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LUCIANA DIAS GHIRALDI-LOPES

Perfil proteico de *Mycobacterium tuberculosis* após a exposição a Etambutol e Eupomatenóide-5

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Tese de Doutorado apresentado ao Programa de Pós-Graduação em Ciências da Saúde da Universidade Estadual de Maringá como requisito para a obtenção do título de Doutor em Ciências da Saúde.

Orientadora: Prof[a]. Dr[a]. Rosilene Fressatti Cardoso.

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2017

FOLHA DE APROVAÇÃO

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Universidade Estadual de Maringá (Presidente)

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Dra. Jane Marta Graton Mikcha
Universidade Estadual de Maringá

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DEDICATÓRIA

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“Felizes aqueles que na escuridão da noite acreditaram no resplendor da luz”.

(Trecho do livro O silêncio de Maria- Inácio Larrañaga)

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Perfil proteico de *Mycobacterium tuberculosis* após a exposição a etambutol e eupomatenóide-5

RESUMO

A tuberculose (TB) é uma doença infectocontagiosa crônica, causada principalmente pelo bacilo *Mycobacterium tuberculosis (M. tb)*. Nos últimos anos surgiram poucos compostos efetivos em *M. tb* o que motivou a pesquisa com fármacos já utilizados no tratamento e também a busca por novas opções terapêuticas. O Etambutol (EMB) é um fármaco utilizado no esquema de primeira escolha da TB e atua inibindo a enzima arabinosil transferase, impedindo a biossíntese de arabinogalactana. A substância eupomatenóide-5 (EUP-5), é uma neoligana isolada de plantas do gênero *Piper* com atividade antimicrobiana, inclusive atividade anti-*M. tb*. O objetivo deste estudo foi avaliar o perfil proteômico de *M. tb* após a exposição a EMB e EUP-5 utilizando eletroforese bidimensional e espectrometria de massas e contruir redes de interação proteica utilizando o banco de dados STRING-10. Essa avaliação foi dividida em dois trabalhos. O primeiro, consiste em um manuscrito 'Abordagem proteômica traz novos conhecimentos sobre os alvos de Etambutol em *Mycobacterium tuberculosis*' em que a cepa de referência *M. tb* $H_{37}Rv$ foi exposta a concentração subinibitória de EMB por 24 h e 48 h. As principais alterações proteicas ocorreram em proteínas de metabolismo intermediário e respiração indicando que essas proteínas também podem ser exploradas como alvos. O segundo trabalho é um artigo científico nomeado 'Perfil protéico de *Mycobacterium tuberculosis* após indução por eupomatenóide-5 revela potenciais alvos de fármacos' em que realizamos a análise proteômica e morfológica de *M. tuberculosis* $H_{37}Rv$ após 12 h, 24 h e 48 h de exposição a concentração subinibitória de EUP-5. Nessa condição, EUP-5 foi capaz de promover o arredondamento e rugosidades nos bacilos. As principais alterações proteicas ocorreram após 24 h em proteínas relacionadas ao metabolismo intermediário, metabolismo lipídico, virulência e detoxificação, proteínas de informação e processos celulares, sendo algumas micobactéria-específicas. A construção de redes de interações de proteínas pelo banco de dados STRING-10 é uma ferramenta eficiente e permite uma melhor visualização do impacto causado por EMB e EUP-5 em *M. tb* e demonstrou a interação de múltiplas proteínas que foram responsáveis por distúrbios no metabolismo e consequente morte do bacilo.

Palavras-chave: Tuberculose. Proteômica. Etambutol. Eupomatenóide-5. Espectrometria de massas.

Proteome profile of *Mycobacterium tuberculosis* after ethambutol and eupomatenoid-5 induction

ABSTRACT

Tuberculosis (TB) is a chronic infectious disease caused by the bacillus *Mycobacterium tuberculosis* (*M. tb*). In recent years, just a low number of new effective compounds against *M. tb* had emerged, which motivates researches with drugs already used in the treatment of TB and also the search for new therapeutic options. Ethambutol (EMB) is a drug used in the first scheme of TB treatment and works inhibiting arabinosyl transferases which prevents the biosynthesis of arabinogalactan. The substance eupomatenoid-5 (EUP-5), is a neoligan isolated extracted from genus *Piper* with antimicrobial activity including anti-*M. tb* activity. The aim of this study was to evaluate the proteomic profile of *M. tb* after EMB and EUP-5 induction using two-dimensional electrophoresis coupled to mass spectrometry and to construct protein interaction networks using the STRING-10 database. This evaluation was divided into two papers. In the first manuscript 'Proteomic approach brings new knowledge about the targets of Ethambutol in *Mycobacterium tuberculosis*' the reference strain *M. tb* H_{37}Rv was induced to sub inhibitory concentration of EMB for 24 h and 48 h. The major protein changes occurred in proteins of intermediate metabolism and respiration indicating that these proteins can also be explored as drug targets. The second article was named 'Protein profile of *Mycobacterium tuberculosis* after induction by eupomatenoid-5 reveals potential drug targets'. A proteomic and morphological analysis of *M. tb* H_{37}Rv was performed after 12 h, 24 h and 48 h of EUP-5 induction to the sub inhibitory concentration. EUP-5 was able to promote the rounding and wrinkled appearance of bacilli and the main protein changes occurred after 24 h in proteins related to intermediate metabolism, lipid metabolism, virulence and detoxification, information proteins and cellular processes with some mycobacteria-specific proteins. The construction of networks of protein interactions by the STRING-10 database is an efficient tool which allows a better visualization of the impact caused by EMB and EUP-5 in *M. tb* and demonstrated the interaction of multiple proteins that were responsible for disorders in the metabolism and consequent death of the bacilli.

Key words: Tuberculosis. Proteomics. Ethambutol. Eupomatenoid-5. Mass spectrometry.

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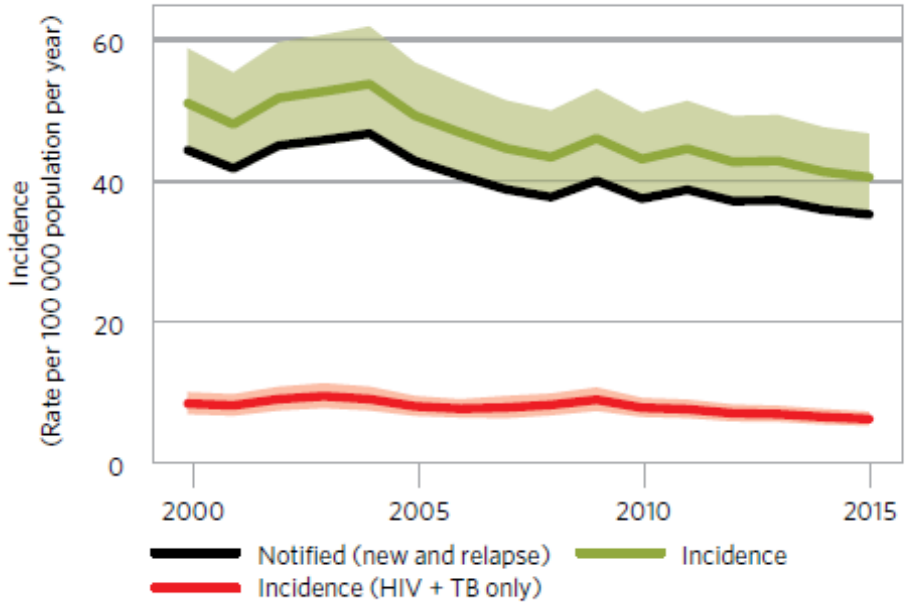
CAPÍTULO I

TUBERCULOSE

A tuberculose (TB) é uma doença infectocontagiosa crônica, causada principalmente pelo bacilo *Mycobacterium tuberculosis (M. tb)*. É considerada um importante problema de saúde pública devido à alta incidência, prevalência e mortalidade. Estima-se que em 2015 houve 10,4 milhões de pessoas infectadas e 1,4 milhões de mortes, sendo a TB uma das principais causas de morte por doenças infecciosas no mundo, situando-se acima do número de mortes por HIV/ SIDA (WHO, 2016).

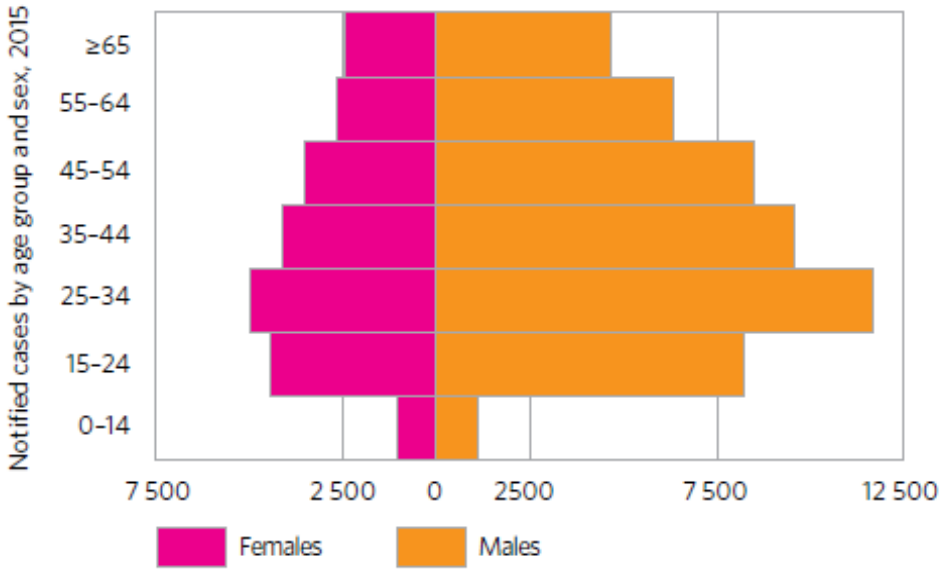
O Brasil faz parte dos 22 países priorizados pela Organização Mundial da Saúde (OMS) que concentram 80 % dos casos de tuberculose e apesar da incidência ser decrescente ao longo dos anos o país ainda apresentou incidência de 84 mil casos e taxa de 41/100.000 habitantes em 2015 (Figura 1). Os casos são mais comuns em indivíduos do sexo masculino na faixa etária correspondente a plena capacidade produtiva, o que acarreta enorme prejuízo econômico ao país (Figura 2) (WHO, 2016).

FIGURA 1- INCIDÊNCIA DA TUBERCULOSE NO BRASIL. TAXA POR 100.000 HABITANTES/ANO



Fonte: Organização Mundial da Saúde (WHO, 2016)

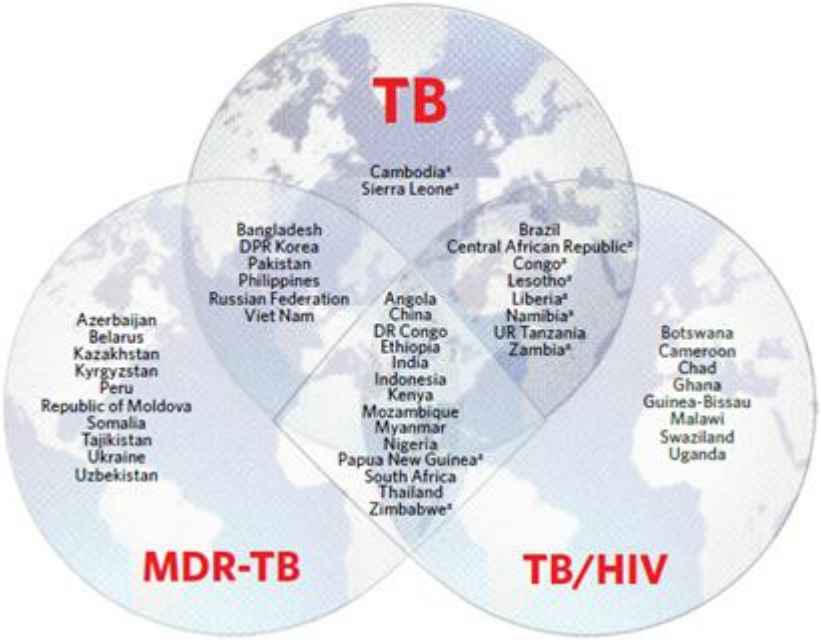
FIGURA 2- CASOS NOTIFICADOS POR GÊNERO E IDADE EM 2015, NO BRASIL



Fonte: Organização Mundial da Saúde (WHO, 2016)

De acordo com a classificação da OMS em grupos de países conforme a incidência de TB, incidência de TB-MDR e incidência da coinfecção TB-HIV, o Brasil faz parte dos 20 países com as maiores taxas de incidência de TB e de coinfecção TB-HIV, não classificado apenas no grupo de países que apresentam altas taxas de resistência ao tratamento (Figura 3).

FIGURA 3- GRUPOS DE PAÍSES COM ALTA INCIDÊNCIA DE TUBERCULOSE NO MUNDO E AS SUAS ÁREAS DE SOBREPOSIÇÕES



Fonte: Organização Mundial da Saúde (WHO, 2016)

A TB é transmitida via aérea e geralmente afeta os pulmões (TB pulmonar) mas outros sítios/órgãos também podem ser afetados (TB extrapulmonar). A TB pulmonar é a forma clínica mais frequente e a mais relevante para a saúde pública, pois o bacilo quando expelido pela tosse por gotículas de saliva de indivíduos bacilíferos, perpetuam a cadeia de transmissão via aérea (BRASIL. MINISTÉRIO DA SAÚDE, 2011; WHO, 2016). Em geral, apenas 5 a 15% dos indivíduos infectados irão desenvolver a TB ao longo da vida, no entanto, indivíduos coinfectados com HIV e *M. tb* possuem 26 vezes mais chances de desenvolver a doença. No Brasil, a cada aumento de um caso de indivíduos com síndrome da imunodeficiência adquirida (AIDS) por 100.000 habitantes, houve um aumento de 1,5% na incidência da TB, o que faz do HIV a condição clínica mais intimamente relacionada a TB (BRASIL. MINISTÉRIO DA SAÚDE, 2016; WHO, 2016).

Apesar de o Brasil ter atingido a meta proposta de diminuição da incidência da TB em 2015 (BRASIL. MINISTÉRIO DA SAÚDE., 2016), as medidas de controle não são suficientes para interromper a transmissão. Fatores como aglomerados humanos, medidas de controle ineficazes, fatores comportamentais e socioeconômicos do paciente como etilismo, tabagismo, desnutrição, diabetes mellitus, baixa resistência imunológica e abandono do tratamento são situações existentes em todo o território brasileiro e são determinantes no sucesso do controle da TB (LACERDA et al., 2014; WHO, 2016).

O tratamento recomendado para a TB, em casos novos, é bastante efetivo com alta taxa de cura, desde que utilizada as doses corretas e por tempo suficiente, apesar dos efeitos colaterais (HASSAN et al., 2015; HOAGLAND et al., 2016; WHO, 2016). Em geral, após duas a três semanas de tratamento, a maior parte dos pacientes deixam de ser bacilíferos, diminuindo a possibilidade de transmissão da doença (BRASIL. MINISTÉRIO DA SAÚDE, 2011). O primeiro antimicrobiano efetivo no tratamento da TB foi a estreptomicina (SM), introduzida em 1944. A isoniazida (INH) foi introduzida em 1952 e até hoje é considerada o fármaco mais eficiente na eliminação bacilar. Em 1965, a rifampicina (RIF) e em 1968 o etambutol (EMB) passaram a integrar a terapia e a pirazinamida (PZA) passou a compor a poliquimioterapia da doença em 1970 (DE SOUZA; VASCONCELOS, 2005).

Atualmente, o esquema de primeira escolha para o tratamento da TB consiste no uso combinado de INH, RIF, PZA e EMB por dois meses, seguido de quatro meses com INH e RIF em um único comprimido (dose fixa combinada) (Figura 4). INH e RIF são os medicamentos de maior poder bactericida, atuando em todas as populações bacilares sensíveis (intracavitárias, granulomas, intracelulares). PZA é ativo somente em meio ácido (bacilos no interior de granulomas) e EMB é um fármaco bacteriostático que tem atividade em bacilos em multiplicação, utilizado para prevenir

a emergência de bacilos resistentes. Este elevado tempo de administração dos medicamentos anti-TB, evita a seleção de bacilos resistentes, uma vez que bacilos naturalmente resistentes a um medicamento podem ser sensíveis a outro e a tomada de um único comprimido combinado favorece a adesão do paciente ao tratamento (BRASIL. MINISTÉRIO DA SAÚDE, 2011).

FIGURA 4- ESQUEMA BÁSICO PARA O TRATAMENTO DA TB EM ADULTOS

Regime	Fármacos	Faixa de peso	Unidade/dose	Meses
2 RHZE Fase Intensiva	RHZE 150/75/400/275 comprimido em dose fixa combinada	20kg a 35kg	2 comprimidos	2
		36kg a 50kg	3 comprimidos	
		> 50kg	4 comprimidos	
4 RH Fase de manutenção	RH Comprimido ou cápsula de 300/200 ou de 150/100 ou comprimidos de 150/75*	20 a 35kg	1 comprimido ou cápsula de 300/200mg ou 2 comprimidos de 150/75*	4
		36kg a 50kg	1 comprimido ou cápsula de 300/200mg + 1 comprimido ou cápsula de 150/100mg ou 3 comprimidos de 150/75*	
		> 50kg	2 comprimidos ou cápsulas de 300/200mg ou 4 comprimidos de 150/75*	

Fonte: Brasil. Ministério da Saúde, 2011

A falha no tratamento pode levar a emergência de isolados clínicos resistentes. Em 2015, foram reportados 480.000 casos de resistência a RIF (RR-TB) e resistência a múltiplos fármacos (MDR-TB). O surgimento de isolados resistentes a RIF, INH acrescida de resistência a uma fluoroquinolona e a um fármaco injetável de segunda linha (amicacina, canamicina ou capreomicina) (XDR-TB) é uma realidade em todo o mundo e o esquema de tratamento deve ser composto por, pelo menos, quatro fármacos com atividades efetivas que, preferencialmente não tenham sido utilizados anteriormente. Fármacos pioneiros utilizados no tratamento a TB, tais como a SM e outros como etionamida (Et), ofloxacina, terizidona e clofazimina, também têm ação contra *M. tb*, sendo empregados, entretanto, apenas em situações especiais, como na TB resistente (BISAGLIA et al., 2003).

A capacidade de desenvolver resistência e de adotar um estado latente tem mantido a TB no topo das mortes por doenças infecciosas (CHETTY *et al.*, 2016). Existe um esforço global para o desenvolvimento de novos fármacos que se sejam efetivos em bacilos ativos, resistentes e latentes.

A introdução de fármacos como delamanida, bedaquilina (atualmente em ensaios clínicos fase IV), rifamicinas, oxazolidinonas, nitroimidazopirano, SQ109 e ATB 107, considerados apenas candidatos ainda, além da reutilização de outros demonstra esse crescente interesse na busca por medicamentos eficientes e menor tempo de tratamento (JIA et al., 2005; SHEN et al., 2010; CHETTY et al., 2016).

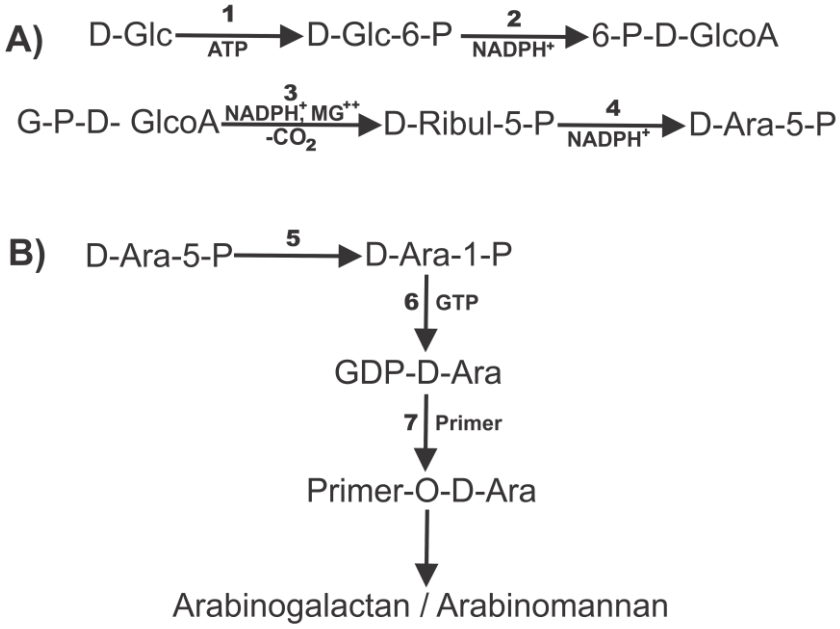
ETAMBUTOL

EMB foi implantado na terapia anti-TB em 1966, mas somente em 2009 passou a fazer parte do esquema primário de tratamento na fase intensiva (dois primeiros meses) devido ao aumento da resistência a INH (de 4,4 % para 6,0 %) (BRASIL. MINISTÉRIO DA SAÚDE, 2011). É um fármaco ativo contra bacilos em multiplicação por afetar a integridade da parede celular de *M. tb*. EMB atua inibindo a enzima arabinosil transferase codificada pelo operon *embCAB* impedindo, dessa forma, a biossíntese de arabinogalactana (TAKAYAMA; KILBURN, 1989; CHETTY et al., 2016). Esse bloqueio da síntese do principal polissacarídeo da parede celular leva a perda da integridade e aumento da permeabilidade celular (XU et al., 2015; CHETTY et al., 2016; WHO, 2016).

As primeiras investigações da ação de EMB propuseram que este medicamento interferia no papel de poliaminas e cátions divalentes no metabolismo de RNA (FORBES; KUCK; PEETS, 1962, 1965), mas estudos posteriores em *M. smegmatis* não confirmaram a existência de um alvo catiônico específico para EMB (BEGGS; ANDREWS, 1973). Após, KILBURN & GREENBERG, (1977) mostraram que EMB ocasionava uma redução da coesão celular transformando grandes aglomerados de células de *M. smegmatis* em clusters menores, possivelmente por uma redução de lipídios na parede celular. Em seguida TAKAYAMA *et al.* (1979), mostraram que o EMB inibia simultaneamente a transferência de ácido micólico para a parede celular e estimulava a síntese de dimicolato de trealose em *M. smegmatis*. Estudos posteriores (KILBURN, JAMES O.; TAKAYAMA, 1981) mostraram que EMB provocava uma acumulação ainda mais precoce de monomicolato de trealose, dimicolato de trealose e ácido micoico livre e que esse desequilíbrio na síntese lipídica era letal ao *M. smegmatis.* Por último, TAKAYAMA, K.; KILBURN (1989), demonstraram que a incorporação de glicose marcada com ^{14}C na arabinana de parede celular foi imediatamente inibida após a adição de EMB e que esse efeito ocorria primeiramente na arabinana de arabinogalactana (AG) e posteriormente na arabinana de lipoarabinomanana (LAM), sugerindo,

portanto, que EMB não atuava nos estágios iniciais da síntese de arabinana, mas sim na polimerização final e a enzima arabinosil transferase foi implicada como alvo (Figura 6).

FIGURA 5- PROVÁVEL MECANISMO DE AÇÃO DE ETAMBUTOL EM *Mycobacterium smegmatis*



Abreviações: Glc: glicose; Ara: arabinose; GlcoA: ácido glicônico; Ribul: ribulose; Rib: ribose; P: fosfato.
Fonte: Takayama, K.; Kilburn, 1989.

EUPOMATENÓIDE-5

As espécies vegetais são uma fonte rica de muitos compostos biologicamente ativos. Substâncias extraídas de plantas tem sido utilizadas em medicamentos tradicionais para o tratamento de várias doenças em todo o mundo. Aproximadamente 60% população confia em plantas medicinais para seus cuidados primários e a utilização de extratos brutos ou substancias isoladas de plantas tem sido usados para esses fins (GAUTAM; SAKLANI; JACHAK, 2007). A ação anti-TB dos produtos naturais é uma área de investigação com vasto potencial, principalmente nos países com grande biodiversidade, como é o caso do Brasil e até agora poucas plantas foram testadas em micobactérias (GUPTA et al., 2010).

O gênero *Piper,* pertencente à família *Piperaceae,* é constituído por aproximadamente 700 espécies e é distribuído em regiões tropicais e subtropicais. Esse gênero vem despertando interesse da comunidade científica por seus resultados químicos e biológicos promissores de diferentes

classes de compostos bioativos, tais como: alcalóides, amidas, chalconas, diidrochalconas, flavonas, flavononas, terpenos, esteróides, kavapironas, fenilpropanóides, lignanas e neolignanas (PARMAR et al., 1997). Estudos com extratos de diferentes espécies de *Piper* têm mostrado uma grande diversidade de metabólitos com marcantes atividades biológicas. A grande importância deste gênero está baseada não somente na utilização de suas inúmeras espécies como plantas medicinais, mas também como fonte de matéria-prima para a indústria farmacêutica, cosmética e de perfumes (DA SILVA, 2006).

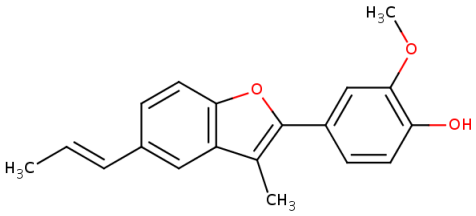
A espécie *Piper solmsianum* C. DC. var *solmsianum* é conhecida popularmente como caapeba ou pariparoba, é um arbusto de 1 a 3 metros de altura e as folhas apresentam formato oval. Ela floresce nos meses de setembro, outubro, novembro e dezembro. A espécie tem distribuição geográfica no Sudeste e Sul do Brasil, comum em matas com luz difusa, planícies alagadiças ou brejos (DA SILVA, 2006).

FIGURA 6- FOLHAS DE *Piper solmsianum* C.DC. variedade *solmsianum*

Fonte: Da Silva, 2006

A substância eupomatenóide-5 (EUP-5) (Figura 8), é uma neoligana isolada de plantas do gênero *Piper* com atividade biológica, inclusive antimicrobiana e inseticida (CHAURET et al., 1996; PESSINI et al., 2003; KOROISHI et al., 2008). As neolignanas são lignóides cujo esqueleto é formado exclusivamente pelo grupo fenilpropânico (C6-C3)n, sendo n restrito a poucas unidades. São metabólitos secundários de plantas produzidos pela dimerização oxidativa de duas unidades fenilpropanóides, e se diferenciam das lignanas principalmente por não apresentarem o carbono gama (C-γ) do resíduo n-propilbenzênico oxigenado (BARBOSA FILHO, 2001).

FIGURA 7- ESTRUTURA DA NEOLIGNANA EUPOMATENÓIDE-5.



A exata ação de EUP-5 não está completamente elucidada. Estudos com protozoários demonstraram que EUP-5 está relacionado à disfunção mitocondrial e dano oxidativo (LUIZE et al., 2006; PELIZZARO-ROCHA et al., 2011). LAZARIN-BIDÓIA et al., 2013 demonstraram que EUP-5 promove peroxidação lipídica e fragmentação do DNA em *T. cruzi* e VENDRAMETTO et al., 2010 observaram atividade *in vitro* do EUP-5 em formas promastigotas, amastigotas axênicas e amastigotas intracelulares de *Leishmania amazonensis*.

A atividade antifúngica do EUP-5 também foi demonstrada em fungos dermatófitos e outros fungos patogênicos com atividade semelhante a antifúngicos utilizados no tratamento convencional (DE CAMPOS et al., 2005; KOROISHI et al., 2008).

EUP-5 também demonstrou atividade antibacteriana em bactérias Gram-positivas e Gram-negativas, assim como em *Staphylococcus aureus* MRSA (methicillin-resistant *S. aureus*) (PESSINI et al., 2003; MARÇAL et al., 2010), mas não possui atividade em bactérias da microbiota intestinal. EUP-5 também apresentou ótima atividade antiproliferativa *in vitro* e parece atuar por diferentes mecanismos relacionados ao dano oxidativo em celulas tumorais de rim, ovário, próstata e mama (LONGATO et al., 2011, 2015).

Recentemente, nosso grupo de pesquisa demonstrou que EUP-5 exibe atividade anti- *M. tb* e apresenta sinergismo com RIF e EMB (SCODRO et al., 2013; LOPES et al., 2014), o que faz dessa substância um promissor candidato a fármaco anti-TB.

PROTEOMA

Uma característica vital dos microrganismos é a sua capacidade de adaptação às alterações do ambiente em que se encontra, o que envolve a regulação da expressão gênica em resposta aos diferentes sinais ambientais (KATO-MAEDA; GAO; SMALL, 2001). A regulação gênica dos fenômenos biológicos pode ser demonstrada pelo transcriptoma e pelo perfil proteômico, uma vez que, a regulação da atividade gênica controla a sub ou superexpressão de um produto celular em um

momento específico (YAMAMOTO et al., 2001). Dessa forma, a análise proteômica é definida pela composição de todas as proteínas expressas pelo genoma de um organismo em um determinado momento (WESTERMEIER; MAROUGA, 2005).

As proteínas são responsáveis pela maioria das atividades em uma célula viva. Todo o metabolismo celular é sinalizado por proteínas o que permite a identificação de alvos de interesse. Estudos moleculares estão em evidência atualmente e ferramentas que permitem essa análise como transcriptoma, proteômica e metabolômica são úteis para identificar e mensurar respostas a um ambiente em constante modificações (BANTSCHEFF et al., 2007). Dessa forma, a proteômica se apresenta como uma ferramenta poderosa na identificação de biomarcadores de doenças, caracterização de processos fisiológicos normais e patológicos, análise de interação entre as proteínas e também no desenvolvimento farmacêutico e toxicológico (WESTERMEIER; MAROUGA, 2005; UNWIN; WHETTON, 2007)

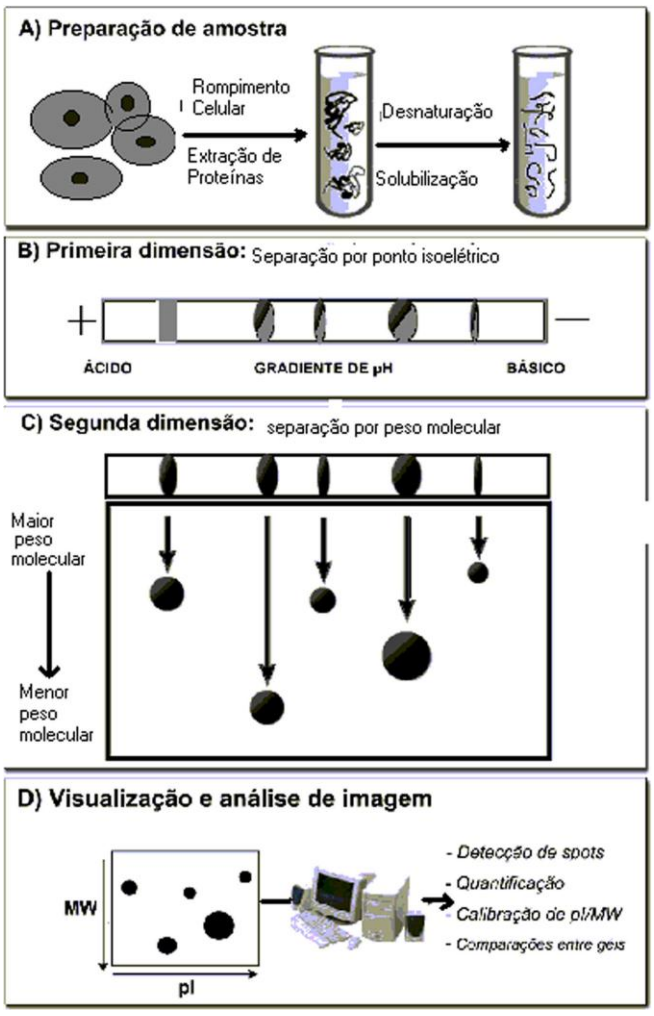
Técnicas que avaliam mRNAs, são altamente sensíveis e passíveis de automação, porém não refletem necessariamente as alterações proteicas, que são de extrema importância no estudo metabólico e bioquímico de células ou micro-organismos (LEROY; RAOULT, 2010). Os estudos proteômicos fornecem informações valiosas sobre alterações na síntese proteica, taxas de degradação, modificações pós-transformacionais, interações proteicas e localização subcelular de proteínas, o que melhora nossa compreensão e conhecimento de fenômenos fisiológicos para uma condição específica. Estudos proteômicos comparativos permitem uma compreensão completa dos processos biológicos que afetam a expressão proteica pela comparação quantitativa de processos que as proteínas estão envolvidas (HAN; LEE; LEE, 2011). Ao contrário de estudos bioquímicos clássicos que se concentram em apenas uma ou algumas proteínas, a proteômica atual tem sido considerada como uma abordagem mais abrangente e sistemática para a investigação de sistemas biológicos (WALGREN; THOMPSON, 2004).

As proteínas são alvo para a a maioria dos fármacos, estudos do perfil protéico de microganismos submetido a concentrações subinibitórias de determinado fármaco, pode ajudar a determinar o potencial quimioterápico e o desenvolvimento de novos medicamentos assim como colaborar na compreensão de mecanismos de resistência e ação em bacilos dormentes (SHARMA et al., 2010).

A eletroforese bidimensional em gel de poliacrilamida (2-D) foi introduzida na década de 70, mas nos últimos 10 anos que ocorreram os grandes avanços nos métodos que possibilitam identificar proteínas separadas por 2-D. Nesta metodologia, as proteínas são separadas em duas etapas consecutivas. Na primeira, denominada focalização isoelétrica (IEF), as moléculas migram

na horizontal em gel de poliacrilamida com gradiente de pH imobilizado até atingirem o pH no qual sua carga seja igual a zero (ponto isoelétrico ou pI). Na segunda etapa, as proteínas são submetidas a uma eletroforese com direção perpendicular a IEF, em gel de poliacrilamida contendo dodecilsulfato de sódio (SDS-PAGE), e então separadas de acordo com sua massa molecular (BARBOSA et al., 2012). A detecção proteica geralmente é realizada por técnicas de coloração utilizando Coomassie Blue ou prata, e em seguida digitalizados e as imagens analisadas por um software. O material de fundo (background) é subtraído, os spots comparados e os dados normalizados e analisados estatisticamente para quantificação de volumes proteicos ou intensidade. Embora a 2-D seja uma técnica trabalhosa, permite a separação de vários milhares de proteínas solúveis com resolução ainda sem igual por outros métodos de separação de proteínas (HUGHES et al., 2006; BARBOSA et al., 2012).

FIGURA 8- ESQUEMA DE PROCEDIMENTOS DA ELETROFORESE BIDIMENSIONAL



Fonte:CIERO; BELLATO, 2002- adaptado.

A identificação das proteínas é realizada por espectrometria de massas. A técnica consiste basicamente na ionização de um composto e na avaliação da razão massa/carga (m/z) dos íons. O equipamento utilizado compreende uma fonte de ionização, um ou mais analisadores de massas e um detector. O primeiro componente é utilizado para gerar íons peptídicos ou proteicos, geralmente transferindo prótons (H^+) para as moléculas sem alterar sua estrutura química. O íon é acelerado por campo elétrico e separado por m/z no analisador de massas, ou então é selecionado de acordo com uma m/z previamente determinada e fragmentado em um processo denominado *em tandem* (MS/MS). Finalmente, os íons passam pelo detector, que está conectado a um computador com programas para análise de dados. Os dois métodos principais de ionização utilizados em proteômica são o MALDI (*Matrix-Assisted Laser Desorption/Ionization*) e o ESI (*Electrospray Ionization*) (BARBOSA et al., 2012). A formação de íons por MALDI ocorre através da co-cristalização de um excesso de matriz com o analito em uma placa de metal. A matriz, em geral pequenas moléculas orgânicas, é capaz de absorver o comprimento de onda emitido pelo laser. O laser atinge os cristais de matriz e analito formados na placa, levando a absorção dessa energia pela matriz e a subsequente dessorção e ionização dos analitos presentes na amostra. Já na ionização por ESI as amostras são dissolvidas em um tampão ou solvente que são bombeadas a um fluxo de microlitros por minuto através de uma agulha hipodérmica que está em uma alta voltagem para dispersar eletrostaticamente, ou eletrospray, gotas de tamanho micrométricos, que são rapidamente evaporadas e transmitem a sua carga para o analito (EMIDIO et al., 2015).

Independentemente do método de ionização utilizado, a sensibilidade e acurácia do espectrômetro de massas está diretamente relacionada com o analisador de massa que irão realizar a separação dos íons gerados através da sua razão m/z (EMIDIO et al., 2015). Os tipos mais comuns de analisadores são o TOF (*Time Of Flight*), o quadrupolo e os analisadores de aprisionamento de íons **(***ion trap)* (BARBOSA et al., 2012).

Nos analisadores TOF, a separação de íons é baseada na velocidade dentro do tubo de vôo. Todos os íons são formados, na fonte de ionização, com a mesma carga e apresentam a mesma energia potencial elétrica quando expostos ao campo, essa energia será então convertida em energia cinética em função da massa da molécula. Portanto, íons com menor valor de m/z alcançarão maior velocidade que íons de maior m/z. Após os íons serem acelerados, eles viajam por uma distância fixa, até chegarem ao detector (EMIDIO et al., 2015). Uma das limitações do sistema MALDI-TOF é a dificuldade de detecção de proteínas de baixo peso molecular que consequentemente geram poucos peptídeos. Para melhorar o desempenho, os analisadores TOF podem ser combinados com analisadores quadrupolos (Qs), caracterizado por um conjunto de quatro hastes em que um campo

elétrico oscilante é aplicado e apenas certos valores de m/z conseguem alcançar o detector, portanto somente íons de uma determinada razão m/z seguirá a trajetória ao detector enquanto os demais são desviados (BARBOSA et al., 2012; EMIDIO et al., 2015). Os analisadores do tipo *ion trap* são relacionados ao quadrupolo. Enquanto o quadrupolo apresenta campos elétricos em duas dimensões (eixos x e y) e os íons movem-se perpendiculares ao campo (eixo z), o analisador *ion trap* filtram e aprisionam os íons de interesse em um campo elétrico tridimensional, e estes são gradualmente liberados em ordem de m/z crescente (BARBOSA et al., 2012).

Apesar do impacto fenomenal da espectrometria de massa e das técnicas de separação de peptídeos na proteômica, a identificação e quantificação de todas as proteínas em um sistema biológico ainda é um desafio técnico (BANTSCHEFF et al., 2007).

Estudos proteômicos em *M. tb* vem sendo realizados há anos com diversos objetivos, como expressão proteica em diferentes condições de incubação (STARCK et al., 2004; ALBRETHSEN et al., 2013), diferenças na expressão proteica entre isolados de *M. tb* resistentes e sensíveis a fármacos (JIANG et al., 2006; KUMAR et al., 2013; LATA et al., 2015; SHARMA et al., 2015; ZHAO et al., 2015), expressão proteica após a indução por medicamentos já utilizados no tratamento da TB (JIA et al., 2005; JIANG et al., 2011; CAMPANERUT-SÁ et al., 2016) e também de candidatos a fármacos (JIA et al., 2005; SHEN et al., 2010). Dessa forma, a proteômica se consolida como uma valiosa ferramenta para a compreensão da adaptação do *M. tb* a diversas situações como as mencionadas acima.

JUSTIFICATIVA

Apesar dos avanços da medicina nos últimos quinze anos, a tuberculose continua sendo um problema de saúde pública com alta mortalidade em todo o mundo. Os fármacos utilizados para o tratamento da TB foram introduzidos há cerca de 50 anos e apesar de promoverem a cura em grande parte dos casos, apresentam desvantagens como terapia prolongada e a elevada toxicidade, favorecendo o abandono por parte dos pacientes.

Nos últimos anos, surgiram poucos compostos efetivos contra *M. tb*, o que motivou o interesse pela comunidade científica em compreender melhor mecanismos de ação de fármacos já utilizados no tratamento da TB, bem como a busca por novas opções terapêuticas.

A proteômica é uma técnica que permite a compreensão do genoma funcional dos microrganismos em determinadas condições. Como as proteínas são os alvos para a maioria dos fármacos, pela análise da expressão proteica é possível compreender e investigar a ação de

fármacos anti-TB, mecanismos de resistência destes fármacos, assim como, buscar novos alvos terapêuticos. A técnica fornece uma avaliação mais precisa das alterações induzidas por fármacos, com achados não previstos na análise genômica.

Dessa forma, estudos proteômicos podem contribuir para uma melhor compreensão das alterações causadas por EMB e por EUP-5 em *M. tb*.

OBJETIVOS

GERAL

Realizar análise do perfil proteico da cepa de referência *Mycobacterium tuberculosis* H_{37}Rv após a indução por EMB e EUP-5.

ESPECÍFICOS

Realizar análise diferencial do perfil proteico em *M. tuberculosis* H_{37}Rv antes e após a indução por EMB e EUP-5.

Construir a rede de interação das proteínas identificadas após a indução por EBM e EUP-5 usando STRING-10 database.

Realizar a microscopia eletrônica de varredura de *M. tuberculosis* H_{37}Rv após diferentes tempos de indução por EUP-5.

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CAPITULO II

Manuscrito 1: “PROTEOMICS APPROACHES BRING NEW INSIGHTS ON ETHAMBUTOL TARGETS IN *Mycobacterium tuberculosis.*”

Proteomics approaches bring new insights on Ethambutol targets in *Mycobacterium tuberculosis*

Ghiraldi-Lopes, LDG[a,b*]; Campanerut-Sá, PAZ[b]; Evaristo, GPC[c]; Meneguello, JE[d]; Fiorini, A[b]; Baldin, VP[d]; Souza, EM[e]; Scodro, RBL[a,b]; Siqueira, VLD[b,d]; Cardoso, RF[a, b, d]

[a]Programa de Pós Graduação em Ciências da Saúde, Universidade Estadual de Maringá, Maringá, Paraná, Brasil
[b]Departamento de Análises Clínicas e Biomedicina, Universidade Estadual de Maringá, Maringá, Paraná, Brasil
[c]Laboratório Brasileiro de Controle de Dopagem (LBCD)/ Laborat;orio de Suporte de Desenvolvimento Tecnológico (LADETEC)/ Instituto de química da Universidade Federal do Rio de Janeiro (IQ/UFRJ)- Rio de Janeiro- RJ
[d]Programa de Pós Graduação em Biosciências aplicadas a Farmácia, Universidade Estadual de Maringá, Maringá, Paraná, Brasil
[e]Departamento de Ciências Biológicas, Universidade Federal do Paraná, Curitiba, Paraná, Brasil

*Corresponding author Luciana Dias Ghiraldi Lopes. Postgraduate Program in Health Sciences, State University of Maringá- PR-Brazil

Av. Colombo 5790, Bloco T-20, Sala 303, CEP 87020-900, Maringá, PR, Brasil.

Phone number:+55 44 3011-5394, Cell phone: +55 44 99825-1064.

e-mail: ldghiraldi@gmail.com

Email authors:

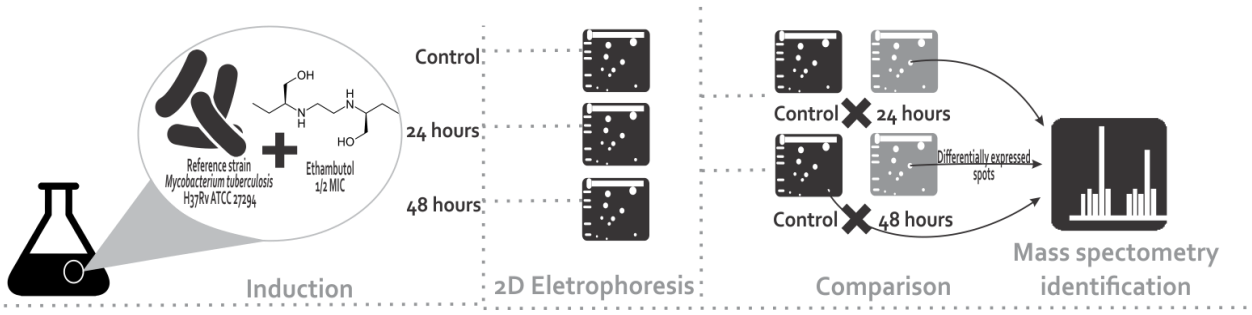
Luciana Dias Ghiraldi Lopes: ldghiraldi@gmail.com
Paula Aline Zanetti Campanerut-Sá: pazcampanerut@gmail.com
Geisa Paulino Caprini Evaristo: geisapc@gmail.com
Jean Eduardo Meneguello: jan.meneguello@gmail.com
Adriana Fiorini: drifiorini@gmail.com
Vanessa P. Baldin: vanessapbaldin@gmail.com
Emanuel M. Souza: souzaem@ufpr.br
Regiane Bertin de Lima Scodro: regianebertin@gmail.com
Vera Lucia Dias Siqueira: vldsiqueira@gmail.com
Rosilene Fressatti Cardoso: rfressatticardoso@gmail.com

ABSTRACT

In recent years, very few effective drugs against *Mycobacterium tuberculosis* (*M. tb*) have emerged which motivates researchers to back attention to the drugs already used in the treatment of tuberculosis. EMB is a drug that affects the integrity of the cell wall by inhibiting arabinosyl transferases encoded by *embCAB* operon. Based on the need to better investigate the complex mechanism of action of ETB, our study presented the proteome profile of *M. tb* after different times of EMB exposure, aiming to comprehend the dynamics of bacilli response to its effects. *M. tb* was exposed to ½ MIC of EMB at 24 and 48 h. The proteins were identified by MALDI- TOF/TOF. The main protein changes occurred in metabolic proteins as dihydrolipoyl dehydrogenase [LpdC] (Rv0462), glutamine synthetase1 [GlnA1] (Rv2220), electron transfer flavoprotein subunit beta [ETF-β] (Rv3029c) and adenosylhomocysteinase [SahH] (Rv3248c). Our results support that the intermediary metabolism and respiration were readily affected by EMB and this disturbance provided proteins that could be explored as drug targets.

Keywords: *Mycobacterium tuberculosis*, Ethambutol, Proteome, Two-dimension gel electrophoresis, MALDI- TOF/TOF, STRING database.

GRAPHICAL ABSTRACT



INTRODUCTION

The treatment of tuberculosis (TB) is centered on the standard regimen of isoniazid (INH), rifampicin (RIF), pyrazinamide (PZA) and ethambutol (EMB). However, the resistance to these drugs has helped to keep TB as a health problem worldwide. In 2015, it was estimated 480,000 multidrug resistant (MDR-TB), which is resistant to INH and RIF, incident cases and 100,000 people with rifampicin-resistant TB (RR-TB) who were also newly eligible for MDR-TB treatment. Although the number of TB deaths fell by 22 % between 2000 and 2015, TB remained one of the top 10 causes of death worldwide in 2015 (WHO, 2016).

In recent years, just a low number of new effective compounds against *Mycobacterium tuberculosis* (*M. tb*) had emerged (Chetty et al., 2016; Hoagland et al., 2016; Kakkar and Dahiya, 2014), which motivates researchers to back attention to the drugs already used in the treatment of TB. So, a better understanding of the action and the resistance mechanism of these drugs can help in designing new active compounds against *M. tb* and also in the discovery of new therapeutic targets (Bernardes-Genisson et al., 2013; Hughes et al., 2006).

The proteomic approach is an interesting tool to investigate *M. tb* response to a certain drug or other external stress factors (Campanerut-Sá et al., 2016; Gopinath et al., 2015; Sharma et al., 2015, 2010; Shen et al., 2010; Hughes et al., 2006; Starck et al., 2004). It can provide changes not predicted by genome analyses as post-translational changes and protein-protein interactions (Unwin and Whetton, 2007; Westermeier and Marouga, 2005).

EMB (Fig. 1) is a drug that affects the integrity of the *M. tb* cell wall and works inhibiting arabinosyl transferases encoded by *embCAB* operon (Chetty et al., 2016). These inhibition leads to the accumulation of free mycolic acids, resulting in bacilli death (WHO, 2016; Xu et al., 2015). EMB exhibit a bacteriostatic activity with moderate bacillary killing (de Steenwinkel et al., 2010).

Proteomic analysis involving EMB in *M. tb* are rare (Jia et al., 2005). There are some proteomic studies with EMB induction in *M. smegmatis* (Jiang et al., 2011; Wang and Marcotte, 2008) which provide clues for further investigation of molecular mechanism of EMB action. Based on this, our study presented the proteome profile of *M. tb* in different times of EMB induction, aiming to clarify the dynamics of bacilli response to EMB effects.

MATERIAL AND METHODS

***Mycobacterium tuberculosis* growth conditions and drug exposition**

Mycobacterium tuberculosis $H_{37}Rv$ (ATCC 27294) reference strain was cultured in Middlebrook 7H9 (Difco Laboratories, Detroit, MD, USA) supplemented with 0.2 % (v/v) glycerol, 0.05 % Tween 80 and 10 % oleic-acid-albumin-dextrose-catalase enriched Middlebroook OADC (BBL/Becton-Dickinson, Sparks, MD, USA) and incubated at 37 °C for 2 weeks in conical flasks. Ethambutol MIC 2 µg/mL was previously determined by REMA assay (Palomino et al., 2002). Ethambutol (Sigma, St, Louis, USA) stock solution (1,000 µg/mL) was added to the cultures to achieve sub-MIC (½ MIC, 1 µg/mL) concentration. The conical flasks were reincubated at 37 °C for 24 and 48 h (de Steenwinkel et al., 2010) under shaking. A not induced EMB culture flask was maintained to the same conditions to be used as protein profile reference.

Protein extraction and two-dimensional gel eletrophoresis (2-D)

Mycobacterial cells were collected by centrifugation, washed three times and suspended in lysis buffer (7 M urea, 2 M tiourea, 4 % CHAPS, 0.5 % immobilized pH gradient (IPG) buffer, 40 mM dithiothreitol (DTT), protease inhibitor cocktail (Amresco, OH, USA). To rupture the bacilli cell wall, sonication was used until the achievement of a uniform suspension. Protein concentration was estimated by Bradford method (Bradford, 1976) using bovine serum albumin as standard. All assays were performed in biological and technical replicates twice in different days. Proteins solution were purified by Clean-up kit (GE Healthcare Life Science, USA) according to manufacturer's instructions. IPG strips pH 4-7, 13-cm length (GE Healthcare Life Science, USA) were used and 500 µg of protein solution were rehydrated overnight at 20 °C in 50 V by each drug induction time. The following four step program was used in Ettan IPGphor 3 (GE Healthcare, USA) at 20°C: first, 500 Volts (V) for 1 h; second 1000 V for 1 h; third 8000 V for 2.5 h and 8000 V for 1 h. The current limit was set at 50 µA per strip. After, IPG strips were equilibrated for 20 min in equilibration buffer I (6 M urea, 2 % SDS, 75 mM Tris-HCl, pH 8.8, 30 % glycerol, 0.002 % bromophenol blue) containing 100 mg DTT and later in equilibration buffer II containing 250 mg of iodoacetamide also for 20 min.

To protein separation in second dimension, 12.5 % SDS-polyacrylamide gels in a vertical electrophoretic unit 600 Ruby (GE Healthcare, USA) at constant voltage of 300 V for 4 hours was used. The gels were stained with Coomassie Blue G-250 according Neuhoff et al., 1988. The gels were scanned by Image Scanner II system (Amersham Biosciences, USA) and analyzed using Image Master Software 6.0 (Amersham Biosciences, USA). All spots were manually checked and those with differential intensity (1.5 fold changes) were selected for identification (Student-t test $p <$

0.05). In order to rule out the possibility of any gel artifact, protein spots showing the same intensity were used as internal control.

In-gel tryptic digest and mass spectrometry analysis

The protein spots of interest were manually excised from the gels, destained thrice with 50 % acetonitrile and 25 mM ammonium bicarbonate pH 8.0 for 30 min, dehydrated with 100 % acetonitrile and allowed to air dry after solvent removal. The gel pieces were rehydrated at 4 °C for 30 min with 400 ng trypsin (Promega, USA) in acetonitrile 10 % and 40 mM ammonium bicarbonate, followed by 12 h incubation in a rotatory shaker at 37 °C and then added 2 % formic acid to quench the reaction. To extract the peptides, the gel pieces were incubated three times with 20 µL of 30 % acetonitrile and 5 % formic acid for 30 min under vortex. All the supernatants were combined and incompletely vacuum dried. The pellets containing the tryptic peptides were resuspended in 0.1 % formic acid for MS analysis. For this, 1 µL of each spot sample of tryptic peptides were mixed 1:1 with freshly prepared α-cyano-4-hydroxycinnamic acid (CHCA) matrix (Sigma, St, Louis, USA) in 50 % acetonitrile and 0,1 % trifluoroacetic acid (TFA), and spotted on MALDI target plate. Peptide mass spectra were obtained on a mass spectrometer MALDI-Tof/Tof *Autoflex II* (Bruker Daltonics, USA) and analyzed on *Flex Analysis 2.0* (Bruker Daltonics). Proteins were identified by peptide mass fingerprinting (PMF) and some peptides primary sequences were confirmed by tandem mass spectrometry (MS/MS) using the program MASCOT V2.1 (Matrix Science, UK) against the NCBInr database and an internal database composed of *Mycobacterium* protein sequences downloaded from Uniprot database. MASCOT protein scoring (based on combined MS and MS/MS spectra) greater than 51, combined with at least 2 identified peptides, were considered statistically significant ($p < 0.05$).

RESULTS

After 24 h of EMB sub-MIC induction, six proteins were differentially expressed comparing the protein profile of *M. tb* H_{37}Rv**.** Multifunctional alpha-ketoglutarate metabolic enzyme [KDH] (Rv1248c) was over-expressed. The 3-phosphoglycerate dehydrogenase [SerA1] (Rv2996c), dihydrolipoyl dehydrogenase [LpdC] (Rv0462), membrane protein (Rv2799) and conserved protein (Rv0831c) were absent and chaperonin GroEL-2 (Rv0440) was under-expressed in this time of exposure in comparison to control gels (Fig. 2B).

Nine differentially expressed proteins were identified in 48 h of EMB sub-MIC induction. Probable PhiRv1 phage protein (Rv1575), possible formamidopyrimidine-DNA glycosylase-like (Rv0944) and LpdC (Rv0462) were absent in this EMB induction time. Conserved protein (Rv0831c), probable PhiRv2 phage protein (Rv2659c), glutamine synthetase 1 [GlnA1] (Rv2220), electron transfer flavoprotein subunit beta [ETF-β] (Rv3029c) and chaperonin GroEL-2 (Rv0440) were under-expressed and adenosylhomocysteinase [SahH] (Rv3248c) was over-expressed (Fig. 2C). All proteins differentially expressed by EMB induction time are listed in Table 1.

We analyzed the differentially expressed proteins by STRING-10 with a medium confidence score threshold of 0.4 and build an interactome network of these set of proteins (Fig. 3). It was observed that proteins belonging to intermediary metabolism and respiration, virulence, detoxification, adaptation classes interacted with each other as well as their partners. There were no interactions with proteins belonging to the classes: information pathway, insertion sequences and phages and proteins with unknown function.

DISCUSSION

EMB, a bacteriostatic drug, was introduced in TB treatment in 1966 and participates in the first line scheme of treatment to minimize the risk of the emergence of resistance to the other anti-TB drugs (Chetty et al., 2016; WHO, 2016). Our research group has been working on alternative anti-*M. tb* strategies, such as combination of used drugs and discovery of new active compounds (Caleffi-Ferracioli et al., 2016; Campanerut-Sá et al., 2016; De Oliveira Demitto et al., 2015; Lopes et al., 2014; Pagliotto et al., 2015; Pires et al., 2014; Scodro et al., 2013). To our knowledge, this is the first study that presented the proteome profile of *M. tb* after different times of EMB induction, which can contribute as an insight into the EMB's action mechanism.

The literature information is centered on changes in cell wall skeleton by inhibition of biosynthesis of arabinogalactan (Wu et al., 2014), but due to global increase of resistant strains, the full understanding of how such drug acts is of paramount importance to new drugs design (WHO, 2016). In this line, studies to understand the dynamics of proteins expression in environmental conditions, such as the induction of *M. tb* to a particular drug, is a valuable tool in the search for new drug targets in the bacillus. Thus the main emphasis of this study was to understand the proteome profile of the *M. tb* H_{37}Rv reference strain induced to sub-MIC concentration of EMB in different times.

After 24 h of EMB induction, enzymes of cellular metabolism were altered. The multifunctional alpha-ketoglutarate metabolic enzyme [KDH] (Rv1248c) was over-expressed in comparison with the not induced control. This enzyme plays a regulatory role in the tricarboxilic acid cycle as E1 component of a canonical alpha-ketoglutarate dehydrogenase complex (KDHC) that produces succinyl CoA via oxidative decarboxylation (WAGNER *et al.*, 2011). KDH is lacking in humans, representing a potential target for new chemotherapy of TB (Tian et al., 2005).

Two enzymes of intermediary metabolism, present in the control, were not detected after 24 h of EMB induction: dihydrolipoyl dehydrogenase [LpdC] (Rv0462) and 3-phosphoglycerate dehydrogenase [SerA1] (Rv2996c). LpdC is the E3 component in KDHC and in pyruvate dehydrogenase complex (PDH) and it is required for the virulence of *M. tb* acting as peroxynitrite reductase/peroxidase enzyme, which helps the bacilli to resist to the reactive nitrogen intermediate hosts (Venugopal et al., 2011). LpdC was also absent in 48 h of EMB induction, which shows that, being continuous induced to this time, the change is maintained, not allowing this intermediate to return to normal levels.

SerA1 (Rv2996c) participates in serine synthesis by D-3-phosphoglycerate from glycolysis (Grant, 2012) and is an essential enzyme in *M. tb* metabolism (Sassetti et al., 2003). This enzyme exhibit at least three different structural motifs that have been referred to as types I, II, and III. The two most well studied SerA1 enzymes are from *M. tb* and *E. coli*, that are types I and II, respectively. *M. tb* SerA1 type I is composed of four domains differing of SerA1 from *E. coli*, composed of three distinct domains, which is speculated to confer a physiological advantage regarding to the persistant stage of TB infection (Burton et al., 2007; Dey et al., 2009, 2005). At this moment, we can infer that EMB affected the intermediary metabolism by altering important enzymes of tricarboxylic acid cycle, such as the over-expression of KDH (E1 component) and absence of LpdC (E3 component) and SerA1. This interruption will contribute to the bacilli death and this target should be more investigated to synthesize new anti TB drugs.

The 24 h EMB induction also disrupted the expression of membrane protein (Rv2799) and conserved protein (Rv0831c), which have unknown expression patterns and functions. The conserved protein (Rv0831) has already been detected in other proteomic studies as *M. tb* plasma proteome (Sinha et al., 2002), *M. tb* proteome in nutrient limitation and hypoxia (Albrethsen et al., 2013) and *M. tb* proteome after INH induction (Campanerut-Sá et al., 2016).

The Chaperonin GroEL-2 (Rv0440) was down-expressed in 24 and 48 h of EMB induction. The known function of this protein is to prevent misfolding and promote refolding and proper assembly of unfolded polypeptides generated under stress conditions (Tuberculist, 2013). This

protein is also considered an immunogenic protein and a stimulator for the synthesis of pro-inflammatory cytokines (Gu et al., 2003; Lewthwaite et al., 2001; Monahan et al., 2001). GroEL-2 was also under-expressed after bacillus induction to ATB 107, a new compound against *M. tb* (Shen et al., 2010), and absent after 48 h of sub-MIC INH induction (Campanerut-Sá et al., 2016). It was reported that its decrease in expression contributes to bacilli weakness and death (Shen et al., 2010).

Most protein changes were observed in 48 h of EMB induction, confirming our time choice to explore differentially expressed proteins by this drug. At this time of EMB induction, probable PhiRv1 phage protein (Rv1575) was absent, and probable PhiRv2 phage protein (Rv2659c) and the conserved protein (Rv0831c) were down regulated. Prophage-like elements Rv1 and Rv2 are predicted in genomes of both *M. tb* H_{37}Rv and CDC1551, while related elements are present in *Mycobacterium bovis* AF2122/97, but absent in *M. bovis* BCG (Cole et al., 1998). This Rv1 and Rv2 elements encode for putative phage proteins such as capsid subunits, prohead proteases and integrases (Bibb et al., 2005). Probable PhiRv2 phage protein (Rv2659c) is an integrase member of the dormancy regulon induced in non-replicating hypoxia state of *M. tb* (Uniprot 2017). This non replicating bacillary population can express antigens that could be used in vaccines that may enhance the ability to prevent active TB and reactivation of disease as the described H56 vaccine (Lin et al., 2012).

Another absent protein in 48 h of EMB exposure was the possible formamidopyrimidine-DNA glycosylase-like (Rv0944). This enzyme seems to play a significant role in DNA damage repair, caused mainly by oxidative damage in the macrophages, which is considered a hostile environment because of the high levels of total reactive oxygen radicals (Olsen et al., 2009). In this sense, the bacillus with activity in repair oxidative and nitrosative DNA damages has greater chances of survival. We believe that the absence of formamidopyrimidine-DNA glycosylase-like, caused by EMB induction, contributes to a greater fragility of the bacillus, which will not be able to repair DNA damage being more exposed to recognition by the immune system of the host.

In the 48 hours of ETB induction we could also observe that the bacillary intermediary metabolism and respiration were affected. Glutamine synthetase 1 [GlnA1] (Rv2220), Electron transfer flavoprotein subunit beta [ETF-β] (Rv3029c) were under-expressed and Adenosylhomocysteinase [SahH] (Rv3248c) was over-expressed. The GlnA1, together with glutamate synthetase are the unique means of ammonia assimilation under nitrogen limiting conditions and play an important role in the biosynthesis of pathogenic mycobacteria cell wall (Chandra et al., 2010; Mowbray et al., 2014). Considering the metabolic change and that the EMB is believed to disturb the cell wall synthesis, after 48 h of EMB induction we had a considerable

decay in the concentration of viable bacilli, justifying the under-expression of the GlnA1. Similar to our results GlnA1 was over-expressed in 12 h, but absent in 24 and 48 hours of INH induction (Campanerut-Sá et al., 2016).

The ETF-β (Rv3029c) is a specific electron acceptor for other dehydrogenases, acting in the main respiratory chain via ETF-ubiquinone oxidoreductase (Tuberculist, 2013). In our study, this protein was under-expressed, indicating a decrease in bacilli respiratory rate caused by EMB action, which is similar to the observed in *M. tb* proteomic after 48 hours of INH exposure (Campanerut-Sá et al., 2016). Jia et al., 2005 observed an increase of this protein in a proteomic study that induced *M. tb* to INH, EMB, and SQ109 an EMB analog, at their MIC concentration, for 24 hours. The authors concluded that ETF-β up-expression is not related with the action of these drugs.

Adenosylhomocysteinase [SahH] (Rv3248c) was over-expressed in 48 h of EMB induction. This enzyme catalyzes the hydrolysis of S-adenosylhomocysteine (SAH) to adenosine and homocysteine and appears to be essential for *in vitro* bacillus growth. In *M. tb,* the levels of SAH and homocysteine are modulated in response to different carbons sources and para-aminosalicylic acid (Chakraborty et al., 2013; Griffin et al., 2011). It is known that SahH plays an important role in metabolic regulation, but the exact mechanism by which this occurs is still unknown (Singhal et al., 2013). This protein was identified in proteome profiles of susceptible and MDR *M. tb* clinical isolates in intracellular macrophage-like THP-1 cell environment (Singhal et al., 2012). The above authors justify their findings considering some common mechanism are adopted by susceptible and resistant mycobacteria for their survival within macrophages, which could serve as drug targets. We believe that our findings on this enzyme can contribute as additional information on this important metabolic target over-expressed by EMB induction. It was recently reported that SahH also has a higher binding affinity to IL-8 than the binding affinity of the chemokine with its specific receptors, which makes this enzyme an exceptional mycobacterial effector engaged in the modulation of pathogen adherence to the target host cells (Dziadek et al., 2016).

STRING analyses revealed that EMB differentially expressed protein categorized in intermediary metabolism and respiration, in virulence, detoxification and adaptation interacted to other proteins involved in the same categories except with proteins involved in information pathways, insertion sequences and phages and hypothetical proteins, which showed no interaction with the others. Jiang et al., 2011 in a proteomic study with 6 h of *M. smegmatis* mc^2155 EMB induction, reported that some of the proteins which modulate mycolic acid synthesis were down-regulated after EMB induction in their protein-protein interaction network analysis by STRING.

Alterations in metabolism, synthesis and modification proteins of macromolecules were also reported by them.

CONCLUSION

Our results support that disturbance on intermediary metabolism and respiration were readily affected by EMB and provide proteins that could be explored as drug targets. Further studies, with susceptible and MDR clinical isolates, are of paramount importance to continue investigating the complex pathways that lead the bacillus to death by EMB.

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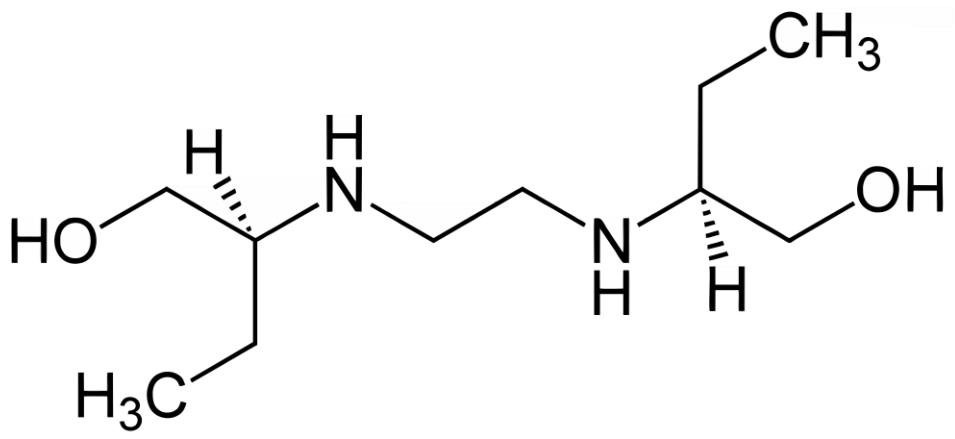

Fig. 1- Ethambutol structure.

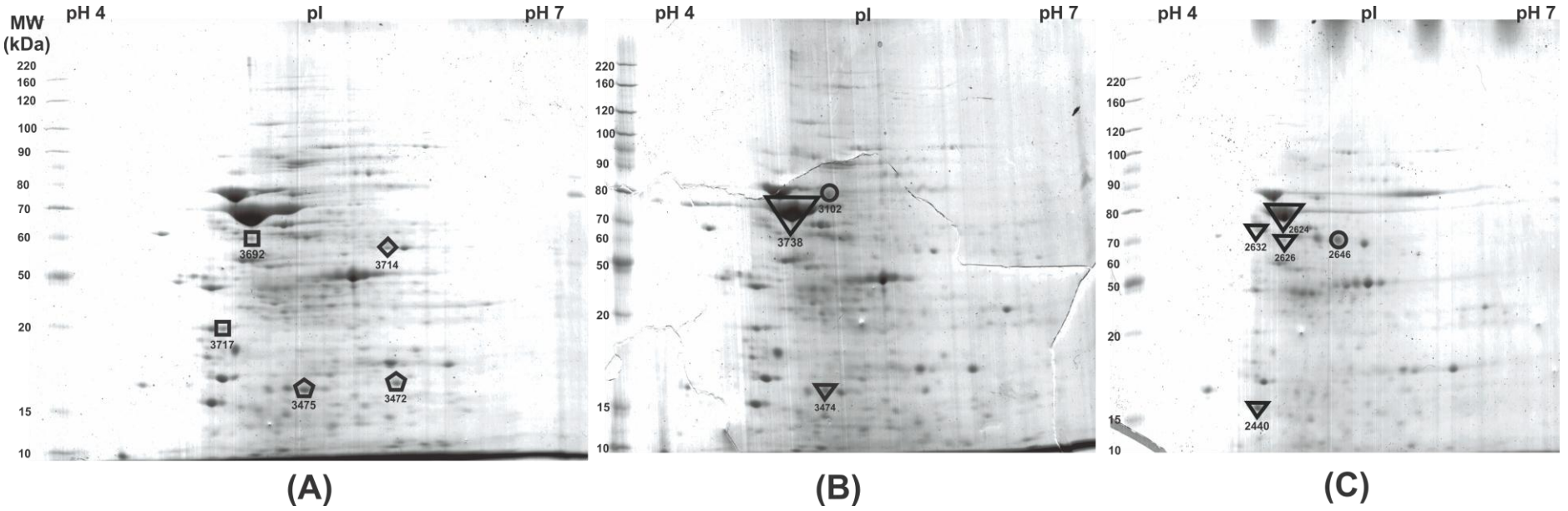

Fig. 2- Two dimensional gel electrophoresis. A: *M. tb* $H_{37}Rv$ without EMB induction. B: 24 h of *M. tb* $H_{37}Rv$ EMB induction. C: 48 h *M. tb* $H_{37}Rv$ of EMB induction.

Table 1. Differentially expressed proteins in *Mycobacterium tuberculosis* H_{37}Rv by EMB induction.

Spot number	ORF number	Fold Change*	Protein identified	Gene	Mascot	Functional Class**
3102	Rv1248c	Present in 24 h	Multifunctional alpha-ketoglutarate metabolism enzyme [KDH]	Rv1248c	24(51)	intermediary metabolism and respiration
3692	Rv2996c	Absent in 24 h	D- 3-phosphoglycerate dehydrogenase [SerA1]	*serA1*	38(51)	intermediary metabolism and respiration
3714	Rv0462	Absent in 24 h and 48 h	Dihydrolipoyl dehydrogenase [LpdC]	*lpdC*	46(51)	intermediary metabolism and respiration
3717	Rv2799	Absent in 24 h	Membrane protein	Rv2799	43(51)	cell wall and cell process
3474	Rv0831c	12.47 Under-expressed in 24 h	Conserved protein	Rv0831c	337 (51)	conserved hypotheticals
3738 2624	Rv0440	4.77/6.42 Under-expressed in 24 h and 48 h	Chaperonin GroEL-2	*groEL2*	71(51)	virulence, detoxification, adaptation
3472	Rv1575	Absent in 48 h	Probable PhiRv1 phage protein	Rv1575	40(51)	insertion sequences and phages
3475	Rv0944	Absent in 48 h	Possible formamidopyrimidine-DNA glycosylase	Rv0944	37(51)	information pathways
2632	Rv2659c	9.75 Under-expressed in 48 h	Probable PhiRv2 prophage integrase	Rv2659c	29(51)	insertion seqs and phages

Spot number	ORF number	Fold Change*	Protein identified	Gene	Mascot	Functional Class**
2626	Rv2220	2.47 Under-expressed in 48 h	Glutamine synthetase 1 [GlnA1]	*glnA1*	38(51)	intermediary metabolism and respiration
2440	Rv3029c	1.54 Under-expressed in 48 h	Electron transfer flavoprotein subunit beta [ETF-β]	*fixA*	67(51)	intermediary metabolism and respiration
2646	Rv3248c	Present in 48 h	Adenosylhomocysteinase [SahH]	*sahH*	68(51)	intermediary metabolism and respiration

***** Fold change after EMB induction. Cutoff value ≥ 1.5 fold changes and $p < 0.05$
****** According to TubercuList (http://genolist.pasteur.fr/TubercuList/)

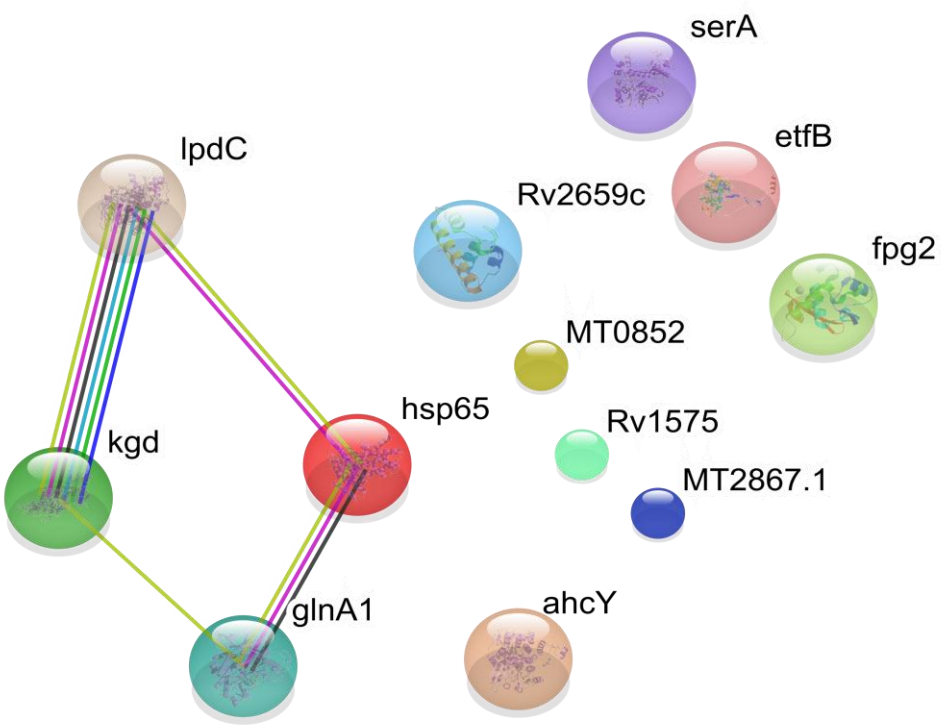

Fig. 3- STRING interactome. Proteins involved intermediary metabolism and respiration, virulence, detoxification, adaptation classes interacted with each other as well as their partners. There were no interactions with proteins belonging to the classes: information pathway, insertion sequences and phages and proteins with unknown function.
lpdC: Dihydrolipoyl dehydrogenase, kgd: Multifunctional alpha-ketoglutarate metabolism enzyme, hsp65: Chaperonin GroEL-2, glnA1: Glutamine synthetase 1, ahcY: Adenosylhomocysteinase, Rv2659c: Probable PhiRv2 prophage integrase, MT0852: Rv0831c, Rv1575: Probable PhiRv1 phage protein, MT2867.1: Rv2799, serA: D- 3-phosphoglycerate dehydrogenase, etfB: Electron transfer flavoprotein subunit beta, fpg2: Possible formamidopyrimidine-DNA glycosylase.

Artigo 2: “PROTEOMIC PROFILE OF *Mycobacterium tuberculosis* AFTER EUPOMATENOID- 5 INDUCTION REVEALS POTENTIAL DRUG TARGETS.”

Proteomic profile of *Mycobacterium tuberculosis* after eupomatenoid- 5 induction reveals potential drug targets

Ghiraldi-Lopes, LDG[a,b*]; Campanerut-Sá, PAZ[b]; Meneguello, JE[c]; Seixas, FAV[d]; Lopes-Ortiz, MA[c,e]; Scodro, RBL[a,b]; Agostinho, CTP[c]; da Silva, R. Z[f]; Siqueira, VLD[b,c]; Nakamura, CV[g] , Cardoso, RF[a,b,c].

[a] Postgraduate Program in Health Sciences, State University of Maringá- PR- Brazil
[b] Department of Clinical Analyses & Biomedicine, State University of Maringá- PR- Brazil
[c] Postgraduate Program in Biosciences Applied to Pharmacy, State University of Maringá- PR- Brazil
[d] Department of Biochemistry, State University of Maringá- Maringá- PR- Brazil
[e] Uningá University Center- Maringá- PR- Brazil
[f] State University of Ponta Grossa- Ponta Grossa- PR- Brazil
[g]Postgraduate Program in Pharmaceutical Sciences, State University of Maringá- PR- Brazil

*Corresponding author: Luciana Dias Ghiraldi Lopes.
Postgraduate Program in Health Sciences, State University of Maringá- PR- Brazil
Av. Colombo 5790, Bloco T-20, Sala 303, CEP 87020-900, Maringá, PR, Brazil.
Phone number: +55 44 3011-5394, Cell phone: +55 44 99825-1064.
e-mail: ldghiraldi@gmail.com

ABSTRACT

Aim: We investigated a proteome profile, protein-protein interaction and morphological changes of *M. tuberculosis* after different times of eupomatenoid-5 (EUP-5) induction to evaluate the cellular response to the drug induced damages. Methods: The bacillus was induced to sub-MIC of EUP-5 at 12, 24 and 48 h. The proteins were separated by 2D gel electrophoresis, identified by LC/MS-MS. Electron scanning microscopy and STRING analyses were performed. Results: EUP-5 impacts mainly in *M. tb* proteins of intermediary metabolism and interactome suggests a multi-site disturbance that contributes to bacilli death. Electron microscopy revealed the loss of bacillary form. Conclusion: Some of the differentially expressed proteins have the potential to be drug targets such as citrate synthase (Rv0896), pgk (Rv1437), ketol-acid reductoisomerase (Rv3001c) and AtpA (Rv1308).

Keywords: Tuberculosis; *Mycobacterium tuberculosis*; Eupomatenoid-5; Proteome; Two-dimension gel electrophoresis; LC/MS-MS; Scanning electron microscopy; STRING database; protein changes; drug targets.

INTRODUCTION

Despite the medicine advances in the latest fifteen years, tuberculosis (TB) remains as one of the world's biggest threats. In 2015, 10.4 million people were estimated to be sick with TB but only 6.1 million new cases of TB were reported. TB ranks alongside HIV as a leading cause of death from an infectious disease. Globally, 11% of new TB cases in 2015 were HIV-positive [1].

The recommended treatment for TB, in new cases, is very effective in bacillary clearance, but depends on fully compliance of the patient, despite the longtime of treatment and its many side effects [1–3]. The treatment failure can lead to the emergence of resistant strains and consequently spread of the resistant form of the disease. Cases of rifampicin resistant (RR-TB), including multidrug- resistant tuberculosis (MDR-TB), which is caused by isoniazid (INH) and rifampicin (RIF) resistant strains, led 480,000 incident cases in 2015 [1]. Few new effective drugs against *Mycobacterium tuberculosis* (*M. tb*) had emerged in recent years [3,4], so there is an urgent need to develop new, safe, effective and affordable anti-TB agents [5].

The research for active ingredients derived from crude extracts, fractions isolated from plants and bioactive compounds with antibacterial activity, has recently shown promising results [6–18]. The eupomatenoid-5 (EUP-5) (Fig. 1) compound extracted from genus *Piper* has shown antibacterial, antifungal, antileishmanial and trypanocidal activity [8,19–26]. The action of EUP-5 is not yet fully understood, however, studies with protozoan parasites showed that the EUP-5 action is associated with mitochondrial dysfunction and oxidative damage [24], lipid peroxidation and DNA fragmentation [27]. Also in different cancer cell lines, EUP-5 seems to act by different mechanisms related to oxidative damage [21], but in *M. tb* no mechanism has been proposed.

The proteomic approach is a tool used by many authors to understand the functional genome of microorganisms. Understanding and investigating anti-TB drug action, drug resistance mechanisms and screening for new therapeutic targets by proteomic analysis provides a more accurate assessment of drug-induced changes, with findings not predicted in genomic analysis [28–33].

Our group recently demonstrated that EUP-5 exhibits anti-*M. tb* activity and showed synergism with RIF and ethambutol (EMB) and no antagonism with the three first-line anti-tuberculosis drugs [22]. Considering the excellent Minimal Inhibitory Concentration (MIC) of EUP-5 in and its potential as anti-*M. tb* agent [18,22], we investigated the proteome profile of *M. tb* after different times of EUP-5 induction aiming to evaluate the bacillary response to drug induced damages. Our results suggest that EUP-5 impacted mainly in a variety of proteins related to detoxification, adaptation and intermediate metabolism.

MATERIAL AND METHODS

1- *Mycobacterium tuberculosis* growth conditions, drug induction and protein extraction

Mycobacterium tuberculosis H_{37}Rv ATCC 27294 was cultured in Middlebrook 7H9 broth (Difco Laboratories, Detroit, MD, USA) supplemented with 0.2 % glycerol, 0.05 % Tween 80 and 10 % oleic-acid-albumin-dextrose-catalase enrichment Middlebroook OADC (BBL/Becton-Dickinson, Sparks, MD, USA) at 37 °C for 2 weeks in conical flasks. EUP-5 MIC 1.9 µg/mL was previously determined using REMA assay [18,34] and 10.000 µg/mL of EUP-5 was added to the cultures to achieve sub-MIC (1/2 MIC, 0.975 µg/mL) concentration. Conical flasks were reincubated at 37 °C for 12, 24 and 48 h with shaking [35]. A not induced EUP-5 culture flask was maintained at the same conditions to be used as protein profile reference.

After the incubation time, the bacilli were collected by centrifugation, washed three times with saline and resuspended in lysis buffer (7 M urea, 2 M tiourea, 4 % CHAPS, 0.5 % IPG buffer, 40 mM dithiothreitol (DTT) and protease inhibitor cocktail (Novex). Sonication was used to help in rupture of the bacilli. Protein concentration was estimated by Bradford method [36] using bovine serum albumin as standard. All assays were carried out in duplicate independently.

2- Two-dimension gel eletrophoresis (2-D)

The Clean-up kit (GE Healthcare Life Science, USA) was used to purify the protein solution according to manufacturer's instruction. To protein separation in first dimension, Immobilized pH gradient (IPG) strips pH 4-7, 13 cm length (GE Helthcare Life Sciences, USA) were used and 400 µg protein were rehydrated overnight at 20 °C in 50 V for each EUP-5 induced time. The gel strips were focused on Ettan IPGphor 3 (GE Healthcare, USA) at 20 °C using the following four step program: a) 500 Volts (V) for 1 h; b) 1000 V for 1 h; c) 8000 V for 2.5 h and d) 8000 V for 1 h. The current limit was set at 50 µA per strip. Prior to the second dimension, the strips were incubated in equilibration buffer (6 M urea, 2% SDS, 75 mM Tris-HCl, pH 8.8, 30 % glycerol, 0.002 % bromophenol blue) containing 100 mg DTT and then 250 mg of iodoacetamide for 20 min respectively. In second dimension, proteins were separated on 12.5 % SDS-polyacrylamide gels in a vertical electrophoretic unit 600 Ruby (GE Healthcare, USA) at constant voltage of 300 V for 4 h. The gels were stained with Coomassie Blue G-250 [37].

Image Scanner II system (Amersham Biosciences, USA) and Image Master Software 6.0 (Amersham Biosciences, USA) were used to scan and analyze the obtained gels, respectively. Student-t test was used to enumerate spots with differential intensity (cutoff value $\geq$ 1.5 fold changes and $p < 0.05$).

3- In-gel tryptic digest

The protein spots of interest were manually excised from the gels, destained thrice with 50 % methanol/ 2.5 % acetic acid in purified water for 60 min, dehydrated with 100 % acetonitrile twice for 5 min and allowed to air dry after solvent removal. The gel pieces were reduced at room temperature for 30 min with DTT 10 mM in 100 mM NH_4HCO_3 and iodoacetamine 50 mM in 100 mM NH_4HCO_3, both removed with a rapid spin. Then, the gel pieces were dehydrated and rehydrated with acetonitrile 100 % and 100 mM NH_4HCO_3. Digestion spots were carried out with trypsin (Promega, USA) in NH_4HCO_3 50 mM for 30 min at 4 °C and rehydrated in NH_4HCO_3 50 mM followed overnight incubation 37 °C. To extract the peptides, the gel pieces were incubated 10 min with 5 % trifluoride acetic acid (TFA) in purified water and 10 min with 5 % TFA in acetonitrile 50 %. All the supernatants were combined and dried incompletely. The pellets containing the tryptic peptides were resuspended in 0.1 % TFA for MS analysis [38].

4- Mass spectrometry analysis and data analysis (LC- MS/MS)

For protein analysis, an aliquot of 4.5 μL of proteins resulting of peptide digestion were separated by C18 (100 mm6100 mm) RP-nanoUPLC (nanoAcquity, Waters) coupled with a Q-Tof Premier mass spectrometer (Waters) with nanoelectrospray source at a flow rate of 0.6 mL/min. The gradient was 2–90 % acetonitrile in 0.1 % formic acid over 45 min. The nanoelectrospray voltage was set to 3.5 kV, a cone voltage of 30 V and the source temperature was 100 °C. The instrument was operated in the ‘top three’ mode, in which one MS spectrum is acquired followed by MS/MS of the top three most-intense peaks detected. After MS/MS fragmentation, the ion was placed on exclusion list for 60 s and for the analysis of endogenous cleavage peptides, a real time exclusion was used [39].

The spectra were acquired using software MassLynx v.4.1 and the raw data files were converted to a peak list format (mgf) without summing the scans by the software Mascot Distiller v.2.3.2.0, 2009 (Matrix Science Ldt.). Peptide mass fingerprint data were searched using Mascot engine v.2.3.01 (Matrix Science Ltd.) *Mycobacterium tuberculosis* Uniprot 2016 protein database, with carbamidomethylation as fixed modifications, oxidation of methionine as variable modification, one trypsin missed cleavage and a tolerance of 0.1 Da for both precursor and fragment ions. Only peptides with at least five amino acid residues which showed significant threshold ($p < 0.05$) in Mascot- based score, were considered in the results. MS/MS spectra were manually validated for the b and y ion series [39].

5- Scanning electron microscopy (SEM)

M. tb cultures were centrifuged and cells washed three-times in PBS, pH 7.4. The cells were fixed at least 2 h with 2.5 % glutaraldehyde and cacodilate 0.1 M at 4 °C for 24 h. The fixed cells were placed on a glass support with poly-l-lysine (Sigma), ethanol dehydrated, subjected to critical-point drying in CO_2, coated with gold. The electron microscopy experiments were performed in duplicate on different days. The reading was carried out in a Quanta 250 (Fei, USA) SEM. Averages of 30 to 50 microscopic fields in each sample were selected by random scanning and photographed.

6- STRING analysis

The protein–protein interaction network of *M. tb* EUP-5 induced proteins was built by using a dataset from STRING database (Search Tool for the Retrieval of Interacting Genes/Proteins, http://string.embl.de/) [40]. STRING-10 server was used to predict the interacting partners of protein-protein interaction. STRING database uses a combination of prediction approaches and an integration of other information (neighborhood, transferred neighborhood, gene fusion, co-occurrence, co-expression, experiments, databases, text mining). Network was made at medium confidence level (0.400) allowing all active prediction methods.

RESULTS

The comparison of the protein profile of *M. tb* H_{37}Rv induced and not induced by sub-MIC EUP-5 at 12, 24 and 48 h was carried out by 2D electrophoresis and LC-MS/MS (Fig. 2). Duplicates gels were run for each induced time. The proteins were separated according to their isoelectric point and molecular mass. Approximately 84 spots were detected in each exposure time by ImageMaster 6.0 software. All proteins differentially expressed by EUP-5 induction time are listed in Table 1.

In the EUP-5 induction 12 h gels, four altered proteins were observed. Cell wall synthesis protein Wag 31 (Rv2145c) was absent, chaperonin GroEL-2 (Rv0440) was down-expressed, propionyl-CoA carboxylase [AccD5] (Rv3280) was present only at this time, and one spot could not be identified.

The EUP-5 induction 24 h gels showed eight differentially expressed proteins. Four proteins were absent: glyceraldehyde 3-phosphate dehydrogenase [Gapdh] (Rv1436), succinyl-CoA synthetase alpha chain [SucD] (Rv0952), cell wall synthesis protein Wag 31 (Rv2145c) and ketol-acid reductoisomerase (Rv3001c). Elongation factor Tu [Ef-Tu] (Rv0685), universal stress protein [Usp] (Rv2028c), chaperone protein Dnak (Rv0350), citrate synthase I [GltA2] (Rv0896) and

phosphoglycerate kinase [Pgk] (Rv1437) were present when compared to not EUP-5 induced assay. Chaperonin GroEL-2 was under-expressed in this EUP-5 induction time.

In EUP-5 induction 48 h gels, two additional proteins were identified, probable ATP synthase alpha chain [AtpA] (Rv1308) and D-3-phosphoglycerate dehydrogenase [SerA1] (Rv2996c). Cell wall synthesis protein Wag 31 (Rv2145c) and ketol-acid reductoisomerase (Rv3001c) were absent and chaperonin GroEL-2 was under-expressed in this induction time.

The morphological changes of *M. tb* cells were evaluated in each EUP-5 induced time. EUP-5 promoted an alteration in bacillary form and in multiplication. Comparison between not induced cells and after 12 h of EUP-5 induction demonstrated that the bacillus became swollen, and, by increasing the induction time (24 h and 48 h), the bacilli lost the bacillary form, assuming a round and wrinkled appearance. Structures similar to those of outer membrane were observed, as can be seen in Fig. 3.

We analyzed the differentially expressed proteins by STRING-10 with a medium confidence score threshold of 0.4 and build and interactome network of these set of proteins (Fig. 4). We found that proteins involved in intermediary metabolism and respiration, lipid metabolism, virulence/ detoxification/ adaptation and information pathway interacted with each other as well as their partners, except the cell wall synthesis protein Wag 31 (Rv2145c).

DISCUSSION

The action of EUP-5, obtained from *Piper solmsianum* C. DC. var. *solmsianum,* has been demonstrated against Gram-positive and Gram-negative bacteria [41]. Since 2013, our research group has concentrated efforts in the search for effective and less toxic alternatives for the treatment of TB [17,18,22,42–44]. Scodro *et al.* [18] demonstrated that EUP-5, obtained from *Piper regnellii,* has an excellent MIC against *M. tb* and selectivity index (SI=20), which makes it a promising candidate for new anti-TB drugs and EUP-5 had demonstrated to interacted synergistically with drugs already used in TB treatment [22]. In the present study, we focused on better understanding how EUP-5, at fixed sub-MIC, acts in *M. tb* by analyzing the protein profile, protein-protein interactions and morphological changes caused by this new TB drug candidate in different induced times.

After 12 h of EUP-5 induction, the cell wall protein Wag 31 (Rv2145c) was absent. This protein is originally identified as a cell wall antigen in *M. tb* and is known to be involved in bacterial shape and division [45–47], although its pathophysiologic function still mostly unknown [48]. The cell wall is the first barrier encountered by EUP-5, therefore we observed this protein absent within 48 h of the EUP-5 induction. Mukherjee *et al.* [47] demonstrated that Wag 31 plays

an important role in protecting the *M. tb* against deleterious effects of oxidative stress by interaction with penicillin-binding protein 3 (PBP3), an important protein for the synthesis of peptidoglycan, preventing cleavage by proteases. Its absence promotes a greater exposition of PBP3 to free radicals, which affects the cell wall integrity. It is also known that Wag 31 interacts with XCL2 chemokine of T cells blocking its secretion to extracellular environment, which affects the chemotactic signalization toward T cells, allowing the bacilli to evade immune response [48]. This mechanism can occur in cases of restrictions of oxygen and carbon or amino acid source suggesting a potential connection between Wag 31 and *M. tb* virulence [49]. Fig. 1 demonstrates bacillary changes observed by SEM, performed at 12, 24 and 48 h of EUP-5 induction. It is evident the progressive changes in the bacillary form to a round shape, as well as changes in the cell wall with wrinkles and loss of integrity. Similar results were observed at different times of INH sub-MIC induction (0.03 µg/mL) by scanning electron micrographs [32].

In this proteome analysis, the chaperonin GroEL-2 (Rv0440) was under-expressed in all EUP-5 induced times. GroEL-2 is an immunoreactive protein related with the correct proper assembly of unfolded polypeptides generated under stress conditions and participates in detoxifications and adaptations process in the bacillus [50]. A new candidate for anti-TB drug, ATB 107, also promoted GroEL-2 under-expression and the authors suggested that it could cause weakening of the self-restoration function under stress conditions [51]. Starck *et al.* [52] demonstrated the presence of GroEL-2 in proteolytic fragments only in *M.tb* in anaerobic compared with aerobic conditions. In our study, GroEL-2 showed a progressive under-expression in all EUP-5 inducing times, suggesting that the bacillus in contact with EUP-5 could respond with a decrease in respiratory rate, which would lead to death.

In 12 h of EUP-5 induction, the propionyl-CoA carboxylase [AccD5] (Rv3280) was the only protein that was over-expressed in comparison with not induced control. This protein is related with lipid metabolism and belongs to the essential complex of *M. tb* acetyl coenzyme A carboxylase (ACC). This complex has both propionyl-CoA carboxylase and acetyl-CoA carboxylase activities [53]. The carboxylation of propionyl-CoA is one of the two putative metabolic pathways that *M. tb* could use to synthesize the methylmalonyl-CoA, which is necessary for the synthesis of the complex lipids characteristic of *M. tb* [54] and essential to variability, virulence and formation of biofilms in mycobacteria [55]. It was reported that members of ACC complex (AccA3, AccD4 and AccD5) participate together with Wag 31 during mycobacteria cell elongation when nascent peptidoglycan is synthesized and deposited at the poles [55]. In our study, the propionyl-CoA carboxylase over-expression may be related to an attempt, by the bacillus in association of Wag 31 absence, to increase the production of fatty acids in the cell membrane and maintain the mycobacteria duplication.

The intermediate metabolism and respiration were affected after 24 h of induction by EUP-5. Glyceraldehyde 3-phosphate dehydrogenase [Gapdh] (Rv1436), a key enzyme in anaerobic glycolysis, was absent in this induction time. Gapdh is also involved in a variety of cellular processes, acting as a transcription factor, as a microtubule-binding protein, as lactoferrin receptor and as an apoptosis inducer[56,57]. The Gapdh absence observation lead us to infer that EUP-5 could affect not only the production of ATP, through the glycolytic pathway, but also other routes of duplication, acquisition of nutrients and essential factors to bacillus survival. Phosphoglycerate kinase [Pgk] (Rv1437), also involved in glycolysis, was present in 24 h of EUP-5 induction. Recent studies showed that Pgk was increased in proteomic profile of a patient who developed MDR-TB during the course of anti-TB therapy [58,59]. The above authors suggest the useful of Pgk as a promising biomarker to serological diagnosis and probably for detecting drug resistance in the future. The presence of this protein in 24 h, but not in 48 h of EUP-5 induction, deserves better attention in additional studies for considering EUP-5 as an anti-TB drug candidate.

The absence of succinyl-CoA synthase alpha [SucD] (Rv0952), observed only within 24 h of EUP-5 induction, may impact in an evident decrease in the oxidative metabolism of *M. tb*. This enzyme is involved in the conversion of succinate to succinyl-CoA in the tricarboxylic acid cycle and also in the destruction of the ketone body [50]. As Starck *et al.* [52] and Kumar *et al.* [60] detected over-expression of this protein in *M. tb* cultured under anaerobic conditions, which probably triggers the ketone pathway, and in isolates resistant to Kanamycin (KM) and Amikacin (AM) respectively, the absence of SucD in our study induces us to think that this is related only to the decrease in the growth of *M. tb*. In contrast, at this time of induction of EUP-5, the presence of citrate synthase I [GltA2] (Rv0896) can be interpreted as an attempt by the bacillus to acquire nutrients from carbohydrate degradation products, fatty acids and ensure the production of ATP by the tricarboxylic acid cycle [61].

Ketol-acid reductoisomerase (Rv3001c) is a bifunctional enzyme that catalyzes the second and third reaction of the branched-chain amino acid (BCAA) pathway and it was absent after 24 h of EUP-5 induction. This pathway is present only in microorganisms and plants, not in human hosts, which makes it a specific target [62]. Grandoni *et al*. [63], had already reported that inhibitors of ketol-acid reductoisomerase had an anti-TB activity in reference strain ATCC 35801 and in resistant clinical isolates. The specificity of this protein turn possible the development of new drugs that inhibit the BCAA pathway [62]. In our study, ketol-acid reductoisomerase was absent after 24 and 48 h of EUP-5 induction, indicating that the pathway to mycobacterial survival is blocked at this time, putting it in a position to be considered for further investigation and better understanding of its action in the bacillus.

The 24 h EUP-5 induction caused over-expression of Elongation factor Tu [Ef-Tu] (Rv0685) which is a translation factor with ribosome-dependent GTPase activity. It is known that this factor is related to the interaction with RNA during mycobacteria protein biosynthesis, regulation of cell growth in nutrient deprivation condition [50,64,65]. The Ef-Tu phosphorylation is involved in the setting of the bacillus to stress conditions during the course of the infection. The *M. tb* phosphorylated Ef-Tu has a decreased affinity for GTP, thus, the low GTP production in response to the oxidative stress present in this environment leads to a reduction in protein synthesis. [64]. Other drugs used in TB treatment, like INH, KM and AM also promoted an over-expression of this protein [30,32], suggesting that in drug stress conditions, also observed in our study by EUP-5 induction, bacillus has an interruption of translational steps of unnecessary proteins [64].

Universal stress protein [Usp] (Rv2028c) and chaperone protein Dnak (Rv0350) are proteins related with virulence, detoxification and adaptation [50] and were over-expressed in 24 h of EUP-5 induction. The Usp family (Rv1996, Rv2005c, Rv2026c and Rv2028c) proteins are regulated by DosR-DosT regulon expressed under stress conditions as hypoxia, nitric oxide and carbon monoxide production [66–68]. So, by the first time, we can suggest that over-expression of Usp could be in response to stress induced by EUP-5. Although, the exact function of the Usp family proteins is not fully elucidated, it is known to be present during the persistence of hypoxia and probably in nonreplicating state of the bacilli [68,69]. Recently, Sharma & Bisht [33] discussed about the importance of conducting further studies about hypothetical proteins and proteins of unknown function due to their possible involvement in drug resistance mechanisms, such as causing neutralization or compensation of drugs effects.

DnaK, a chaperonin of heat shock proteins (Hsp70) is involved in several processes related to bacterial virulence and host defense [70]. Prado-Rosales *et al.* [71], reported the presence of DnaK in vesicles membranes of *M. tb*, *M. bovis* bacille Calmette-Guérin and other virulent and nonvirulent mycobacterial species. In Fig. 3-C we can observe by SEM, in *M. tb* EUP-5 induction, some structures similar to those of the outer membrane vesicles. In mycobacteria, these vesicles are responsible by lipids and proteins transport, and are involved in immune response of the host. [71–73]. Similar to EUP-5 induction, over-expression of DnaK was also observed in SM susceptible and resistant *M. tb* isolates induced to SM sub-MIC, and *in silico* docking analysis showed significant interaction of SM and DnaK [74,75].

The role of most differentially expressed proteins at 48 h of EUP-5 induction has been discussed previously. The novelty is the presence of two proteins, ATP synthase alpha chain [AtpA] (Rv1308) and D-3-phosphoglycerate dehydrogenase [SerA1] (Rv2996c). AtpA, a regulatory subunit in production of ATP [50], is encoded by *atpA* gene, which is considered an emerged hub gene that could be used as promising candidate for drug targets, due to its importance in

microorganism survival and multiplication [76,77]. Sharma *et al*. [30], observed upregulation of AtpA in KM and AM resistant isolates, which indicates that the development of resistance may also involves the increased energy production in response to bacilli death.

D-3-phosphoglycerate dehydrogenase [SerA1], a NADH cofactor dependent, is the first enzyme in the L-serine biosynthetic pathway [78,79]. There are three types of SerA1, which differs in size and domain composition. *M. tb* SerA1 Type 1 is composed of four distinct domains that works as a specific ligand binding site and provides an important potential target for drug development [78,80,81]. We can speculate that the over-expression of SerA1 in EUP-5 induction promotes a rise in the production of L-Serine that increases the uptake of sulfur to cysteine formation. In turn, it will participate in glutathione synthesis, an agent used in the recovery of oxidized proteins.

STRING analyses revealed that EUP-5 over-expressed proteins of virulence, detoxification and adaptation, intermediary metabolism and respiration, information pathways and lipid metabolism interacted to other proteins involved in the same categories, except with cell wall and cell process category, which showed no interaction with the others. Thus, the over-expressed proteins of EUP-5 and its interactive partners lead us to suggest that this compound could promote a multi-site disturbance in the metabolism of *M. tb* that is responsible for the death of the bacilli (Fig. 4).

The characterization of the pathways that are required for the *M. tb* growth of is of paramount importance for the development of more effective anti-TB agents. Some of the proteins observed in this study present considerable potential to be drug targets due their specificity in *M. tb*, with active enzyme sites that differ from other microorganisms and humans.

To the best of our knowledge, this is the first study to assess proteomic profile and bacillary morphology by SEM induced by EUP-5 in *M. tb*. The greatest impact on *M. tb* metabolism was observed in 24 hours of EUP-5 induction with changes in proteins related to intermediary metabolism and respiration, lipids metabolism, virulence and detoxification, information pathways and cell process. The most frequent morphological changes were at 24 and 48 h of EUP-5 induction, which were swollen, rounded and wrinkled bacillary appearance.

The differential expression of *M. tb* proteins that arose from the EUP-5 induction constitutes an attempt of cellular response to the damages caused by this compound. Some of the differentially expressed proteins have the potential to be drug targets such as citrate synthase [82,83], pgk [84], ketol-acid reductoisomerase [63] and AtpA [85,86]. In this sense, drugs that act on these proteins, in synergistic association with EUP-5, may represent a treatment option that would affects *M. tb* by different mechanisms. Thus, our data support further studies of EUP-5 in dormancy bacillus, resistant isolates (MDR and XDR) and intramacrophages cells cultures.

EXECUTIVE SUMMARY

- *Mycobacterium tuberculosis* is the main etiological agent of tuberculosis, a disease that still represents a serious health problem worldwide.
- Recently our team demonstrated that EUP-5 exhibits anti-*M. tb* activity and showed synergism with RIF and EMB and no antagonism with the three first-line anti-tuberculosis drugs.
- An evaluation of the protein profile, protein-protein interactions and morphological changes, induced by EUP-5 sub-MIC in *M. tuberculosis* at different times was made.
- The proteins were separated by 2D gel electrophoresis and identified by LC-MS/MS analysis.
- The greatest impact of EUP-5 on bacterial metabolism was at 24 h, which involved alterations in proteins in different settings as intermediary metabolism and respiration, lipids metabolism, virulence and detoxification, information pathways and cell process.
- The most frequent changes, observed by SEM, were at 24 and 48 h of EUP-5 sub-MIC induction with swollen, rounded and wrinkled bacillary appearance.
- Some of these proteins present considerable potential to drug targets due to be specific for mycobacteria.

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662 **Table 1. Details of proteins differentially expressed in *Mycobacterium tuberculosis* H_{37}Rv after EUP-5 induction**

Induction time	ORF number	Gene	Spot number	Protein identified	Mascot	Matched peptides	Fold Change*	Functional Class**
12 hours	Rv2145c	*wag31*	1555	Cell wall synthesis protein Wag 31 P9WMU1	259	10	Absent in 12 h	cell wall and cell processes
	Rv0440	*groEL2*	3814	Chaperonin groEL-2 P9WPE7	1277	31	2.85 Under-expressed in 12 h	virulence, detoxification, adaptation
	Rv3280	*accD5*	3846	Propionyl-CoA carboxylase [AccD5] P9WQH7	53	3	Present in 12 h	lipid metabolism
	Not identified	-----	3844	-----	-----	-----	-----	-----
24 hours	Rv0440	*groEL2*	3524	Chaperonin groEL-2 P9WPE7	2300	45	1.87 Under-expressed in 24 h	virulence, detoxification, adaptation
	Rv2145c	*wag31*	1555	Cell wall synthesis protein Wag 31 P9WMU1	259	10	Absent in 24 h	cell wall and cell processes
	Rv1436	*gap*	1537	Glyceraldehyde 3-phosphate dehydrogenase [Gapdh] P9WN83	78	2	Absent in 24 h	intermediary metabolism and respiration
	Rv0952	*sucD*	1531	Succinyl-CoA synthetase alpha chain [SucD] P9WGC7	104	6	Absent in 24 h	intermediary metabolism and respiration
	Rv3001c	*ilvC*	1520	Ketol-acid reductoisomerase P9WGC7	39	3	Absent in 24 h	intermediary metabolism and respiration
	Rv0685	*tuf*	3465	Elongation factor Tu [Ef-Tu]	323	6	Present in 24 h	Information

				P9WNN1				pathways
	Rv2028c	Rv2028c	3466	Universal stress protein [Usp] P9WFD9	95	3	Present in 24 h	virulence, detoxification, adaptation
	Rv0350	*dnaK*	3367, 3467	Chaperone protein Dnak P9WMJ9	2408	55	Present in 24 h	virulence, detoxification, adaptation
	Rv0896	*gltA2*	3480	Citrate synthase I [GltA2] P9WPD5	137	5	Present in 24 h	intermediary metabolism and respiration
	Rv1437	*pgk*	3495, 3496	Phosphoglycerate kinase [Pgk] P9WID1	760	15	Present in 24 h	intermediary metabolism and respiration
48 hours	Rv0440	*groEL2*	1944	Chaperonin groEL-2 P9WPE7	2300	45	2.54 Under-expressed in 48 h	virulence, detoxification, adaptation
	Rv2145c	*wag31*	1555	Cell wall synthesis protein Wag 31 P9WMU1	259	10	Absent in 48h	cell wall and cell processes
	Rv3001c	*ilvC*	1519	Ketol-acid reductoisomerase P9WKJ7	39	3	Absent in 48h	intermediary metabolism and respiration
	Rv1308	*atpA*	1932	ATP synthase alpha chain [AtpA] P9WPU7	303	9	Present in 48h	intermediary metabolism and respiration
	Rv0350	*dnaK*	1858	Chaperone protein Dnak P9WMJ9	984	30	Present in 48h	virulence, detoxification, adaptation

	Rv2996c	*serA1*	1957	D-3-phosphoglycerate dehydrogenase [SerA1 P9WNX3	68	5	Present in 48h	intermediary metabolism and respiration

***** Cutoff value ≥ 1.5 fold changes and $p < 0.05$

****** According to TubercuList (http://genolist.pasteur.fr/TubercuList/)

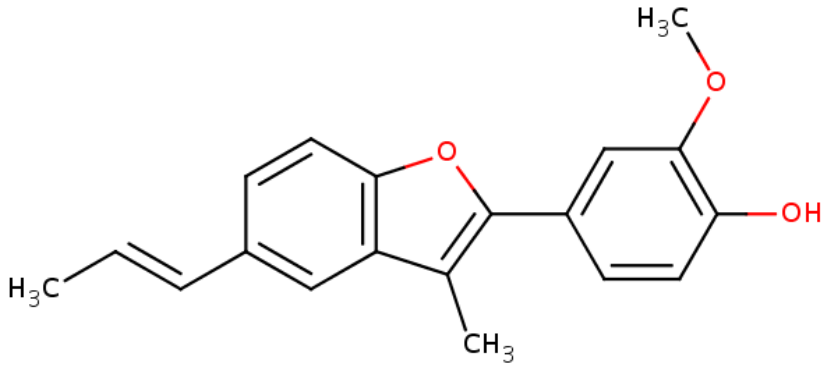

Fig. 1 Chemical structure of eupomatenoid-5.

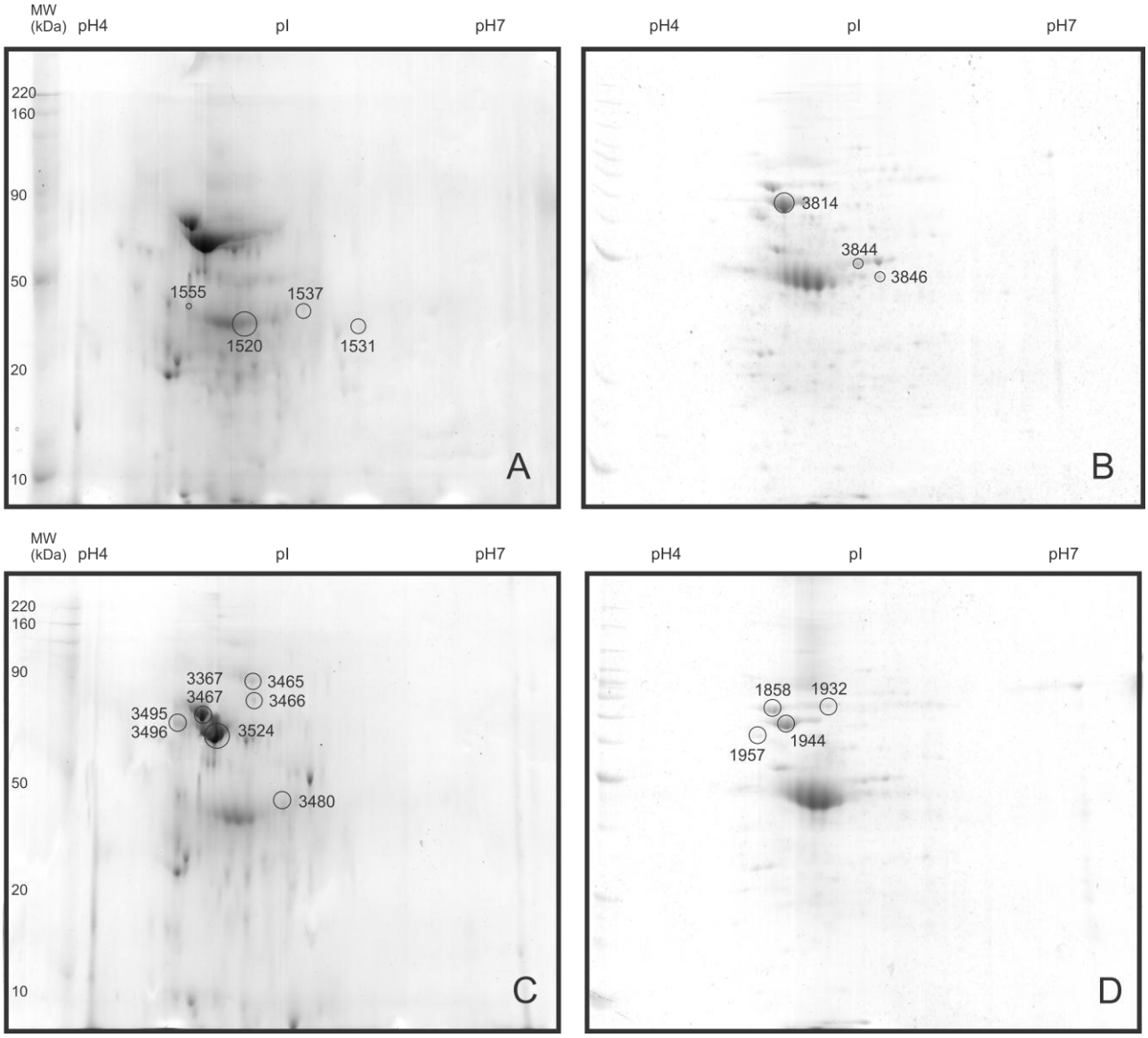

Fig. 2. Two- dimensional gels profile of *Mycobacterium tuberculosis* $H_{37}Rv$ (*M. tb*) induced to sub-MIC of EUP-5. (A) Not induced gel (control). (B) 12 h of EUP-5 induction gel (C). 24 h of EUP-5 induction gel. (D) 48 h of EUP-5 induction gel. Circled spots indicate proteins differentially expressed (Table 1).

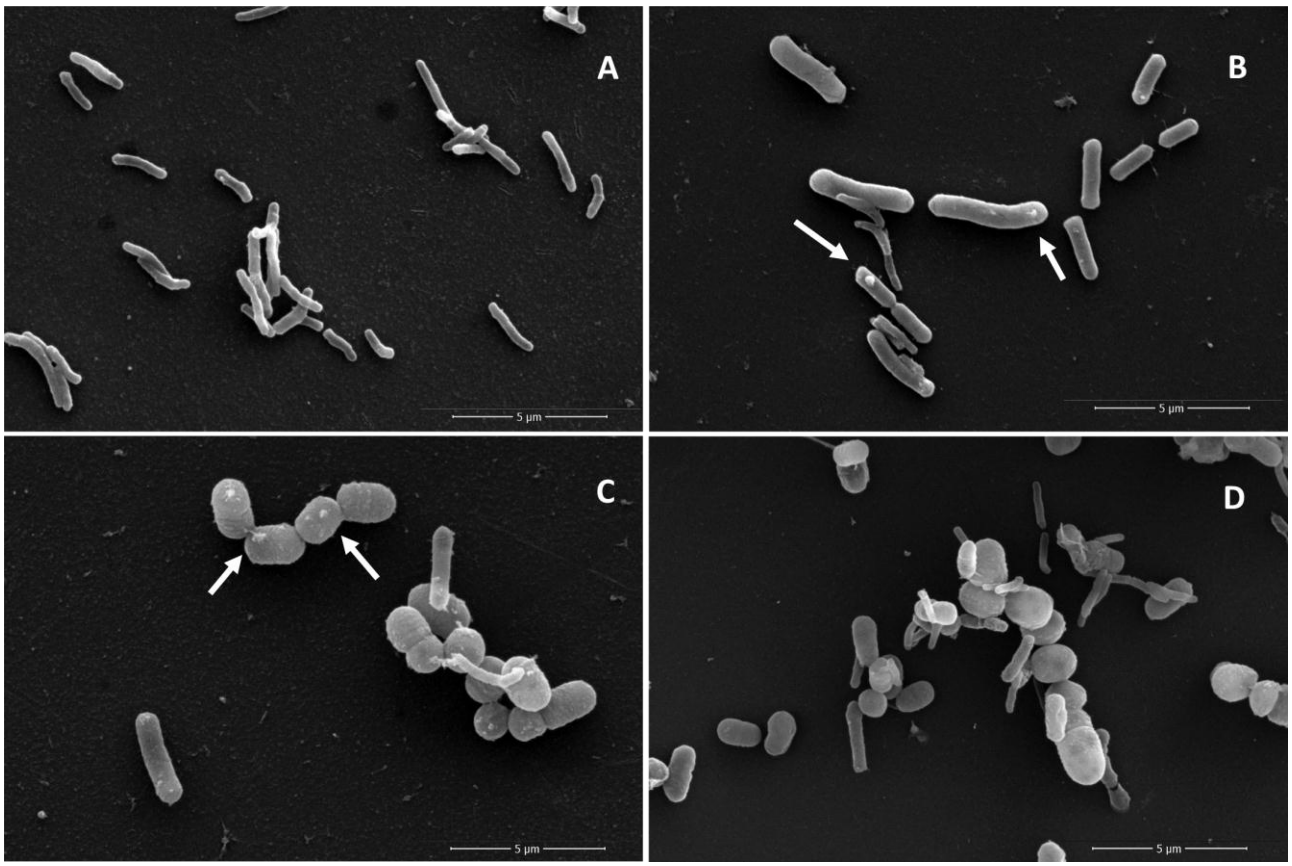

Fig. 3. Scanning electron microscopy of *Mycobacterium tuberculosis* $H_{37}Rv$ (*M. tb*) induced to sub-MIC of EUP-5. (A) Not induced cells (control). (B) 12 h of EUP-5 induction (C). 24 h of EUP-5 induction. (D) 48 h of EUP-5 induction. The white arrows indicate structures similar to outer membrane vesicles.

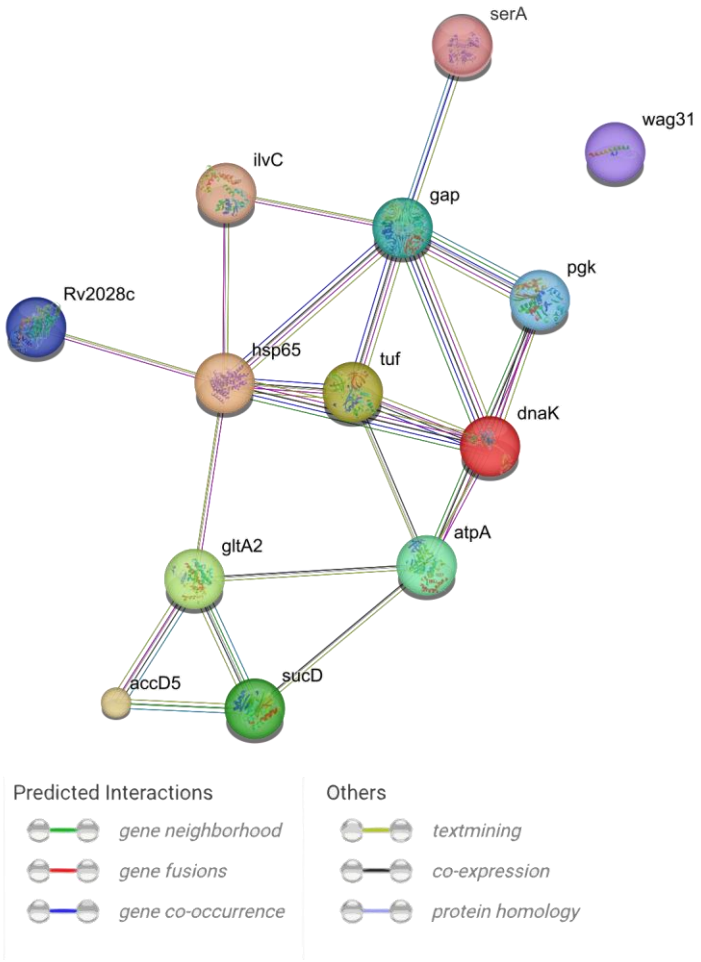

Fig. 4. STRING analyses reveals that proteins involved in intermediary metabolism and respiration, lipid metabolism, virulence/ detoxification/ adaptation and information pathway interacted with each other as well as their partners, except the cell wall synthesis protein Wag 31 (Rv2145c).

CAPÍTULO III

CONCLUSÕES

1) A proteômica é uma ferramenta que auxilia na compreensão da ação de fármacos em *Mycobacterium tuberculosis* (*M. tb*).

2) As condições de cultivo de *M. tb* H_{37}Rv e exposição a EMB e EUP-5 em diferentes tempos foram padronizadas e aplicadas à análise proteômica.

3) A metodologia de proteômica foi útil para reconhecimento das proteínas expressas pela cepa *M. tb* H_{37}Rv após a exposição a EMB e EUP-5.

4) As principais alterações proteicas relacionadas a exposição de *M. tb* H_{37}Rv ao EMB ocorreram após 48 horas e foram proteínas relacionadas ao metabolismo intermediário e respiração, sugerindo que estas poderiam ser exploradas como alvos.

5) As principais alterações proteicas relacionadas a exposição de *M. tb* H_{37}Rv a EUP-5 ocorreram após 24 h e foram proteínas relacionadas ao metabolismo intermediário, metabolismo lipídico, virulência e detoxificação, proteínas de informação e processos celulares. Algumas dessas proteínas são consideradas alvos para fármacos por serem micobactérias específicas.

6) As principais alterações morfológicas causadas por EUP-5 em *M. tb* H_{37}Rv, observadas pela microscopia eletrônica de varredura, foram arredondamento caracterizando perda da forma bacilar e bacilos com aparência enrugada.

7) A construção de redes de interações de proteínas por STRING-10 database é uma ferramenta eficiente e permite uma melhor visualização do impacto causado por EMB e EUP-5 em *M. tb* H_{37}Rv.

PERSPECTIVAS

Diante dos resultados obtidos neste trabalho, o EUP-5 se apresenta como um potencial candidato a fármaco anti-TB e o mecanismo de ação de EMB ainda não foi totalmente explorado. Desta forma, a avaliação da expressão proteica tem grande importância na continuidade dessas investigações em micobactérias. Estudos proteômicos com isolados clínicos sensíveis e resistentes aos fármacos de primeira linha, com micobactérias não-tuberculosas e com bacilos em estado dormente podem contribuir para este objetivo. Além disso, novas ferramentas de bioinformática como o *Cytoscape* e *molecular docking* podem contribuir para um melhor entendimento de novos alvos proteicos ou marcadores de resistência.

ANEXOS

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The corresponding author will, at no cost, receive a customized Share Link providing 50 days free access to the final published version of the article on ScienceDirect. The Share Link can be used for sharing the article via any communication channel, including email and social media. For an extra charge, paper offprints can be ordered via the offprint order form which is sent once the article is accepted for publication. Both corresponding and co-authors may order offprints at any time via Elsevier's Webshop. Corresponding authors who have published their article open access do not receive a Share Link as their final published version of the article is available open access on ScienceDirect and can be shared through the article DOI link.

AUTHOR INQUIRIES

Visit the Elsevier Support Center to find the answers you need. Here you will find everything from Frequently Asked Questions to ways to get in touch. You can also check the status of your submitted article or find out when your accepted article will be published.

Future Medicine Author Guidelines

This document outlines how to prepare articles for submission. We recommend you read these guidelines in full before submitting your article or making an article proposal.

Table of Contents

Journal aims & scope

Aims and scope information can be found on the individual journal webpages, along with information regarding Editorial Board members and indexing:
Biomarkers in Medicine
Epigenomics
Future Cardiology
Future Microbiology
Future Neurology
Future Oncology
Future Virology
Immunotherapy
Journal of 3D Printing in Medicine
Journal of Comparative Effectiveness Research
Nanomedicine
Personalized Medicine
Pharmacogenomics
Regenerative Medicine
For the following journals, please see the separate Management Series Author Guidelines:
Breast Cancer Management
CNS Oncology
Colorectal Cancer
Hepatic Oncology
International Journal of Hematologic Oncology
International Journal of Endocrine Oncology
Lung Cancer Management
Melanoma Management
Neurodegenerative Disease Management
Pain Management Version: 4th May 2016

Audience

The audience for Future Medicine titles consists of clinicians, research scientists, decision-makers and a range of professionals in the healthcare community. Authors should bear in mind the multidisciplinary status of the readership when writing the article.
Future Medicine articles have been engineered specifically for the time-constrained professional. The structure is designed to draw the reader's attention directly to the information they require. Version: 4th May 2016

Future Medicine Ltd, Unitec House, 2 Albert Place, London, N3 1QB, UK; T: +44 (0)20 8371 6090; F: +44 (0)20 8371 6089; www.futuremedicine.com Future Medicine Ltd is part of the Future Science Group www.future-science-group.com

At-a-glance article formatting checklist	**Abstract**	**Keywords**	**Future Perspective**	**Executive Summary**	**Reference limit**	**Figures & Tables permitted**	**Supporting cover letter required**
Word count range							
Review	4000–6000	✓	✓	✓	✓	~80	✓	✗
Perspective	4000–6000	✓	✓	✓	✓	~80	✓	✗

Special Report	1500–3000	✓	✓	✓	✓	~50	✓	✗
Research articles	Varies by journal	✓	✓	✗	Summary points	~80	✓	✓
Case Study/ Case Series	1500–3000	✓	✓	✗	Summary points	~50	✓	✓
Editorial	1500	✗	✓	✗	✗	20	✗	✗
Commentary	1500–3000	✗	✓	✗	✗	20	✗	✗
Priority Paper Evaluation	1500	✓	✓	✓	✓	20	One of each max.	✗
Conference Scene	1500	✓	✗	✗	✗	20	✗	✗
Company Profile	2000	✓	✓	✗	Summary points	20	One of each max.	✗
Letter to the Editor	1500	✗	✗	✗	✗	20	✗	✗

Article types

Future Medicine publishes a range of article types, descriptions of which are outlined below. Authors are encouraged to consult the 'at-a-glance formatting checklist' for details on word counts and other formatting requirements. The information below gives an overview of the requirements for each article type published by Future Medicine. However, authors should consult the ICMJE "*Recommendations for the Conduct, Reporting, Editing, and Publication of Scholarly Work in Medical Journals*" (http://www.icmje.org/icmje-recommendations.pdf), in particular the section on "*Preparing a Manuscript for Submission to a Medical Journal*" prior to submitting to a Future Medicine journal, for more detailed information.

Reviews

Reviews aim to highlight recent significant advances in research, ongoing challenges and unmet needs; authors should be concise and critical in their appraisal of the subject matter, and strive for clarity. The focus should be on key, defining developments rather than providing a comprehensive literature survey. Reviews should provide balanced coverage of the field and not focus predominantly on the author's own research. Authors are encouraged to include their own perspective on current trends and future directions.

Systematic Reviews:

Systematic reviews should be conducted following the recommendations of PRISMA (http://www.prisma-statement.org/).

Word limit: 4000–6000 words (excluding abstract, executive summary, references and figure/table legends)

Required sections (for a more detailed description of these sections see Article sections):

- Title
- Author(s) names & affiliations
- Abstract
- Keywords
- Body of article
- Future perspective
- Executive summary
- References: target of approximately 80 references
- Reference annotations

 Financial disclosure/Acknowledgements

Perspectives

Perspectives have the same basic structure and length as review articles; however, they should be more speculative and forward-looking, even visionary. They offer the author the opportunity to present criticism, address controversy or provide a personal angle on a significant issue. Authors of perspectives are encouraged to be opinionated, with all positions concisely and clearly argued and referenced. Referees will be briefed to review these articles for quality and relevance of argument only. They will not necessarily be expected to agree with the author's position.

Word limit: 4000–6000 words (excluding abstract, executive summary, references and figure/table legends)

Required sections (for a more detailed description of these sections see Article sections):

 Title
 Author(s) names & affiliations
 Abstract
 Keywords
 Body of article
 Future perspective
 Executive summary
 References: target of approximately 80 references
 Reference annotations
 Financial disclosure/Acknowledgements

Special Reports

Special reports are short review-style articles that highlight a particular niche area, be it a specific emerging field, novel hypotheses or method. Articles are categorized as Special Reports at the discretion of the Editorial team.

Word limit: 1500–3000 words (excluding abstract, executive summary, references and figure/table legends)

Required sections (for a more detailed description of these sections see Article sections):

 Title
 Author(s) names & affiliations
 Abstract
 Keywords
 Body of article
 Future perspective
 Executive summary
 References: target of approximately 50 references
 Reference annotations
 Financial disclosure/Acknowledgements

Original Research Articles

Authors of original research **must** provide a supporting Cover Letter on submission briefly detailing:

 Relevance to the journal's audience
 Where the novelty in the study lies
 How the study advances understanding of the field
 Direct and potential implications of the findings

NB. All Future Medicine journals will consider studies presenting positive, negative or inconclusive data.

Authors are also advised to consult the **Methods Reporting Checklist for Authors**, available here.

Experimental details & data: Only where a novel experimental procedure has been employed full details must be provided, such that a skilled scientist would be able to reproduce the results presented. The synthesis of all new compounds must be described in detail. Details of routine or previously reported experimental procedures should be provided via references only. Experimental procedures and/or data running to more than two Word document pages should be placed in a supplementary information file.

Data sharing: If requested by the Editor or reviewers, authors should be able to provide additional relevant original data underpinning their research. Version: 4th May 2016

Clinical Trial reporting: For authors presenting the results of clinical trials, the guidelines recommended by CONSORT (http://www.consort-statement.org/) and GPP3 (http://www.ismpp.org/gpp3) should be followed. In addition, where available the clinical trial registration number should be included at the end of the abstract, and on the first mention of the trial in the main body of text. Unregistered clinical trials should be declared as such, and the reason for nonregistration should be provided. Mention of other trials should also include the relevant registration number, where available.

Secondary outcomes, exploratory analyses, and *post hoc* analyses should be clearly identified as such; these may be included in the primary publication or published separately, in which case they should clearly reference the primary publication and should not be published before it.

Observational studies: where observational research has been carried out, authors should follow the recommendations of STROBE (http://www.strobe-statement.org/).

Data deposition: where data have been deposited in a public repository, authors should state at the end of the abstract the data set name, repository name and number.

Word and figure/table limit: Limits vary depending on journal title. Please contact the relevant Commissioning Editor for further details.

Required sections (for a more detailed description of these sections see Article sections):

- Title
- Author(s) names & affiliations
- Structured abstract
- Keywords
- Introduction
  - Should only cite directly pertinent references
  - Should not include data of conclusions from the work being reported
- Patients & methods/Materials & methods
  - Where an organization was paid or otherwise contracted to help conduct the research (e.g., data collection and management), this should be detailed
  - Should include information indicating that the research was approved or exempted from the need for review by the responsible review committee (institutional or national). Where no formal ethics committee is available, a statement indicating that the research was conducted according to the principles of the Declaration of Helsinki should be included
  - Information on the selection and description of participants should define how authors measured race or ethnicity and justify their relevance
- Results
  - Numeric results should be given not only as derivatives (e.g., percentages) but also as the absolute numbers from which the derivatives were calculated
  - Statistical significance of results should be specified, if any
- Discussion

- Authors should distinguish between clinical and statistical significance, and avoid making statements on economic benefits and costs unless the manuscript includes the appropriate economic data and analyses
- Authors should avoid claiming priority or alluding to work that has not been completed

 Conclusions

 Summary points

 References

 Reference annotations

 Financial disclosure/Acknowledgements

 Ethical conduct of research

Four types of research article are accepted:

Full research article

Research articles should present novel work that makes a significant impact within the scope of the journal, and which represents an important advancement in knowledge or understanding. Routine or incremental work is not suitable for full research papers. Research should be reported succinctly; the inclusion of detailed background discussion is to be avoided. Supporting data or further experimental details can be submitted as Supplementary Information.

Preliminary communication

Preliminary communication articles are intended for short reports of studies that present promising improvements or developments on existing areas of research. The significance and potential implications of the developments must be explicit.

Short communication

Short communication articles are short, peer-reviewed articles that build on a previously published study, document partial research results from an ongoing study, or discuss results from studies limited in scope.

Methodology

Methodology articles should provide an overview of a new experimental or computational method, test or procedure. The method described may be either completely novel, or may offer a demonstrable improvement on an existing method. The significance and potential implications of the developments must be explicit.

Case Studies/Case Series

Case studies/series present a notable medical case or series of related cases of interest, and aim to further the reader's understanding of the issues relating to such situations.

Word limit: 1500–3000 words

Required sections (for a more detailed description of these sections see Article sections):

 Title

 Author(s) names & affiliations

 Abstract

 Keywords

 Body of the article. A suggested structure could be:

- Presentation of case – setting and patient details/history
- Initial diagnosis/assessment
- Treatment/management
- Outcome and implications

 Discussion/conclusion

 Summary points

 References

 Reference annotations

 Financial disclosure/Acknowledgements
 Ethical conduct of research

Editorials

Editorials are short articles that provide an insight into, or snapshot of issues of topical importance to the journal's target audience or researchers and other professionals. The intention is that the article should offer an expert perspective on a topic of recent interest. More detailed discussions can take the form of Commentary articles.

Word limit: 1500 words maximum (excluding keywords and references).

Required sections (for a more detailed description of these sections see Article sections):

 Title
 Author(s) names & affiliations
 Photo (headshot) of authors (including up to one co-author if desired)
 Keywords
 Body of article
 References: **Please note:** A maximum of **20 references** are permitted
 Financial disclosure/Acknowledgements
 Please note: No figures, tables or boxes are permitted in editorials

Commentaries

Commentaries are short articles that are similar to Editorials, yet provide a more detailed discussion of a topic.

Word limit: 1500–3000 words (excluding keywords and references).

Required sections (for a more detailed description of these sections see Article sections):

 Title
 Author(s) names & affiliations
 Photo (headshot) of authors (including co-authors if desired)
 Keywords
 Body of article
 References: **Please note:** A maximum of **20 references** are permitted
 Financial disclosure/Acknowledgements
 Please note: No figures, tables or boxes are permitted in commentaries

Interviews

Interviews are conducted with key opinion leaders in the field, and can include a look back over their career and achievements to date, a discussion on their current research, and their thoughts and observations on the field as a whole.

Word limit: 1500 words

Required sections:

 Title
 Interviewee name & affiliation
 Photo (headshot) of the interviewee
 Summary/biographical paragraph
 Series of questions for discussion (provided by the journal's Commissioning Editor)
 Response from the author to each point
 Additional reference sources for the interested reader

Priority Paper Evaluations

Priority paper evaluations review significant, recently published original research articles carefully selected and assessed by specialists in the field (not a paper from the author's own group). The original research detailed in the chosen paper is discussed with the aim of keeping readers informed of the most promising discoveries/breakthroughs relevant to the subject of the journal through review and comment from experts. Priority Paper Evaluations are intended to extend and expand on the information presented in the original publication, putting it in context and explaining why it is of importance. The ideal article will provide both a critical evaluation and the author's opinion on the quality and novelty of the information disclosed.
Word limit: 1500 words maximum (excluding abstract, keywords and references).
Required sections (for a more detailed description of these sections see Article sections):
 Title
 Author(s) names & affiliations
 Abstract
 Keywords
 Summary of methods and results
 Discussion
 Future perspective
 Executive summary
 References: **Please note:** a maximum of **20 references** are permitted in priority paper evaluations
 Reference annotations
 Financial disclosure/Acknowledgements
 Figures/tables: if necessary, only **one of each** is permitted

Research Highlights

Research highlights discuss a number of recent original research papers, summarizing and commenting on each paper to give readers a real sense of the cutting edge of research in the field.
Word limit: 3–4 brief summaries on recent research of 200–500 words each (excluding references).
Required sections:
 Citation of original research paper
 Summary paragraph
 References: **Please note:** A maximum of **20 references** are permitted
 Financial disclosure/Acknowledgements
 Please note: No figures, tables or boxes are permitted in research highlights

Conference Scenes

Conference scenes aim to summarize the most important research presented at a recent relevant meeting or event. It is not usually feasible to attempt comprehensive coverage of the conference; authors should therefore focus on those presentations that are most topical, interesting or thought-provoking.
Word limit: 1500 words maximum (excluding abstract, conference details and references).
Required sections:
 Conference details (title, date, location)
 Abstract/overview of meeting (120 words maximum)
 Body of article
 References: **Please note:** A maximum of **20 references** are permitted
 Financial disclosure/Acknowledgements
 Please note: No figures, tables or boxes are permitted in conference scenes

Company Profiles

Company profiles allow representatives from pharmaceutical, biotechnology, etc. companies to describe the work currently being carried out within their particular organization, relevant to the field of the journal in question. These reports are intended to provide an insight into the history and strategy of a company and profile its corporate capabilities, advanced technologies and future potential.

Word limit: 2000 words

Required sections (for a more detailed description of these sections see Article sections):

- Title
- Author(s) names & affiliations
- Abstract
- Keywords
- Introduction – brief factual account of the history and strategy of the company including background information e.g., the year the company was founded, number of employees etc.
- Body of article
- Summary points
- Financial disclosure/Acknowledgements
- **Please note:** A maximum of **20 references** are permitted
- Figures/tables: if necessary, only **one of each** is permitted

Letters to the Editor

Readers may submit Letters to the Editor, commenting on an article published in the journal.

Word limit: 1500 words

Inclusion of Letters to the Editor in the journal is at the discretion of the Editor. All Letters to the Editor will be sent to the author of the original article, who will have 28 days to provide a response to be published alongside the Letter.

Drug, Device & Vaccine Evaluations

Separate author guidelines for the submission of these article types are available. Please contact the Drug Evaluations Commissioning Editor to request a copy.

Clinical Trial Protocols & Clinical Trial Evaluations

Separate author guidelines for the submission of these article types are available. Please contact the Drug Evaluations Commissioning Editor to request a copy.

Article sections

The following list provides notes on the key article sections; authors should consult the 'at-a-glance formatting checklist' to determine which sections are required for their submission.

Title

Concisely and clearly conveys the scope/novelty of the article; not more than 120 characters.

Author(s) names & affiliations

Including full name, address and e-mail.

Guidance on author sequence:

Author sequence is at the authors' discretion; however, Future Medicine journals suggest following the recommendations in GPP3 Appendix Table 2 (http://www.ismpp.org/gpp3), whereby authors are listed either in order of the level of their contribution, or alphabetically. The corresponding author should always be indicated.

Guidance on a change of affiliation during writing:

Where an author has changed their affiliation prior to the publication of an article, the affiliation should reflect where the major part of the work was completed. Current affiliation and contact information should be listed in an acknowledgement.

Abstract

Not more than 120 words; no references should be cited in the abstract. The abstract should highlight the importance of the field under discussion within the journal's scope, and clearly define the parameters of the article.

Structured abstract (for Research articles)

Not more than 120 words, broken down into Aims, Patients & Methods/Materials & Methods, Results and Conclusions. For authors presenting the results of clinical trials, the guidelines recommended by CONSORT should be followed when writing the abstract (http://www.consort-statement.org/), and the clinical trial registration number included at the end of the abstract, where available. Data deposition: where data have been deposited in a public repository, authors should state at the end of the abstract the data set name, repository name and number.

Keywords

Up to 10 keywords (including therapeutic area, mechanism[s] of action etc.) plus names of drugs and compounds mentioned in the text.

Body of the article

The article content should be arranged under relevant headings and subheadings to assist the reader.

Future perspective

The author is challenged to include speculative viewpoint on how the field will have evolved 5–10 years from the point at which the article was written.

Executive summary

A series of bulleted summary points that illustrate the main topics or conclusions made under each of the main headings of the article.

Summary points (Research articles & Company profiles only)

8–10 bullet point sentences highlighting the key points of the article.

Priority Paper Evaluations

Priority paper evaluations review significant, recently published original research articles carefully selected and assessed by specialists in the field (not a paper from the author's own group). The original research detailed in the chosen paper is discussed with the aim of keeping readers informed of the most promising discoveries/breakthroughs relevant to the subject of the journal through review and comment from experts. Priority Paper Evaluations are intended to extend and expand on the information presented in the original publication, putting it in context and explaining why it is of importance. The ideal article will provide both a critical evaluation and the author's opinion on the quality and novelty of the information disclosed.

Word limit: 1500 words maximum (excluding abstract, keywords and references).

Required sections (for a more detailed description of these sections see Article sections):

- Title
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- Keywords
- Summary of methods and results
- Discussion
- Future perspective
- Executive summary
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 Summary points
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Including full name, address and e-mail.

Guidance on author sequence:

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Guidance on a change of affiliation during writing:

Where an author has changed their affiliation prior to the publication of an article, the affiliation should reflect where the major part of the work was completed. Current affiliation and contact information should be listed in an acknowledgement.

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Not more than 120 words; no references should be cited in the abstract. The abstract should highlight the importance of the field under discussion within the journal's scope, and clearly define the parameters of the article.

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A series of bulleted summary points that illustrate the main topics or conclusions made under each of the main headings of the article.

Summary points (Research articles & Company profiles only)

8–10 bullet point sentences highlighting the key points of the article.

Financial disclosure/Acknowledgements

Disclosing any financial and/or material support that was received for the research or the creation of the work. Also disclosing any relationships any authors have (personal, academic or financial relationships that could influence their actions) or financial involvement with an organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. If writing assistance has been used in the creation of the manuscript, this should also be stated and any sources of funding for such assistance clearly identified.

Ethical conduct of research

For studies involving data relating to human or animal experimental investigations, authors should obtain appropriate institutional review board approval and state this within the article (for those investigators who do not have formal ethics review committees, the principles outlined in the Declaration of Helsinki should be followed). In addition, for investigations involving human subjects, authors should obtain informed consent from the participants involved and include an explanation of how this was obtained in the manuscript.

References

Key points

 Authors should focus on recent papers and papers older than 5 years should not be included except for an over-riding purpose.

 Primary literature references, and any patents or websites, should be numerically listed in the reference section in the order that they occur in the text (including any references that only appear in figures/tables/boxes).

 Information from manuscripts submitted but not accepted should be cited in the text as “unpublished observations” with written permission from the source.

 Avoid citing a “personal communication” unless it provides essential information not available from a public source, in which case the name of the person and date of communication should be cited in the text, with written permission from the source.

 References should be denoted numerically and in sequence in the text, using Arabic numerals placed in square brackets, i.e., [12].

 Quote first six authors’ names. If there are more than six, then quote first three *et al*.

 Reference annotations: 6–8 references should be highlighted that are of particular significance to the subject under review as “* of interest” or “** of considerable interest”, along with a brief (1–2 line) synopsis.

 The Future Medicine Reference Manager and EndNote styles can be downloaded from our website at: www.futuremedicine.com/page/authors.jsp

Format

 Author’s names should appear without full stops in their initials

 Quote first six authors’ names. If there are more than six, then quote first three *et al*.

 A full stop follows authors’ names

 Journal name should be in italics and abbreviated to standard format

 Volume number followed by comma, not bold

 Page number range separated by a hyphen with no spaces, followed by the year in brackets, and then a full stop

Examples

Journal example:

Fantl JA, Cardozo L, McClish DK *et al*. Estrogen therapy in the management of urinary incontinence in postmenopausal women: a meta-analysis. *Obstet. Gynecol.* 83(1), 12–18 (1994).

Book example:
De Groat WC, Booth AM, Yoshimura N. Neurophysiology of micturition and its modification in animal models of human disease. In: *The Autonomic Nervous System (Volume 6).* Andrews WR (Ed.), Harwood Academic Publishers, London, UK, 227–289 (1993).
Meeting abstract example:
Smith AB, Jones CD. Recent progress in the pharmacotherapy of diseases of the lower urinary tract. Presented at: *13th International Symposium on Medicinal Chemistry*. Atlanta, GA, USA, 28 November–2 December 1994.
Patent example:
Merck Frosst Canada, Inc. WO9714691 (1997).
(Use the following formats for patent numbers issued by the World, US and European patent offices, respectively: WO1234567, US1234567, EP-123456-A).
Website example (organization homepage):
US Food and Drug Association.
www.fda.gov
Website example (specific webpage/document):
American Cancer Society. Cancer Facts and Figures 2015 (2015). www.cancer.org/research/cancerfactsstatistics/cancerfactsfigures2015/index
Milenkovic M, Russo CA, Elixhauser A. Hospital stays for prostate cancer, 2004. HCUP Statistical Brief #30. Agency for Healthcare Research and Quality, MD, USA (2007).
www.hcup-us.ahrq.gov/reports/statbriefs/sb30.pdf

Reference annotations

Papers or of particular interest should be identified using one or two asterisk symbols:
 * = of interest
 ** = of considerable interest

Each of the chosen references should be annotated with a brief sentence explaining why the reference is considered to be of interest/particular interest.

Figures, tables & boxes

Summary figures, tables and boxes are very useful, and we encourage their use in certain article types (see above section on Article types for details on which articles can include figures/tables/boxes). The author should include illustrations to condense and illustrate the information they wish to convey. Commentary that augments an article and could be viewed as 'stand-alone' should be included in a separate box. An example would be a summary of a particular trial or trial series, a case study summary or a series of terms explained. Figures, tables and boxes should be numbered consecutively according to the order in which they have been first cited in the text. All abbreviations used within them should be defined in the legend. All figures, tables and boxes should be submitted in an **editable format**. Ideally these should be sent as Microsoft Word, Microsoft Excel or Adobe Illustrator files. If you are uncertain whether the format of your files is appropriate, please check with the Commissioning Editor. It is unnecessary to incorporate copies into the body of the manuscript; however, they should be included at the end of the document where possible. Please ensure that **scale bars** are included in figures where appropriate (i.e., photomicrographs). Symbols, arrows or letters used in photomicrographs should contrast with the background. Please explain internal scale and identify the method of staining in photomicrographs.
If any of the figures, tables or boxes used in the manuscript requires permission from the original publisher, it is the author's responsibility to obtain this. More details on obtaining permissions can be found in the copyright section below.

Supplementary information

Figure, tables and boxes larger than one A4 page will be included as online-only supplementary information. At the Editor's discretion data or experimental details can also be included.

Color figure charge

Future Medicine has a charge for the printing of color figures (i.e., each color page) in the print issue of the journal. We have no page charges, unlike some other publishers, and aim to keep our color charge to a minimum. This charge does not apply to the online (including PDF) version of articles, where all figures appear in color at no charge.

Chemical structures

If possible, please submit structures drawn in ISISDraw or Chemdraw format. However, chemical structures can be redrawn in-house. Please use the following conventions:

 Always indicate stereochemistry where necessary – use the wedge and hash bond convention for chiral centers and mark cis/trans bonds as such.

 Draw small peptides (up to five amino acids) in full; use amino acid abbreviations (Gly, Val, Leu, etc.) for larger peptides.

 Refer to each structure with a number in the text; submit a separate file (i.e., not pasted throughout the text) containing these numbered structures in the original chemical drawing package that you used.

Electronic files

Please submit any other illustrations/schemes in an editable electronic format such as Illustrator, PowerPoint, Excel or as postscripted/encapsulated postscripted (.ps/.eps) files. Photos should be provided at a resolution of 600 dpi, or as high as possible.

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