) Universidade Federal do Rio de Janeiro, UFRJ, Rio de Janeiro, Brasil.

2004 - 2004 Extensão universitária em Bioinformática e Modelagem Molecular. (Carga horária: 40h) Associação Brasileira de Química, ABQ-SUL, Rio Grande do Sul, Brasil.

2004 - 2004 Extensão universitária em Escola de Inverno Em Química Orgânica. (Carga horária: 30h) Universidade Federal do Rio Grande do Sul, UFRGS, Rio Grande do Sul, Brasil.

2004 - 2004 Extensão universitária em Delineamentos Experimentais em Biologia. Carga horária: 24h) Pontifícia Universidade Católica do Rio Grande do Sul, PUCRS, Rio Grande do Sul, Brasil.

2003 - 2003 Atuação do Farmacêutico na Pesquisa Clínica. (Carga horária: 4h)
Associação dos Farmacêuticos do Rs, AFARGS, Rio Grande do Sul, Brasil.

2003 - 2003 Química Medicinal. (Carga horária: 6h)
Sociedade Brasileira de Química, SBQ, São Paulo, Brasil.

2002 - 2002 Estabilidade de Produtos Farmacêuticos/Cosméticos. (Carga horária: 7h)
Associação dos Farmacêuticos do Rs, AFARGS, Rio Grande do Sul, Brasil.

2002 - 2002 Radicais Livres e Nutracêuticos. (Carga horária: 7h)
Associação dos Farmacêuticos do Rs, AFARGS, Rio Grande do Sul, Brasil.

2001 - 2001 As Bases da Oncologia. (Carga horária: 8h)
Hospital de Clínicas de Porto Alegre, HCPA, Rio Grande do Sul, Brasil.

Aprovação em Língua Estrangeira

1 Proficiência em Lingua Inglesa Test of English as a Foreign Language (TOEFL) - Score 250. 2002.

2 Proficiência em leitura de lingua inglesa – UFRGS. 2005

3 Proficiência em leitura de lingua francesa – UFRGS. 2006