**UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL FACULDADE DE ODONTOLOGIA**
**PROGRAMA DE PÓS - GRADUAÇÃO NÍVEL DE MESTRADO**
**ÁREA DE CONCENTRAÇÃO EM CLÍNICA ODONTOLÓGICA**
**CARIOLOGIA/DENTÍSTICA**

**Guilherme Stein Porto Alegre**

# CARACTERIZAÇÃO DO POTENCIAL ANTIFÚNGICO DE NOVOS SAIS IMIDAZÓLICOS EM *CANDIDA ALBICANS*: ESTUDO IN VITRO

Porto Alegre, dezembro de 2018

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# CARACTERIZAÇÃO DO POTENCIAL ANTIFÚNGICO DE NOVOS SAIS IMIDAZÓLICOS EM *CANDIDA ALBICANS*: ESTUDO IN VITRO

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*“Don’t raise your voice, improve your argument.”*

***Desmond Tutu.***

## Sumário

*Resumo* ..... *8*
*Abstract* ..... *9*
*Antecedentes e Justificativa* ..... *10*
**Candida albicans** ..... **10**
**Candidíase Oral** ..... **12**
**Estomatite relacionada à dentadura** ..... **14**
**Tratamentos convencionais** ..... **16**
**Liquidos lônicos** ..... **17**
*Objetivos* ..... *19*
*Artigo 1* ..... *20*
*Referências* ..... *29*
*Artigo 2* ..... *33*
*Referências* ..... *40*
*Considerações Finais* ..... *43*
*Referências bibliográficas* ..... *44*

## Resumo

O aumento de episódios de infecções fúngicas acarreta diversos fatores que impactam diferentes esferas no âmbito da área da saúde. Dentre as infecções fúngicas mais recorrentes da cavidade bucal, a candidíase oral é a patologia mais frequente destas. O microrganismo isolado mais observado nessas infecções é a *Candida albicans*, o qual vive em uma relação comensal em inúmeras localidades do corpo humano, até mesmo em indivíduos com um bom estado de saúde geral. Quando, por algum fator predisponente, ocorre um aumento no crescimento das taxas deste microrganismo, este, então pode causar a candidíase oral, uma infecção fúngica oportunista. Ao comparar o número de terapias disponíveis para o profissional realizar o manejo de uma infecção fúngica com o número de medicamentos antibióticos, por exemplo, o primeiro é bem mais restrito do que o segundo. Isso pode acarretar, ao tratar uma infecção, resistência por parte do patógeno ao tratamento realizado, mesmo que de forma correta. A necessidade de alternativas a terapias convencionais foi o que motivou a realização deste trabalho, visando explorar uma nova alternativa como conduta nesses casos. Estudos prévios realizados apontaram características com um grande potencial de sais imidazólicos como forma de tratamento em algumas situações. Neste estudo foi observado um efeito fungicida e fungistático por parte destas substâncias quando avaliadas com relação a um isolado laboratorial de *Candida albicans* (ATCC90008). Ao avaliar os compostos $C_{18}$MImCl, $C_{10}$MimCl, $C_{16}$MimMeS, $C_{16}$MimCl e $C_{16}$DMImMeS, a menor concentração inibitória mínima (CIM) e concentração fungicida mínima (CFM) foram encontradas pelo primeiro composto nos testes de suscetibilidade. Nenhuma diferença estatística no teste de biofilme em formação foi encontrada entre os compostos e no teste de biofilme pré-formado, por mais que tenha sido observada diferença estatística dentre os compostos, em testes posteriori, não encontrou-se diferença entre $C_{16}$MImCl e $C_{16}$DMImMeS. Ao caracterizar sua ação em biofilme em formação de *Candida albicans* em blocos de resina acrílica, simulando uma base de prótese dentária, encontrou-se um melhor controle da formação de biofilme no composto $C_{18}$MImCl sendo estatisticamente diferente dos outros compostos e grupos controles.



**Palavras-chave:**

Sais imidazólicos; antifúngicos; candidíase oral.

**Abstract**

The rise of episodes of fungal infections evolves several factors that affect different spheres in the health area. Among the most frequent fungal infections of the oral cavity, oral candidiasis is the most frequent pathology among all. The isolated microorganism most observed in these infections is *Candida albicans*, which lives in a commensal relation in numerous localities of the human body, even in individuals with a good general health state. When, for some reason some predisposing factor, increases growth rates of this microorganism, oral candidiasis may occur, considered an opportunistic fungal infection. When comparing the number of therapies available for the professional to manage a fungal infection with the number of antibiotic medications, for example, the first scenario is much more restricted than the second. This can cause, when treating an infection, resistance by the pathogen to the treatment performed, can happen, even if correctly. The need for alternatives to conventional therapies was what motivated the accomplishment of this work, aiming to explore a new alternative as conduct in these cases. Previous studies have pointed out characteristics with a great potential of imidazolium ionic liquids as a form of treatment in some situations. In this study a fungicidal and fungistatic effect of these substances was observed when evaluated in relation to a laboratory isolate of *Candida albicans* (ATCC90008). Evaluating the compounds $C_{18}$MImCl, $C_{10}$MimCl, $C_{16}$MimMeS, $C_{16}$MimCl and $C_{16}$DMImMeS the lowest minimum inhibitory concentration (CIM) and minimum fungicidal concentration (MFC) were found by the first compound in the susceptibility tests. No statistical difference in the formation biofilm test was found between the compounds and in the pre-formed biofilm test, even though a statistical difference was observed among the compounds in posteriori tests, there was no difference between $C_{16}$MimCl and $C_{16}$DMImMeS. Characterizing its biofilm action in the formation of *Candida albicans* in blocks of acrylic resin, simulating a dental prosthesis base, a better control of the biofilm formation in the $C_{18}$MImCl compound was found to be statistically different from the other compounds and control groups.

**Key words:**

Imidazolium salts; antifungal; oral candidiasis.

## Antecedentes e Justificativa

### *Candida albicans*

O gênero *Candida* é considerado um fungo frequentemente encontrado em diversas localidades do corpo, considerado um microrganismo normal do microbioma humano encontrado na cavidade oral, mesmo em pacientes saudáveis. A incidência deste microrganismo ser encontrado nesta cavidade pode variar entre toda população, sendo reportados taxas de variando entre 35%-80% Dentre todas as localidades do corpo humano, a *Candida albicans* representa o microrganismo com maior taxa de incidência dentre todas espécies de *Candida*, sendo isolado com uma taxa de 70% (Odds, 1988).

Infecções fúngicas causadas pela espécie de *Candida*, continua sendo a causa mais comum no mundo todo. Essas infecções causadas por *Candida* não são somente prevalentes, mas também podem ser associadas com consideráveis taxas de morbidade e mortalidade como apresentados em um estudo realizado por Wey *et al.* em 1988 na cidade de Iowa, nos Estados Unidos em um hospital da cidade, onde a taxa de mortalidade de infecções no sistema sanguíneo causadas por *Candida* foi de 38%, neste mesmo estudo, os autores constataram que dos casos avaliados, 61% das infecções foram causadas por *Candida albicans*.

Um estudo realizado por Soll no ano de 2002 afirmou que a infecção fúngica sistêmica mais comum é a candidíase, além disso é apontada como mais da metade dos casos de micoses invasivas. A *C. albicans* é considerada como sendo responsável pela causa da maioria destas infecções e que tal fato pode ser atribuído a capacidade deste microrganismo viver em uma relação comensal benéfica em diferentes localizações do corpo humano, mas que pode ser facilmente encontrada na cavidade bucal, por exemplo.

A *Candida albicans*, patógeno fúngico oportunista, pode ser encontrada em mucosa oral de pacientes com bom estado de saúde e em trato gastrointestinal normal de pacientes considerados como saudáveis. Porém, alguns fatores como, a relação da deficiência ou excesso de nutrientes provindos da dieta, podem estar ligados com alterações endógenas na flora microbiana, fatores mecânicos também podem desencadear uma alteração no sistema e permitir com que o número de microrganismos

se replique. Estas alterações, em pacientes imunocomprometidos como HIV positivo, nascidos prematuros, transplantados são extremamente facilitadas devido à alta suscetibilidade à infecções fúngicas (Biswas *et al*. 2007).

Um microrganismo polimórfico pode ser considerado quando esse, tem a capacidade de crescer em diferentes formas, alterando significativamente sua morfologia. Esse mecanismo que permite com que o microrganismo tenha o poder em alternar formas, possui uma grande relação quanto à sua patogenicidade, por exemplo. Fatores como pH e temperatura apresentam grande impacto no controle da morfologia da *Candida albicans*, podendo ser esses, considerados fatores ambientais (Yokoyama *et al*. 1994), estudos demonstram também que dependendo do pH a morfologia da *Candida* pode alterar, um pH mais ácido (pH<6) as células *de Candida albicans* tem a tendência de crescimento em forma de levedura, já em um pH mais básico (ph>7) associasse mais frequentemente o crescimento em forma de hifa (Odds, 1988; Sudbery, 2011).

A transição da forma de crescimento (levedura ou hifa) está fortemente ligado por uma rede de sinais que pode estimular sua ativação por desencadeamento de algum tipo de estímulo como inanição e aderência. Essas alterações morfológicas fazem com que a *C. albicans* possa ser considerado um fungo com a característica de dimorfismo, mas, especialmente, falando desse microrganismo, tem o poder de adotar um considerável espectro de morfologias, pode também ser considerado um fungo polimórfico ou pleomórfico pois pode crescer nas formas de levedura, hifas e também em pseudo-hifas. Alterações morfológicas deste microrganismo podem ser consideradas como respostas a estímulos ambientais, permitindo uma adaptação em variados nichos biológicos (Biswas *et al*. 2007).

O aumento do número de pacientes com alta suscetibilidade a infecções fúngicas tem um grande impacto sobre a razão do aumento dessas taxas de décadas passadas para o presente e não pelo motivo da existência de maiores variedades de patógenos (Balkis *et al.*, 2002).

No mesmo estudo de (Soll, 2002), o autor explica que pelo fato deste microrganismo ser um patógeno oportunista que vive em uma relação benéfica de comensalismo, pode acabar tendo como resposta às alterações do sistema do hospedeiro um mecanismo que permite um alto crescimento nas cavidades e pode penetrar nos tecidos, comprometendo todo sistema imunológico alterando a fisiologia dos tecidos do hospedeiro. Quando em condições normais, esse microrganismo é considerado

inofensivo, uma vez que é encontrado, como citado anteriormente, em pacientes saudáveis.

Uma característica importante da *Candida albicans* que deve ser citada é o conjunto de proteínas específicas que conferem a aderência deste microrganismo a outros, células do hospedeiro e até mesmo superfícies, estas proteínas são chamadas de adesinas (Garcia *et al.* 2011).

Dentre as adesinas estudadas, a mais relacionada com *C. albicans* são as aglutininas (ALS) que consistem em oito diferentes proteínas, na fase de adesão a mais importante das aglutininas é a Als3 que tem relação com a forma de hifa, essas proteínas possuem a capacidade de codificar moléculas de glicosilfosfatidilinositol (GPI) que possuem a capacidade de se ligar com glicoproteínas da superfície de diferentes células (Zordan, 2012). Outra adesina que vale ressaltar é a Hwpl que também tem associação com a forma de hifa e também possui ligação com GPI e acaba servindo como substrato para que ocorra uma reação de ligação covalente com transglutaminases (Staab *et al.* 1999). Ambas adesinas citadas anteriormente estão relacionadas com a contribuição de formação do biofilme atuando, então, como adesinas complementares (Nobile *et al.* 2008).

A *Candida albicans* pode utilizar dois mecanismos distintos para invadir células do hospedeiro, pode invadir por penetração ativa ou endocitose enduzida (Zakikhany, 2007).

Outro fator de virulência que deve ser citado sobre a *C. albicans* é a capacidade deste microrganismo em formar biofilme tanto em superfícies bióticas quanto abióticas (Fanning & Mitchell, 2012), para a ocorrência deste diferente ecossistema que é o biofilme, a formação ocorre por uma sequência lógica, começando com a etapa de adesão de células da levedura a um substrato, a partir dessa aderência, ocorre então a proliferação de células e então uma mudança na sua morfologia para a forma de hifa na parte superior do biofilme, ocorre também um acúmulo de material na matriz extracelular e então uma dispersão destas células no complexo que é o biofilme (Finkel &Mitchell, 2011).

### *Candidíase Oral*

A candidíase oral pode ser classificada como uma infecção fúngica causada pelo patógeno oportunista do gênero *Candida*, sendo muito comum em pacientes idosos,

porém pode acometer também crianças, com pouco ou ausência de um bom estado de saúde oral, podendo ocorrer também mais facilmente em indivíduos imunocomprometidos, como pacientes diabéticos, HIV positivos ou em pacientes oncológicos. Essa patologia ocorre pelo aumento no crescimento de *Candida spp.* sendo o microrganismo isolado mais comum a *Candida albicans* (Epstein, 1990).

O impacto causado pelo aumento no crescimento de *Candida spp.* pode ser observado através de um desconforto local, disfagia (resultando até mesmo em má nutrição) e alterações gustativas, por exemplo, se não tratada corretamente, através desta porta de entrada, esse microrganismo pode se disseminar para o sistema sanguíneo e então causar infecções mais severas com consideráveis taxas de morbidade e mortalidade (Fraser *et al*. 1992).

Cuidado com o paciente durante o manejo de infecções é de extrema importância para o sucesso do tratamento, um estudo realizado por Morgan *et al.* 2001, constatou que por requisição da equipe de enfermagem, trinta porcento da equipe médica estudada prescreveria nistatina para o tratamento da candidíase oral, sem mesmo avaliar o paciente.

Diversos fatores de risco podem predispor a ocorrência de candidíase oral, podendo ser divididos quanto ao patógeno, onde a *Candida spp.* é considerada um microrganismo eucariótico com uma parede celular recobrindo a membrana plasmática, onde nesta membrana, possui uma alta quantidade de ergosterol, este microrganismo possui a capacidade de metabolizar glicose em ambientes tanto aeróbios quanto anaeróbios (Lehmann, 1998).

Fatores relacionados ao hospedeiro, tanto locais quanto sistêmicos, também possuem um grande impacto, como o funcionamento regular das glândulas salivares onde estas, tem um efeito de tamponar, e remover microrganismo da cavidade oral e mucosa (Peterson, 1992), medicamentos que possam reduzir a imunidade ou fluxo salivar, fagocitose e alterar a microflora oral também podem influenciar na ocorrência de candidíase oral (Milne & Crompton, 1974). Outros fatores como fumar, diabetes, algumas síndromes com condições de imunossupressão (HIV, leucemia e alterações nutricionais) também apresentam impacto no aparecimento desta enfermidade (Phillips *et al*. 1996).

Como citado anteriormente, uma disfunção nas glândulas salivares pode predispor a condição de ocorrer candidíase oral, uma vez que a saliva possui uma quantidade rica em lactoferrina, polipeptídeos, lisozimas e sialoperoxidaze, substâncias as quais auxiliam na atuação da inibição do crescimento exagerado de *Candida spp.* (Turner & Ship, 2008).

Substâncias com ação tópica as quais possuam altos níveis de corticosteroides, o uso irracional de bochechos antimicrobianos, podem temporariamente alterar e suprimir a microbiota da cavidade oral e facilitar a ocorrência desta patologia (Jainkittivong *et al*. 2007).

Estudos prévios como o de Lalla *et al*. em 2013, constatou que candidíase oral pode ser encontrada em 5-7% de pacientes infantis, 20% em pacientes com câncer e varia entre 9 até 31% de pacientes HIV positivos.

A candidíase oral pode se manifestar em diferentes formas de infecção, um diagnóstico correto deve ser realizado pelo profissional para que o tratamento seja eficaz, uma dessas formas é a candidíase pseudomembranosa, pode ser apresentada como uma infecção aguda ou também crônica, devido a casos recorrentes (Patil *et al*. 2015). Na mucosa oral ela apresenta uma placa de coloração branca-amarelada com textura cremosa, essa placa, que pode ser removida superficialmente, porém revelando uma superfície eritematosa que frequentemente pode ocorrer o sangramento, é formada por principalmente células epiteliais descamadas, uma rede emaranhada de fibrina, material necrótico e microrganismos no formato de hifa (Lalla *et al*. 2013).

Candidíase eritematosa pode aparecer de ambas formas, aguda ou crônica, normalmente é relacionada com o uso prolongado de antibióticos de amplo espectro (Farah *et al.* 2010), clinicamente possui sintomatologia dolorosa localizada na área eritematosa, é a única forma de candidíase que possui estreita relação com dor, lesões no dorso da língua podem ocorrer com áreas com ausência de papilas, porém, pode se localizar também no palato (Dodd *et al.* 1991).

A candidíase hiperplásica se apresenta geralmente de forma crônica, clinicamente, pode ser observado uma placa esbranquiçada aderida ou de forma eritematosa, porém diferentemente da candidíase pseudomembranosa, essa, a placa não sai à raspagem superficial. É comumente encontrada de maneira bilateral em comissura labial na mucosa bucal (Sanketh *et al.* 2015).

### *Estomatite relacionada à dentadura*

Lesões na mucosa relacionadas com contato da superfície de uma prótese dentária estão fortemente ligadas com características relacionadas à uma infecção por *Candida*, como placa na superfície desta prótese, desadaptação da mesma ou até mesmo algum tipo de trauma na cavidade bucal (Freitas *et al*. 2008).

Dentre essas, estomatite causada pelo uso de prótese é a lesão que deve ser considerada de maior importância clínica e com uma forte ligação à infecções causadas por *Candida spp*. Visto que o uso de dentadura pode predispor pacientes a possuírem lesões na mucosa devido a diversos fatores etiológicos (Fleishman *et al*. 1985).

Um estudo desenvolvido por Jainkittivong *et al.* 2002, constatou que ao se comparar indivíduos usuários de próteses totais com indivíduos com próteses fixas, lesões na mucosa bucal eram muito mais prevalentes no primeiro caso do que no segundo, devido ao desenvolvimento de um ambiente mais propício para tal condição.

Outro estudo realizado no ano de 2013 por Altarawneh *et al*. encontrou como resultados do trabalho uma quantidade que chegou a ser vinte vezes maior o número de *Candida albicans* de pacientes com candidíase relacionada à dentadura do que pacientes sem essa condição.

O material que constitui a superfície de uma prótese, pode servir como uma espécie de nicho ou de meio de cultura para que se cultive ali e gere um foco de infecção fúngica (Campos *et al.* 2008).

Pode ser considerada como uma inflamação crônica da mucosa que está em contato com a área que abrange uma prótese dentária, associadas com candidíase (Lund *et al*. 2010).

Com altas taxas de prevalência, essas lesões chegam a ser observadas em aproximadamente entre 50 e 65% dos usuários de algum tipo de prótese. Clinicamente podem ser observados pontos de hiperemia, eritemas difusos, ou lesão com aspecto granular, pacientes com essa condição, frequentemente podem relatar uma sintomatologia dolorosa ou até mesmo uma sensação de queimadura. Diferentes fatores como uma pobre higiene oral e deficiente desinfecção protética, uso noturno da prótese, má adaptação da mesma e fluxo salivar reduzido são alguns dos fatores etiológicos que podem estar presentes (Williams & Lewis, 2011).

As propriedades da superfície do substrato de uma prótese como energia livre de superfície, rugosidade e hidrofobicidade são características extramente correlacionadas com a fase inicial de adesão de microrganismos. A relação entre medidas como ângulo de contato em diferentes superfícies e *Candida albicans* possuem uma ligação, é considerado que quanto maior a energia livre de superfície, maior a adesão dos microrganismos nessas e também, quanto mais hidrofóbica uma superfície, menor é esperado a adesão dos mesmos (Pereira et al. 2008).

***Tratamentos convencionais***

Ao analisar e realizar uma comparação da disponibilidade de terapias ao tratar uma infecção fúngica com o número de antibióticos disponíveis no mercado, o número do primeiro é bem menor do que o segundo (Lewis & Williams, 2017).

Medicamentos com ação antifúngica podem ser agrupados segundo seu modo de ação e então classificados quanto a suas atividades temos drogas como a amfotericina e nistatina, as quais possuem a capacidade de causar uma ruptura da membrana da célula fúngica, classificados como antifúngicos poliênicos. Outra classe são as substâncias classificadas como equinocandinas que possuem mecanismo de ação interrompendo a síntese de glicanos da parede celular fúngica pois acabam inibindo a enzima chamada 1,3- β – glicano sintase, como a caspofungina. Podem também interferir o processo de síntese de RNA e replicação de DNA como a flucitosina. Outro grupo que possui características muito importantes são classificados como antifúngicos do grupo azol (dentre eles os mais conhecidos nesse caso são o fluconazol e itraconazol), atuam na inbição no processo de síntese do ergosterol (Lewis & Williams, 2017).

Diferentes condutas podem ser adotadas ao iniciar o tratamento de pacientes com uma infecção fúngica, uso de 100 mg ou 200 mg de fluconazol por dia durante um período de 7 até 14 dias, outros autores consideram também a opção de tomar uma dose única contendo 750 mg de fluconazol seria equivalente ao tratamento citado anteriormente (Pappas *et al.* 2009; Hamza *et al*. 2008).

Equinocandinas, com diferentes maneiras de administração, também podem ser utilizadas como forma de tratamento destas situações, porém quando comparados com antifúngicos do grupo azol, apresentam maior resistência a tratamento (Villanueva et al. 2001).

Em um estudo realizado por Thompson *et al*., 2010, afirma que quando realizar a escolha ao tratar uma infecção com fluocitosina, embora essa apresente atividade antifúngica, rapidamente pode ser adquirida resistência a mesma e então deve ser utilizada como uma solução auxiliar para potencializar seu tratamento e não atuar de maneira protagonista.

Neste mesmo estudo feito por Thompson *et al*. 2010, foi constatado que a

resistência a terapias antifúngicas convencionais é um problema que continua sendo observado principalmente nas infecções lideradas por espécies de *Candida*, e por este motivo, pela capacidade de criar resistência até mesmo durante o tratamento correto dessas infecções, testar a resistência por parte dos microrganismos patogênicos deve ser considerada em tratamentos de casos refratários.

Outro fator que gera resistência desses microrganismos pode ser observado por exemplo na *C. albicans* durante tratamentos, com utilização de substâncias do grupo azol, com duração de longos períodos utilizando medicamentos com baixas dosagens (White *et al*. 1998). O produto resultante do gene ERG11, a enzima 14 – α – demetilase, enzima que atua na síntese de lanosterol em ergosterol, pode sofrer pequenas mutações e então ao invés de tratar, gerar resistência ao tratamento quando do uso dessas substâncias (White *et al*. 2002).

Quando avaliado métodos convencionais de desinfecção de próteses, em pacientes que são usuários, diversos métodos podem ser elencados e estudados com auxílio de diferentes substâncias/soluções, uma revisão sistemática realizada em 2017 por Papadiachou e Polyzois, onde avaliou-se, em 25 artigos, diferentes técnicas como por exemplo, uso de ultrassom, com/sem escovação com/sem agentes químicos, escovação com agente químico, somente escovação, somente uso de agentes químicos, irradiação. Os autores chegaram a conclusão de que intervenções mecânicas associadas com a utilização de agentes químicos alcançam melhores desfechos quando buscados uma boa desinfecção de uma prótese total.

### *Liquidos Iônicos*

Líquidos iônicos são considerados da família dos sais, os quais apresentam em sua fórmula um cátion orgânico e um ânion que pode ser tanto orgânico quanto inorgânico com diferentes tamanhos de cadeia, sob pressão com condições ambientais, esses compostos apresentam seu ponto de fusão sendo inferior que cem graus Celsius (Jing et al. 2016).

Estes líquidos iônicos são produtos químicos obtidos através de processos com objetivo de substituir substâncias perigosas e danosas, encontram-se dentro da classe considerada como “Green Chemistry” e possuem diferentes aplicações em diversas áreas (Plechkova & Seddon, 2008).

Em estudos previamente realizados, a nível mundial, devido à suas propriedades

desejáveis como baixo ponto de fusão, não serem inflamáveis, estabilidade química e térmica. Uma variedade de líquidos iônicos são testados devido a possibilidade de alteração de suas propriedades físico-químicas, quando alterados o tipo e a estrutura das cadeias (ânion e cátion) (Dupont *et al.* 2000).

Estes líquidos podem ser classificados de acordo com o tipo cátion orgânico e podem ser divididos entre diversas categorias, dentre elas a categoria dos líquidos iônicos imidazólicos (Wasserscheid & Waffenschmidt, 2000).

As características como hidrofobicidade e hidrofilicidade podem ser alteradas conforme alteração do tipo e estrutura do ânion e do cátion, como já citado anteriormente. Mas, geralmente, uma cadeia lateral mais curta tem a tendência de apresentar maior potencial hidrofílico (Cocalia *et al.* 2006).

Estes sais imidazólicos, possuem, em sua estrutura química, um anel imidazólico, o que lhes confere uma capacidade de interação com sistemas biológicos electrostaticamente (Anderson & Long, 2010).

Estudos prévios já foram executados com estas substâncias, porém com um olhar mais direcionado a suas características e potencial antimicrobiano, por exemplo (Petkovic, 2009).

Sua atividade visando caracterizar e analisar suas propriedades antifúngicas foram estudadas em trabalhos como no de Schrekker *et al.* 2013, onde exemplifica e gera uma hipótese para explicar seu mecanismo de ação através da enzima 14- α -demetilase, também observa a capacidade de inibição de diferentes compostos em diversas cepas de diferentes espécies de microrganismos e encontra resultados promissores utilizando pequenas concentrações. Bem como Bergamo *et al.* 2014, onde ao testar o composto $C_{16}$MImCl comparando com fluconazol, por exemplo, encontrou concentrações baixas como 0,028 *μg*/mL, capazes de inibir o crescimento celular e possuir também atividade antibiofilme.

# Objetivos

## Objetivo Geral

O objetivo do presente estudo foi de avaliar o potencial antifúngico de líquidos iônicos imidazólicos em relação cepa de *Candida albicans*.

## Objetivos Específicos

Avaliar a suscetibilidade de um isolado laboratorial de *Candida albicans* por meios de teste visando determinar concentrações inibitórias e fungicidas mínimas.

Determinar o efeito de diferentes concentrações frente a um biofilme pré-formado e biofilme em formação deste microrganismo.

Verificar o poder de desinfecção de diferentes sais imidazólicos frente a um biofilme pré-formado de *Candida albicans* em corpos de prova simulando uma base de prótese em resina acrílica.

**Artigo**

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# IMIDAZOLIUM SALTS AND ITS ANTIFUNGAL POTENTIAL AGAINST *CANDIDA ALBICANS*: AN IN VITRO STUDY

IMIDAZOLIUM SALTS AND ITS ANTIFUNGAL POTENTIAL AGAINST *CANDIDA ALBICANS*: AN IN VITRO STUDY

**Significance and impact of the study**

Necessity for new treatment alternatives against pathogens due to the rise of resistance fungus, for example, is increasingly noticeably. Oral infections caused by *Candida albicans* can be related to several factors, if this condition is recurrent, treatment resistance can be developed by these yeasts and a series of alternatives should be available to elect a conduct to cease this infection. So, this *in vitro* study, aims to evaluate the capacity of a new type of antifungal agent that could be an alternative to conventional therapies when these conditions are present.

**Abstract**

Oral candidiasis, the most frequent infection of the oral cavity, has *Candida albicans* as the main isolate, that can be found alone or in association to other species in the colonization of this condition. The widespread use of broad-spectrum antifungals can be harmful in high dosages and cause resistance by fungus during treatment of infections. Due to the small number of drugs available to be chosen by the professional, new therapies must be explored to serve as an alternative to conventional treatment of fungal infections. Imidazolium salts (IMS) has been studied before, as an alternative in different types of strains due to the capability to interact to biological systems and promising characteristics that can be interesting to pharmacological industries. Five imidazolium salts were tested, $C_{18}$MImCl, $C_{10}$MimCl, $C_{16}$MimMeS, $C_{16}$MimCl and $C_{16}$DMImMeS, towards characterizing the antifungal effect against a *Candida albicans* laboratory strain, susceptibility tests, minimum inhibitory (MIC) and minimum fungicide concentrations (MFC), biofilm formation and pre-formed biofilm tests were performed. Lowest MIC and MFC was find by $C_{18}$MimCl, 32.0±15.0*μg/mL* both. No statistical difference was found between IMS in biofilm formation even though all had fungistatic characteristics, in pre-formed biofilm $C_{16}$MImCl and $C_{16}$DMImMeS presented statistical difference between the others salts and after a post hoc (Tukey test) no difference was encountered between them towards finding which concentration was the best.

**Key words:**

Ionic liquids; antifungal; oral candidiasis.

**Introduction**

Numerous factors can justify the rise of fungal infections taxes, some of these factors are the use of corticosteroids, broadspectrum antibiotics and immunosupressants (Ortega *et al*. 2011; Holzeihemer, 2002; Lass-Florl, 2009). Due to the expansion of people immunocompromised, this scenario also increases the number of cases of fungal infections, one factor that can develop this condition is caused by mucosal or cutaneous barrier disruption, being able to cause fatal conditions on these patients (Segal *et al.* 2006). The most frequent cause of this opportunistic infections worldwide is by Candida spp. (Pfaller & Diekema, 2007). The therapeutic conduct on the treatment of these infections generally varies depending on the severity of the problem and immune condition of the patient (White *et al*. 1998).

One of the most frequent oral disease associated with *Candida albicans* is denture stomatitis. Oral candidiasis is the most common type of fungi infection of the oral cavity, candida is present in the oral flora of 53% of the population. Eighty percent of the isolates found in the oral flora corresponds to *Candida albicans*. This microorganism can colonize alone or in association with *Candida glabrata* and/or *Candida tropicalis* (found in 7% of healthy people and 80% in patients with candidiasis) (Castellote & Soriano, 2013).

*Candida albicans,* a biphasic fungus (Odds, 1988), can grow in two different forms, yeasts and hypha and can become pathogenic due to some factors that could possibly such as response to a physiological change in the oral cavity, initiating the infection process of oral candidiasis (Hebecker *et al*. 2014). The balance between the immunological mechanisms of the host, funghi and other conditions of oral microbiota maintained stable, oral colonization of *C. albicans* cannot be considered as maleficent. If this balance becomes unstable, the development of oral candidiasis may occur (Soysa & Ellepola, 2008). When its location is on the skin surface or mucous membrane, yeast form is encountered, if this microorganism invades tissues is more likely to find the hyphal form because its bigger pathogenicity (Odds, 1988).

Some predisposing factors may collaborate on this process of oral candidiasis such as the widespread use of broadspectrum of antibiotics that may disturb the oral flora (Soysa & Ellepola, 2008) and unregulated immune response due to any disorder (Farah *et al*. 2000), such as HIV (Klein *et al*. 1984), use of chemotherapy and its aggressive treatment that can promote changes of mucosa (Thompson *et al*. 2010). Oral candidiasis risk can be also increased by the reduced flow of saliva (Bratic *et al*. 2013).

The management of this condition in many cases has been controlled by topical applications or systemic administration of antifungals. The therapy to be administrated may be chosen based on several factors as oral symptoms and previous medical history of the patient. Some common conducts are nystatin oral suspension, amphotericin and miconazole (Hebecker *et al*. 2014; Farah *et al*. 2000).

Biofilm of *C. albicans* is formed typically as an association of yeasts and hyphal forms, forming a mixed biofilm (Andes *et al*. 2004), its pathogenicity is much greater then planktonic state (Nett *et al*. 2010). The power to switch from yeast to hyphal form is a well-known characteristic from *C. albicans,* increasing the virulence considerably (Brand, 2012). The definition of biofilm can be considered as a microorganism community that can be attached to surfaces (Fuolli & Mellado, 2014). Initial attachment of *Candida* cells to form biofilm is mediated by hydrophobicity and the action of electrostatic forces and from specific adhesins presents on fungal cells such as fibrinogen and fibronectin (De las Penas *et al*.

2003). This type of biofilm can cause clinical problems because it is able to increase resistance to most antifungal treatments and can be a threat to the host because of the challenge of finding an effective solution towards its problem (Cavalheiro *et al.* 2018).

The resistance by *Candida albicans* strains is observed and concerns as epidemiological studies are performed (Gulgun *et al*. 2015). It is important to know how to manage a fungal infection as seen on antifungal resistance cases, otherwise the clinical consequence could be treatment failure in patients with this condition (Sanglard & Odds, 2002). The development of new drug alternatives should continue as its observed that fungi have performed a notable capacity to inhibit the effect of antifungals (Canuto & Rodero, 2002). Based on antifungal action, there is a small number of antifungal classes available for the treatment of these infections (Sanglard & Odds, 2002). New antifungal drugs have been created and presented to use as an alternative on the treatment of oral infections (Gulgun *et al*. 2015).

Imidazolium compounds can be found in biological active substances such as some antifungal agents, considered as ionic liquids are classified as salts that has fusion point below 100ºC, has thermal and chemic stability, low viscosity and low vapor pressure (Braunstein *et al.*, 1971). These liquids also known as imidazolium salts has in its chemical structure an imidazolium ring that makes them able to interact electrostatically with biological systems (Anderson & Long, 2010). These salts are produced by the alkylation of imidazole, creating a discreet pair of anion and cation (Hough *et al*. 2007). Some studies were done towards observing interesting characteristics of these salts such as anti-yeasts, anti-inflammatory and anti-fungal (Frade & Afonso, 2010; Schrekker *et al*. 2013). The main mechanism of action of these substances is the capacity to inhibit the process of conversion of lanosterol to ergosterol through enzyme lanosterol 14 α-demethylase causing disruption of cell membrane by decreasing ergosterol formation (Schrekker *et al.* 2013).

The objective of this in vitro study was to identify if there was an antifungal potential and evaluate the effect of imidazolium salts and its capability to inhibit or even kill a strain of *Candida albicans*.

**Results and discussion**

As a pioneer research on stablishing the effect of IMS on *C. albicans*, there will be some comparisons throughout interaction of these compounds and different species. All five of the imidazolium salts ($C_{18}$MImCl, $C_{10}$MImCl, $C_{16}$MImMeS, $C_{16}$MImCl and$C_{16}$DMImMeS) tested were able to inhibit the in vitro growth of *Candida albicans* yeasts and resulted on different MIC between each salt. These values varied between compounds, after four repetitions in different times, MIC of the five IMS against a laboratory strain of *C. albicans* yeast are shown in table 1. The lowest MIC encountered was from $C_{18}$MImCl, 32.0±15.0*μg/mL*. The highest concentration in susceptibility test was 55.0±30.0 and 80.0±0.0*μg/mL* by $C_{16}$MImMeS and $C_{10}$MImCl, respectively. In 2013, a study described by Schrekker H.S.*et al*., was tested multiple IMS as antifungal agents and its toxicity, including $C_{10}$MImCl and $C_{16}$MImCl against *Candida parapsilosis, Candida tropicalis and Candida glabrata*.

The MIC of $C_{10}$MImCl against *C. tropicalis* varied between 0.9 *μg/ml* to 31.2 *μg/ml*, against *C. glabrata* the MIC ranged from 7.8 *μg/ml* to 31.2 *μg/ml*, when tested in *C. parapsilosis* this concentration variation was 1.9 *μg/ml* to 15.6 *μg/ml*. In the same study, $C_{16}$MImCl was tested against *C. glabrata, C. tropicalis and C. parapsilosis* and the MIC found did not vary against these three species, MIC being 0.9 *μg/ml*.

Even though these are different species of *Candida*, when compared studies, $C_{10}$MImCl was able to inhibit growth of *C. albicans* yeasts at 80.0±0.0*μg/mL.* The increase of MIC comparing these two species of *Candida* could be explained due to the fact that *C. albicans,* could have a morphogenesis phenomenon, the power to convert reversibly its yeast cells to pseudohyphal or hyphal growth, this growth of hyphae has an important role in tissue invasion and an increase of resistance to phagocytosis, a virulence mechanism. Clinically, *C. glabrata* is the second most found specie of the *Candida* genre in human oral cavity, this specie is isolated frequently from patients with dental prosthetics (Jorge, 2012), Vandenbussche and Swinne in 1984 isolated *C. glabrata* in 48% of the patients with denture meanwhile, *C. albicans* was found in 82% of the same patients, association between these two species can also be found. Dalla Lana *et al*. 2015, also studied $C_{10}$MImCl against 45 clinical isolates from four dermatophytic species of human origin, Microsporun canis, Microsporum gypseum, Tricophyton mentagrophytes and Triclophyton rubrum, a geometric mean was calculated and values of MIC50 (values of MIC that can inhibit 50% of growth) varied between the mean to one hundred times higher (MIC), the lower geometric mean of MIC50 found by $C_{10}$MImCl was 0.39 *μg/ml* and the higher 12.50*μg/ml*.

Bergamo *et. al*, in 2014 tested $C_{16}$MImClwith five strains of *C. tropicalis* planktonic, the MIC found, again did not vary and this concentration was 0.014 *μg/ml*. Study by Dalla Lana *et al.* 2015, already cited, $C_{16}$MImCl against those four species of dermatophytes, the lowest geometric mean of MIC50 was 0.02*μg/mL* and the highest 0.039*μg/mL.* In this study, against a strain of *C. albicans*, the result of this IMS was promising, being one of the lowest concentrations tested between all five salts, this concentration was 40.0±28.3*μg/mL*.

According to Schrekker, 2013, cation side length plays an important role through finding MIC and MFC, according the increase of length of this side chain there was a trend that these concentrations tended to be lower than compounds with smaller or longer side chains. Even though there is a difference between *Candida* species of the studies, this concept was verified as observed in $C_{10}$MImCl with the highest concentration in this assay and $C_{18}$MImCl as the lowest.

Between all five imidazolium salts that were able to inhibit growth of *Candida albicans*, all of them also had a fungicide effect, as well, in its yeast. The MFC assay, also after three repetitions, resulted on a variety of concentrations that were able to kill the yeasts when tested, this concentration range varied again between compounds, being the lowest concentration identified as 32.5±15*μg/mL* again from $C_{18}$MImCl. The highest concentration was found again also in $C_{10}$MImCl being 120.0±46.2*μg/mL* (Table 1). In 2015, a study conducted by Dalla Lana and collaborators verified fungicide effect towards different species of dermatophytes, for all 45 dermatophytes, $C_{16}$MImCl and $C_{16}$MImMeS demonstrated gradual decrease of fungal activity, but at four times MIC a total absence of activity could be detected.As seen on table 1, in this study $C_{18}$MImCl and $C_{16}$DMImMeS not only showed to be fungistatic but also had a fungicide behavior in the same concentration.

When studying the effectiveness of imidazolium salt in biofilm, all five compounds studied were able to inhibit biofilm formation of *C. albicans*. Some of these IMS had almost one hundred percent tax of inhibition of growth by *C. albicans* biofilm such as $C_{18}$MImCl and $C_{10}$MImCl. Between these two, $C_{18}$MImCl were capable to inhibit any growth of *C. albicans* biofilm on a lower concentration, 0.04mg/ml, while $C_{10}$MImCl inhibited growth of biofilm at 0.08mg/ml, double the concentration of the first. Even though the others did not have the same result as these two, $C_{16}$MImMeS had a power of inhibition of ninety-eight percent at 0.32mg/ml, $C_{16}$MImCl a ninety-seven percent at 0.4mg/ml and $C_{16}$DMImMeS also a ninety-eight percent inhibition at 0.4 mg/ml.

Table 2 represents means and deviation error between all groups of concentrations and compounds of inhibition growth in biofilm formation the higher value was found by $C_{18}$MImCl being capable to inhibit 99.6 ± 0.8(%) of biofilm activity. The smaller tax of biofilm activity was found by $C_{16}$MImCl as 89.3 ± 15.3 (%).

In pre-formed biofilm, the highest success rate by the capacity of inhibition was by $C_{16}$DMImMeS and $C_{16}$MImCl with the capacity of 71.9 ± 11.1 (%), at 0.8mg/ml and 71.1 ± 14.3 (%) at 0.32mg/ml respectively. It is important to cite that all of five, again, had the potential to inhibit growth of biofilm, this time pre-formed. A 58.3 ± 30.3 percent rate by $C_{18}$MImCl was observed at 0.32 mg/ml, seventy-six percent by $C_{16}$DMImMeS also at 0.32 mg/ml and a 57.2 ± 23.0 percent rate of inhibition by $C_{10}$MImCl at 0.8 mg/ml, as seen on Table 2.

Bergamo *et. al* in 2015, studied a few strains of *C. tropicalis* biofilm and $C_{16}$MImCl and found a variation of concentrations in this IMS that varied between 0.028 $\mu$g/ml to 0.225 $\mu$g/ml and its activity. In biofilm formation assay MIC of $C_{16}$MImCl was considered 0.4 mg/ml and in pre-formed biofilm was 0.32 mg/ml. This could be attributed to the fact that are different strains from different species of *Candida* biofilm.

Schrekker *et. al*, in 2016 analyzed the antibiofilm potential of $C_{16}$MImCl and $C_{16}$DMImMeS when incorporated in Poly(L-lactide) biomaterials and noticed that when concentration of IMS increased, the percentage of inhibition of *C. tropicalis* biofilm would follow and success tax of inhibition would be higher, in this study the percentage of antibiofilm impediment for $C_{16}$MImCl and $C_{16}$DMImMeS ranged between 32-92% and 0-94%, respectively.

None of the five compounds demonstrated statistical difference between concentrations on any of the assays, except $C_{16}$MImCl and $C_{16}$DMImMeS in pre-formed biofilm assay considering value of $p<0.05$. As expected, a post hoc, Tukey test between these two compounds were performed and no statistical difference between them was found.

Even though this experiment was an in vitro study, this research also had some limitations. Some limitations that can be cited is about the strain used, it was a laboratorial one and not a clinical one. It was performed in only one strain of *Candida albicans* yeast and values found could vary between samples. Despite this, since the study was an in vitro one, all the steps could be well controlled, the strain used was a stablished one. A part of this study was performed planctonically, reminding that clinically could be produced biofilm. It's necessary to promote experiments in clinical strains.

Since this study is the first and pioneer stablishing the effect of imidazolium salts on *Candida albicans* and its biofilm, more researches in this area must be performed, results obtained in the field towards finding new alternatives to conventional therapies.

**Materials and methods**

To answer these questions, tests were performed and concentrations of five imidazolium ionic solutions were studied against planktonic cells and biofilms of a laboratory strain of *Candida albicans,* ATCC 90008, yeast, in tests that provides a parameter that evaluate the capacity of this salts to inhibit the growth and kill these strains of yeast. These experiments were performed with at least three repetitions in different times each.

Imidazolium salts

Five imidazolium salts were synthesized by a chemist in (Laboratory of Technological Process and Catalysis of Institute of Chemistry of Universidade Federal do Rio Grande do Sul) guided by protocols that are standardized in the literature 1-*n*-decyl-3-methylimidazolium chloride ($C_{10}$MImCl), 1-*n*-hexadecyl-3-methylmidazolium chloride ($C_{16}$MImCl), 1-*n*-octadecyl-3-methylimidazolium chloride ($C_{18}$MImCl), 1-*n*-hexadecyl-3-methymidazolium methanesulfonate ($C_{16}$MImMeS) and 1,2-dimethyl-3-hexadecylimidazolium methanesulfonate ($C_{16}$DMImMeS). The molecular weights (g/mol) of $C_{10}$MImCl, $C_{16}$MImCl, $C_{18}$MImCl, $C_{16}$MImMeS and $C_{16}$DMImMeS are respectively: 244.80, 328.86, 375.02, 388.61 and 234.32.

A series of microdilutions of these salts was made before the experiment. These series of dilution based on document M27-A3 published in 2008 by Clinical Laboratory Standard Institute (CLSI) with some modifications, instead of DMSO as diluent in the microdilutions phase was used PBS. Starting from the stock solution the preparation of dilution was performed as one hundred times higher of the desired final concentration to be studied. After that a dilution of 1/50 was done where 100 $\mu$L of each stock solution was transferred to a tube containing 4,9 mL of PBS, so the concentration was two times higher than the final concentration wanted. In this study, the range of concentration of the six imidazolium salts tested varied between 160 $\mu$g/mL to 5$\mu$g/mL.

Inoculum Preparation

The strain of *Candida albicans* (ATCC 90008) was cultivated by twenty-four hours on Saboraud Dextrose Agar Himedia M1067-500G as growth medium in Petri plates at 37ºC on the incubator. After this period, colonies of the strain were suspended in a tube with saline solution (NaCl 0,9%) and agitated to incorporate the colonies on this solution. With a spectrophotometer (Milton Roy Spectronic 21D) with wave length of 530 nm the optic density was adjusted to 0,5 in McFarland scale, this solution had approximatively concentration between $3x10^6$ UFC/ml. A 1:1000 dilution was done taking 10 $\mu$L of this solution and added to a tube containing 10ml of Mueller-Hinton Broth (KASVI K25-610034).

Inoculation of microtiter plates

For this experiment, a ninety-six wells microtiter plate (KASVI K12-096) was used, the inoculation of this plate occurred in the following way: the wells in column number one of the six salts was identified as sterility control and filled with 200 $\mu$L of Mueller Hinton Broth, as growing control in the wells of column number eight filled with 100 $\mu$L of the suspension of the inoculum and 100 $\mu$L of PBS. Columns number two through seven filled with 100 $\mu$L of the inoculum suspension and 100 $\mu$L of each dilution of antifungal in each well. Line A was $C_{18}$MImCl, line B - $C_{10}$MImCl, line C - $C_{16}$MImMeS, line D - $C_{16}$MImCl and line E - $C_{16}$DMImMeS. After the filling of all wells of the microtiter plate, a forty-eight-hour period of incubation at 37ºC was respected then the minimum inhibitory concentration assay was evaluated.

Minimum inhibitory concentration

As mentioned previously, after 48 hours of incubation, with the help of one inverted mirror, was visually analyzed the concentrations of each well of each salt. The minimum inhibitory concentration (MIC) was defined as the concentration that produced at least a fifty percent reduction at least of the growing of the yeasts when compared to the other wells. All of the concentrations data were recorded by a trained individual.

Minimum fungicide concentration

As MIC was determined, one concentration less diluted than MIC and one concentration more diluted than MIC of each imidazolium salt to be performed a minimum fungicide concentration (MFC) assay. In this phase of the study 25 $\mu$L of these three concentrations of each salt were sown in duplicates in Petri plates with SDA as growing medium with previous identifications. These plates were incubated for a twenty-four-hour period at 37ºC. As respected this period of incubation, the plates were removed from incubator and visually analyzed, the MFC was considered as the minimum concentration capable of providing none yeast growth. All these data were recorded for further evaluation.

Biofilm formation

To evaluate the effect of the same IMS against biofilm formation of *Candida albicans* were tested. Biofilms were prepared in 96-well microtiter plates KASVI K12-096. After a 24-hour cultivation of *C. albicans* strain (ATCC 90008) in SDA from the same brand and reference, at 37ºC, 100 $\mu$L of the inoculum of *Candida albicans*, was transferred to these microtiter plates and then incubated for an hour and a half period again at 37 ºC.

These inoculums are pre-determined stock solutions of cells by Jin et.al, in 2004, the final concentration must be $10^7$cells/ml in PBS, adjusting this solution until reaches 0,5 McFarland Scale standard.

After this adhesion phase respected, the liquid was aspirated, each well was washed two times with 150 $\mu$L of PBS and 200$\mu$L of Yeast Nitrogen Base HIMEDIA M139-100G (YNB) added with different concentrations of IMS, these concentrations ranged from 0.04mg/ml to 0.8mg/ml, were placed in each well. This microtiter plate was incubated for 24 hours at 37ºC.

Pre-formed biofilm

*Candida albicans* biofilms were prepared following the same protocol descripted in "Biofilm formation" but, the step of washing the wells with PBS and adding YNB with different concentrations of IMS was done after 24 hours of incubation at 37ºC, so, after the adhesion phase, the liquid was aspirated and then each well filled with 200$\mu$L of pure YNB.

Twenty-four hours of incubation respected with YNB with different concentrations of IMS, the supernatant was aspirated in each well, then, biofilms were washed two times with 200 $\mu$L of PBS and colored with 110 $\mu$L of crystal violet (In Lab IC – 42555) 0.4% by 45 minutes this substance penetrates in the wall cells and stays withheld in the cytoplasm. This step was followed by washing each well with 350 $\mu$L of distilled sterile water and immediately discolored with 200 $\mu$L of ethanol 95%, again by 45 minutes.

In the end, after these steps, 100 $\mu$L of each well on the plate was collected and placed in its correspondent well on a new microtiter plate to be analyzed spectrophotometrically by ELISA (Reader Thermo Scientific Multiscan GO) at 595nm of absorbance. This experiment had two controls, one positive and one negative. In the positive, 200 $\mu$L of pure YNB was added after adhesion phase of *Candida albicans*, and in the negative control, where 200 $\mu$L of pure and just YNB was added, this absorbance number of negative control served to subtract from the number of the other wells to subtract any interferences.

**Statistical analysis**

After all results were collected a data base was created in the software IBM SPSS Statistics v.24 then an ANOVA test performed and post hoc tests as Tukey test when applicable to verify any statistical difference between groups considering $p<0.05$.

**Conclusions**

As resistance to antifungal treatments by *Candida albicans* rise, managements alternatives to control opportunistic infections have to be available. Five imidazolium salts were studied, all of them had antifungal characteristics in its formulation. Positive results were found in susceptibility tests and biofilm assays and could been evaluated that a small quantity of IMS against planktonic cells of a strain of *Candida albicans* can inhibit growth or even stop this production of yeasts, for biofilm as well, all of IMS presented power to inhibit formation or had fungicide effect at pre-formed biofilm.

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**Ethical approval**

This article does not contain any studies related to humans neither to animals performed by any authors.

**Conflict of Interests**

This study has no conflicts of interests.

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**Table 1** Susceptibility Tests of Imidazolium Salts Against *Candida albicans*

| Compounds | MIC (μg/ml) | MFC (μg/ml) |
|---|---|---|
| $C_{18}$MImCl | 32.5 ±15.0 | 32.5 ±15.0 |
| $C_{10}$MImCl | 80.0 ±0.0 | 120.0 ±46.2 |
| $C_{16}$MImMeS | 55.0±30.0 | 60.0±23.1 |
| $C_{16}$MImCl | 40.0±28.3 | 45.0±25.2 |
| $C_{16}$DMImMeS | 45.0±25.2 | 45.0±25.2 |

Minimun Inhibitory and Fungicide Concentrations against *C. albicans*; MIC: Minimum Inhibitory Concentration and MFC: Minimum Fungicide Concentration

**Table 2** Imidazolium Salts' Inhibition Taxes in Biofilm Formation and Pre-Formed Biofilm of *Candida albicans*

| Compounds | Biofilm Formation | Pre-Formed Biofilm |
|---|---|---|
| $C_{18}$MImCl | 99.6 ± 0.8 | 58.3 ± 30.3 |
| $C_{10}$MImCl | 96.6 ± 7.2 | 57.2 ± 23.0 |
| $C_{16}$MImMeS | 95.3 ± 15.2 | 66.9 ± 22.7 |
| $C_{16}$MImCl | 89.3 ± 15.3 | 71.1 ± 14.3 |
| $C_{16}$DMImMeS | 93.8 ± 11.2 | 71.9 ± 11.1 |

Descriptive analysis of success rate of inhibition by imidazolium salts in both cases (Biofilm formation and Pre-formed biofilm of *C. albicans*) both results are presented by percentage and deviation error.

**Artigo**

# IMIDAZOLIUM SALTS' DISINFECTION EFFECT IN PROSTHETIC DENTURE BASE ACRYLIC RESIN WITH *CANDIDA ALBICANS* BIOFILM: AN IN VITRO STUDY

IMIDAZOLIUM SALTS' DISINFECTION EFFECT IN PROSTHETIC DENTURE BASE ACRYLIC RESIN WITH *CANDIDA ALBICANS* BIOFILM: AN IN VITRO STUDY

**Significance and impact of the study**

Prosthetic denture disinfection is a major step during daily oral hygiene. None or poor disinfection of prosthetics can create a favorable environment for growth of several microorganisms and if this condition persists can lead to serious problems, for example, oral infections. The most frequent oral infection to occur is oral candidiasis, this scenario is caused by a biofilm of cells attached to a surface that could happen on prosthetic denture base. Previous studies demonstrated that *Candida albicans* frequently is the most microorganism isolated from this type of biofilm and can colonize alone or in association to others. The need for alternatives to conventional disinfection is evident since this specie of *Candida* could develop resistance to these known methods.

**Abstract**

Denture stomatitis related to denture is the most frequent fungal infection encountered in denture wearers population, with different causes known, *Candida albicans* biofilm is one of the most important reason to cause this condition. Poor denture hygiene or low denture disinfection could initiate an infection process that could reflect in this condition. Due to the necessity of development of new disinfection substances, previous studies have shown that imidazolium salts (IMS), ionic liquids, have interesting characteristics and good potential treating fungal infections. Since studies have focused to stablish the relationship between these solutions and other microorganisms, this research focus on the interaction between *C. albicans* and IMS. Five IMS was evaluated and different concentrations between compounds was analyzed, based on previous studies, and compared to a positive control (chlorhexidine digluconate 0.12%) and a negative control (saline solution 0.9%). The highest biofilm activity inhibition was observed in $C_{18}$MImCl at 0.32 mg/mL demonstrating better results when compared to the positive control. Statistical difference between substances compared to the negative control was found and a post hoc, Tukey test, performed demonstrated that $C_{18}$MImCl was statistically different when compared to the other groups.

**Key words:**

Ionic liquids; antifungal; oral candidiasis

**Introduction**

Oral health is considered an element towards achieving quality of life (Locker, 2000), but, stablishing and maintaining this condition is not considered in most protocols of personal hygiene for elderly in hospitals, for example (Peltola *et al*. 2000). United Kingdom has fifteen million denture wearers being twenty-five percent of population wearing partial or total types of denture (Coulthwaite & Verran, 2007).

With the diversity of textures and surfaces, in denture wearers oral cavity, the growth support of microorganism and plaque or biofilm could happen (Neill, 1968). Palatal mucosa can respond to bad stimulations as generating a chronical inflammatory condition in denture wearers (Frenkel *et al*. 2000). Mucosal lesions related to denture have several causes such as denture plaque, infection, prothesis bad adaptation, allergy or any mucosal trauma (Freitas *et al*. 2008).

A number of serious problems/infections can be associated with denture wearing, one of them is denture-related stomatitis (Sumi *et al*. 2002). Denture stomatitis can be considered a form of oral candidiasis that can happen alone or in association to other infections events (Holmstrup, 1990). Prevalence of mucosal lesions can be considered higher in denture wearers (Budtz, 1981), denture prothesis base can be frequently colonized by *C. albicans* and lead to denture stomatitis (Pereira *et al*. 2008).

The material used in the fabrication of denture prothesis is frequently polymehylmethacrylate, a type of polymer, lined with some soft materials to enhance comfort to the patient, these materials are more porous that could serve as a retainable niche for microorganism colonization (Marsh, 2004).

Previous studies have already showed that through microscopic analysis, the fitting surface of dental prothesis could be intensively colonized by *C. albicans* due to depressions and porous surfaces, offering a mechanical retention to this microorganism and propitiate *Candida* overgrowth (Allison & Douglas, 1973).

Unlike dental plaque, the microbiology of denture plaque it is not so studied. This second differs from the first in its location and composition, if associated to bad denture hygiene could lead to oral infections caused frequently by *Candida* (Coulthwaite & Verran, 2007).

Opportunistic oral infections lead by fungus presents higher prevalence, even though the progress towards therapeutic conduct have been more accurate. These infections can be especially noted in denture wearers. And one of the main reasons of these infections happen could be attributed to the adherence of yeast cells of Candida species in irregularities surfaces of a denture material and associated to the lack of oral hygiene and other systemic factors (Pereira *et al*. 2008).

Several factors can predispose microbial hazards condition to patients with partial or total denture prosthetics, during a professional adjustment/repair interior particle of acrylic resin could be exposed for example, and if none or poor disinfection to this dispositive done, oral infections lead by fungus may be at risk (Lin *et al*. 1999).

Denture related stomatitis is strongly associated to *Candida* infection and is the most common lesion between denture wearers (Jainkittivong et al. 2009). The first stage of a fungal infection lead by Candida on denture wearers, is considered by the capability of adherence of this microorganism on these surfaces and it may contribute to deterioration of the devices (Cannon & Chaffin, 2000),

its adherence can be either located through a plaque on the surface or directly on the denture base confectioned with polymethylmethacrylate (Edgerton *et al*. 1993).

In countries where social differences can be observed, a considerable number of edentulous people can be detected and so, the number of denture wearers are higher, such as in Brazil. In 2002/2003, an epidemiological survey of oral health was conducted by the Ministry of Health, data found by this survey showed that average DMFT of almost twenty-eight in the age group of sixty-five to seventy-four, and of this number, sixty-five percent consisted by missing teeth. This survey also presented that approximately eighty percent of the elderly are edentulous of superior arch and forty-eight percent of lower arch (Freitas *et al*. 2008).

Hashizume *et al.* 2015 evaluated the disinfection effect of several substances on *Candida albicans* adhered to acrylic resin and found that two-percent chlorhexidine solution and one-percent hypochlorite solution had the best disinfection effect among substances analyzed. A systematic review by Emami *et al*. 2013, evaluated through previous articles several disinfection methods such as chlorhexidine digluconate, amphotericin B, mouthwashes, photodynamic therapy and nystatin suspension had no statistical difference between them as disinfection methods.

Imidazolium salts are considered ionic compounds and represent a class of substances that have in its structure an organic cation (imidazolium ring) and either organic or inorganic anion (Riduan & Zang, 2013). These compounds have extremely interesting characteristics desirable for a drug low vapour pressure, high chemical a thermal stability (Pham *et al*. 2010) and the capacity to interact with biological systems (Schrekker *et al*. 2013). Schrekker *et al.* 2013 considered imidazolium salts, ionic liquids, as more effective and less injurious than substances already known and commercialized.

The objective of this in vitro study was to evaluate disinfection effect of imidazolium salts on *Candida albicans* pre-formed biofilm on acrylic resin blocks, simulating the condition of a denture prothesis of denture wearers population.

**Results and discussion**

As this study is one of the first stablishing the relationship between *C. albicans* and IMS, different concentrations of different solutions were analyzed, in this particular test, where evaluated the effect of these solutions in pre-formed biofilm of *C. albicans* in acrylic resin blocks, simulating a denture base, some substances were better than others.

It is shown on Figure 1, a box plot, of all groups evaluated on this study inhibition taxes (%) when in contact of a pre-formed biofilm of *C. albicans*, compared to negative control (Saline solution 0.9%) where there was maximum growth of the strain and no inhibition effect. In the statistical analysis it was considerate that disinfection effect when compared to negative control was statistically different because p value was 0.018.

The highest inhibition tax of this study was observed by $C_{18}$MImCl, 78.1 ± 16.4 %, presenting great potential working under these conditions, this tax was even higher than Chlorhexidine Digluconate 0,12%, positive control, that had 40.4 ± 31.2 %. Besides these solutions, only one more of the IMS studied had a positive average, $C_{10}$MImCl, 38.1 ± 55.0 %. No inhibition effect was observed to $C_{16}$MImCl, $C_{16}$MImMeS and $C_{16}$DMImMeS on the concentration evaluated.

Although $C_{10}$MImCl had a positive effect towards inhibiting biofilm growth of *C. albicans*, it is important to emphasize that the concentration of this compound analyzed was more than two times higher than $C_{18}$MImCl concentration. Dalla Lana *et al*. 2015 tested different concentrations IMS against multidrug-resistant dermatophytes and observed that cation and anion of these substances had an important role towards its effect, in this study, authors found that higher and shorter alkyl segments had higher minimum inhibition concentrations (one of the IMS studied was $C_{18}$MImCl) and demonstrated an optimum chain length of $C_{16}$ for efficacy. Regarding IMS-anion, volume, water solubility and coordination strength was considered important characteristics of evaluation and if increased anion volume of IMS, higher MIC values would be found.

According to previous studies, IMS have fungistatic and fungicide effects when treating a fungal infection (Dalla Lana *et al*. 2015; Schrekker *et al*. 2013), this study corroborate with previous ones, effect differences of compounds were observed from these studies, but could be justified due to the fact that this is a pioneer research studying IMS on *C. albicans*, once that virulence of this microorganism is notoriously higher than *Candida non-albicans* because its capability of phospholipase and proteinase production that are assessed to *C. albicans* (Hannula *et al*. 2000) and the circumstance that this microorganism is considered a biphasic fungus and has the power to switch between forms either yeast to hyphae and vice versa (Odds, 1988), increasing considerably its harmfulness.

Also, previous studies have shown that biofilm growth in dentures intend to increase antifungal resistance once that this biofilm is stablished, mature *C. albicans* biofilms have a considerable increased heterogeneous architecture and morphology, when analyzing the distribution of fungal cells and when compared to other surfaces biofilm growths, irregulate surface of polymethimethacrylate are different than the ones that develops on hydrophobic and flat surfaces such as on silicone materials (Chandra *et al*. 2001).

This study agrees with Schrekker *et al*. 2013 where authors affirm that further studies with different substances and different strains would help to get more answers and observe if this pattern of antifungal activity and concentrations will follow these studies that are already in the literature.

**Materials and methods**

A series of steps were performed towards finding IMS effect on disinfection of acrylic resin denture base associated with *C. albicans* biofilm.

Acrylicic resin blocks

To test disinfection of IMS, seventy acrylic resin block patterns were confectioned, simulating denture prosthesis base, heat-curing acrylic resin system is typically chosen as first choice to denture prosthetics, and as well, to this study. Vipicril Plus powder 450g (ref. 525168) and Vipicril liquid 120ml (ref. 525335), material elected to this confection was mixed according fabricants' proportions, with the assistance of a silicone matrix, resin was placed in it and this set to and included to a muffle with plaster. After closing the muffle, with a thousand kilograms-force, polymerization phase was started for ninety minutes on a pan at sixty-five degrees Celsius.

With this stage completed, acrylic resin blocks were cut onto 5x5x2 mm dimension on Isomet Low Speed Saw (Buehler – 11-1280-160), then chemical polishing with methyl methacrylate monomer base (POLI-QUIM Clássico) at chemical polisher (Termotron PQ – 9000) performed by ten seconds each block at 85ºC and with the help of sandpaper blocks was sanded and roughness measured and adjusted to approximately 0.2-0.3 (Mitutoyo SJ-201). Sterilization process was by plasma hydrogen peroxide, in order to maintain polymeric properties of acrylic resin.

*Candida albicans* suspension

After a 24h incubation in Saboraud Dextrose Agar (Himedia M1067-500G medium growth, *Candida albicans,* (ATCC 90008) a laboratorial sample, colonies were collected and added to 10 mL of Brain-Heart Infusion Broth (BHI) (Kasvi K25-610008) and adjusted to 0,5 in McFarland scale, after quantifying in Brain-Heart Infusion Agar the medium found was $3{,}1x10^6$ UFC/ml. With this inoculum prepared and adjusted, one sterile acrylic resin block was placed in a well of a ninety-six well microtiter plate (KASVI K12-096) and a 24h contamination period, at 37ºC, was respected to enable growth of *C. albicans* biofilm on an incubator (FANEM LTDA MODEL 002 CB).

Substances studied

Based in previous studies performed, 5 imidazolium salts were elected, synthtized by a chemist in (Laboratory of Technological Process and Catalysis of Institute of Chemistry of Universidade Federal do Rio Grande do Sul) according to protocols published, 1-*n*-decyl-3-methylimidazolium chloride ($C_{10}$MImCl), 1-*n*-hexadecyl-3-methylmidazolium chloride ($C_{16}$MImCl), 1-*n*-octadecyl-3-methylimidazolium chloride ($C_{18}$MImCl), 1-*n*-hexadecyl-3-methymidazolium methanesulfonate ($C_{16}$MImMeS) and 1,2-dimethyl-3-hexadecylimidazolium methanesulfonate ($C_{16}$DMImMeS). The molecular weights (g/mol) of $C_{10}$MImCl, $C_{16}$MImCl, $C_{18}$MImCl, $C_{16}$MImMeS and $C_{16}$DMImMeS are respectively: 244.80, 328.86, 375.02, 388.61 and 234.32.

Dilutions of these substances were necessary and performed according Clinical Laboratory Standard Institute (CLSI) document M27-A3 published in 2008. But, instead using DMSO as diluent for these compounds, PBS buffer was used. Concentrations of each salt was based in previous minimum inhibitory concentration (MIC) studies and one concentration per salt was analyzed. Concentrations for $C_{16}$MImCl, $C_{16}$DMImMeS, $C_{18}$MImCl, $C_{10}$MImCl and $C_{16}$MImMeS were respectively 0.16mg/ml, 0.32 mg/ml, 0.32

mg/ml, 0.8 mg/ml and 0.8 mg/ml.

For control groups, was used, saline solution (NaCl 0,9%) as negative control, and as positive control, chlorhexidine digluconate 0,12%.

Treatment solutions

Each block was washed with saline solution and randomized to a ten-minute treatment period for each substance was performed, after the twenty-four-hour contamination stage. Two hundred microliters of each substance were placed in a new ninety-six well microtiter plate and then, one block immersed in each well of treatment.

Each row of the microtiter plate had a letter "A" through "H", that in this case was considered a substance, either imidazolium salt or control groups. For letter "A" through "E" was placed $C_{18}$MImCl, $C_{10}$MImCl, $C_{16}$MImMeS, $C_{16}$MImCl and $C_{16}$DMImMeS respectively. Row letter "F" positive control and "G" negative control. Letter "H" contained pure BHI broth utilized to subtract any interference of the medium during analysis. Columns "1" to "10" was filled with the correspondent substance evaluated.

With treatment stage completed, in a new ninety-six microtiter plate, containing two hundred microliters of pure BHI broth each block was immersed in its respective well and this plate incubated for twenty-four hours at thirty-seven degrees Celsius.

Analysis

Inhibition taxes analysis was executed with help of an ELISA spectrophotometer (Reader Thermo Scientific Multiscan GO) at 530 nm of absorbance. After the result given by the spectrophotometer, all wells were subtracted the value of "H" row that had pure BHI, this way, any interference by medium growth could be minimized.

**Statistical analysis**

A data base was created, after all results collected and an ANOVA test performed in the software IBM SPSS Statistics v.24 to verify any statistical difference between groups, followed by Tukey test, considering statistical difference $p<0.05$.

**Conclusions**

The urge towards finding new therapies when treating a fungal infection is clinically relevant because could be extremely convenient and helpful when situations such as recurrent or resistant fungal infections are present. The number of antifungal drugs, protocols of disinfection and therapeutic conducts are extremely smaller when compared to the number available of widespread antibiotics to choose when treating a bacterial infection.

This necessity and development of new antifungal solutions have a great impact to conventional treatments already known/used and could be associated to these therapies in order to facilitate fungal infection treatment. This study observed a better disinfection effect by $C_{18}$MImCl than even the positive control in *C. albicans* biofilm on acrylic resin blocks simulating a denture base, further studies would help stablish this characteristic, since this research is pioneer studying disinfection effect of IMS on these conditions.

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**Ethical approval**

This article does not contain any studies related to humans neither to animals performed by any authors.

**Conflict of Interests**

This study has no conflicts of interests.

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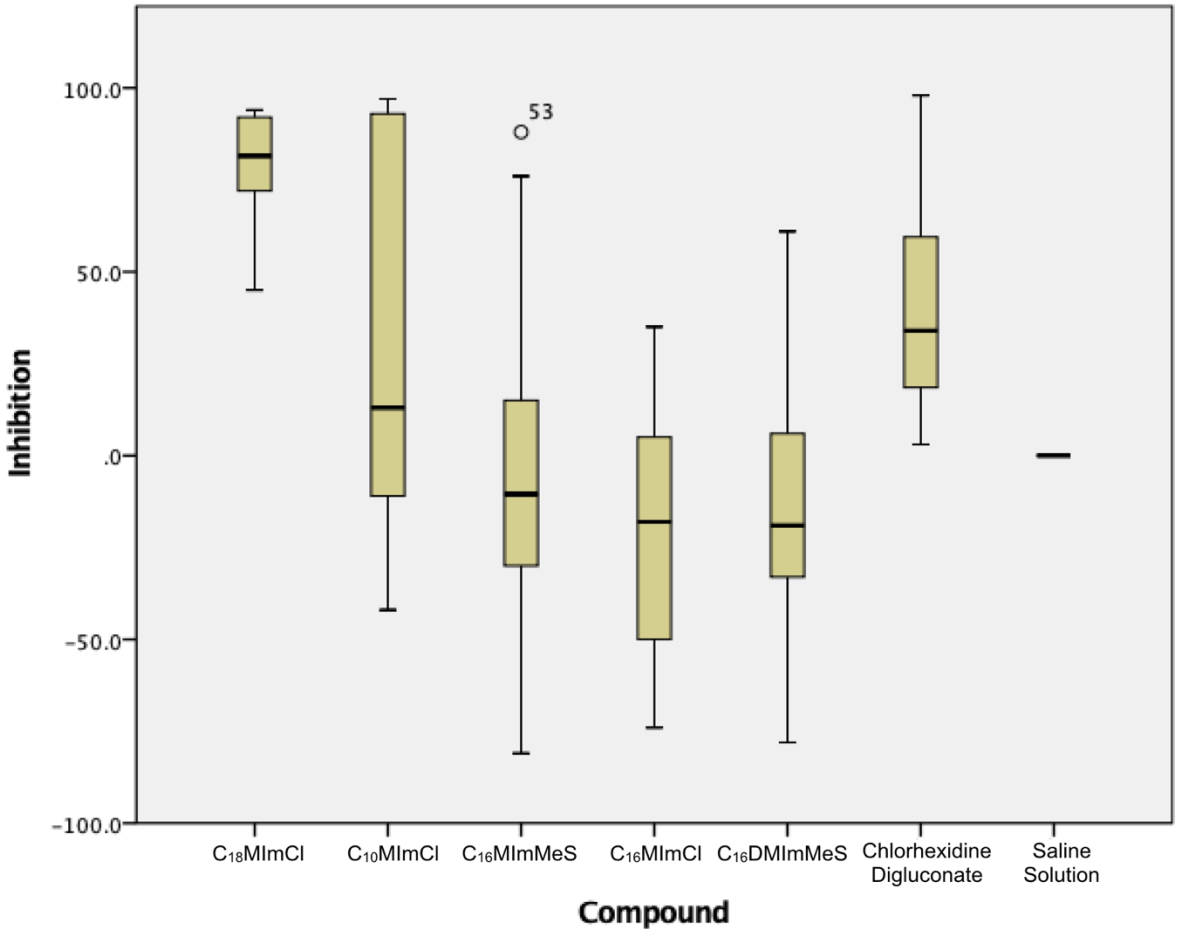


**Figure 1**. Demonstrates statistical analysis of IMS and positive control compared to negative control when evaluating the disinfection effect of these substances in acrylic resin blocks with pre-formed *Candida albicans* biofilm.

## Considerações Finais

De acordo com os resultados obtidos, é possível afirmar que líquidos iônicos imidazólicos possuem um efeito antifúngico quando relacionados com a *Candida albicans*, possuindo um efeito fungicida e fungistático tanto em biofilme em formação, quanto em biofilme pré-formado, possui também ação desinfectante quando simuladas características de uma base de prótese em resina acrílica em associação com um biofilme pré-formado de *Candida albicans*.

Devido ao fato deste estudo ser pioneiro em avaliar essa relação, novos estudos devem ser realizados para corrobar com os achados do mesmo. Uma vez que a *Candida albicans* possui uma morfologia complexa, com diferente fator de virulência quando comparados com outros microrganismos.

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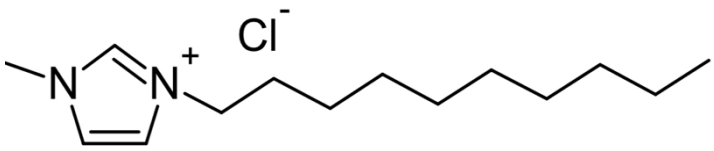
## Anexos

*Estrutura Química dos Compostos Avaliados no Presente Estudo*

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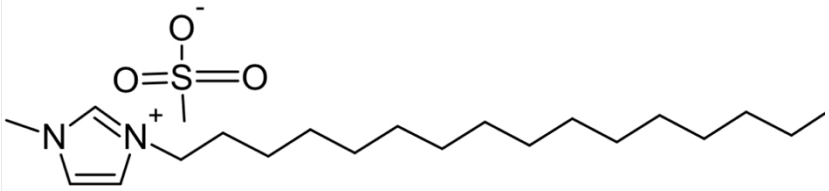


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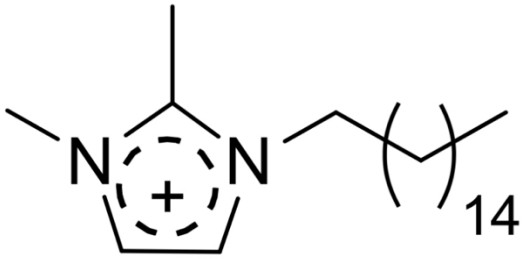


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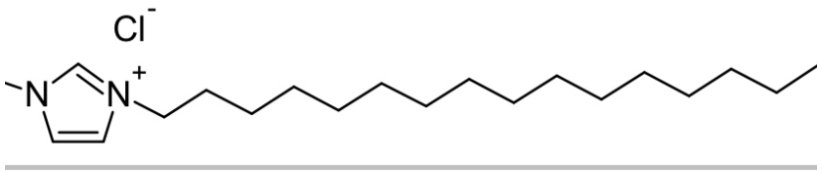


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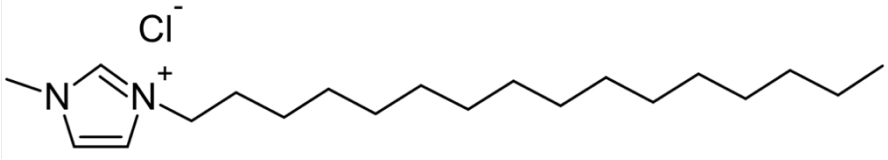


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