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AVALIAÇÃO DO POTENCIAL CITOTÓXICO E ANTIBACTERIANO *IN VITRO* DE COMPLEXOS TRIAZENIDO E BENZOTRIAZENIDO-ONA DE Au(I) E Cu(II)

Santa Maria, RS
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Tese apresentada ao Curso de Doutorado do Programa de Pós-Graduação em Ciências Farmacêuticas, Área de Concentração em Análises Clínicas e Toxicológicas, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para a obtenção do grau de **Doutor em Ciências Farmacêuticas**.

Orientadora: Prof[a]. Dr[a]. Rosmari Hörner

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A minha família, especialmente a meus amados pais Elíane e José, a meu irmão Marcel e a meu esposo Thiago. Esta conquista também é de vocês!! Obrigada por tudo!!

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"Porque dignificas a vida e buscas a cura, tua recompensa brotará nos sorrisos carregados de esperança. Bendizem-te os lábios convalescentes, e porque és como o brilho do sol depois de uma longa tempestade, tu sempre serás bem-aventurado"

Naír Carpenedo

RESUMO

AVALIAÇÃO DO POTENCIAL CITOTÓXICO E ANTIBACTERIANO *IN VITRO* DE COMPLEXOS TRIAZENIDO E BENZOTRIAZENIDO-ONA DE Au(I) E Cu(II)

AUTORA: Maísa Kräulich Tizotti
ORIENTADORA: Rosmari Hörner

Nos dias atuais, há uma crescente ascensão das doenças neoplásicas e das infecções bacterianas ocasionadas por microrganismos multirresistentes em todo o mundo, representando grandes problemas para a saúde pública. Dessa forma, novos agentes antineoplásicos e antibacterianos têm sido extensivamente investigados. Notavelmente, os metalofármacos são reconhecidos por apresentarem inúmeras possibilidades, as quais são escassas para compostos orgânicos tradicionais, devido ao aumento da resistência a medicamentos contendo tais moléculas. Por estes motivos, o presente estudo teve como propósito avaliar o potencial biológico *in vitro* de quatro novos complexos metálicos contendo átomos de ouro(I) ou cobre(II): $[(L)Au(PPh_3)]$, onde PPh_3 = trifenilfosfina e HL = 1-(4-amidofenil)-3-(4-acetilfenil)triazeno; $[(L)_2(Py)_2(OH_2)Cu(II)]$, $[(Bipy)_2(L)_4Cu(II)_2]$ e $[(Phen)_2(L)_4Cu(II)_2]$, onde Py = piridina; Bipy = 2,2'-bipiridina, Phen = 1,10-fenantrolina e HL = 1,2,3-benzotriazina-4(3*H*)-ona. Assim, após a caracterização por técnicas espectroscópicas e difração de raios X em monocristal, estes compostos foram avaliados *in vitro* quanto a sua atividade citotóxica (valores de CI_{50} - concentração que causa 50% de inibição no crescimento celular comparada ao controle) e antibacteriana (valores de CIM - concentração inibitória mínima). Os efeitos citotóxicos dos compostos frente a células de medula óssea de pacientes não tratados, com suspeita de malignidades hematológicas, foram investigados por meio de ensaio de citotoxicidade, utilizando o reagente brometo de 3-(4,5-dimetiltiazol-2-il)-2,5-difeniltetrazólio (MTT). As propriedades antibacterianas destes complexos foram avaliadas frente a diferentes cepas bacterianas potencialmente patogênicas, utilizando o método de microdiluição em caldo Mueller Hinton. Além disso, os complexos de Cu(II) foram testados quanto ao seu potencial citotóxico em linhagens celulares tumorais (K562, MCF-7 e B16F10) e linhagem celular não tumoral (VERO), por meio do ensaio com MTT. Os resultados obtidos para $[(L)Au(PPh_3)]$ demostraram que este complexo triazenido de Au(I) foi citotóxico frente a células de paciente com síndrome mielodisplásica (CI_{50} = 7,72 μM) e apresentou atividade expressiva frente a cepas bacterianas Gram positivas, incluindo microrganismos multirresistentes. Por sua vez, o complexo dinuclear de Cu(II) contendo co-ligantes phen na esfera de coordenação $[(Phen)_2(L)_4Cu(II)_2]$ apresentou elevada citotoxicidade em células tumorais (até 16 vezes maior do que a obtida para o metalofármaco Cisplatina), sendo altamente citotóxico nas linhagens de melanoma murino B16F10 (CI_{50} = 4,37 μM) e adenocarcinoma de mama MCF-7 (CI_{50} = 6,16 μM), bem como em amostras de pacientes com leucemia mieloide crônica (CI_{50} = 7,76 μM) e síndrome mieloproliferativa (CI_{50} = 7,01 μM). Interessantemente, ambos os complexos de Cu(II) contendo co-ligantes bipy ou phen apresentaram atividade antibacteriana frente a cepas Gram positivas e Gram negativas. Por conseguinte, estas conclusões indicam claramente que $[(L)Au(PPh_3)]$ e $[(Phen)_2(L)_4Cu(II)_2]$ possuem promissoras propriedades citotóxicas e antibacterianas *in vitro*.

Palavras-chave: Triazeno. 1,2,3-benzotriazina-4(3*H*)-ona. Complexos de ouro(I). Complexos de cobre(II). Atividade biológica.

ABSTRACT

IN VITRO STUDY OF CITOTOXIC AND ANTIBACTERIAL ACTIVITY OF TRIAZENIDE AND BENZOTRIAZENIDE-ONE COMPLEXES OF Au(I) AND Cu(II)

AUTHOR: Maísa Kräulich Tizotti
ADVISOR: Rosmari Hörner

Nowadays, the neoplastic diseases and bacterial infections caused by multidrug-resistant (MDR) microorganisms are ascending worldwide, representing great problems to public health. Thus, new antitumor and antimicrobial agents have been largely investigated. In this regard, metallodrugs open an array of possibilities, which traditional organic molecules cannot fulfill any longer due to growing drug resistance. For this reason, the present study aimed to evaluate the *in vitro* biological potential of four new metal complexes containing gold(I) or copper(II) íons: $[(L)Au(PPh_3)]$, where PPh_3 = triphenylphosphane and HL = 1-(4-amidophenyl)-3-(4-acetylphenyl)triazene; $[(L)_2(Py)_2(OH_2)Cu(II)]$, $[(bipy)_2(L)_4Cu(II)_2]$ and $[(Phen)_2(L)_4Cu(II)_2]$, where Py = pyridine; bipy = 2,2'-bipyridine, phen = 1,10-phenanthroline and HL = 1,2,3-benzotriazine-4(3*H*)-one. So, after characterization by means of spectroscopic techniques and single-crystal X-ray diffraction, these compounds were evaluated for their cytotoxic (IC_{50} values – concentration that causes 50% inhibition in the cell growing compared to control) and antibacterial (MIC values – Minimal Inhibitory Concentration) *in vitro* activities. Cytotoxic effects of compounds on bone marrow cells from untreated patients with suspected hematological malignancies were investigated by cytotoxicity assay, using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) as reagent. The antibacterial properties of these complexes against potentially pathogenic bacterial strains were evaluated by the Mueller-Hinton broth microdilution method. In addition, Cu(II) complexes were tested for their cytotoxix potential towards tumor cell lines (K562, MCF-7 and B16F10), as well as one non tumor cell (Vero) by MTT assay. The results obtained for $[(L)Au(PPh_3)]$ showed that this Au(I) triazenide complex was strongly cytotoxic on cells from patient with myelodysplastic syndrome (IC_{50} = 7.72 µM) and presented distinguished activity against Gram-positive bacterial strains, including MDR microorganisms. In turn, dinuclear Cu(II) complex containing phen co-ligands in coordination sphere $[(Phen)_2(L)_4Cu(II)_2]$ showed considerable cytotoxic effects on tumor cells (up to 16 times bigger than the one obtained to the metallodrug Cisplatin). This complex was highly cytotoxic on B16F10 melanoma (IC_{50} = 4.37 µM) and MCF-7 breast cancer (IC_{50} = 6.16 µM) lineages, as well as on bone marrow samples, especially from patients with chronic myeloid leukemia (IC_{50} = 7.76 µM) and myeloproliferative syndrome (IC_{50} = 7.01 µM). Remarkably, both Cu(II) complexes containing bipy or phen co-ligands presented antibacterial effects against Gram-positive and Gram-negative strains. Therefore, these findings clearly indicate that $[(L)Au(PPh_3)]$ and $[(Phen)_2(L)_4Cu(II)_2]$ have promising *in vitro* cytotoxic and antibacterial properties.

Keywords: Triazene. Gold(I) complexes. 1,2,3-benzotriazine-4(3*H*)-one. Copper(II) complexes. Biological activity.

SUMÁRIO

APRESENTAÇÃO

As seções primárias que integram esta tese de doutorado (elaborada no forma de artigos integrados) encontram-se dispostas da seguinte maneira: **INTRODUÇÃO**, **OBJETIVOS**, **PUBLICAÇÕES CIENTÍFICAS**, **DISCUSSÃO**, **CONCLUSÃO** e **REFERÊNCIAS**.

A **INTRODUÇÃO** descreve sucintamente o tema da pesquisa e sua relevância, bem como, abrange uma breve revisão da literatura sobre o assunto investigado.

Na seção **PUBLICAÇÕES CIENTÍFICAS** os itens Material e Métodos, Resultados, Discussão, Conclusão e Referências estão contidos em um ARTIGO e em um MANUSCRITO, representando a íntegra deste estudo:

ARTIGO

Abrange a versão aceita para a publicação do artigo intitulado "**X-ray characterization and *in vitro* biological evaluation of 1-(4-amidophenyl)-3-(4-acetylphenyl)triazene and the gold(I) triazenide complex {Au(I)[RPhNNNPhR'][PPh$_3$]} [R = (C=O)NH$_2$, R' = (C=O)CH$_3$]"**, o qual foi publicado no periódico **Inorganica Chimica Acta** (v. 441, p. 78-85, 2016 - DOI: 10.1016/j.ica.2015.10.038).

MANUSCRITO

Abrange o manuscrito intitulado "***In vitro* cytotoxic and antibacterial properties of three new copper(II) complexes containing 1,2,3-benzotriazine derivative and N-donor heterocyclic ligands**", redigido de acordo com as normas do periódico **Dalton Transactions** ao qual será submetido.

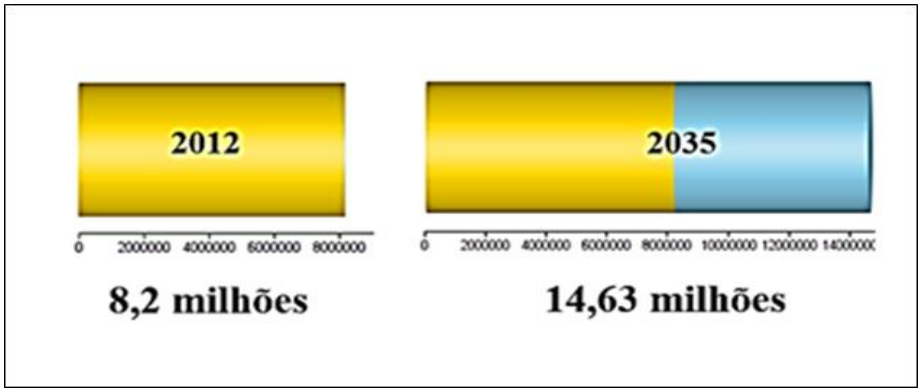
A **DISCUSSÃO** contempla uma análise integrada dos principais resultados contidos no ARTIGO e no MANUSCRITO.

As **REFERÊNCIAS** remetem apenas a citações contidas nas seções **INTRODUÇÃO** e **DISCUSSÃO** desta tese.

1 INTRODUÇÃO

Nos últimos anos, importantes avanços foram alcançados no que diz respeito à prevenção, diagnóstico e tratamento das doenças neoplásicas. No entanto, apesar dos recentes progressos, as contínuas transições demográficas e epidemiológicas indicam que nas próximas décadas haverá um aumento significativo no ônus global do câncer, representando um evidente problema de saúde pública (BRAIN, 2014; BRASIL, 2016; AMERICAN CANCER SOCIETY, 2016). Com base nos dados da última estimativa mundial sobre incidência do câncer e mortalidade, realizada pela *International Agency for Research on Cancer* (Iarc) da Organização Mundial da Saúde (OMS), as neoplasias estão entre as principais causas de óbito no mundo e já superam o número de óbitos provocados pelas doenças coronárias. Esta estimativa apontou que, no ano de 2012, ocorreram 8,2 milhões de mortes em decorrência do câncer no mundo, sendo esperados para o ano 2035, aproximadamente 24 milhões de novos casos e 14,6 milhões de óbitos relacionados a doença (Figura 1) (WORLD HEALTH ORGANIZATION, 2014a; FERLAY et al., 2015).

Figura 1 – Número de óbitos por câncer no mundo no ano de 2012 e predições para o ano de 2035, considerando o efeito demográfico



Fonte: Adaptação de World Health Organization, 2014a.

Além da problemática do câncer, outro assunto que têm gerado grande preocupação na comunidade médica e científica consiste no rápido aparecimento e disseminação de bactérias multirresistentes (MDR, do inglês *multiple drug resistance*). A emergência de cepas bacterianas MDR se deve principalmente ao uso generalizado e, muitas vezes, inadequado dos medicamentos antibacterianos, levado à pressão seletiva (CARLET; RAMBAUD; PULCINI,

2014; HOLMES et al., 2016). Infecções causadas por estes patógenos estão frequentemente associadas a complicações clínicas onerosas, resultando em aumento da morbidade e mortalidade (RICE, 2008; BOUCHER et al., 2009; BRASIL, 2010). Essa situação tem sido agravada pela escassez de novos agentes terapêuticos. Embora alguns fármacos inovadores estejam em desenvolvimento (LING et al., 2015) grande parte dos medicamentos lançados na última década são derivados de classes terapêuticas já utilizadas e, dessa forma, sujeitos aos mesmos mecanismos de resistência (APPELBAUM, 2012; PIDDOCK, 2012; CHOPRA, 2013; KARAM et al., 2016).

Nas últimas décadas, inúmeros esforços têm sido despendidos para que novas moléculas com propriedades antitumorais e antibacterianas sejam descobertas, no intuito de superar as limitações terapêuticas existentes. Além da exploração de produtos naturais (GUO et al., 2014), o estudo da atividade farmacológica de compostos sintéticos tem sido amplamente realizado como estratégia para a identificação e desenvolvimento de novos medicamentos. Na área da química inorgânica medicinal, destaca-se a coordenação de metais de transição a ligantes orgânicos bioativos, em razão da sua possível aplicabilidade biológica (THOMPSON; ORVIG, 2006; GASSER; OTT; METZLER-NOLTE, 2011; GAYNOR; GRIFFITH, 2012; MARTINS et al., 2014).

Desde a descoberta dos efeitos antiproliferativos da Cisplatina, há quase 50 anos (ROSENBERG; VAN CAMP; KRIGAS, 1965) um vasto número de complexos metálicos tem sido desenvolvidos e investigados quanto ao seu potencial biológico (MJOS; ORVIG, 2014). Notavelmente, complexos de ouro(I) e cobre(II) têm se destacado devido a ampla versatilidade farmacológica demonstrada em ensaios *in vitro* e *in vivo*. No geral, a atividade desses complexos foi modulada pelos ligantes coordenados, demonstrando que moléculas mais planares e lipofílicas exercem um papel fundamental na permeação celular e na ligação a alvos biológicos (MARZANO et al., 2009; DUNCAN et al., 2012; SANTINI et al., 2014; BERTRAND; CASINI, 2014; ZOU et al., 2015).

Primeiramente empregado na medicina chinesa e árabe, o ouro tem sido utilizado como um agente terapêutico a milhares de anos. A partir da identificação da sua utilidade no tratamento da artrite reumatoide, em meados do século 20, inúmeros estudos têm sido conduzidos com o propósito de desenvolver novas moléculas contendo ouro e descobrir novas aplicações terapêuticas para os compostos contendo este metal de transição. Nas últimas décadas, a atividade biológica de diferentes complexos de Au(I) contendo fosfinas ou ligantes nitrogenados tem sido descrita na literatura, sendo que, grande parte desses complexos tem apresentado promissores efeitos antitumorais e antibacterianos. Esses estudos também

evidenciaram que a atividade citotóxica dos complexos pode estar relacionada a inibição de importantes enzimas, tais como a tiorredoxina redutase (TrxR), sendo os mecanismos de ação provavelmente diferentes daqueles da cisplatina (BERTRAND; CASINI, 2014; ZOU et al., 2015).

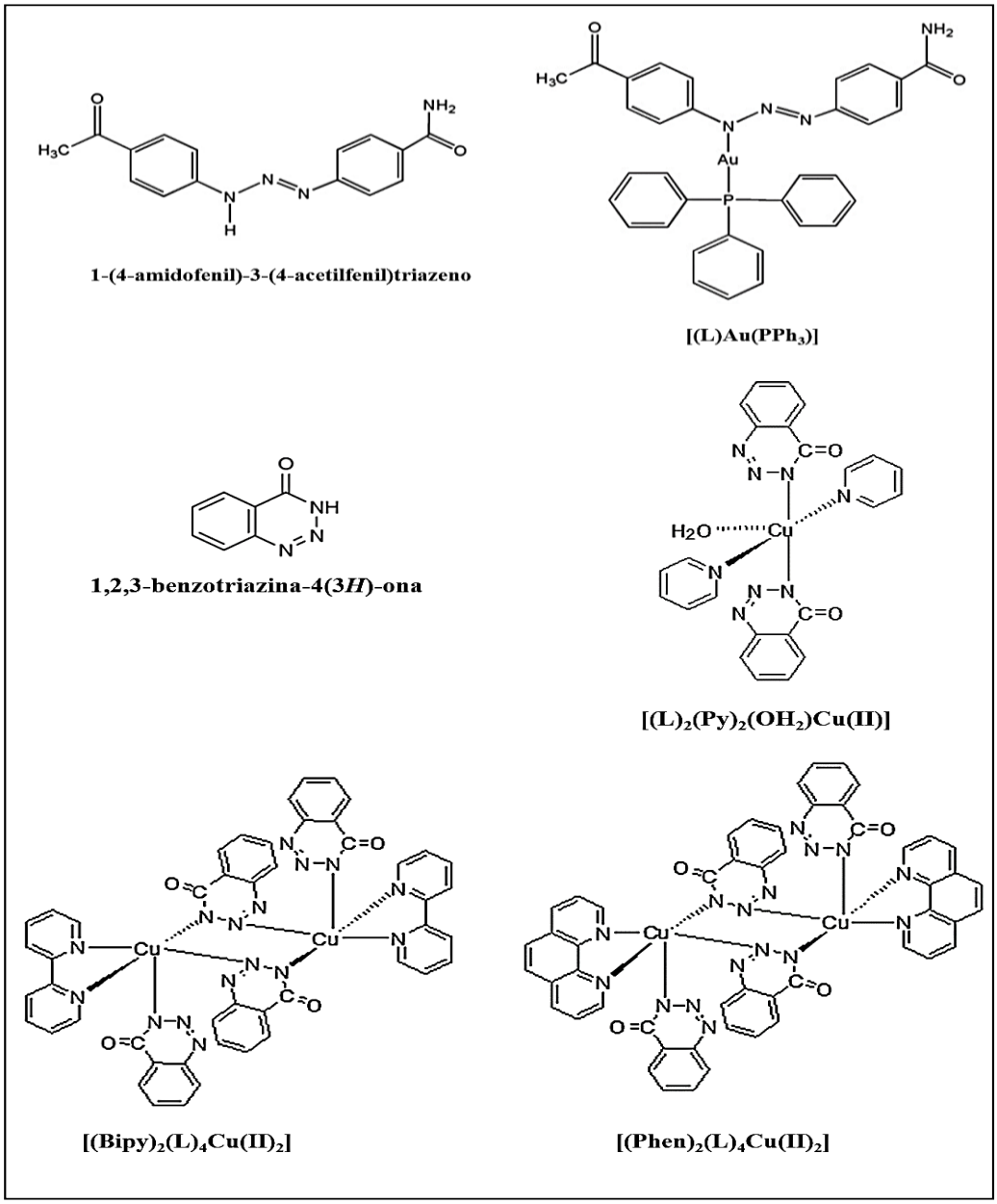
Além do ouro, outro metal de transição que tem despertado grande interesse nos pesquisadores é o cobre, especialmente devido a essencialidade deste elemento para os sistemas biológicos e a sua importante participação em reações de oxidação-redução (DENOYER et al., 2015). Em razão dos promissores resultados obtidos e publicados nos últimos anos, complexos de cobre(II) vêm sendo amplamente visados como potenciais agentes terapêuticos, especialmente aqueles contendo ligantes diimínicos como 1,10-fenantrolina (phen). Estes complexos apresentam um amplo espectro de atividades, incluindo atividade antitumoral, antibacteriana e de nuclease sintética, sendo capazes de induzir a hidrólise ou clivagem oxidativa do DNA superenovelado. Além disso, demonstram ter um efeito antitumoral superior ao dos complexos de coordenação da platina (GAMA et al., 2011; RAMAKRISHNAN et al., 2011; GAYNOR; GRIFFITH, 2012; SANTINI et al., 2014).

Compostos nitrogenados como os triazenos (TZCs) representam potenciais ligantes bioativos, uma vez que vários derivados desta classe possuem considerável atividade biológica, incluindo propriedades antitumorais (KATSOULAS et al., 2005; MARCHESI et al., 2007; CAPORASO et al., 2007; SEITER et al., 2009; FUKUSHIMA, TAKESHIMA, KATAOKA, 2009; DOMINGUES et al., 2010; CIMBORA-ZOVKO et al., 2011; JOHNSON, CHANG, 2012; BONMASSAR et al., 2013; ADIBI et al., 2013; AGNIESZKA et al., 2014; BROZOVIC et al., 2014) e antibacterianas (GOSWAMI, PUROHIT, 2001; HÖRNER et al., 2008; DOMINGUES et al., 2010; PARAGINSKI et al., 2014). TZCS aromáticos, contendo a estrutura 1,2,3-benzotriazina-4(3*H*)-ona também representam moléculas atrativas, tendo em vista as suas promissoras propriedades farmacológicas descritas na literatura (CALIENDO et al., 1999; CALIENDO et al., 2000; CHOLLET et al., 2002; LE DIGUARHER et al., 2003). Por conseguinte, na qualidade de ligantes, os TZCs são capazes de satisfazer exigências estereoquímicas de uma ampla variedade de complexos metálicos (BACK et al., 2012), apresentando substancial aplicabilidade na química de coordenação e na química medicinal.

Cosiderando estas particularidades, este estudo objetiva avaliar o potencial citotóxico e antibacteriano *in vitro* de um complexo triazenido de Au(I) contendo o fragmento trifenilfosfina (PPh_3), bem como, de três complexos de Cu(II) contendo ligantes 1,2,3-benzotriazenido-4-ona e co-ligantes heterocíclicos, tais como, piridina (py), 2,2’-bipiridina (bipy) e 1,10-fenantrolina (phen) (Figura 1). A atividade citotóxica do complexo de Au(I) e

seus precursores foi investigada por meio de ensaio de citotoxicidade *in vitro*, utilizando-se células derivadas de pacientes com suspeita de malignidades hematológicas. Para os complexos de cobre(II), além dos ensaios de citotoxicidade em células derivadas de pacientes, foram realizados ensaios adicionais frente a quatro diferentes linhagens celulares, sendo três delas tumorais (K562, MCF-7 e B16F10) e uma não tumoral (VERO). Além disso, o potencial antibacteriano, tanto do complexo de Au(I) quanto dos complexos de Cu(II), foi avaliado frente a várias espécies patogênicas.

Figura 1 – Estrutura dos pré-ligantes e complexos de Au(II) e Cu(II)



Fonte: Autor.

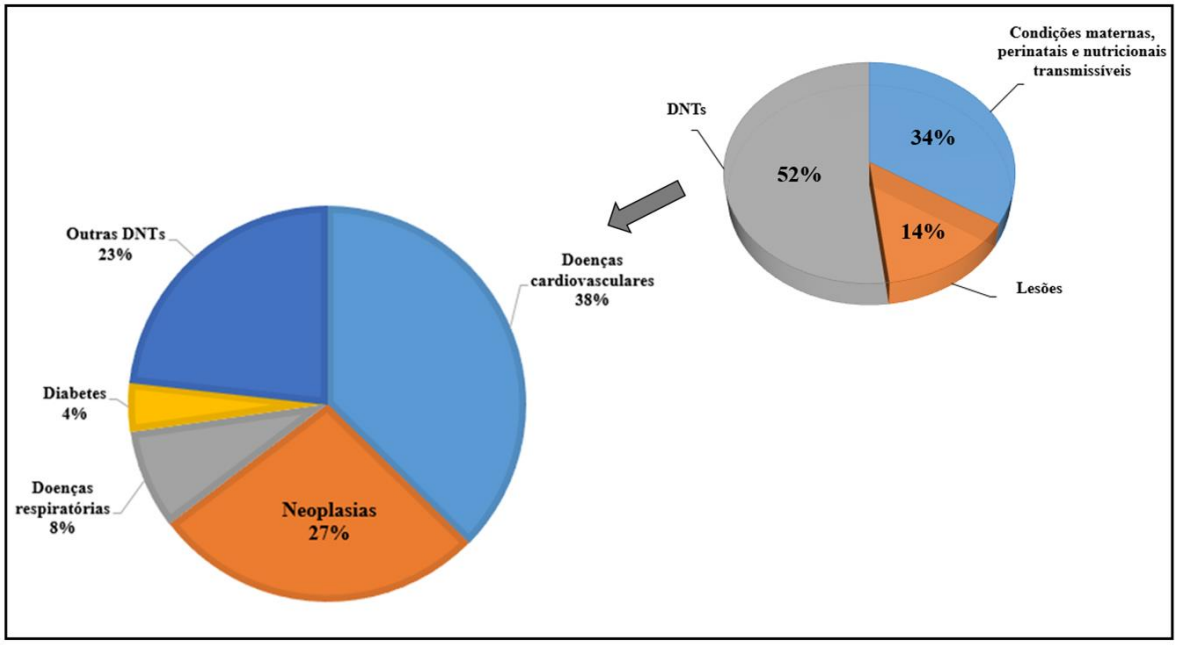
1.1 REVISÃO DA LITERATURA

1.1.1 Doenças neoplásicas: estimativas e tratamento

De acordo com a Organização Mundial da Saúde (OMS), dos 56 milhões de óbitos ocorridos no mundo em 2012, 38 milhões (68%) foram decorrentes de doenças não transmissíveis (DNTs), especialmente, doenças cardiovasculares (17,5 milhões), neoplasias (8,2 milhões), doenças respiratórias crônicas (4,0 milhões) e diabetes (1,5 milhões). Dessa forma, as DNTs ocasionaram mais mortes do que todas as outras causas combinadas, sendo projetado um aumento substancial no número de óbitos no ano de 2030 (52 milhões). Ressalta-se que, de todas as mortes por DNTs em 2012, 42% (16 milhões) ocorreram antes dos 70 anos de idade. A morte prematura é uma consideração importante ao avaliar o impacto das DNTs em uma determinada população, visto que a maioria dos óbitos prematuros (82%) ocorrem em regiões menos desenvolvidas, tais como, a África, Ásia, América Central e América do Sul (WORLD HEALTH ORGANIZATION, 2014b).

A Figura 2 ilustra as principais causas de óbitos no mundo em 2012, entre os indivíduos com menos de 70 anos, demonstrando a elevada porcentagem de mortes ocasionadas pelas neoplasias.

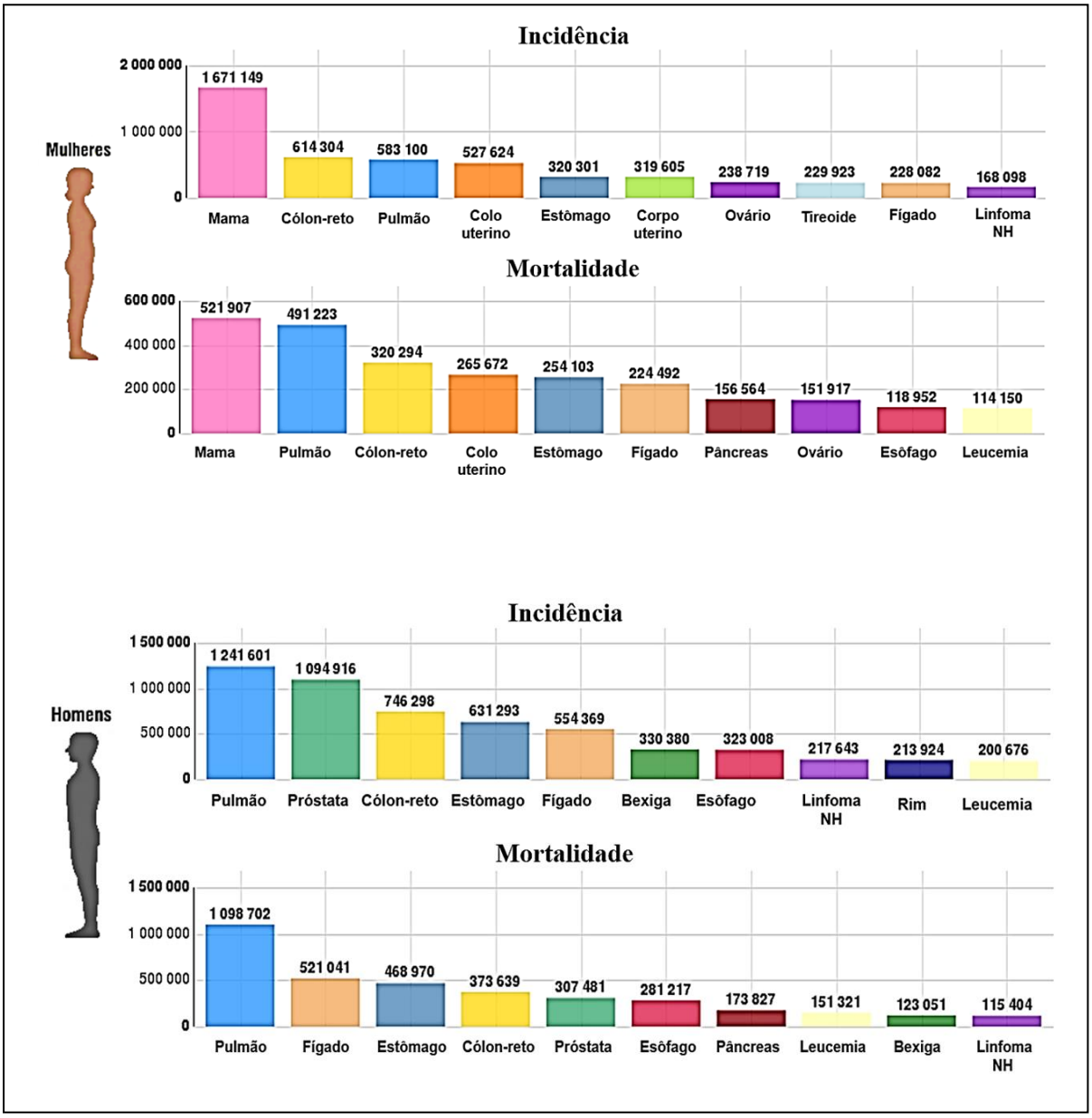
Figura 2 - Percentuais de morte por causa, para a faixa etária inferior a 70 anos, no ano de 2012.



Fonte: Adaptação de World Health Organization (2014b).

Na Figura 3 podem ser observados os 10 tipos de neoplasias mais incidentes e mais letais no mundo, por sexo, no ano de 2012. Entre as mulheres o câncer de mama foi o mais incidente, sendo também responsável pelo maior número de óbitos. Entre os homens o câncer de pulmão foi o mais incidente e o mais letal (WORLD HEALTH ORGANIZATION, 2014a).

Figura 3 – Distribuição proporcional dos 10 tipos de câncer mais incidentes e mais letais no mundo, por sexo, no ano de 2012.

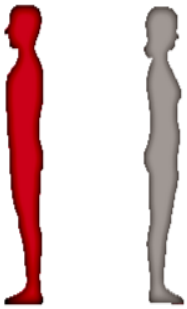


Fonte: Adaptação de World Health Organization (2014a).

Na Figura 4 podem ser visualizados os 10 tipos de câncer mais incidentes no Brasil, para o ano de 2016, por sexo (BRASIL, 2016). Comparando estes dados com a estimativa mundial pode-se dizer que no Brasil a neoplasia mais incidente no sexo feminino também é o câncer de mama, já para os homens o câncer de próstata supera o câncer de pulmão em número de casos novos.

Figura 4 – Distribuição proporcional dos dez tipos de câncer mais incidentes no Brasil (exceto pele não melanoma), estimados para 2016, por sexo.

Localização Primária	Casos	%	Localização Primária	Casos	%
Próstata	61.200	28,6%	Mama feminina	57.960	28,1%
Traqueia, Brônquio e Pulmão	17.330	8,1%	Cólon e Reto	17.620	8,6%
Cólon e Reto	16.660	7,8%	Colo do útero	16.340	7,9%
Estômago	12.920	6,0%	Traqueia, Brônquio e Pulmão	10.890	5,3%
Cavidade Oral	11.140	5,2%	Estômago	7.600	3,7%
Esôfago	7.950	3,7%	Corpo do útero	6.950	3,4%
Bexiga	7.200	3,4%	Ovário	6.150	3,0%
Laringe	6.360	3,0%	Glândula Tireoide	5.870	2,9%
Leucemias	5.540	2,6%	Linfoma não Hodgkin	5.030	2,4%
Sistema Nervoso Central	5.440	2,5%	Sistema Nervoso Central	4.830	2,3%



Fonte: Brasil (2016).

O câncer corresponde a um conjunto distinto de doenças caracterizadas pelo crescimento descontrolado e disseminação de células neoplásicas. A metástase (disseminação de células malignas do tumor primário para outros tecidos e órgãos) é a característica mais letal dessas doenças, sendo responsável por mais de 90% das mortes (HANAHAN; WEINBERG, 2011). Embora as causas que conduzem ao aparecimento do câncer ainda não estejam totalmente definidas, evidências têm demonstrado que tanto fatores intrínsecos (mutações hereditárias, hormônios, condições imunológicas, entre outros) quanto fatores externos/adquiridos (tabagismo, dieta pouco saudável, microrganismos infecciosos, alcoolismo, obesidade, exposição a produtos químicos, radiação, poluentes ambientais, entre outros) podem estar associados. Esses fatores podem agir em conjunto ou em seqüência, sendo que dez ou mais anos, muitas vezes, se passam entre a exposição a fatores externos e o aparecimento do câncer (ANAND et al., 2008; AMERICAN CANCER SOCIETY, 2016).

Na maioria das doenças neoplásicas, as alterações genéticas são adquiridas como eventos somáticos, porém, em um menor número de neoplasias, são encontradas alterações

genéticas hereditárias. Uma vez que os mesmos genes podem estar envolvidos tanto em mutações adquiridas quanto hereditárias, a compreensão dos processos moleculares e celulares envolvidos pode ser aplicada a totalidade dos cânceres (CASSIDY et al., 2010).

Durante o processo de carcinogênese ocorre uma série de alterações celulares, as quais levam ao descontrole da proliferação, da diferenciação e processo de morte celular programada (apoptose). Em tecidos normais há um controle cuidadoso da produção e liberação de sinais de promoção do crescimento que orientam a proliferação e a diferenciação celular, garantindo a homeostase do número de células, a manutenção da arquitetura e da função dos tecidos. A principal característica das células neoplásicas consiste na sua capacidade de sustentar a proliferação celular de forma crônica. Esse desequilíbrio entre a taxa de morte celular e a formação de novas células está relacionado tanto às anormalidades genéticas das células tumorais quanto à incapacidade do hospedeiro em detectar e destruir tais células. Assim, falhas no processo de apoptose, estimulação inapropriada da proliferação, bem como, anormalidades em genes supressores de tumor constituem as principais causas do crescimento descontrolado no câncer (CASCIATO; LOWITZ, 2009; HANAHAN; WEINBERG, 2011; CHAFFER; WEINBERG, 2015).

Nos dias atuais, várias modalidades terapêuticas têm sido empregadas no tratamento do câncer (cirurgia, radioterapia, quimioterapia, imunoterapia, terapias hormonais e terapias direcionadas ao alvo molecular). Diferentemente das abordagens terapêuticas locais, como a extirpação cirúrgica e a radioterapia, a quimioterapia é uma abordagem terapêutica sistêmica que recorre a agentes químicos citotóxicos capazes de interagir com as células neoplásicas, erradicando ou controlando o crescimento do câncer. Atualmente, cerca de 50 diferentes medicamentos antineoplásicos estão disponíveis para uso clínico e diversas novas moléculas estão sendo testadas em ensaios clínicos (RAO; LAIN; THOMPSON, 2013).

De modo geral, para que um agente anticancerígeno seja considerado ideal ele deve ser capaz de destruir as células neoplásicas, deixando as células normais ilesas. Contudo, a maioria dos medicamentos antineoplásicos convencionais atua de forma não específica, lesando tanto células cancerosas quanto células normais de crescimento rápido e, consequentemente, ocasionando inúmeros efeitos adversos. Além disso, muitos agentes citotóxicos são altamente mutagênicos, o que pode conduzir a um risco maior de tumores secundários (RAO; LAIN; THOMPSON, 2013).

Nas últimas décadas, importantes progressos foram alcançados na compreensão acerca dos mecanismos moleculares envolvidos no câncer. Informações obtidas por meio do sequenciamento do genoma humano têm servido de base para a descoberta de novos alvos

moleculares e terapias direcionadas, tais como, os inibidores da proteína quinase e anticorpos monoclonais (GRANT, 2009; GARRAWAY; LANDER, 2013; SLIWKOWSKI; MELLMAN, 2013).

Apesar dos recentes avanços, a resistência aos fármacos antitumorais ainda é a principal causa de falhas terapêuticas durante o tratamento antineoplásico. Na maioria das vezes, o rápido aparecimento de resistência torna necessária a utilização de altas doses de quimioterapia e combinações de agentes citotóxicos, levando a um aumento dos efeitos adversos. Além disso, as células neoplásicas são capazes de desenvolver resistência simultânea a diferentes agentes antineoplásicos, bem como, às novas terapias direcionadas ao alvo molecular (LANGE, 2008; HOLAHAN et al., 2013; IZAR et al., 2013) Assim, constantes estudos têm sido realizados no intuito de otimizar os resultados da terapia antineoplásica.

1.1.2 Infecções bacterianas: o problema da multirresistência

Os medicamentos antimicrobianos são, indiscutivelmente, os fármacos mais notáveis e essenciais na medicina. Com o desenvolvimento das sulfonamidas e das penicilinas no século XX, houve uma revolução no tratamento de infecções bacterianas, levando a uma drástica redução na morbidade e mortalidade (VON NUSSBAUM et al., 2006; SILVER, 2011). Consequentemente, a quimioterapia antibacteriana tornou-se fundamental na prática médica e tem contribuído significativamente para a saúde da sociedade moderna e uma maior expectativa de vida (WHITE, 2011; PIDDOCK, 2012).

No entanto, a rápida evolução e propagação de bactérias multirresistentes aos antimicrobianos, aliada ao desenvolvimento insuficiente de novos agentes antibacterianos, poderá afetar seriamente o futuro da terapia anti-infecciosa, especialmente para infecções bacterianas, causadas por patógenos Gram negativos (BOUCHER et al., 2009). Especialistas estimam que até à próxima década, o mundo terá testemunhado a ampla disseminação de infecções hospitalares e comunitárias incuráveis (GRUNDMANN et al., 2011).

A resistência crescente e inexorável de patógenos hospitalares, como o *Staphylococcus aureus*, a diversos antibióticos de primeira escolha, bem como, a rápida emergência de bactérias Gram negativas resistentes aos carbapenêmicos têm afligido a comunidade médica. Os microrganismos são considerados multirresistentes, quanto apresentam resistência a diferentes classes de antimicrobianos testados em exames microbiológicos de rotina. Dentre os patógenos multirresistentes considerados críticos estão: *Enterococcus* spp. resistente aos

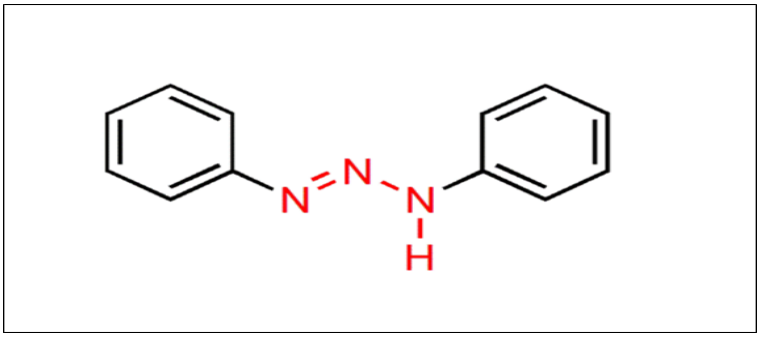
glicopeptídeos, *Staphylococcus* spp. resistente ou com sensibilidade intermediária à vancomicina, *Pseudomonas aeruginosa*, *Acinetobacter baumannii* e Enterobactérias resistentes a carbapenêmicos (ertapenem, meropenem ou imipenem) (BOUCHER et al., 2009; BRASIL, 2010; KARAM et al., 2016).

Dessa forma, o fracasso em descobrir novas classes de antimicrobianos tem levado a previsões de uma catástrofe médica e um retorno à era pré-antibiótica. Embora novos fármacos estejam em desenvolvimento, não satisfazem adequadamente as crescentes necessidades médicas (APPELBAUM, 2012; CHOPRA, 2013). Assim, são necessárias novas classes de agentes antimicrobianos que possuam mecanismos de ação inovadores e que sejam eficazes frente a patógenos emergentes.

1.1.3 Aspectos farmacológicos dos triazenos

Os compostos triazenos (TZCs) são caracterizados por uma cadeia alifática composta por três átomos de nitrogênio interligados em sequência (-N=N-N-). Sua química data de 1859, quando Peter Griess sintetizou o primeiro TZC, 1,3-*bis*(fenil)-triazeno (Figura 5) (MOORE; ROBINSON, 1986).

Figura 5 – Estrutura molecular do composto 1,3-*bis*(fenil)-triazeno

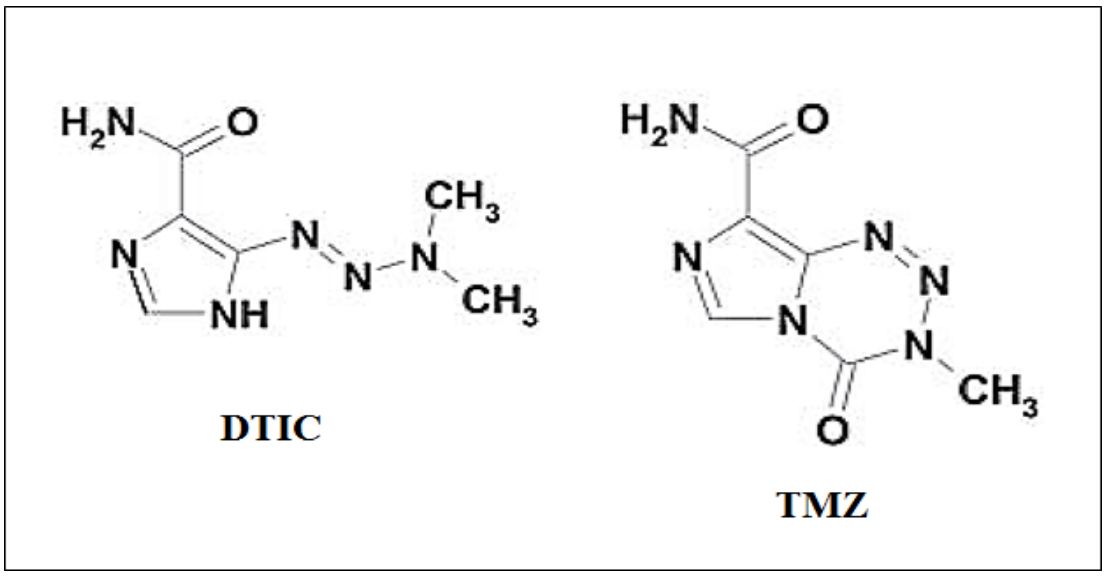


Tais compostos têm sido estudados a mais de um século devido ao relativo interesse estrutural, propriedades reativas e propriedades biológicas, especialmente, atividade antitumoral, antibacteriana e de nuclease, ou seja, capacidade de clivar o DNA de forma semelhante às nucleases naturais (KIMBALL; HERGES; HALEY, 2002; GADJEVA, 2002; HÖRNER, 2003; BURUIANA; STROEA; BURUIANA, 2009; HANUSEK et al., 2009; DOMINGUES et al., 2010; BACK et al., 2012; ADIBI et al., 2013; BONMASSAR et al.,

2013; SANTOS et al., 2014, PARAGINSKI et al., 2014). Ressalta-se que há um grande interesse da comunidade científica por moléculas que interajam com o DNA, especialmente, aquelas capazes de clivá-lo, devido as suas promissoras aplicações clínicas e tecnológicas (COWAN, 2001; SILVA et al., 2011).

Classificados no grupo dos agentes antineoplásicos alquilantes monofuncionais, dacarbazina (DTIC) e temozolomida (TMZ) (Figura 6) representam compostos triazenos utilizados atualmente no tratamento clínico de diversos tipos de tumores (CAPORASO et al., 2007; BONMASSAR et al., 2013). O mecanismo de citotoxicidade destes TZCs envolve principalmente a metilação do DNA na posição O^6 da guanina (MARCHESI et al., 2007). Durante a replicação do DNA, esta metilação desencadeia uma cascata de sinais intracelulares que conduzem ao bloqueio do ciclo celular e apoptose (CAPORASO et al., 2007).

Figura 6 – Estrutura molecular dos agentes antineoplásicos triazenos DTIC a TMZ



A dacarbazina (5-(3,3-dimetiltriazeno)imidazol-4-carboxamida) (DTIC), um derivado imidazol-carboxamida, foi sintetizada em 1959 e após vários estudos *in vivo* e *in vitro* foi aprovada para tratamento intravenoso de melanoma metastático, sarcoma de tecidos moles, linfomas Hodgkin e não Hodgkin, entre outros (D'INCALCI; SOUTEYRAND, 2001). Ela é considerada um pró-fármaco, pois é ativada por N-desmetilação no sistema microssomal do fígado formando 5-(3-Monometil-1-triazeno)-imidazol-4-carboxamida (MTIC), que constitui o seu metabólito ativo (MARCHESI et al., 2007).

Ao contrário da DTIC, o triazeno cíclico TMZ possui a vantagem de ser administrado por via oral, uma vez que é solúvel e estável em condições ácidas (APPEL et al., 2012). Em razão de sua facilidade em permear a barreira hematoencefálica, TMZ mostra-se potencialmente ativo contra tumores primários e metastáticos do cérebro, sendo utilizado como fármaco de primeira escolha no tratamento do glioblastoma multiforme (GBM - astrocitoma de grau IV pela OMS), melhorando relativamente as taxas de sobrevida (STUPP et al., 2005; CAPORASO et al., 2007). Os astrocitomas são as neoplasias malignas intracranianas mais frequentes, sendo responsáveis por 60% dos casos de tumores cerebrais primários (ZHU et al., 2002). Dentre os astrocitomas, o GBM é o mais agressivo e com pior prognóstico, ocasionando índices elevados de morbidade e mortalidade (COLMAN; ALDAPE, 2008).

De acordo com a literatura, TMZ sofre conversão espontânea no organismo para o metabólito ativo MTIC. Em condições fisiológicas, ao penetrar nas células neoplásicas, TMZ sofre hidrólise para MTIC, o qual metila as nucleobases do DNA ocasionando danos citotóxicos e, consequentemente, apoptose (WESOLOWSKI; RAJDEV; MUKHERJI, 2010; APPEL et al., 2012). Nos últimos anos, TMZ encontra-se em fase de ensaios clínicos para o tratamento de melanoma e outras neoplasias, incluindo metastases cerebrais, leucemias refratárias, câncer de pulmão, linfomas e tumores neuroendócrinos (RIZZIERI et al., 2010; VON MOSS et al., 2012; TATAR et al., 2013; BONMASSAR et al., 2013).

Apesar da necessidade de uma maior experiência clínica quanto à utilização de TZCs no tratamento da leucemia aguda e mielodisplasia, alguns estudos têm mostrado que tanto a DTIC quanto TMZ possuem atividade antineoplásica em pacientes leucêmicos. Embora apresentem uma citotoxicidade notável na maioria das células tumorais, estes medicamentos ocasionam danos no material genético que são muitas vezes corrigidos por mecanismos de reparo do DNA. Dessa forma, fármacos capazes de inativar as diferentes vias de reparo têm sido amplamente pesquisados como terapia de combinação (SEITER et al., 2002; SEITER et al., 2004; SEITER et al., 2009; TURRIZIANI et al., 2006; CAPORASO et al., 2007; RIZZIERI et al., 2010; BONMASSAR et al., 2013).

Tendo em vista a possível aplicabilidade dos TZCs, especialmente em leucemias agudas, Domingues et al. (2010) estudaram a atividade de alguns compostos TZCs inéditos frente a células mononucleares da medula óssea de pacientes diagnosticados com LMA. Os autores demonstraram que tanto o medicamento DTIC quanto três compostos TZCs inéditos apresentaram pronunciada atividade citotóxica em todas as amostras testadas.

Estudos realizados entre 2001 e 2005, envolvendo compostos triazenos e linhagens celulares tumorais de LMC, evidenciaram que a inibição da tirosina quinase pode representar um dos mecanismos da ação antitumoral de compostos dessa classe (MATHESON et al., 2001; MATHESON et al., 2004; KATSOULAS et al., 2005).

1.1.4 Química inorgânica medicinal: desenvolvimento de novos metalofármacos

A Química Inorgânica Medicinal é uma área em crescente expansão, apresentando um enorme potencial para desenvolver novos agentes terapêuticos e de diagnóstico. Dessa forma, complexos de coordenação representam uma classe de compostos que têm sido amplamente visados como potenciais agentes antitumorais e antimicrobianos. Em grande parte, isto se deve a eficácia terapêutica dos complexos de coordenação da platina em vários tipos de neoplasias (GIELEN; TIEKINK, 2005), tornando-se a base para o tratamento de tumores testiculares, ovarianos, de bexiga, cabeça, pescoço, cólon e esôfago e pulmão. Estes antineoplásicos são também conhecidos como agentes alquilantes bifuncionais, pois são capazes de interagir com o DNA formando ligações cruzadas intrafilamentares e interfilamentares, apresentando elevada citotoxicidade (MJOS; ORVIG, 2014; MUGGIA et al., 2015).

Apesar do sucesso clínico e comercial da Cisplatina e seus derivados, desvantagens como a resistência tumoral e significativa toxicidade têm restringido a utilização destes fármacos (KELLAND, 2007; WHEATE et al., 2010). Assim, há esforços consideráveis na busca por novas abordagens terapêuticas baseadas em metais, mais eficazes e menos tóxicas. Algumas estratégias têm sido aplicadas para a concepção de novos agentes potencialmente ativos, tais como o uso de outros tipos de ligantes e metais de transição, no intuito de encontrar metalofármacos mais potentes e menos tóxicos (GIELEN; TIEKINK, 2005).

O cobre é reconhecido como um micronutriente essencial para os sistemas biológicos (UAUY; OLIVARES; GONZALEZ, 1998), onde é comumente encontrado na forma reduzida (Cu^{1+}) ou oxidada (Cu^{2+}). Estes íons se mantêm fortemente ligados a proteínas de transporte/armazenamento e, devido a sua habilidade para catalizar reações de oxidação-redução (redox), são ativados *in loco* para atuarem como cofatores de diferentes enzimas vitais, como a citocromo *c* oxidase (COX), Cu/Zn superóxido dismutase (SOD1), dopamina β-hidroxilase (DBH), lisina oxidase, metionina sintetase e a tirosinase. Sendo assim, o cobre apresenta importantes funções estruturais, regulatórias e catalíticas no organismo, participando de processos como o metabolismo energético, homeostase do ferro (oxidação de

Fe^{2+} para Fe^{3+}), mecanismos de proteção antioxidante, transporte de oxigênio, sinalização celular para neurotransmissão, síntese de tecidos conectivos (ligações cruzadas entre o colágeno e a elastina), síntese de melanina nos melanócitos, entre outros. (LABBE; THIELE, 1999; PUIG; THIELE, 2002; KIM; NEVITT; THIELE, 2008, DENOYER et al., 2015).

Todavia, a homeostase do cobre requer um amplo controle uma vez que em níveis aumentados apresenta elevada toxicidade, sendo capaz de gerar espécies reativas de oxigênio (EROs) que danificam biomoléculas (HALLIWELL; GUTTERIDGE, 1984). É digno de nota, que tanto a deficiêcia quanto o excesso de cobre podem desempenhar um papel fundamental em vários estados patológicos, como por exemplo, na Síndrome de Menkes que é ocasionada pela deficiência sistêmica de cobre (KALER, 2011; TÜMER, 2013), na doença de Wilson, isto é, uma doença genética rara que compromete o metabolismo do cobre (CRISPONI et al., 2010; PATIL et al., 2013), em distúrbios neurodegenerativos (CERPA et al., 2005; GREENOUGH; CAMAKARIS; BUSH, 2013), entre outros.

Interessantemente, têm sido relatado que os níveis de cobre presentes em diferentes tecidos tumorais excedem aqueles dos tecidos normais. Apesar dessa evidência, os mecanismos envolvidos no aumento das concentrações de cobre em células neoplásicas ainda não foram totalmente elucidados (GUPTE; MUMPER, 2009). Outras investigações têm demonstrado que o cobre possui um efeito estimulatório na angiogênese, isto é, no processo de formação de novos vasos sanguíneos, favorecendo o crescimento tumoral, invasão e metástase. Dessa forma, o desenvolvimento de pequenas moléculas capazes de quelar o excesso de cobre e controlar a progresão do câncer tem tornado-se o ponto central de muitas pesquisas (HASSOUNEH et al., 2007; XIE; KANG, 2009). Além dessas, outras relações existentes entre o cobre e as células tumorais vêm sendo descritas na literatura. Um exemplo representativo seria a associação entre o acúmulo intracelular de cobre e a geração de alterações na proteína X ligada a inibição da apoptose. Segundo os autores, a interação entre os íons cobre e a proteína X aumenta a susceptibilidade das células neoplásicas a estímulos apoptóticos, ocasionando morte celular (MUFTI; BURSTEIN; DUCKETT, 2007).

Nas últimas décadas, um número considerável de complexos de Cu(II) têm sido desenvolvidos e avaliados quanto a suas propriedades biológicas no intuito de descobrir novas estratégias terapêuticas, especialmente para o câncer. Notavelmente, vários destes complexos têm apresentado um amplo espectro de atividades, mostrando-se, na maioria das vezes, mais ativos e menos tóxicos do que importantes agentes antineoplásicos utilizados na prática clínica. Dessa forma, tem sido sugerido que tais compostos possam desempenhar seu efeito antitumoral por meio de mecanismos diferentes daqueles descritos para os complexos de

coordenação da platina, os quais formam ligações covalentes com o DNA. Apesar deste pressuposto, inúmeros complexos continuam sendo investigados quanto a sua habilidade de interagir com o material genético, sendo que grande parte deles tem apresentado promissora capacidade de clivar a dupla hélice do DNA e induzir a morte celular programada (MARZANO et al., 2009; SANTINI et al., 2014).

Complexos de cobre(II) contendo ligantes heterocíclicos doadores de nitrogênio, particularmente diiminas bidentadas como 2,2-biperidina (bipy), 1,10-fenantrolina (phen) e seus derivados substituídos, despertam o interesse dos químicos medicinais devido a sua elevada capacidade de ligação com o DNA plasmidial e eficácia antitumoral *in vitro* (SANTINI et al., 2014).

Nos estudos envolvendo tais compostos, características como a geometria de coordenação, planaridade e a natureza dos ligantes foram críticas na determinação do modo de interação com o DNA. Geralmente, os complexos de Cu(II) com ligantes diimina não substituídos (2,2-biperidina, 1,10-fenantrolina) se ligam ao DNA por meio de intercalação, já aqueles contendo diiminas substituídas foram capazes de formar ligações não covalentes com esta biomolécula. Em muitos casos, a ligação dos complexos promoveu quebras tanto na cadeia simples ou quanto na cadeia dupla do DNA em condições fisiológicas. A cisão das cadeias duplas do DNA superenovelado levou a uma maior letalidade celular, uma vez que tais quebras são menos facilmente reparadas por mecanismos de reparo do DNA. Quanto a via, a clivagem oxidativa foi a mais comumente observada para estes compostos. No entanto, já foram relatados complexos de Cu(II) capazes de clivar o DNA por meio de um mecanismo predominantemente hidrolítico. Dependendo do tipo de dano induzido no DNA pelos complexos de cobre, o processamento celular pode conduzir a célula à ativação de vias de transdução de sinal de apoptose. Apesar de muitos complexos de cobre desencadearem a morte celular apoptótica como consequência do dano ao DNA, poucos artigos relatam elucidações sobre os determinantes moleculares de transdução de sinal ativados em células tratadas com complexos de cobre (KATSAROU et al. 2008; THOMADAKI et al., 2008; RAMAKRISHNAN et al,, 2009; GAMA et al., 2011; RAMAKRISHNAN et al. 2011; SILVA et al., 2011; ZIVEC et al., 2012; FERNANDES et al. 2013; SILVA et al., 2014, PIVETA et al., 2014; SANTINI, 2014).

Além do DNA, outros constituintes celulares tais como as topoisomerases I e II ou o complexo multiproteico proteassoma estão emergindo como supostos alvos desses compostos (SANTINI, 2014).

2 OBJETIVOS

2.1 OBJETIVO GERAL

Avaliar a atividade citotóxica e antibacteriana *in vitro* de um complexo triazenido de Au(I) e de três complexos benzotriazenido-ona de Cu(II), bem como, dos sais de metais precursores e ligantes livres relacionados.

2.2 OBJETIVOS ESPECÍFICOS

Investigar o efeito citotóxico de complexos de Au(I) e Cu(II), bem como, dos ligantes não coordenados frente a células tumorais obtidas de pacientes com suspeita de malignidades hematológicas.

Verificar a citotoxicidade dos complexos de Cu(II) e dos ligantes livres frente as linhagens celulares tumorais K562 (leucemia), MCF-7 (câncer de mama) e B16F10 (melanoma murino).

Analisar a seletividade de todos os complexos e ligantes livres por meio do ensaio de citotoxicidade frente a células não tumorais.

Comparar o efeito citotóxico e a seletividade dos complexos de Cu(II) aos resultados obtidos para o antineoplásico Cisplatina, sob as mesmas condições experimentais.

Avaliar a atividade antibacteriana dos complexos de Au(I) e Cu(II), bem como, dos ligantes não coordenados frente a diferentes cepas potencialmente patogênicas.

3 PUBLICAÇÕES CIENTÍFICAS

3.1 ARTIGO

Esta seção abrange a última versão do artigo intitulado "**X-ray characterization and *in vitro* biological evaluation of 1-(4-amidophenyl)-3-(4-acetylphenyl)triazene and the gold(I) triazenide complex {Au(I)[RPhNNNPhR'][PPh$_3$]} [R = (C=O)NH$_2$, R' = (C=O)CH$_3$]**", aceita para publicação no periódico **Inorganica Chimica Acta** (v. 441, p. 78-85, 2016 - DOI: 10.1016/j.ica.2015.10.038)

A primeira página contendo o título da publicação e os autores pode ser visualizada abaixo:

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X-ray characterization and *in vitro* biological evaluation of 1-(4-amidophenyl)-3-(4-acetylphenyl)triazene and the gold(I) triazenide complex {Au(I)[RPhNNNPhR′][PPh$_3$]} [R = (C=O)NH$_2$, R′ = (C=O)CH$_3$]

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X-ray characterization and *in vitro* biological evaluation of 1-(4-amidophenyl)-3-(4-acetylphenyl)triazene and the gold(I) triazenide complex {Au(I)[RPhNNNPhR'][PPh_3]} [R = (C=O)NH_2, R' = (C=O)CH_3]

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Abstract

The asymmetric 1,3-disubstituted biaryl triazene 1-(4-amidophenyl)-3-(4-acetylphenyl)triazene (**1**) and the corresponding complex [1-(4-amidophenyl)-3-(4-acetylphenyl)triazenido-κN^3](triphenylphosphane-κP)gold(I) (**2**) were characterized by single-crystal X-ray structure analysis and, the antibacterial and the cytotoxic activities of both compounds were investigated. In the solid state the free molecule **1** shows a 2-D arrangement *via* classical N–H···N and N–H···O hydrogen bonds, while the linear coordinated gold(I) complex **2** reveals a 1-D arrangement *via* centrosymmetric pairs of molecules generated by classical N–H···O bifurcated hydrogen bonding. The cytotoxic potential of **1** and **2** on the bone marrow of patients with a clinical suspicion of leukemia compared with samples of an healthy patient was evaluated. Complex **2** exhibited expressive antiproliferative effects, especially on cells from patient with myelodysplastic syndrome. Antibacterial activities (minimum inhibitory concentration, MIC) of **1** and **2** against 16 bacteria were evaluated. Compound **2** presented an high level activity (MIC = 8 μg × mL^{-1}) against *Enterococcus faecalis* ATCC 29212, *E. faecalis* ATCC 51288 and *Staphylococcus epidermidis* ATCC 12228.

Keywords: Triazene; Gold(I)-triphenylphosphane triazenide complex; Antibacterial activity; Leukemia

1. Introduction

The substantial cancer increase [1] associated with the emergence of infections caused by multidrug-resistant (MDR) bacterial pathogens [2, 3] has led to the expansion of innovative therapies research. Considering this fact, there is an ongoing research project in view of the development of new metal-based complexes containing triazenide ligands and their *in vitro* biological evaluation, especially considering antibacterial and antileukemic activities.

In 2011, Curado et al. [4] published that leukemia is a leading cancer-related cause of death among children, adolescents, and young adults in Latin America. In spite of the considerable progress in leukemia treatment, relapse remains a common problem and most of the chemotherapy regimens used do not lead to a cure [5, 6, 7].

Antibiotics have also become an important component in medical practice. However, the widespread and inappropriate use of such drugs has resulted in the selection of resistant bacterial strains. Although many antimicrobial agents are still effective, therapeutic options for some infections are extremely limited. Remarkably, the increasing multidrug resistance and failure to discover new antibacterial drugs candidates have led to predictions of a medical catastrophe [2, 3]. Thereby, both novel targets and novel molecules that inhibit established targets through a different mode of action have been the main objective of studies addressing resistance [8].

Introduction of orally bioavailable gold compound Auranofin® as an alternative for arthritis treatment has conducted to a great interest of medicinal chemists in the development of new gold complexes synthesis [9]. Nevertheless, this promising discovery has not been materialized in an antineoplastic drug approved for clinical use, in spite of the great efforts made toward designing new molecules with this metal [10].

In recent years, the synthesis of molecules able to bind or react with DNA is an outstanding goal in the development of cytotoxic drug candidates [11]. Alkylating agents are electrophilic compounds that exert their effects *via* the transfer of alkyl carbon groups to several cellular constituents. However, DNA alkylation probably represents the major alteration leading to cell death [12,13]. Derivatives of triazenes in which the hydrogen of the diazoamino [-N=N(H)-] nitrogen triad is substituted by an alkyl group belongs to the class of alkylating agents. It is well established that such compounds presents technological potential [14] and pharmacological properties such as antitumor [15-22] and antibacterial [18,23,24] activities. From this point of view, Dacarbazine (DTIC) and Temozolomide (TMZ) are

examples of clinically very important triazenes. Synthesized in 1987, TMZ is an imidazotetrazine derivative from the DTIC, which can be administrated by both parenteral and oral routes [16]. Various preclinical and clinical studies have documented the substantial activity of the TMZ and other triazene-derived compounds against a wide range of tumors, including hematological neoplasms [15-22].

Taking in account that the $[(Ph_3P)Au]^+$ and H^+ cations are isolobal species, our goal was compare the biological activity of the title complex, **2**, with that of the free triazene **1** by substitution of H^+ on the diazoamine group of 1-(4-amidophenyl)-3-(4-acetylphenyl)triazene by the $[(Ph_3P)Au]^+$ fragment. It is worth to mention here that according of the best of our knowledge, only one complex of the type $[(L)Au(PPh_3)]$ (L = triazenido ligand), namely {[1,3-*Bis*(4-nitrophenyl)triazenido](triphenylphosphane)gold(I)} is described in the literature in analogy of complex **2** [25].

In this context, this work reports the crystal structure analysis by X-ray diffraction on a single crystal of the free triazene **1** and the related complex **2**, as well as, the evaluation of the *in vitro* antitumoral and antimicrobial activities of these compounds.

2. Experimental section

2.1 Synthesis of 1-(4-amidophenyl)-3-(4-acetylphenyl)triazene (HL), **1** and, of [1-(4-amidophenyl)-3-(4-acetylphenyl)triazenido-κN^3](triphenylfosphane-κP)gold(I) $(L)Au(PPh_3)]$, **2**.

The protonated triazene (HL) **1** and the related gold(I) complex $[(L)Au(PPh_3)]$ **2** were synthesized and characterized by spectroscopic methods as previously described [26]. In addition to the spectroscopic characterization, the elemental analysis of **1** $C_{15}H_{14}N_4O_2$ (282.30): calcd. C 63.82, H 5.00, N 19.85; found C 62.59, H 5.03, N 18.98 and, of **2** $C_{33}H_{28}AuN_4O_2P$ (740.54): calcd. C 53.52, H 3.81, N 7.57; found C 52.72, H 3.80, N 7.58 were carried out, respectively.

2.2 X-ray Crystal structure analysis

The X-ray diffraction experiments for the free triazene **1** and the related complex **2** were carried out using a single crystal fixed on a glass fiber for data collection. Data were collected at 20°C with a Bruker APEX II CCD area–detector diffractometer and graphite

monochromatized Mo-K$_\alpha$ radiation using the COSMO program. No intensity decay was observed during data collection. Crystal system and space group were unequivocally determined from the data sets and reciprocal space images. Cell refinement, data reduction, and absorption correction were performed applying software *SAINT* and *SADABS*, respectively [27]. The structure of both compounds were solved by direct methods and refined on F^2 with anisotropic temperature parameters for all non-hydrogen atoms [28, 29]. The positional parameters of the H atoms of the free triazene **1** were obtained from a Fourier map difference and refined with an isotropic displacement parameter. For complex **2,** the positional parameters of the H atoms bonded to C and N atoms were obtained geometrically, with the C–H and N–H distances fixed (0.96 Å for Csp^3 methyl; 0.93 Å for Csp^2 phenyl; 0.86 Å for Csp^2 N$_{amide}$), and refined as riding on their respective C and N atoms, with U_{iso}(H) = 1.5U_{eq}(Csp^3 methyl), U_{iso}(H) = 1.2U_{eq}(Csp^2 phenyl), and with U_{iso}(H) = 1.2U_{eq}(Csp^2 N$_{amide}$), respectively [29]. Graphical representations were drawn applying *DIAMOND* software [30].

2.3 *In vitro* antitumor activity evaluation

2.3.1 Cells and culture conditions

Six bone marrow samples were obtained at the Hematology-Oncology Laboratory of the Santa Maria University Hospital (HUSM), Santa Maria, Brazil. The samples were collected from different patients, four of them with suspicion of hematological malignancies (before antineoplastic treatment); and one from a patient without hematological malignancies (model of healthy cells). After isolation by Ficoll density-gradient centrifugation (Ficoll-Paque PLUS – GE Healthcare), the mononuclear cells were maintained in RPMI-1640 (Sigma-Aldrich) medium, supplemented with 20% fetal bovine serum (Cultilab, Brazil), 1 % (w/v) penicillin (100 U mL^{-1}) and streptomycin sulfate 100 µg mL^{-1} (Gibco, Invitrogen, Italy) at 37 ºC in a humidified 5% CO_2 atmosphere. Cell viability was estimated by Trypan Blue exclusion and cell number was determined by hemocytometry.

2.3.2 Cytotoxicity assay

The growth inhibitory effect toward bone marrow mononuclear cells was evaluated by means of an MTT assay [31]. The compounds were dissolved in 50% ethanol and diluted to the required concentrations (12.5, 50 and 100 µmol mL^{-1}). Exponentially growing cells were

added to each well of a flat-bottom in 96-well microplates (90 µL/well) at a density of 3 x 10^6 cells mL^{-1}. Then, the cells were exposed to different concentrations of compounds for 24 h in incubator at 37°C and 5% CO_2 humidified atmosphere. Control cultures received cells in the absence of assayed compounds. After 24 h of incubation, each well was treated with 10 µL of MTT dye (0.5 mg mL^{-1}). Four hours later, formazan product was dissolved by the addition of 100 µL of dimethyl sulfoxide. The inhibition of cell growth has been detected by measuring the absorbance at 570 nm using a Fisher Bio-Tek BT2000 MicroKinetics reader. The percentage of cell survival was defined as the relative absorbance of treated cells in relation to the respective controls. The IC_{50} value, defined as the drug concentration that reduced the mean absorbance at 570 nm to 50% of those in the untreated control wells, was calculated for each compound.

2.4 *In vitro* antibacterial activity evaluation

2.4.1 Bacterial strains

Sixteen bacterial strains were used in the study, which includes nine reference bacterial strains of the American Type Culture Collection (ATCC) (*Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 700603, *Staphylococcus aureus* ATCC 29213, *Staphylococcus aureus* ATCC 25923, *Staphylococcus epidermidis* ATCC 12228, *Enterococcus faecalis* ATCC 51288, *Enterococcus faecalis* ATCC 29212 and *Bacillus cereus* ATCC 10987) and seven multidrug-resistant (MDR) clinical bacterial isolates at Santa Maria University Hospital (*P. aeruginosa*, *Acinetobacter baumannii*, *Stenotrophomonas maltophilia*, *K. pneumoniae*, *K. oxytoca*, *Enterobacter brevis*, *S. aureus*, *S. epidermidis*). The guidelines of the Technical Note n° 1/2010, issued by the National Sanitary Surveillance Agency (ANVISA) [32], were followed for the characterization of MDR bacteria. According to them, the resistance to the different classes of antimicrobials in routine microbiological tests characterizes multidrug-resistant microorganisms.

2.4.2 Antimicrobial susceptibility testing

Minimal inhibitory concentrations (MIC) were determined in cation-adjusted Mueller Hinton broth (Difco, Detroit, MI) following the standard microdilution method [33].

Inoculates were prepared in the same medium at a density adjusted to a 0.5 McFarland turbidity standard (1.5 x 10^8 colony forming units - CFU mL^{-1}). The assayed compounds (**1** – **2**) were dissolved in 50% of ethanol. A total of 10 concentrations of the compounds were tested in each assay (0.25 to 128 µg mL^{-1}). The 96 well microplates were incubated without agitation at 35 ± 2 °C for 24 h. After incubation, 10 µL of 2% 2,3,5-triphenyltetrazolium chloride (TTC) were added. Live microorganisms reduce TTC by enzymatic action, originating formazan, kept inside granules in the cells, which become red [34]. All assays were performed in triplicate.

MIC value is defined as the lowest concentration of the compound that, under established *in vitro* conditions, inhibits visible growth of a bacterial culture compared with compound-free control wells [33].

3. Results and Discussion

3.1 Chemistry: Crystal structures

Crystal data and experimental parameters for the molecule **1** (hereinafter also designed as **HL**) and the complex **2** (in the following also designed as [(L)Au(PPh_3)]) are summarized in Table 1. Selected bond lengths (Å) and bond angles (°) are listed in Table 2.

Insert Table 1 here

Insert Table 2 here

The molecular structure of the $C_{15}H_{14}N_4O_2$, **HL,** and of [(L)Au(PPh_3)] are given in Figs. 1 and 2 in a thermal ellipsoid representation and atom-numbering scheme.

Insert Figure 1 here

Insert Figure 2 here

3.1.1 Crystal structure of ($C_{15}H_{14}N_4O_2$, **HL**)

Fig. 1 shows that the molecular structure of **HL** presents the characteristic stereochemistry *trans* about the N=N double bond in the diazoamino group of neutral free triazenes. Deviations observed from normal N-N and C_{aryl}-N bond lengths indicate a delocalization of the π electrons on the triazene group toward the terminal 4-amidophenyl and 4-acetylphenyl substituents of the N=N–N(H) moiety. N11=N12 bond [1.2720(17) Å] is longer than the typical value for a double bond (1.236 Å), whereas N12–N13 bond [1.3252(17) Å] is shorter than the typical value for a single bond (1.404 Å) [35, 36]. In addition, C11–N11 [1.4125(18) Å] and C21-N13 [1.3873(17) Å] bonds are shorter than characteristic N–C_{aryl} single bonds (secondary amines, R_2NH, R = Csp^2; 1.452 Å) [37].

The crystal structure of **HL** reveals molecules related by an axial 2_1 screw-axis parallel to the crystallographic direction [010], connected *via* intermolecular classical hydrogen bonds N2–H3···N11″ and N13–H13···O2‴, symmetry code (″) 1.5-*x*, -0.5+*y*, 0.5-*z*; (‴) 1.5-*x*, 0.5+*y*, 0.5-*z*. Such infinite chains are connected *via* intermolecular classical hydrogen bonds N2–H2···O1′ along the [30-1] base vector resulting in a two-dimensional network of **HL** molecules, symmetry code (′) -*x*, -*y*, 1-*z*. Figure 3 presents the unit cell of **HL** toward plane (103) including a section of the two-dimensional arrangement *via* secondary N-H···O and N-H···N interactions. The geometric details of the hydrogen bonding are listed in Table 3 and were calculated applying the *PLATON* program [38].

Insert Figure 3 here

Insert Table 3 here

On the other hand, the interplanar angles between the molecular fragments identified by letters (**a**), (**b**), (**c**), (**d**) and (**e**) of the free **HL** ligand (Fig. 4, Table 4) indicates that the delocalization of the π electrons on the triazene group toward the terminal substituents of the N=N–N(H) moiety is not hindered, resulting a slight deviation of planarity of the whole molecule.

Insert Figure 4 here

Insert Table 4 here

3.1.2 Crystal structure of ($C_{33}H_{28}AuN_4O_2P$, [(L)Au(PPh_3)])

The crystal structure of [(L)Au(PPh_3)] shows discrete neutral asymmetric two-coordinate mononuclear Au(I) complexes. The deprotonated triazenide ion L^- acts as an N13-η^1 monodentate (two-electron donor) ligand, while one neutral triphenylphosphane molecule completes the coordination sphere of the metal ion to an almost linear arrangement (Figure 2). The Au-N13 bond distance of 2.062(6) Å is similar to the sum of the covalent radii (2.144 Å) [35,36], and corresponds to a covalent single bond. The observed Au-P distance of 2.2243(19) Å agrees with that bond distance of 2.2524(13) Å observed in complex {[1,3-*Bis*(4-nitrophenyl)triazenido](triphenylphosphane)gold(I)} [25].

Similar to the observed on the free HL molecule, variances observed from normal N-N and C_{aryl}-N bond lengths demonstrate a delocalization of the π electrons on the triazene group toward the terminal aryl substituents of the deprotonated N=N-N moiety. The N11=N12 bond [1.274(8) Å] is longer than the typical value for a double-bonded N=N atoms, whereas the N12–N13 bond [1.320(8) Å] is shorter than the typical value for single-bonded N-N atoms. On the other hand the C11–N11 [1.421(8) Å] and C21-N13 [1.380(8) Å] bond distances are shorter than characteristic N–C_{aryl} single bonds as pointed out in the discussion of the structure features of HL.

The crystal structure of [(L)Au(PPh_3)] reveals centrosymmetric pairs of molecules, generated *via* classical N1–H1A···O1′ intermolecular hydrogen bonding, symmetry code (′) 2-*x*, -*y*, -*z*. In addition, such centrosymmetric pairs are within an infinite one-dimensional chain along the base vector [111] *via* intermolecular hydrogen bonds with a bifurcated geometry (D_1–H_1,D_2–H_2)···(*A*) (*D* = donor atom, *A* = acceptor atom), (N1–H1B,C15–H15)···O2″, symmetry code (″) -1+*x*, -1+*y*, -1+*z*. Fig. 5 shows the unit cell of [(L)Au(PPh_3)] toward the crystallographic direction [010] including a section of the one-dimensional arrangement *via* secondary N-H···O and C-H···O interactions.

Insert Figure 5 here

In comparison with the free molecule **HL**, the interplanar angles between the molecular fragments of the complex [(L)Au(PPh_3)] (Table 4, Fig. 4) demonstrate that the delocalization of the π electrons on the deprotonated triazenide group $[N_3]^-$ toward the terminal substituents of the N-N-N triad is hindered due to the significant deviation of the planarity of the whole coordinated L^- ligand.

3.2 Biological activities

3.2.1 *In vitro* cytotoxic studies

The cytotoxicity of triazene **1** and related (triphenylphosphane)gold(I)-complex **2** has been investigated through a standard bioassay. Before presenting cytotoxic effects of the compounds, the clinical diagnoses of the patients studied are shown below: patient **1** (63 year-old, male) has been diagnosed with acute myeloid leukemia (AML), M2 subtype according to the French-American-British (FAB) classification, showing normal karyotype (46,XY); patient **2** (35 year-old, female) has been diagnosed with chronic myeloid leukemia (CML) with karyotype 46,XX,t(1;9;22)(p22;q34;q11); patient **3** (9 year-old, male) has been diagnosed with T-lineage acute lymphocytic leukemia (T-ALL) showing karyotype 46,XY,del(3)(q25); patient **4** (54 year-old, female), inconclusive karyotype, has been diagnosed with myelodysplastic syndrome (MDS); patient **5** (38 year-old, male) has been diagnosed with CML showing karyotype 46,XY,t(9;22)(q34;q11).

In order to compare the observed cytotoxic activity of compounds **1** and **2** with the precursor complex chloro(triphenylphosphane)gold(I), assays including this last compound were carried out and evaluated in view to the growth inhibitory effect toward bone marrow mononuclear cells of the patient **5**.

Table 5 summarizes the results of cytotoxic effect of the compounds **1** and **2** on AML cells, CML cells, ALL cells, MDS cells, and normal cells of bone marrow.

Insert Table 5 here

Based on the concentration-response curve, cell survival did not depend on the compound concentration for **1** and **2.** The triazenide complex **2** showed better cytotoxic activity when compared to triazene molecule **1,** which proved to be ineffective against most of the samples tested. Compound **1** presented promising antiproliferative effect toward CML

cells (IC_{50} = 61.02 µmol mL^{-1}) and AML cells (IC_{50} = 77.47 µmol mL^{-1}) whereas compound **2** demonstrated a pronounced antitumor activity, especially against bone marrow cells from patient with MDS (IC_{50} = 7.72 µmol mL^{-1}) and CML (IC_{50} = 19.22 µmol mL^{-1}). The antiproliferative effect of chloro(triphenylphosphane)gold(I) toward bone marrow mononuclear cells of the patient **5** (IC_{50} = 109.80 µmol mL^{-1}) was significantly lower than that observed for complex **2**. The data also showed the small cytotoxic effect of **1** and **2** on normal cells. These results demonstrate that the coordination of the triazenide ligand to $[(triphenylphosphane)gold(I)]^+$ cation increase significantly the antiproliferative activity of the resulting complex **2** in comparison with that observed for the free triazene **1** against bone marrow mononuclear cells analyzed in this trial.

Thereby, our findings confirm that metal centers are very important for the cytotoxic activity as observed in the classic example Cisplatin®, in which the metal center coordinated to the DNA coil potentializes the antineoplasic activity of the metallodrug [39]. Therefore, it is clear that a general metal-biomolecule interaction is important for the anticancer activity of a metal based-drug [40]. However, biological properties of metal complexes are determined not only by the presence of the metal itself, but also by the number and types of ligands bound, oxidation state and the coordination geometry of the metal center in its respective complex. Also, many metal-based-drugs act as pro-drugs that undergo ligand substitution and redox reactions before they reach their targets [40, 41].

There are already studies with several novel gold(I)-phosphane complexes that display a wide spectrum of antineoplastic activity, especially in some cisplatin-resistant cell lines [42]. These recent surveys stimulate the search for other cytotoxic metal-based agents with improved pharmacological properties. In this context, the use of non platinum central atoms and the incorporation of different organic ligands into metal complexes showed encouraging perspectives.

The cytotoxic activity of complexes including phosphanes and diphosphanes ligands was investigated and is already well given in literature. It was found that this activity was preserved when Au(I) was coordinated by phosphine derivatives, but was generally reduced when the phenyl groups on the phosphine ligand were replaced by other substituents [43].

The precise mechanism related to the cytotoxicity of gold(I) derivatives in form of lipophilic complex cations remain not completely elucidated [44]. In order to advance in this context, the investigation of Au(I) and Ag(I) bidentate pyridyl phosphine complexes against cisplatin-resistant human cancer cells has demonstrated that in vitro cytotoxicity potencies depends strongly upon their lipophilicity [45].

3.2.2 *In vitro* antibacterial activity

The MIC values obtained can be visualized in Table 6. The results demonstrate that triazenide complex **2** proved more active in comparison with the free ligand **1**. This complex was capable to inhibit the growth of several strains, presenting excellent MIC values, especially for Gram-positive bacteria. In contrast, free ligand 1 showed MIC values higher than 128 µg mL^{-1} against all the bacteria assessed.

Insert Table 6 here

It is well documented in other studies that triazenes have better activity against Gram-positive than Gram-negative bacteria [18,24]. It can be partially explained by the fact that the outer membrane of Gram-negative species is known to present a barrier to the penetration of several antimicrobials molecules and the appearance of a mechanism of β-lactamases that are enzymes capable of hydrolyzing cephalosporins, penicillins, and carbapenems, antibiotics often used as antimicrobial therapy to treat nosocomial infections. The hydrolysis of β-lactam antimicrobial by β-lactamases is the most common mechanism of resistance for this class of antibacterial agents in clinically significant Gram-negative bacteria [46]. Although the mode of action of these alkylators is still not detailed, some studies have shown that triazenes are able to cleave plasmid DNA [18].

4. Conclusion

This research has presented one triazene derivative (compound **2**) which shows significant cytotoxic activity against three types of leukemia and MDS. Similarly to other gold(I)-phosphane complexes cited in the literature that display a wide spectrum of antineoplasic activity, in our case, the anticancer activity of the investigated molecules was favored by the presence of metal and by the phosphane. Both compounds **1** and **2** presented small cytotoxicity toward normal cells. The antibacterial properties of **2** indicate that this compound presents potential activity against most Gram-positive pathogens. The results presented suggest that compound **2** could be considered a potential candidate for the development of new antitumor and antibacterial bioactive agents.

Acknowledgments

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Supplementary material

CCDC 898763 and 898764 contains the supplementary crystallographic data for this paper. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif.

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Captions for Figures

Fig. 1. View of the molecular structure with atom–labeling scheme of **HL**. Displacement ellipsoids are drawn at the 50% probability level and H atoms are drawn with arbitrary radii.

Fig. 2. View of the molecular structure with atom–labeling scheme of [(L)Au(PPh_3)]. Displacement ellipsoids are drawn at the 50% probability level and H atoms are drawn with arbitrary radii.

Fig. 3. View of the supramolecular, 2-D arrangement of **HL** towards the plane (103). Infinite two-dimensional arrangement occurs along the crystallographic directions [010] and [30-1] *via* classical hydrogen bonds (indicated as dashed lines) N2-H2···O1′, N2-H3···N11″ and, N13-H13···O2‴; symmetry codes (′): -*x*, -*y*,1-*z*, (″): 1.5-*x*, -0.5+*y*, 0.5-*z*, (‴): 1.5-*x*, 0.5+*y*, 0.5-*z*. Atoms are drawn with arbitrary radii.

Fig. 4. Molecular fragment sections of the free triazene **1 (**X = H) and complex **2** (X = PPh_3).

Fig. 5. View of supramolecular structure, 1-D arrangement of [(L)Au(PPh_3)] toward the crystallographic direction [010]. The one-dimensional arrangement occurs along the base vector [111] *via* classical hydrogen bonds N1–HA···O1′ and non-classical hydrogen bonds C15–H15···O2″ (indicated as dashed lines); symmetry codes (′): 2-*x*, -*y*, -*z*. Atoms are drawn with arbitrary radii.

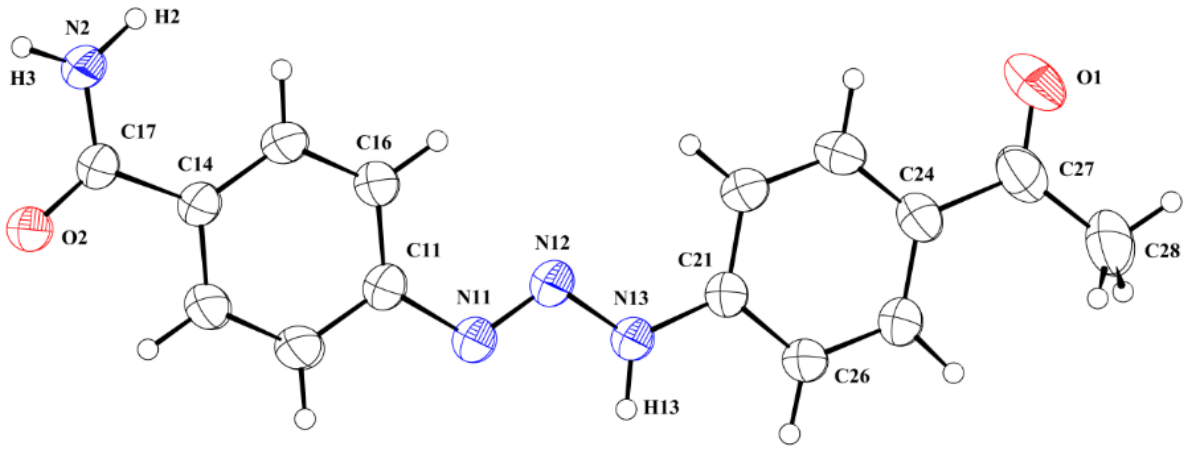

Figure 1 (*Maísa K. Tizotti, et al.*)

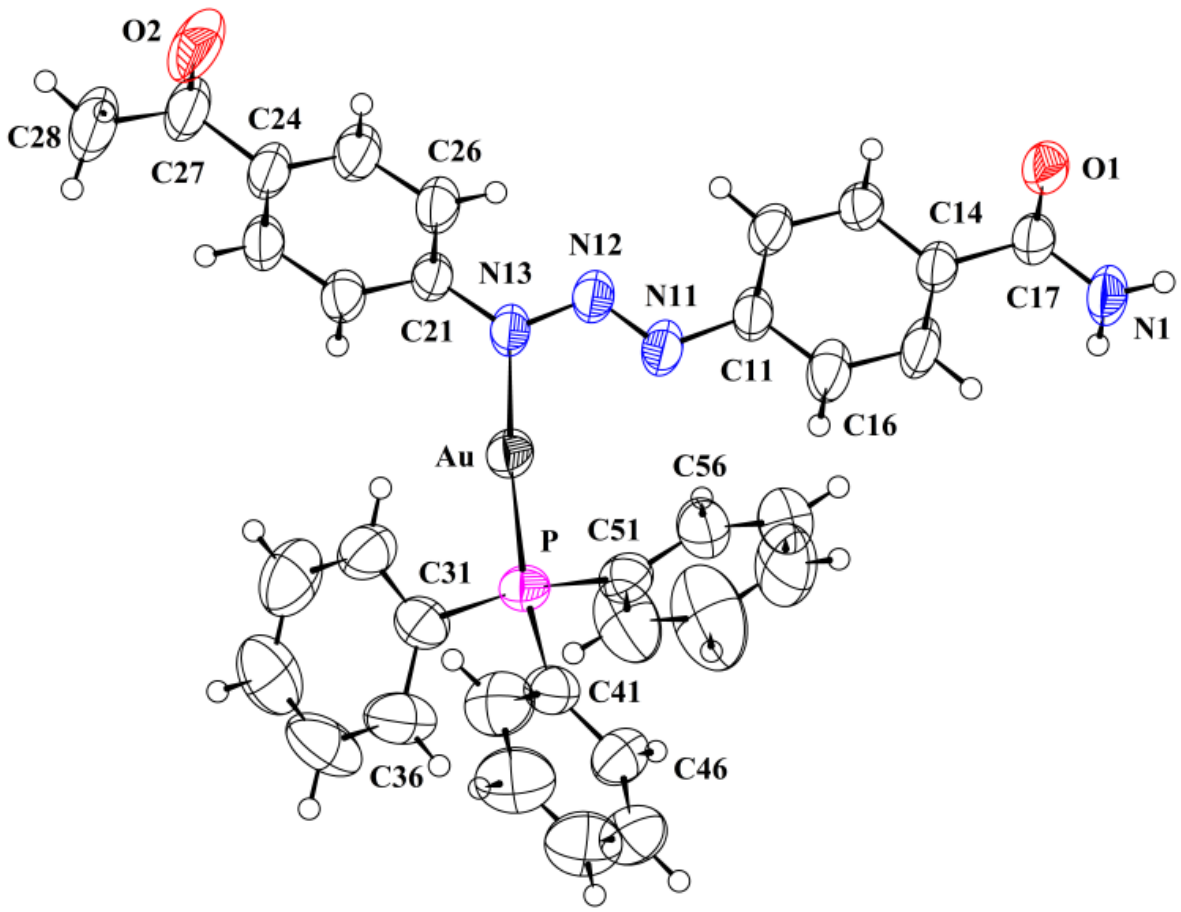

Figure 2 (*Maísa K. Tizotti, et al.*)

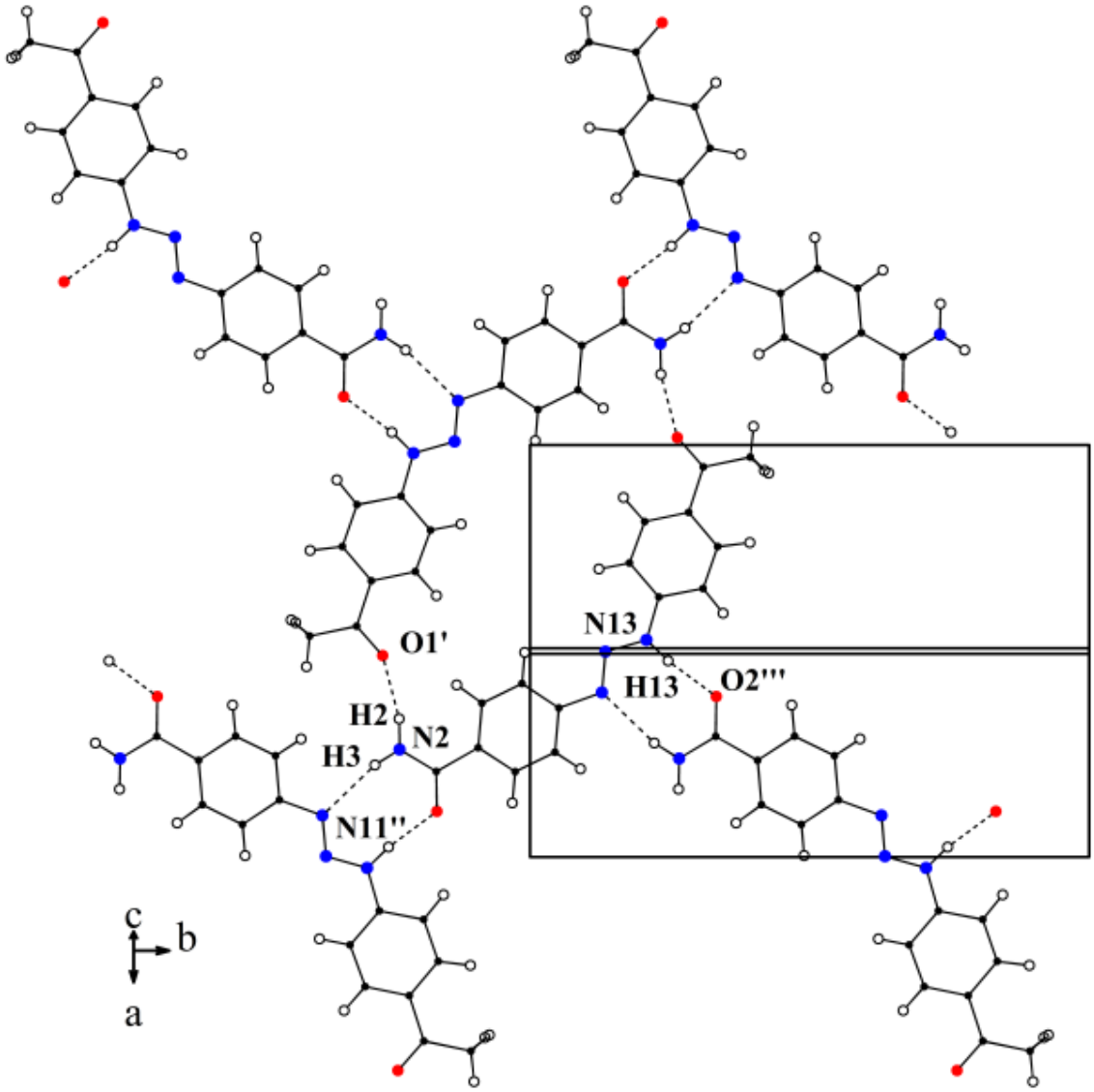

Figure 3 (*Maísa K. Tizotti, et al.*)

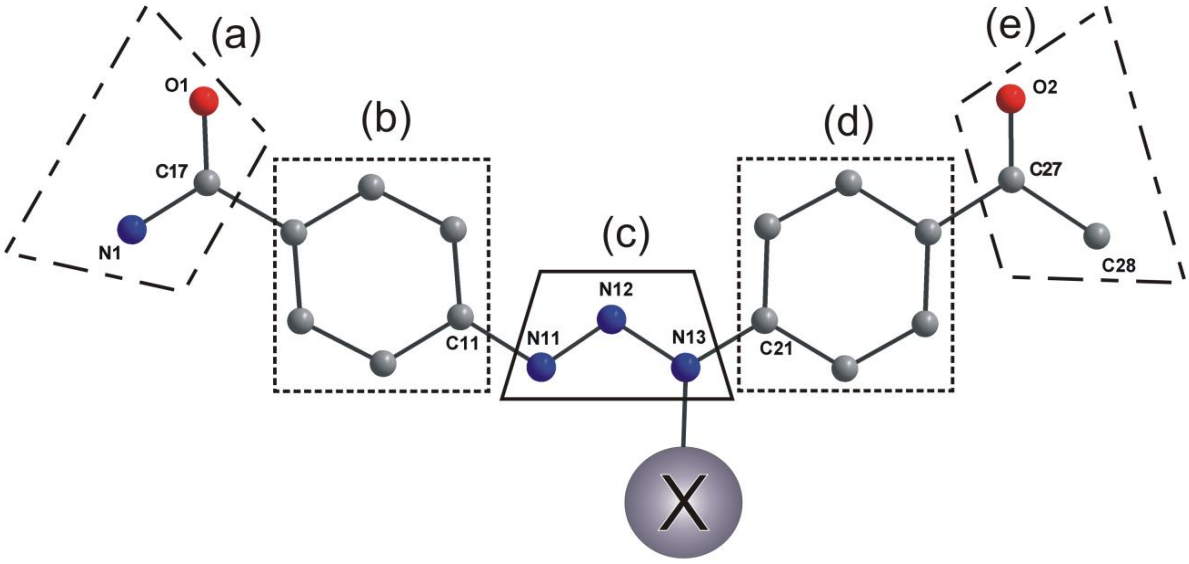

Figure 4 (*Maísa K. Tizotti, et al.*)

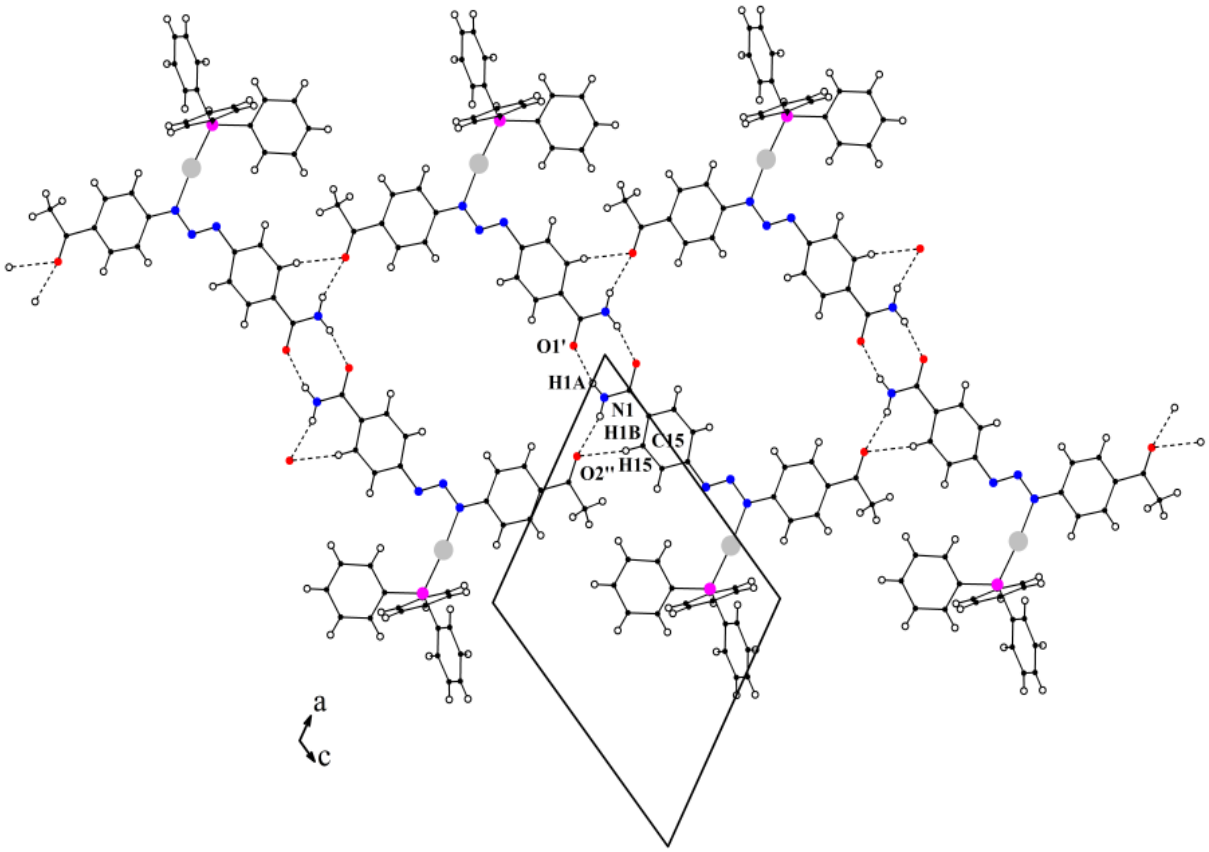

Figure 5 (*Maísa K. Tizotti, et al.*)

Table 1

Crystal data and structure refinement for the free triazene **1** and complex **2.**

	1	**2**
Formula	$C_{15}H_{14}N_4O_2$	$C_{33}H_{28}AuN_4O_2P$
M (g·mol^{-1})	282.30	740.53
Crystal size (mm^3)	0.41 x 0.24 x 0.16	0.20 x 0.14 x 0.12
Crystal system	Monoclinic	Triclinic
Space group	$P2_1/n$	$P(-1)$
a (Å)	7.2645(4)	12.1752(4)
b (Å)	17.2890(7)	12.3736(4)
c (Å)	11.6891(5)	14.2270(4)
α (°)	90	109.540(2)
β (°)	95.833(2)	112.488(2)
γ (°)	90	98.160(2)
V (Å^3)	1460.50(12)	1774.39(10)
Z	4	2
D (calc) (g·cm^{-3})	1.284	1.386
μ (MoKα)(mm^{-1})	0.089	4.221
F(000)	592	728
Temperature (K)	293(2)	293(2)
Radiation (MoKα)(Å)	0.71073	0.71073
Index ranges	$-9 \leq h \leq 5$	$-16 \leq h \leq 16$
	$-23 \leq k \leq 21$	$-15 \leq k \leq 16$
	$15 \leq l \leq 15$	$-17 \leq l \leq 18$
Reflections collected	25958	30480
Completeness to theta (%)	99.7 (θ = 28.38)	99.6 (θ = 27.91)
Max. and min transmission (%)	0.9859 and 0.9645	0.6313 and 0.4856
Unique data, *R*(int)	3645 (0.0316)	8463 (0.0414)
θ range (°)	2.11 to 28.38	1.71 to 27.91
Observed data [(I > 2σ(I)]	2335	6150
R	0.0445	0.0450
wR_2	0.1132	0.1463
S (F^2)	1.020	1.121
Largest diff. peak and hole [e·Å^{-3}]	0.197 / -0.182	1.826 / -0.645

Table 2

Selected geometric parameters (Å, °) for the free triazene **1** and complex **2.**

Bond lengths		Bond angles	
1			
C11 – N11	1.4125(18)	O2-C17-N2	121.79(13)
C17-O2	1.2418(17)	O2-C17-C14	119.03(12)
C17-N2	1.3238(19)	N2-C17-C14	119.17(13)
C21-N13	1.3873(17)	O1-C27-C24	120.76(17)
C27-O1	1.215(2)	O1-C27-C28	119.41(16)
C27-C28	1.491(3)	C24-C27-C28	119.82(16)
N2-H2	0.96(2)	H2-N2-H3	121.4(18)
N2-H3	0.91(2)	N12-N11-C11	114.64(12)
N11-N12	1.2720(16)	N11-N12-N13	110.80(12)
N12-N13	1.3252(17)	N12-N13-C21	121.81(12)
N13-H13	0.93(2)	N12-N13-H13	119.7(12)
2			
C11-N11	1.421(8)	O1-C17-N1	121.2(6)
C17-O1	1.235(9)	O1-C17-C14	121.4(6)
C17-N1	1.343(9)	N1-C17-C14	117.4(6)
C21-N13	1.380(8)	O2-C27-C24	121.9(7)
C27-O2	1.202(11	O2-C27-C28	121.8(8)
C31-P	1.821(8)	C24-C27-C28	116.0(8)
C41-P	1.823(8)	H1A-N1-H1B	120.0 (8)
C51-P	1.820(8)	N12-N11-C11	113.0(6)
N11-N12	1.274(8)	N11-N12-N13	111.8(6)
N13-Au	2.062(6)	N12-N13-C21	114.9(6)
P-Au	2.2243(19)	N12-N13-Au	117.7(4)
		N13-Au-P	174.48(15)

Table 3

Hydrogen-bonding geometric parameters (Å, °) for the free triazene **1** and complex **2**.

D–H···*A*	*D*–H	H···*A*	*D*···*A*	∠*D*–H···*A*
Ligand (**1**)				
N2-H2···O1′	0.96(2)	2.02(2)	2.960(2)	165.5(19)
N2-H3···N11″	0.91(2)	2.25(2)	3.1471(18)	167.8(19)
N13-H13···O2‴	0.93(2)	1.86(2)	2.7794(17)	170(2)
Complex (**2**)				
N1-H1A···O1′	0.86	2.12	2.956(9)	164
N1-H1B···O2″	0.86	2.15	2.973(11)	161
C15-H15···O2″	0.93	2.50	3.419(12)	171

(*D* = donor atom, *A* = acceptor atom)

Symmetry codes: (′) -*x*, -*y*, 1-*z*, (″) 1.5-*x*, -0.5+*y*, 0.5-*z* , (‴) 1.5-*x*, 0.5+*y*, 0.5-*z* for ligand **1**

Symmetry codes: (′) 2-*x*, -*y*, -*z*, (″) -1+*x*, -1+*y*, -1+*z* for complex **2**

Table 4

Interplanar angles (°) between molecular fragments of the free triazene **1** and complex **2**.

Fragments	(a)/(b)	(b)/(c)	(c)/(d)	(d)/(e)	(b)/(d)
HL	7.2(2)	4.2(2)	1.4(2)	6.3(3)	3.8(1)
1	5(1)	6.8(1)	12.0(1)	8(2)	13.2(4)

Table 5

Cytotoxic activity expressed as IC_{50} of compounds **1** and **2** (MTT-dye reduction assay).

Samples	**1**			**2**		
	Concentration (μmol mL^{-1})	% cell death	IC_{50} [(a)] (μmol mL^{-1})	Concentration (μmol mL^{-1})	% cell death	IC_{50} [(a)] (μmol mL^{-1})
1 AML [(b)]	100	45.33	77.47	100	66.17	23.38
	50	31.56		50	51.39	
	12.5	51.82		12.5	60.93	
2 CML [(c)]	100	21.07	306.4	100	52.33	50.69
	50	12.52		50	53.10	
	12.5	26.18		12.5	34.10	
3 ALL [(d)]	100	27.28	205.2	100	71.10	35.36
	50	19.25		50	57.60	
	12.5	29.08		12.5	30.12	
4 MDS [(e)]	100	23.42	131.9	100	81.92	7.72
	50	35.58		50	82.30	
	12.5	49.40		12.5	67.03	
5 CML [(f)]	100	55.77	61.02	100	77.57	19.22
	50	42.70		50	72.58	
	12.5	31.67		12.5	42.70	
CP [(g)]	100	15.78	453.9	100	21.67	315.2
	50	7.22		50	12.50	
	12.5	24.64		12.5	19.68	

[(a)] IC_{50} (dose required to achieve 50% decrease in cell growth). [(b)] 1 AML = acute myeloid leukemia cells of patient number 1. [(c)] 2 CML = chronic myeloid leukemia cells of patient number 2. [(d)] 3 ALL = acute lymphocytic leukemia cells of patient number 3. [(e)] 4 MDS = myelodysplastic syndrome of patient number 4. [(f)] 5 CML = chronic myeloid leukemia cells of patient number 5. [(g)] CP = control patient cells of bone marrow.

Table 6

Minimum inhibitory concentration values (μg mL^{-1}) for the triazene **1** and complex **2.**

Bacterial strain	**1**	**2**
	MIC (μg mL^{-1})	
Staphylococcus aureus ATCC 25923	> 128 μg mL^{-1}	16 μg mL^{-1}
Staphylococcus aureus ATCC 29213	> 128 μg mL^{-1}	16 μg mL^{-1}
Enterococcus faecalis ATCC 29212	> 128 μg mL^{-1}	8 μg mL^{-1}
Enterococcus faecalis ATCC 51288	> 128 μg mL^{-1}	8 μg mL^{-1}
Staphylococcus epidermidis ATCC 12228	> 128 μg mL^{-1}	8 μg mL^{-1}
Bacillus cereus ATCC 14579	> 128 μg mL^{-1}	16 μg mL^{-1}
Escherichia coli ATCC 25922	> 128 μg mL^{-1}	32 μg mL^{-1}
Klebsiella pneumoniae ATCC 700603	> 128 μg mL^{-1}	> 128 μg mL^{-1}
Pseudomonas aeruginosa ATCC 27853	> 128 μg mL^{-1}	> 128 μg mL^{-1}
Staphylococcus aureus (MDR*)	> 128 μg mL^{-1}	16 μg mL^{-1}
Staphylococcus epidermidis (MDR)	> 128 μg mL^{-1}	16 μg mL^{-1}
Klebsiella oxytoca (MDR)	> 128 μg mL^{-1}	16 μg mL^{-1}
Pseudomonas aeruginosa (MDR)	> 128 μg mL^{-1}	> 128 μg mL^{-1}
Stenotrophomonas maltophilia (MDR)	> 128 μg mL^{-1}	> 128 μg mL^{-1}
Empedobacter brevis (MDR)	> 128 μg mL^{-1}	> 128 μg mL^{-1}
Acinetobacter baumannii (MDR)	> 128 μg mL^{-1}	> 128 μg mL^{-1}

* MDR = multidrug-resistant bacteria

3.2 MANUSCRITO

Esta seção compreende um manuscrito científico intitulado "***In vitro* cytotoxic and antibacterial properties of three new copper(II) complexes containing 1,2,3-benzotriazine derivative and N-donor heterocyclic ligands**", redigido de acordo com as normas do periódico **Dalton Transactions**, ao qual será submetido.

In vitro cytotoxic and antibacterial properties of three new copper(II) complexes containing 1,2,3-benzotriazine derivative and N-donor heterocyclic ligands

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Abstract

Three new copper(II) mixed-ligand complexes $[(L)_2(Py)_2(OH_2)Cu(II)]$ (**1**), $[(bipy)_2(L)_4Cu(II)_2]$ (**2**) and $[(Phen)_2(L)_4Cu(II)_2]$ (**3**), where HL = 1,2,3-benzotriazine-4(3*H*)-one; Py = Pyridine; bipy = 2,2'-Bipyridine; phen = 1,10-Phenanthroline, were synthesized and characterized by means of elemental analysis and various spectroscopy (IR, ESI-MS, NMR). In addition, the single crystal structure analysis of compounds **1-3** were carried out and results were presented. The *in vitro* biological evaluation of compounds comprised the study of their cytotoxicity against one non-tumor (VERO) and three tumor cell lines (K562, MCF-7 and B16F10), as well as bone marrow cells from patients with clinical suspicion of hematological malignancies. The antibacterial activity was also tested. Notably, our results revealed that binuclear Cu(II) complexes **2-3** presented remarkable cytotoxic effects towards two tumor cell lines with a potency higher than Cisplatin (IC_{50} = 69.32 to 73.55 µM), used as standard drug. Complex **3** was highly cytotoxic on B16F10 melanoma (IC_{50} = 4.37 µM) and MCF-7 breast cancer (IC_{50} = 6.16 µM) lineages, as well as, on bone marrow samples, especially from patients with chronic myeloid leukemia (IC_{50} = 7.76 µM) and myeloproliferative syndrome (IC_{50} = 7.01 µM). Moreover, complexes **2** and **3** also exhibited moderate antibacterial effect against both Gram-positive and Gram-negative strains. Thus, these findings clearly demonstrated the promising biological properties of complex **3**, particularly its high cytotoxic activity.

Keywords: Cu(II) complexes; 1,2,3-benzotriazine-4(3*H*)-one; 1,2,3-benzotriazenide-4-one ligand; Cytotoxicity; Antibacterial activity.

Introduction

Cancer is widely recognized as a growing global public-health burden and its consequences are particularly devastating. According to a World Health Organization (WHO) report, neoplastic diseases were responsible for 8.2 million deaths in 2012. More than 70% of them occurred in Africa, Asia and Latin America.[1] Although considerable progress have been achieved from diagnosis to cancer treatment[2], the effectiveness of most conventional chemotherapeutic agents (*e.g.* platinum-based anticancer drugs) is limited due to serious side effects and resistance phenomena.[3]

In addition, another world concern is the widespread emergence of multidrug-resistant bacteria strains because of improper antibiotic administration in treatment of human or livestock infections, resulting in increased rates of morbidity and death.[4] At the same time, there is a distinct lack of new antimicrobial agents in development and untreatable infections are becoming a reality.[5]

To overcome these issues, much effort has been dedicated in order to identify innovative molecules with anticancer and antibacterial properties. In this regard, the development of metal-based compounds is the current focus of many medicinal chemists[6] and could be an effective strategy to discovery new therapeutic options.[7] Among transition metals, copper has received considerable attention mainly due to its critical roles in biological systems.[8] During the last decade, a large number of copper complexes were synthesized and screened for their biological potential.[9-10] Notably, several mixed-ligands Cu(II) complexes, in particular those containing heterocyclic diimines (phen or bipy and substituted analogs), have exhibited promising anticancer and antibacterial activities, as well as higher efficiency to cleave DNA.[10-28]

In recent years, our emphasis has been on designing and testing biological activity of triazenes[29] and triazenide complexes[30] because of their unique chemical properties and versatile pharmacological effects. Noteworthy, aromatic nitrogen heterocycles like 1,2,3-triazines has been recognized as other biologically interesting ligands.[31] Some 1,2,3-benzotriazine derivatives display wide range of activities such as anesthetic[32], central nervous system effects[33], antidiarrheal[34], anti-inflammatory[35], antitubercular[36] and anticancer.[37]

Thereby, this paper describes, for the first time, the synthesis, characterization and *in vitro* biological properties of three Cu(II) complexes containing 1,2,3-benzotriazenide-4-one moieties and different pyridyl co-ligands (Scheme 1). The cytotoxicity of these compounds

was tested on 4-panel cell lines and human bone marrow mononuclear cells (BMMCs) using MTT assay. Furthermore, their antibacterial properties were also investigated.

Insert Scheme 1 here

Experimental section

Materials and physical measurements

All reagents and solvents were of analytical grade and used as received, without further purification. For the biological activity assays, sterile ultrapure water (18.2 MΩ) was obtained by purification, through Milli-Q® water purification system (Millipore®), as well as by autoclaving. The elemental analyses were performed using a varioMICRO CHNS analyzer. Infrared absorption spectra were obtained using KBr pellets on Bruker IFS 113v infrared spectrometer. ^{1}H and ^{13}C NMR spectra were obtained by a Bruker DPX-400 spectrometer and recorded at 400 MHz. Electrospray Ionization Mass Spectrometry (ESI-MS) analyses was performed on a Bruker micrOTOF-Q mass spectrometer.

Syntheses

Synthesis of 1,2,3-benzotriazine-4(3*H*)-one (HL). To a mixture of concentrated HCl (3 mL), acetic acid (20 mL) and H_2O (30 mL), pulverized anthranilamide (20.0 mmol, 2.72 g) was added. After almost complete solubilization under vigorous stirring, the temperature was adjusted between 0 and 2 °C. Then, $NaNO_2$ (21.0 mmol, 1.45 g) previously dissolved in H_2O (5 mL) was added slowly. The reaction was maintained for 30 min. Adjustment of the pH of the reaction medium between 6.5 and 7.0 was followed by aqueous solution Na_2CO_3 (20%), resulting in a white precipitate which was filtered off and washed with H_2O (300 mL). The product was purified by recrystallization in ethanol (Scheme 2A). Yield: 93% (2.74 g, 18.6 mM) based on anthranilamide; m.p. 218 °C (210-212 °C). Anal. Calcd for $C_7H_5N_3O$: C, 57.14; H, 3.43; N, 28.56. Found: C, 57.20; H, 3.59; N, 28.81. IR (KBr, ν_{max} cm^{-1}): 3130 [s, ν(N-H)], 3039 [s, ν(C-H)], 2998 [s, ν(C-H)], 2903 [s, ν(C-H)], 2862 [s, ν(C-H)], 2820 [m, ν(C-H)], 2787 [s, ν(C-H)], 1700 [vs, ν(C=O)], 1605 [m, ν(C=C)], 1574 [m, ν(C=C)], 1527 [m, ν(C=C)], 1457 [m, ν(C=C)], 1386 [w], 1333 [m, ν_{as}(N=N)], 1289 [m], 1254 [m], 1228

[m], 1135 [s], 1108 [m], 1096 [m, δ(N-H)], 1062 [w], 1049 [w], 1019 [w], 948 [s], 842 [m, δ(C-H) out of plane], 813 [s, δ(C-H) out of plane], 784 [s, δ(C-H) out of plane], 681 [s, δ(C-H) out of plane] 593 [m], 548 [s], 495 [m], 486 [m]. EM (ESI(-)-TOF), *m/z* = 315.1 $[2M+Na-2H]^+$; 299.1 $[2M-N-4H]^+$; 293.2 $[2M-H)]^+$; 282.0; 255.2; 249.0 $[2M-(3N+3H)]^+$; 236.1 $[2M-(2N+2H)]^+$; 221.2 $[M+Ph-2H]^+$; 205.0; 200.1; 188.0; 180.0; 171.1; 155.0; 147.0382 [calculated mass 147.0433; $M]^+$; 146.0349 [base peak, $M-H]^+$; 139.0 $[M-N_2-H]^+$; 90.0 $[PhN]^+$. ^{1}H NMR (DMSO-d_6/TMS, 400 MHz) δ = 14.89 (s, 1*H*, N-*H*); 8.25 (d, *J*=7.8 Hz, 1*H*, Ar-*H*); 8.18 (d, *J*=8.1 Hz, 1*H*, Ar-*H*); 8.10 (t, *J*=7.7 Hz, 1*H*, Ar-*H*); 7.93 (t, *J*=7.5 Hz, 1*H*, Ar-*H*). ^{13}C NMR (DMSO-d_6, 100 MHz) δ = 155.21 (*C*(=O)NH_2); 143.90; 134.72; 123.05; 127.48; 123.92; 119.94.

Synthesis of [*trans-bis*(1,2,3-benzotriazenide-4-one-*κN*^{1})*trans-bis*(pyridine-*κN*)(aquo-*κO*)cobre(II)], [(L)$_2$(Py)$_2$(OH$_2$)Cu(II)] (1). To a solution of **HL** (2.0 mmol, 295 mg) in dimethylformamide (10 mL) and methanol (2 mL) was added KOH pellets (3.0 mmol, 168 mg) under magnetic stirring, followed by addition of copper(II) acetate dihydrate $[Cu(AcO)_2{\cdot}H_2O]_2$ (1.0 mmol, 199.6 mg) dissolved in 5 mL of a mixture of methanol and pyridine (1:1). After stirring for 15 min, H_2O (2.0 mL) was added. The reaction medium was kept under stirring for 16 h, while a blue-violet solution was observed. This was filtered to remove possible solid impurities. Blue needle-shaped crystals suitable for X-ray diffraction were observed directly in the reaction medium after one day. The light blue crystals were separated prior to the total evaporation of the solvent mixture from the reaction medium, washed with methanol (10 mL) and air dried. A light blue monocrystal was isolated for analysis by X-ray diffraction. Note: Drying the light blue crystals under vacuum turns them green, demonstrating decomposition of the original structurally characterized complex (Scheme 2B). Yield: 72% (383 mg, 0.72 mM) based on $[Cu(AcO)_2{\cdot}H_2O]_2$; m.p. 238 °C (explosive decomposition). Anal. Calcd for $C_{24}H_{20}CuN_8O_3$: C, 54.18; H, 3.79; N, 21.06. Found: C, 53.44; H, 4.05; N, 21.17. IR (KBr, ν_{max} cm^{-1}): 3530 [m, ν(O-H)], 3466 [m, ν(O-H)], 3104 [w, ν(C-H)], 3070 [w, ν(C-H)], 1643 [w, ν(C=O)], 1598 [vs, ν(C=C)], 1567 [s, ν(C=C)], 1482 [m, ν(C=C)], 1460 [m, ν(C=C)], 1450 [s, ν(C=C)], 1395 [m, ν(N=N)], 1357 [w], 1343 [w], 1236 [w], 1218 [m, ν(N–N)], 1157 [m], 1142 [s], 1062 [s], 1041 [m], 1017 [m], 959 [m], 782 [s, δ(C-H) out of plane], 762 [m, δ(C-H) out of plane], 736 [m, δ(C-H) out of plane], 696 [s, δ(C-H) out of plane], 627 [m], 502 [m], 438 [m]. EM (ESI(+)-TOF), *m/z* = 903.7 $[M+2L_1+py+H]^+$; 885.0 $[M+2L_1+py-OH]^+$; 868.1 $[M+Cu+2L_1-H_2O]^+$; 763.4

[M+Cu+L_1+Na]$^+$; 716.0 [M+Cu+H_2O+Na+py]$^+$; 703.1; 685.4 [M+2py-4H]$^+$; 643.2; 619.5 [M+N_3CO+OH]$^+$; 591.5 [M+Cu-3H]$^+$; 550.0 [M+H_2O+H]$^+$; 534.0 [base peak, M+3H]$^+$; 473.3 [M-2N-CO-2H]$^+$; 442.0 [M-NPh+H]$^+$; 413.3 [M-NPhCO]$^+$; 393.3; 365.0 [M-(L_1+H_2O+2H)]$^+$; 337.0 [M-(py+NPhCO)+3H]$^+$.

Synthesis of {*trans-bis*[2,2'-bipyridine-κ^2N^1,N^2)(1,2,3-benzotriazenide-4-one-κN^1)(μ_2-1,2,3-benzotriazenide-4-one-κ^2N^1,N^2)]}, [(bipy)$_2$(L)$_4$Cu(II)$_2$] (2). To a solution of **HL** (2.0 mmol, 295 mg) in dimethylformamide (15 mL) and methanol (2 mL) was added KOH pellets (3.0 mmol, 168 mg) under magnetic stirring, followed by the addition of [Cu(AcO)$_2$·H_2O]$_2$ (1.0 mmol, 199.6 mg) and 2,2'-bipyridine (1.0 mmol, 156 mg) dissolved in methanol (10 mL). After 15 min of magnetic stirring, H_2O (2.0 mL) was added. The reaction medium was maintained under magnetic stirring for 16 h, while a blue solution was observed. Blue block crystals were observed after 5 days, which were separated by filtration prior to the total evaporation of the solvent mixture from the reaction medium, washed with three portions of methanol (10 mL) and air dried. A light blue monocrystal was isolated for analysis by X-ray diffraction (Scheme 2C). Yield: 78% (399 mg, 0.39 mM) based on [Cu(AcO)$_2$·H_2O]$_2$, m.p. 239-240 °C. Anal. Calcd for $C_{48}H_{32}Cu_2N_{16}O_4$: C, 56.30; H, 3.15; N, 21.89. Found: C, 56.14; H, 3.35; N, 22.04. IR (KBr, ν_{max} cm^{-1}): 3118 [w, ν(C-H)], 3106 [w, ν(C-H)], 3077 [w, ν(C-H)], 3034 [w, ν(C-H)], 1639 [s, ν(C=O)], 1621 [vs, ν(C=O)], 1607 [vs, ν(C=C)], 1568 [s, ν(C=C)], 1496 [w, ν(C=C)], 1475 [m, ν(C=C)], 1467 [m, ν(C=C)], 1443 [s, ν(C=C)], 1393 [m, ν(N=N)], 1353 [w], 1340 [m], 1314 [w], 1254 [w], 1220 [s, ν(N–N)], 1156 [m], 1147 [m], 1138 [s], 1107 [m], 1065 [m], 1030 [s], 1019 [m], 953 [m], 774 [s, δ(C-H) out of plane], 731 [s, δ(C-H) out of plane], 693 [m, δ(C-H) out of plane], 650 [w], 636 [m], 623 [m], 505 [m], 424 [m]. EM (ESI(+)-TOF), *m/z* = 1234.1 [M+9Na+5H]$^+$; 1210.1 [M+8Na+4H]$^+$; 1095.1 [M+3Na+4H]$^+$; 1077.0 [M+3Na-N]$^+$; 1054.1 [M+2Na-N]$^+$; 1013.3 [M-(2N+CO)+2Na+1H]$^+$; 1001.3; 985.3; 973.3; 957.3 [M-Cu+2H]$^+$; 935.1; 878.1 [M-L_1+2H]$^+$; 845.0 [M-(L_1+CO)+3H]$^+$; 761.1 [M-(L_1+NPhCO)+3H]$^+$; 720.0 [M-(L_1+bipy)]$^+$; 697.1; 605.0 [M-(L_1+bipy+NPhCO)+3H]$^+$; 568.0 [M-(L_1+2bipy)+4H]$^+$; 518.0; 474.2 [Cu+H+3L_1-CO]$^+$; 438.0 [Cu+L_1+bipy+N_3CO+3H]$^+$; 410.1; 365.0 [base peak, Cu+L_1+bipy]$^+$; 337.0 [Cu+L_1+bipy-N_2]$^+$.

Synthesis of {*trans-bis*[1,10-phenantroline-κ^2N^1,N^2)(1,2,3-benzotriazenide-4-one-κN^1)(μ_2-1,2,3-benzotriazenide-4-one-κ^2N^1,N^2)]}, [(Phen)$_2$(L)$_4$Cu(II)$_2$] (3). The complex was

prepared by employing the procedure employed for **2** and using 1,10-phenanthroline (1.0 mmol, 180 mg) in place of 2,2′-bipyridine. A light blue monocrystal was isolated for analysis by X-ray diffraction (Scheme 2D). Yield: 82% (440 mg, 0.41 Mmol) based on $[Cu(AcO)_2 \cdot H_2O]_2$; m.p. 244-245 °C. Anal. Calcd for $C_{52}H_{32}Cu_2N_{16}O_4$: C, 58.26; H, 3.01; N, 20.91. Found: C, 57.36; H, 3.12; N, 20.75. IR (KBr, ν_{max} cm^{-1}): 3071 [w, ν(C-H)], 3059 [w, ν(C-H)], 3034 [w, ν(C-H)], 1640 [s, ν(C=O)], 1625 [vs, ν(C=O)], 1606 [vs, ν(C=C)], 1588 [s, ν(C=C)], 1519 [m, ν(C=C)], 1495 [w, ν(C=C)], 1479 [m, ν(C=C)], 1468 [m, ν(C=C)], 1456 [m, ν(C=C)], 1427 [s, ν(C=C)], 1391 [m, ν(N=N)], 1355 [w], 1337 [m], 1245 [w], 1220 [s, ν(N–N)], 1159 [m], 1136 [m], 1107 [m], 1071 [m], 1065 [m], 1053 [w], 1038 [s], 1017 [m], 954 [m], 874 [m], 851 [s], 786 [s], 774 [s, δ(C-H) out of plane], 724 [s, δ(C-H) out of plane], 692 [m, δ(C-H) out of plane], 648 [w], 623 [m], 503 [m], 432 [m]. EM (ESI(+)-TOF), *m/z* = 1033.3 $[M\text{-}NCO+5H]^+$; 983.1 $[M\text{-}NPh+3H]^+$; 926.1009 $[M\text{-}L_1+2H]^+$; 924.1025 [calculated mass 924.1030; $M\text{-}L_1]^+$; 891.1 $[M\text{-}phen+H]^+$; 859.5 $[M\text{-}(phen+2N+3H]^+$; 815.0 $[M\text{-}(phen+Ph)+H]^+$; 778.1; 727.5 $[M\text{-}(L_1+Phen+N+3H)]^+$; 683.4; 639.4 $[M\text{-}(L_1+Phen+PhCO+H)]^+$; 569.1 $[M\text{-}(2L_1+Phen+CO+H)]^+$; 526.3 $[Cu+3L_1+2N\text{-}3H]^+$; 498.2 $[Cu+3L_1\text{-}3H]^+$; 423.1 [base peak, $Cu+2Phen]^+$; 391.0 $[Cu+Phen+L_1+2H]^+$; 361.0 $[Cu+Phen+L_1\text{-}2N]^+$; 317.0 $[Cu+2L_1\text{-}NCO+4H]^+$; 288.0 $[Cu+Phen+NCO+4H]^+$; 243.0 $[Cu+Phen]^+$.

Insert Scheme 2 here

Single crystal structure analysis

Diffraction data for Cu(II) complexes were collected using Mo-Kα (k = 0.71073 Å) radiation on a Bruker D8 APEX2 diffractometer equipped with a CCD area detector at room temperature 293(2) K. The structures were solved by the direct methods using *SHELXS*97 or *SIR*2004 programs.[38,39] Non-hydrogen atoms were refined anisotropically with the full-matrix least-squares technique based on F^2 using the *SHELXL*2014 program.[40] The positions of the hydrogen atoms were calculated geometrically based on the model of the atom H coupled to the respective atom C, with refinement including isotropic thermal parameters U_{iso}(H) = 1.2U_{eq}(Csp^2-phenyl) and the bond length C-H = 0.93 Å.[40] The graphical representations involved the DIAMOND program version 3.1.a.[41]

Citotoxicity studies

Cells lines and growth conditions. The tumor cell lines K562 (human chronic myeloid leukemia), MCF7 (human breast adenocarcinoma) and B16F10 (murine melanoma), as well as non-tumoral cell line VERO (established from the kidney of a normal adult African green monkey) were obtained from the Rio de Janeiro Cell Bank (RJ, Brazil). Cells were grown in Dulbecco's Modified Eagle Medium (DMEM, Sigma-Aldrich, USA), supplemented with 10% (v/v) Fetal Bovine Serum (FBS, Cultilab, Brazil) and 1% (v/v) antibiotic antimycotic solution (Sigma-Aldrich, USA). All cultures were maintained at 37 °C in a humidified atmosphere with 5% CO_2.

Isolation of bone marrow mononuclear cells and culture conditions. Diagnostic samples of bone marrow aspirates from five untreated patients with suspected hematological malignancies, surplus to clinical requirements, were obtained at the Hematology-Oncology Laboratory of the University Hospital of Santa Maria (HUSM). The bone marrow mononuclear cells (BMMCs) were isolated by density gradient centrifugation using Ficoll-Paque PLUS (GE Healthcare, Germany). In brief, fresh bone marrow samples were diluted with an equal volume of phosphate buffered saline (PBS) and carefully layered upon Ficoll. After centrifugation (400 g for 20 min), BMMCs were collected, washed three times and cultured in RPMI 1640 medium (Sigma-Aldrich, USA) supplemented with 20% (v/v) fetal bovine serum (Cultilab, Brazil) and antibiotic antimycotic solution (Sigma-Aldrich, USA) at 37 °C in a humidified atmosphere with 5% CO_2.

Subsequent to the diagnosis completion, the following data were collected from each patient: patient **1** [female, 74-year-old, diagnosed with acute myeloid leukemia (AML), M2 subtype according to the French-American-British (FAB) classification, karyotype 46,XX]; patient **2** [female, 7-year-old, diagnosed with T-lineage acute lymphocytic leukemia (T-ALL), karyotype 46,XX]; patient **3** [female, 67-year-old, diagnosed with chronic lymphocytic leukemia (CLL) in blast crisis, karyotype 46,XX]; patient **4** [female, 62-year-old, diagnosed with chronic myeloid leukemia (CML), karyotype 46,XX,t(9;22)(q34;q11)]; patient **5** [male, 81-year-old, diagnosed with myeloproliferative syndrome (MPS), karyotype 46,XX].

This experimental protocol (reference number: 23081.012084/2008-32) has been approved by the Research Ethic Committee of the Federal University of Santa Maria (UFSM).

Evaluation of cell viability by MTT assay. The cytotoxic effects of test compounds on cell cultures were determined by metabolic method, using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) as reagent.[42] Briefly, 5 x 10^4 cells per well were seeded in 96-well plates. For adherent cells lines (i.e. B16F10, MCF-7, VERO), plates were incubated at 37 °C overnight to allow cell attachment prior to test drug treatment. The Cu(II) complexes **1-3** and the corresponding uncoordinated ligands were dissolved in 50% ethanol and diluted with fresh culture medium to obtain the following final concentrations in wells: 100, 50, 25, 12, 6, 3 and 1 µM. Cisplatin (Libbs Farmacêutica, Brazil), a metal-based antineoplastic drug, was used as reference. All cell lines were then incubated with different concentrations of compounds for 24 h at 37 °C in a humidified atmosphere with 5% CO_2. Afterwards, each well was treated with 20 µL of a MTT saline solution (5 mg/mL in PBS) and the plates were incubated for 4 more hours. The formazan crystals formed by the living cells were solubilized with 200 µL of dimethylsulfoxide (DMSO) and the absorbance at 570 nm was measured by spectroscopy (Epoch, BioTek Instruments inc., USA). IC_{50} values (concentration of test compound that reduced cell viability by 50% compared to untreated control cells) were estimated by non-linear regression analysis using GraphPad Prism software (version 5.0). Data were reported considering an average of three individual experiments performed in triplicates.

For patient-derived BMMCs, MTT assay was carried out as previously reported.[30]

Screening to antibacterial activity

The antibacterial properties of ligands, complexes and antimicrobial drug tigecycline (Tygacil®, Pfizer) were evaluated against 12 clinically important pathogens. All reference strains were purchased from the American Type Culture Collection (ATCC): *Enterococcus faecalis* ATCC 29212, *E. faecalis* ATCC 51299, *E. casseliflavus* ATCC 700327, *Micrococcus luteus* ATCC 7468, *Staphylococcus aureus* ATCC 25923, *S. aureus* ATCC 29213, *S. epidermidis* ATCC 12228, *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 700603, *Salmonella typhimurium* ATCC 14028 and *Pseudomonas aeruginosa* ATCC 27853. The clinical isolate OXA-23-producing *Acinetobacter baumannii* was obtained from the Special Clinical Microbiology Laboratory (LEMC) of the Federal University of São Paulo (USP), Brazil.

Minimal inhibitory concentration (MIC), defined as the lowest test concentration that completely inhibits visible growth of a microorganism, was determined using Mueller-Hinton broth (MHB) microdilution method.[43] Briefly, bacterial inoculum was prepared using overnight cultures and turbidity adjusted by 0.5 McFarland standard. Subsequently, 96-well microplates containing serial twofold dilutions of the compounds (0.25 to 256 µg mL^{-1}) in MHB and 1 x 10^{-5} colony-forming units per well of exponentially growing bacterial cells were incubated at 35 ± 2 °C for 24 h. Controls were also included in each microplate. After incubation period, the presence of bacterial colonies on wells was visually inspected and the lowest concentration at which there was no growth the MIC value was taken. Three independent experiments were performed in triplicates.

Results and discussion

Crystal structures

The data collection and refinement for the molecules **1-3** are listed in Tables 1-4 and the selected bond lengths (Å) and bond angles (°) are summarized in Table 5. Projections of the molecular structures of **1-3** can be visualized in Fig. 1.

Insert Tables 1-5 here

Insert Fig. 1 here

In vitro cytotoxic activity

The cytotoxic properties of complexes (**1-3**) and uncoordinated ligands on four cells lines were investigated by MTT assay. The results (in terms of IC_{50} values) were compared with those of metal-based anticancer drug Cisplatin under identical experimental conditions and are summarized in Table 6.

Insert Table 6 here

The IC_{50} values reported in Table 6 clearly indicate that free ligands and mononuclear Cu(II) complex **1** were not active on all tested cell lines at a concentration much larger than 100 µM. Nonetheless, dinuclear Cu(II) complexes **2** and **3** presented improved cytotoxic

activity. Out of them, complex **3** showed more potent dose-dependent growth inhibitory effect mainly on cancer *in vitro* models, except in K562 leukemic cells (IC_{50} > 100 μM). As illustrated in Fig. 2 and 3, for B16F10 melanoma and MCF-7 breast cancer cells treated with 3 μM of complex **3**, the percentage of cell survival was 74% and 63% decreasing to 25% and 44% at 6 μM, respectively. In contrast, 47% cell survival was found when Vero cells were exposed at 6 μM of this compound.

Insert Fig. 2 and 3 here

Overall, these results suggest that the cytotoxicity of compounds **2-3** can be derived from themselves and strongly modulated by the nature of coordinated N,N-donor heterocyclic base. This observation is consistent with the evidence that diimine-containing dinuclear Cu(II) complexes can display enhanced pharmacological properties, probably due to synergy between two metal centers and the planar ring systems. In recent years, several studies have revealed the promising anticancer effects of mixed-ligand complexes with 1,10-phen or related diimine chelates. Such findings have been attributed to greater hydrophobicity and planarity of these complexes, which facilitate their permeation through the lipid layer of the cell membrane enabling their interaction with DNA and other cellular constituents.[9,10]

Herein, we found that complex **3** showed significant inhibition in melanoma cells growth. Recently, an interesting evaluation of antiproliferative effects and the influence of tyrosinase activity of Cu(II) complexes on B16F10 cells (high melanin level) has been reported by Nunes and co-workers[44]. Notably, they demonstrated that B16F10 lineage was much more susceptible to dinuclear Cu(II) complexes, which display efficient nuclease activity and tyrosinase mimicking. Thus, the authors suggested that the dinuclear structure of Cu(II) complexes was decisive for their cytotoxic activity and could have substantial influence in the melanogenesis process, simultaneously occurring in melanoma cells.

Remarkably, when we compared the citotoxicity of **2-3** with that of reference drug Cisplatin (Table 6), it was found that both complexes showed superior cytotoxic profile, in particular complex **3** (up to 16 times better). However, like Cisplatin, these complexes were not selective between tumor and non-tumor cells. These findings are in accordance with other studies where dinuclear Cu(II) complexes with phen auxiliary ligands were found to be much more cytotoxic than Cisplatin towards various tumor cell lines.

In general, platinum-based drugs have been used as first-line agents for the several cancer therapeutics (either alone or in combination with other antineoplastic drugs). Their

antitumor activity is associated to cross-linking DNA after aquation of labile groups, causing DNA damage and inducing cell death. In spite of clinical success, high systemic toxicity and inherent or acquired resistance have limited platinum-based drugs use.[3,45] Thus, because of their cytotoxic potential on several tumor cells, Cu(II) complexes have been emerging as most promising alternatives to platinum drugs.[9,10] It is well known that these compounds are able to interfere with different biological processes, showing prominent DNA binding and inducing activation of apoptosis signal transduction pathways.[10,22,24,26,27,46,47]

Having in mind that primary cancer cells offer a more clinically relevant model for drug testing, we decided to investigate the cytotoxic activity of complexes (**1**-**3**) on well-characterized patient-derived samples. Notably, this study takes into account the interaction of tumor cells with the bone marrow stromal microenvironment as well as the genomic background and clinical data of the individuals.[48] Therefore, the cytotoxicity parameters (in terms of IC_{50} values) of tested complexes on BMMCs from newly diagnosed patient are showed in Table 7.

Insert Table 7 here

The results summarized in Table 7 revealed that complex **3** was much more effective towards primary leukemic cells than complex **2**, showing potent cytotoxic effects on CML and MPS samples (IC_{50} = 7.76 and 7.01 μM, respectively). However, both complexes proved to be fully ineffective against AML, T-ALL and CLL blast crisis samples (IC_{50} >100 μM). In addition, cell viability of all primary leukemic cells were not affected by exposing to the varying concentrations of free ligand **HL** and complex **1**.

Interestingly, we have observed considerable differences in cytotoxic activity of complexes **2**-**3**, even cells within the same cancer. For instance, both complexes were cytotoxic on CML sample (Table 7), but were not active against K562 cell line (Table 6). Noteworthy, this tumor lineage was established from patient with CML in terminal blast crisis. It is known that blast crisis represents a great challenge in the management of CML due to limited therapeutic options and unfavorable prognosis. Moreover, a large number of mutations and drug resistance have been associated with progression to blast crisis.[49] Hence, theses results can be related to substantial cancer heterogeneity.[50]

Taken altogether, the results obtained by *in vitro* cytotoxic studies demonstrate that **3** elicited marked activity against various tumor cells with IC_{50} values lower than that of Cisplatin after 24 h of drug exposure. These encouraging findings suggesting that this

compound possess potential to act as anticancer chemotherapeutic. Nevertheless, the high citotoxicity on non-tumor cell must be taken into account in view of a possible therapeutic use of complex **3**.

Antibacterial properties

The antibacterial activity was evaluated by monitoring the growth of different human pathogens in the presence of various concentrations of complexes (**1**-**3**) and uncoordinated ligands, after 24 h treatment. The MIC values obtained in this assay are listed in Table 8. As the expression of the biological activity in μg/mL does not take into account the molecular weight of the individual compounds, MIC values were converted in molarity units (μM) for comparative purposes.

Insert Table 8 here

Considering the activity of the complexes and free ligands in μM, the results (Table 8) clearly show that **2**-**3** displayed considerable inhibitory effects towards both Gram-positive and Gram-negative pathogens, with the former being more susceptible. In case of Gram-positive bacteria, cell wall[51] is composed of a thick layer of peptidoglycan and lipoteichoic acid, which presented more drug permeability. On the other hand, the cell wall of Gram-negative bacteria[52] consists of a thin layer of peptidoglycan in the periplasmic space between the inner and outer lipid membranes. In addition, the periplasmic space contains large amounts of enzymes that protect the cells from attack by antibiotics. It is well established that Gram-negative strains are the most likely to show resistance to multiple drugs, mainly due to distinct mechanisms as closure of porin channels and extrusion of antibiotics by efflux pumps.[4,5]

Remarkably, we found that complex **3** was able to inhibit bacterial growth at concentrations lower than 100 μM, including MDR strains such as carbapenem-resistant *A. baumannii* (MIC = 60 μM) and vancomycin-resistant *E. casseliflavus* (MIC = 15 μM). A possible explanation for the enhanced antibacterial properties of **3** may be the presence of phen groups in the metal coordination sphere, which can promote its increased cell permeability and allow it to reach biological targets inside the cell.

A. baumannii is recognized as an important cause of nosocomial infections, particularly ventilator-associated pneumonia and bacteremia, which have been related with high mortality. In the last years, this common pathogen has become resistant to different classes of traditionally used antimicrobial drugs.[4,5,53] Thus, the development of novel therapeutic strategies for managing *A. baumannii* infections is urgently required.

Conclusions

In this paper, the three new mixed-ligand Cu(II) complexes with benzotriazinone and different N-donor heterocyclic co-ligands (py, bipy and phen) were prepared and fully characterized. All Cu(II) complexes and free ligands were tested for cytotoxic properties in cancerous and normal cells as well as for antibacterial effects in human pathogens. Cytotoxic activity evaluation demonstrate that **3** display improved cytotoxicity on several tumor cells, with IC_{50} values markedly lower than that of Cisplatin. Moreover, this complex showed appreciable antibacterial properties. Thereby, we conclude that the presence of phen co-ligands in complex **3** significantly increased their biological activity making it a really good candidate for further development as potential metal-based drug for anticancer and antibacterial applications.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

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Supplementary data

CCDC 1519731, 1519910 and 1520070 contains the supplementary crystallographic data for complexes **1**, **2** and **3**, respectively. These data can be obtained free of charge via http://www.ccdc.cam.ac.uk/conts/retrieving.html, or from the Cambridge Crystallographic

Data Centre, 12 Union Road, Cambridge CB2 1EZ, UK; fax: (+44) 1223-336-033; or e-mail: deposit@ccdc.cam.ac.uk.

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Captions for Figures

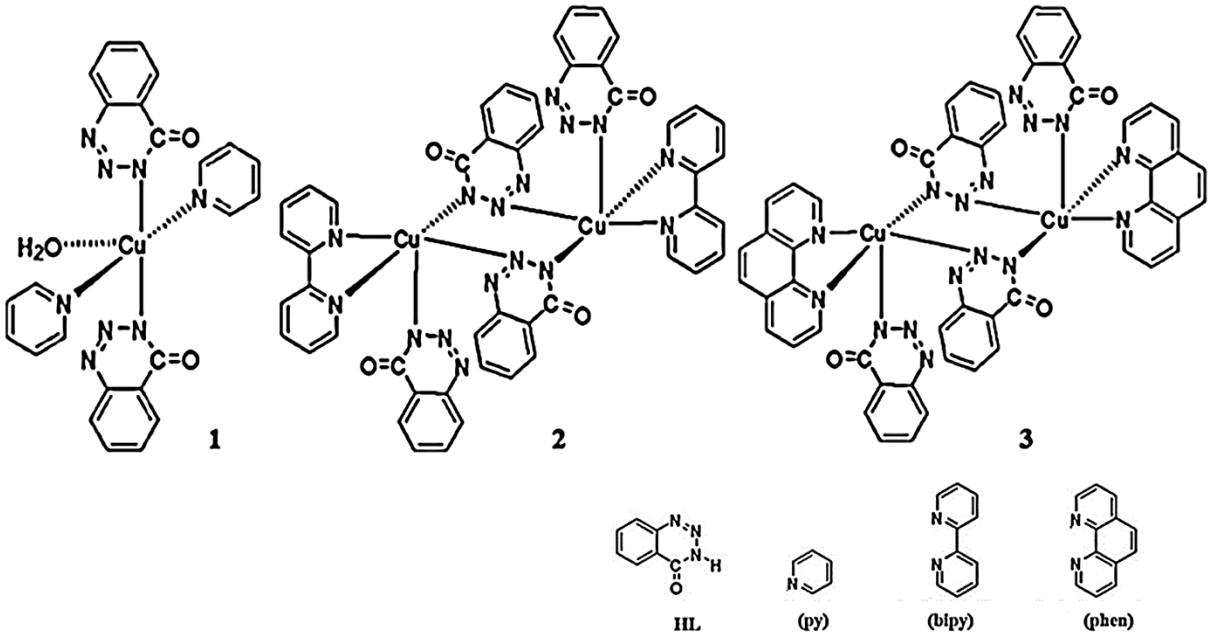
Scheme 1 Chemical structure of ligands and Cu(II) complexes.

Scheme 2 Synthesis of complexes and ligand (A) **HL** (B) complex **1** (C) complex **2** (D) complex **3**.

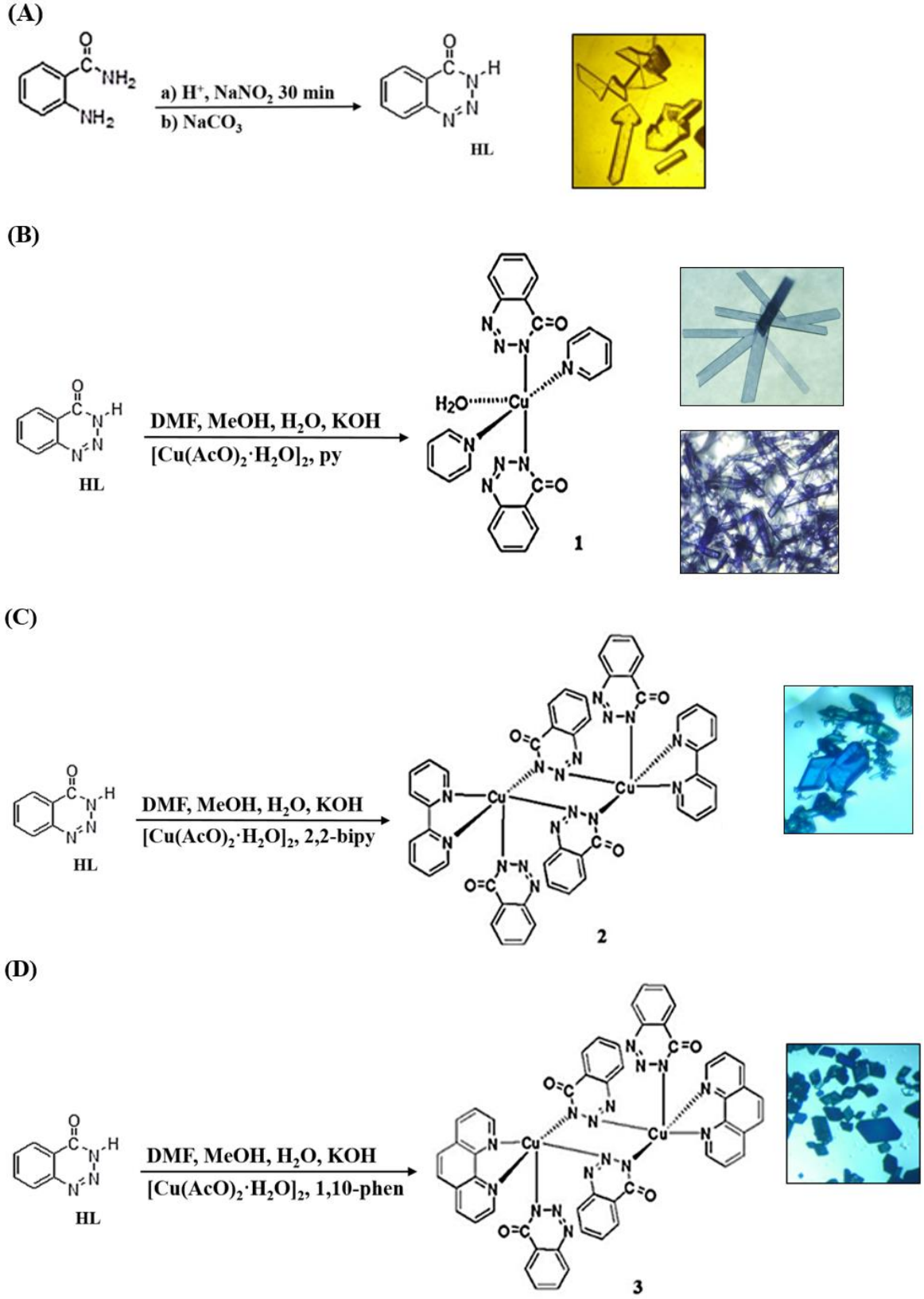
Fig. 1 Projection of the molecular structures of complexes **1-3**. Anisotropic thermal ellipsoids drawn at 30% level.

Fig. 2 Dose-response curves of complex **3** in VERO, B16F10, MCF-7 and K562 cells lines at 24 h. Cell viability is expressed as relative activity of control cells (100%).

Fig. 3 Bar diagram showing the cell viability measurements in non-tumor and tumor cell lines after a 24 h exposure to increasing concentrations (1-100 µM) of complex **3**.



Scheme 1 (*Maísa K. Tizotti, et al.*)



Scheme 2 (*Maísa K. Tizotti, et al.*)

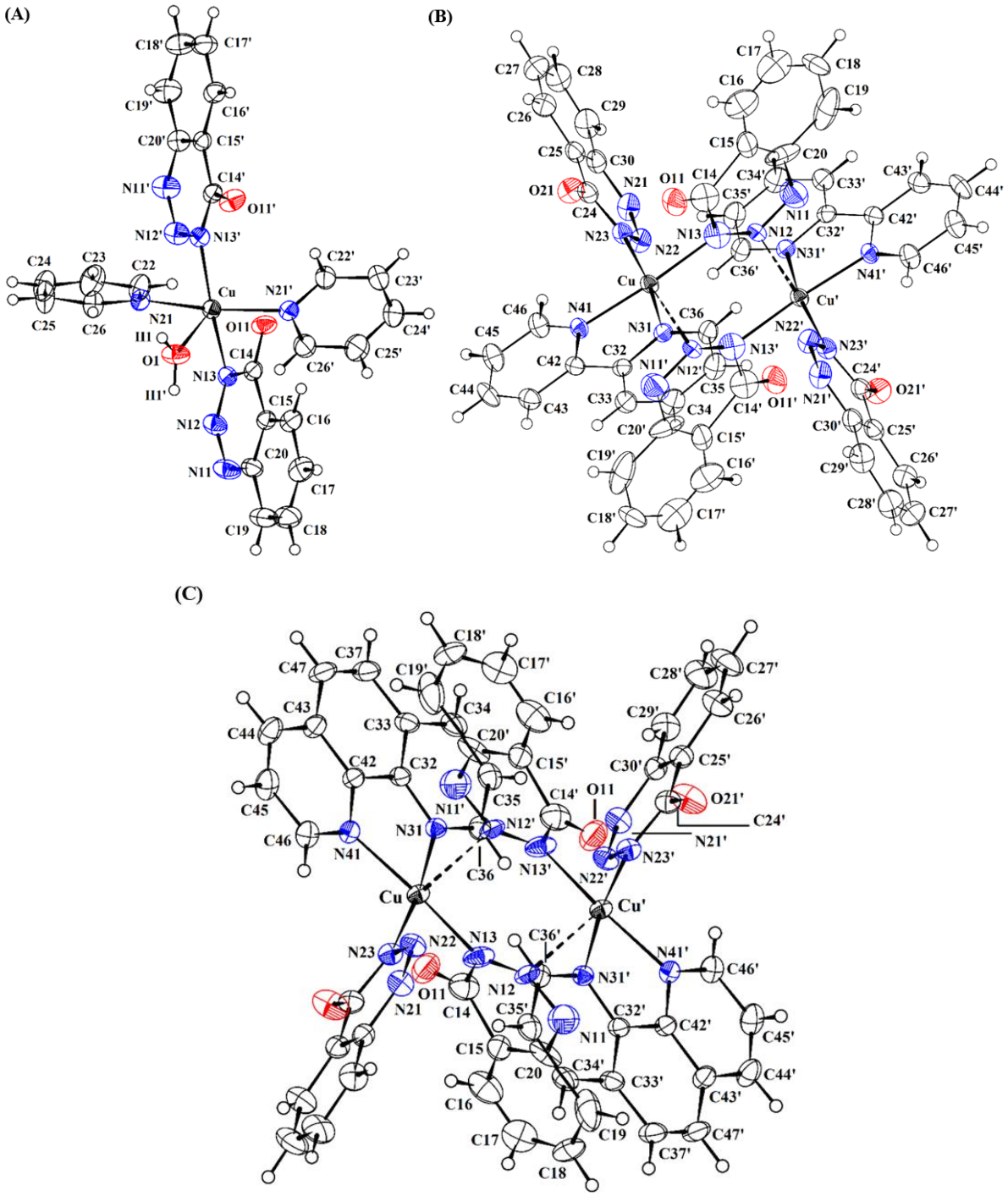


Figure 1 (*Maísa K. Tizotti, et al.*)

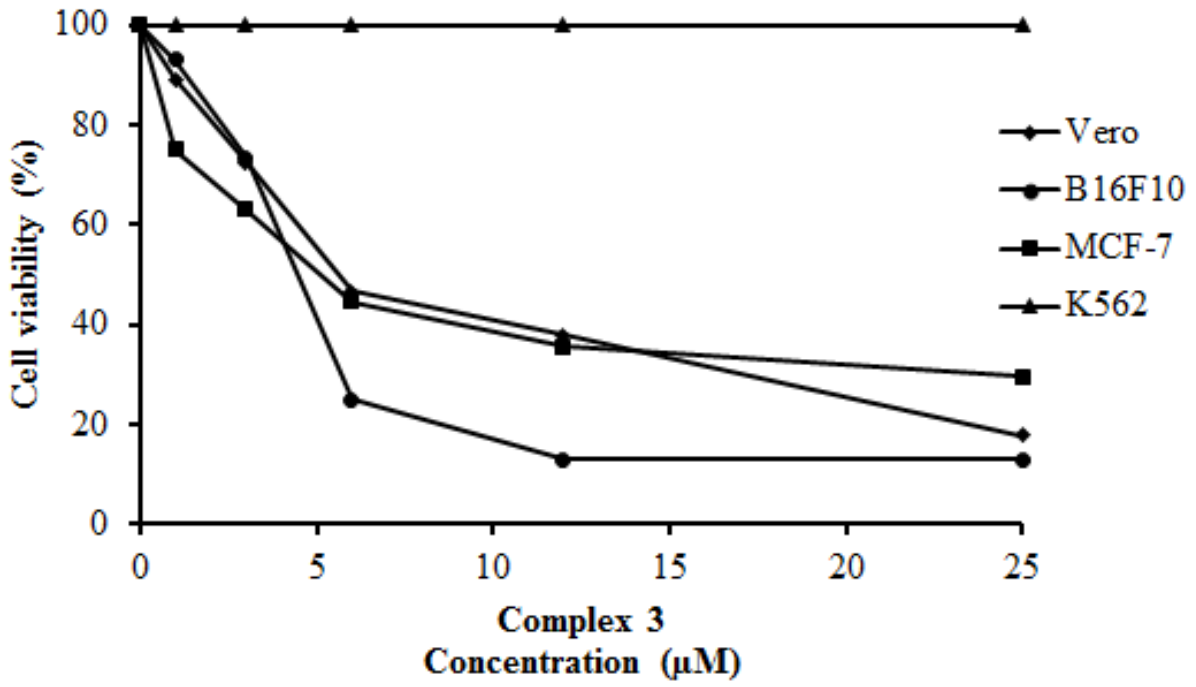


Figure 2 (*Maísa K. Tizotti, et al.*)

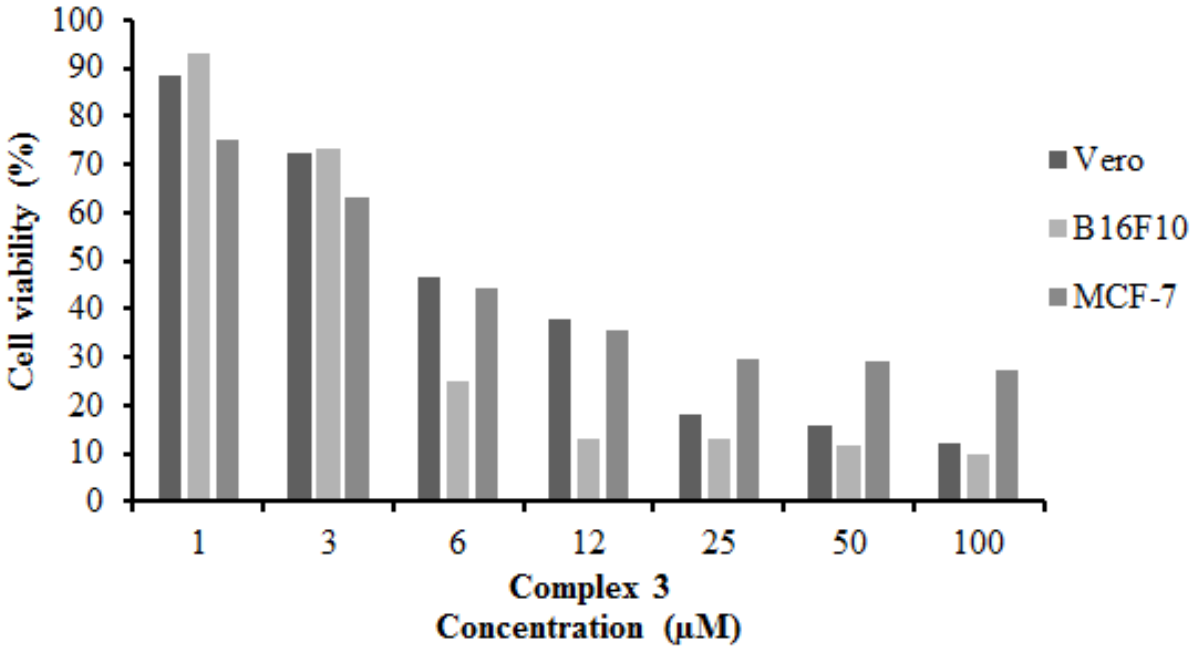


Figure 3 (*Maísa K. Tizotti, et al.*)

Table 1 Crystal data and structure refinement for **1-3**

	1	**2**	**3**
Formula	$C_{24}H_{20}CuN_8O_3$	$C_{48}H_{32}Cu_2N_{16}O_4$	$C_{52}H_{32}Cu_2N_{16}O_4$
*F*w (g mol^{-1})	532.02	1023.98	1072.02
Temperature	293(2) K	293(2) K	293(2) K
Wavelength	0.71073 Å	0.71073 Å	0.71073 Å
Crystal system	Orthorhombic	Triclinic	Triclinic
Space group	*Fdd*2	*P*(-1)	*P*(-1)
Unit cell dimensions	a = 16.4654(4) Å α = 90° b = 41.9157(10) Å β = 90° c = 6.8968(2) Å γ = 90°	a = 10.2236(4) Å α = 90° b = 11.0261(5) Å β = 90° c = 11.9018(5) Å γ = 90°	a = 10.4915(3) Å α = 78.1150(10)° b = 10.8294(3) Å β = 68.2820(10)° c = 11.9746(3) Å γ = 64.0270(10)°
Volume	4759.9(2) Å^3	1093.08(8) Å^3	1134.71(5) Å^3
Z	8	1	1
Density (calculated)	1.485 Mg/m^3	1.556 Mg/m^3	1.569 Mg/m^3
Absorption coefficient	0.962 mm^{-1}	1.041 mm^{-1}	1.007 mm^{-1}
F(000)	2184	522	546
Crystal size (mm^3)	0.31 x 0.26 x 0.24	0.101 x 0.122 x 0.293	0.363 x 0.342 x 0.166
Theta range for data collection	1.94 to 28.29°	1.87 to 28.33°	1.83 to 26.42º
Index ranges	$-21 \le h \le 21$, $-55 \le k \le 55$, $-9 \le l \le 9$	$-13 \le h \le 13$, $-14 \le k \le 14$, $-15 \le l \le 15$	$-13 \le h \le 13$, $-13 \le k \le 13$, $-14 \le l \le 14$
Reflections collected	32005	15739	31559
Independent reflections	2949 [$R_{(int)}$ = 0.0297]	5375 [$R_{(int)}$ = 0.0287]	4631 [$R_{(int)}$ = 0.0194]
Completeness to theta = 28.29°	99.9%	98.6%	99.6%
Absorption correction	Gaussian	-	Gaussian
Max. and min. transmission	0.8020 and 0.7547	-	0.8507 and 0.7114
Refinement method	Full-matrix least-squares on F^2	Full-matrix least-squares on F^2	Full-matrix least-squares on F^2
Data / restraints / parameters	2949 / 1 / 164	5375 / 3 / 306	4631 / 2 / 334
Goodness-of-fit on F^2	1.041	1.081	1.064
Final *R* indices [*I*>2sigma(*I*)]	R_1 = 0.0207, wR_2 = 0.0554	R_1 = 0.0905, wR_2 = 0.2603	R_1 = 0.0676, wR_2 = 0.1877
R indices (all data)	R_1 = 0.0231, wR_2 = 0.0561	R_1 = 0.1079, wR_2 = 0.2763	R_1 = 0.0706, wR_2 = 0.1905
Absolute structure parameter	0.001(9)	-	-
Largest diff. peak and hole	0.175 and -0.167 e.Å^{-3}	3.393 and -1.035 e.Å^{-3}	2.688 and -1.262 e.Å^{-3}

Table 2 Atomic coordinates (x 10^4) and equivalent isotropic displacement parameters ($Å^2$ x 10^3) for complex **1**. U(eq) is defined as one third of the trace of the orthogonalized U^{ij} tensor.

	x	*y*	*z*	*U(eq)*
C(14)	533(1)	-565(1)	2830(2)	39(1)
C(15)	836(1)	-889(1)	2940(2)	38(1)
C(16)	974(1)	-1047(1)	4697(3)	51(1)
C(17)	1278(1)	-1351(1)	4666(3)	63(1)
C(18)	1430(2)	-1505(1)	2937(3)	71(1)
C(19)	1285(1)	-1358(1)	1199(3)	69(1)
C(20)	992(1)	-1040(1)	1197(2)	46(1)
C(22)	1533(1)	174(1)	2789(3)	59(1)
C(23)	2296(1)	305(1)	3052(4)	75(1)
C(24)	2652(1)	462(1)	1540(4)	77(1)
C(25)	2242(1)	491(1)	-142(4)	78(1)
C(26)	1487(1)	354(1)	-337(3)	59(1)
N(11)	876(1)	-894(1)	-568(2)	60(1)
N(12)	618(1)	-603(1)	-623(2)	53(1)
N(13)	452(1)	-439(1)	1016(2)	39(1)
N(21)	1135(1)	194(1)	1116(2)	44(1)
O(1)	0	0	-2501(2)	58(1)
O(11)	348(1)	-401(1)	4255(2)	56(1)
Cu	0	0	824(1)	37(1)

Table 3 Atomic coordinates (x 10^4) and equivalent isotropic displacement parameters (Å^{2}x 10^3) for complex **2**. U(eq) is defined as one third of the trace of the orthogonalized U^{ij} tensor.

	x	*y*	*z*	*U(eq)*
C(14)	6805(13)	-27(12)	2204(10)	84(3)
C(15)	6927(13)	-1505(8)	3207(9)	68(3)
C(16)	5934(14)	-1639(17)	4140(12)	92(4)
C(17)	6164(15)	-2760(19)	4833(14)	103(4)
C(18)	7378(18)	-3879(12)	4690(10)	95(4)
C(19)	8671(13)	-3857(14)	3651(15)	116(6)
C(20)	8336(12)	-2383(14)	2816(7)	81(3)
C(24)	8143(7)	2313(7)	2714(6)	45(1)
C(25)	8898(8)	2090(6)	3628(6)	45(1)
C(26)	8116(10)	2425(9)	4793(8)	62(2)
C(27)	8924(13)	2111(11)	5611(9)	74(2)
C(28)	10496(13)	1500(10)	5281(9)	78(3)
C(29)	11274(11)	1203(10)	4151(10)	70(2)
C(30)	10480(8)	1482(7)	3304(7)	49(2)
C(32)	6226(6)	3553(6)	-1213(5)	36(1)
C(33)	5288(8)	3942(7)	-1956(7)	50(2)
C(34)	4908(9)	2968(9)	-2114(8)	61(2)
C(35)	5477(9)	1608(8)	-1507(8)	58(2)
C(36)	6404(7)	1284(7)	-756(7)	47(1)
C(42)	6736(6)	4505(6)	-1013(5)	36(1)
C(43)	6336(7)	5890(7)	-1562(6)	48(1)
C(44)	6920(8)	6673(7)	-1332(7)	54(2)
C(45)	7852(8)	6065(8)	-556(8)	58(2)
C(46)	8173(7)	4694(7)	-23(7)	49(2)
N(11)	9668(11)	-2437(10)	1871(9)	89(3)
N(12)	9648(8)	-1239(5)	1168(5)	52(1)
N(13)	8273(9)	-143(8)	1297(8)	73(2)
N(21)	11299(7)	1106(7)	2166(7)	57(2)
N(22)	10607(6)	1274(6)	1401(6)	49(1)
N(23)	9054(6)	1820(5)	1664(5)	43(1)
N(31)	6770(5)	2229(5)	-612(4)	37(1)
N(41)	7621(5)	3917(5)	-224(5)	37(1)
O(11)	5854(7)	820(6)	2244(6)	71(2)
O(21)	6771(6)	2867(6)	2851(5)	61(1)
Cu	8092(1)	1863(1)	472(1)	34(1)

Table 4 Atomic coordinates (x 10^4) and equivalent isotropic displacement parameters (Å^{2}x 10^3) for complex **3**. U(eq) is defined as one third of the trace of the orthogonalized U^{ij} tensor.

	x	*y*	*z*	*U(eq)*
C(36)	3511(6)	3835(5)	5605(5)	40(1)
C(35)	4235(7)	3540(7)	6461(5)	50(1)
C(34)	4555(6)	2298(7)	7063(5)	50(1)
C(33)	4160(6)	1314(6)	6832(4)	42(1)
C(37)	4461(7)	-31(7)	7404(5)	51(1)
C(47)	4071(7)	-911(6)	7137(5)	55(2)
C(43)	3303(6)	-550(5)	6255(5)	43(1)
C(44)	2843(7)	-1422(6)	5934(6)	55(2)
C(45)	2132(7)	-967(6)	5089(6)	55(2)
C(46)	1896(6)	350(6)	4533(5)	45(1)
C(42)	3009(5)	754(5)	5669(4)	35(1)
C(32)	3437(5)	1697(5)	5960(4)	33(1)
C(24)	1679(6)	2982(6)	1983(5)	44(1)
C(26)	1395(8)	3002(8)	-7(6)	63(2)
C(27)	476(10)	3191(10)	-651(6)	77(2)
C(28)	-1037(9)	3494(9)	-66(7)	72(2)
C(29)	-1640(7)	3611(7)	1149(6)	56(2)
C(30)	-715(6)	3433(5)	1809(5)	40(1)
C(25)	800(6)	3132(6)	1233(5)	42(1)
C(14)	3182(9)	5170(8)	2813(6)	62(2)
C(16)	4088(9)	6720(11)	1111(7)	79(2)
C(17)	3801(10)	7895(11)	550(8)	80(2)
C(18)	2532(12)	8956(8)	813(7)	80(2)
C(19)	1251(11)	8845(11)	1790(10)	107(4)
C(20)	1608(7)	7419(8)	2424(5)	58(2)
C(15)	3048(7)	6509(6)	2021(6)	51(1)
N(31)	3120(4)	2940(4)	5357(3)	33(1)
N(41)	2328(4)	1196(4)	4808(4)	34(1)
N(21)	-1366(5)	3590(5)	3039(4)	45(1)
N(22)	-575(5)	3488(5)	3672(4)	43(1)
N(23)	906(5)	3217(5)	3173(4)	39(1)
N(11)	343(8)	7342(7)	3304(6)	78(2)
N(12)	495(8)	6147(5)	3808(5)	66(2)
N(13)	1912(7)	5079(5)	3586(5)	56(1)
O(21)	3029(5)	2682(6)	1615(4)	73(2)
O(11)	4290(6)	4281(5)	2725(5)	72(1)
Cu	1946(1)	3207(1)	4251(1)	32(1)

Table 5 Bond lengths [Å] and angles [°] for complexes (**1-3**)

1		**2**		**3**	
N(11)-N(12)	1.292(2)	N(11)-N(12)	1.310(11)	N(31)-Cu	2.022(4)
N(12)-N(13)	1.3515(18)	N(12)-N(13)	1.371(9)	N(41)-Cu	2.045(4)
N(13)-Cu	1.9896(12)	N(12)-Cu#1	2.402(6)	N(21)-N(22)	1.275(6)
N(21)-Cu	2.0476(12)	N(13)-Cu	2.060(8)	N(22)-N(23)	1.359(6)
O(1)-Cu	2.2932(17)	N(21)-N(22)	1.267(8)	N(23)-Cu	1.971(4)
Cu-N(13)#1	1.9896(12)	N(22)-N(23)	1.369(7)	N(11)-N(12)	1.277(9)
Cu-N(21)#1	2.0476(12)	N(23)-Cu	1.978(5)	N(12)-N(13)	1.396(8)
		N(31)-Cu	2.023(5)	N(13)-Cu	2.009(5)
N(11)-N(12)-N(13)	121.54(13)	N(41)-Cu	2.033(5)		
N(12)-N(13)-Cu	119.41(10)	Cu-N(12)#1	2.402(6)	C(36)-N(31)-Cu	128.6(3)
C(14)-N(13)-Cu	117.16(9)			C(32)-N(31)-Cu	113.0(3)
C(22)-N(21)-Cu	120.63(11)	N(11)-N(12)-N(13)	116.8(7)	C(46)-N(41)-Cu	129.6(4)
C(26)-N(21)-Cu	121.36(12)	N(11)-N(12)-Cu#1	103.4(5)	C(42)-N(41)-Cu	112.3(3)
N(13)-Cu-N(13)#1	172.34(7)	N(13)-N(12)-Cu#1	135.3(5)	N(21)-N(22)-N(23)	121.6(4)
N(13)-Cu-N(21)	91.11(5)	N(12)-N(13)-Cu	121.8(5)	N(22)-N(23)-Cu	116.7(3)
N(13)#1-Cu-N(21)	88.14(5)	C(14)-N(13)-Cu	105.7(6)	C(24)-N(23)-Cu	119.7(3)
N(13)-Cu-N(21)#1	88.14(5)	N(21)-N(22)-N(23)	121.7(6)	N(11)-N(12)-N(13)	120.1(6)
N(13)#1-Cu-N(21)#1	91.11(5)	C(24)-N(23)-Cu	118.6(4)	C(14)-N(13)-Cu	118.5(5)
N(21)-Cu-N(21)#1	168.69(8)	N(22)-N(23)-Cu	118.2(4)	N(12)-N(13)-Cu	115.4(4)
N(13)-Cu-O(1)	93.83(4)	C(36)-N(31)-Cu	125.1(4)	N(23)-Cu-N(13)	91.18(18)
N(13)#1-Cu-O(1)	93.83(4)	C(32)-N(31)-Cu	115.5(4)	N(23)-Cu-N(31)	172.91(17)
N(21)-Cu-O(1)	95.65(4)	C(46)-N(41)-Cu	126.1(4)	N(13)-Cu-N(31)	95.18(18)
N(21)#1-Cu-O(1)	95.65(4)	C(42)-N(41)-Cu	115.6(4)	N(23)-Cu-N(41)	92.28(17)
		N(23)-Cu-N(31)	168.8(2)	N(13)-Cu-N(41)	171.2(2)
		N(23)-Cu-N(41)	93.2(2)	N(31)-Cu-N(41)	80.98(16)
		N(31)-Cu-N(41)	79.7(2)		
		N(23)-Cu-N(13)	88.5(3)		
		N(31)-Cu-N(13)	97.5(3)		
		N(41)-Cu-N(13)	172.5(2)		
		N(23)-Cu-N(12)#1	97.4(2)		
		N(31)-Cu-N(12)#1	91.5(2)		
		N(41)-Cu-N(12)#1	91.53(19)		
		N(13)-Cu-N(12)#1	95.4(2)		

Symmetry transformations used to generate equivalent atoms: #1 -*x*,-*y*,*z* (**1**), #1 -*x*+2,-*y*,-*z* (**2**)

Table 6 IC_{50} values (μM) of Cu(II) complexes **1-3** and the corresponding uncoordinated ligands towards different cell lines. All compounds were incubated with cells for 24 h.

Compounds	IC_{50} values (μM)[a]			
	K562	B16F10	MCF-7	VERO
HL	>100	>100	>100	>100
Py	>100	>100	>100	>100
Bipy	>100	>100	>100	>100
Phen	>100	>100	>100	>100
Complex **1**	>100	>100	>100	>100
Complex **2**	>100	29.76	34.01	41.30
Complex **3**	>100	4.26	6.16	6.70
Cisplatin	>100	69.32	73.55	55.03

[a] IC_{50} values are presented as mean values of three independent experiments done in triplicates.

Table 7 Cytotoxic activity of the free ligand **HL** and complexes **1-3** on human BMMCs. All compounds were incubated with cells for 24 h.

Compounds	IC_{50} values (μM)				
	1 (AML)	2 (T-ALL)	3 (CLL Blast crisis)	4 (CML)	5 (MPS)
HL	>100	>100	>100	>100	>100
Complex **1**	>100	>100	>100	>100	>100
Complex **2**	>100	>100	>100	36.23	33.39
Complex **3**	>100	>100	>100	7.76	7.01

Table 8 MIC values of the compounds against several bacterial strains expressed in µg/mL and in µM (the values in parentheses).

Bacteria	MIC µg/mL (µM)							
	1	**2**	**3**	HL	Py	Bipy	Phen	TGC[a]
Gram-positive								
S. aureus	>256	256	32	>256	>256	32	128	<0.25
ATCC 25923	(>481)	(250)	(30)	(>1740)	(>3246)	(205)	(710)	(<0.85)
S. aureus	>256	256	64	>256	>256	32	256	<0.25
ATCC 29213	(>481)	(250)	(60)	(>1740)	(>3246)	(205)	(1420)	(<0.85)
S. epidermidis	>256	256	64	>256	>256	64	256	<0.25
ATCC 12228	(>481)	(250)	(60)	(>1740)	(>3246)	(410)	(1420)	(<0.85)
E. faecalis	>256	64	64	>256	>256	64	128	<0.25
ATCC 29212	(>481)	(62.5)	(60)	(>1740)	(>3246)	(410)	(710)	(<0.85)
E. faecalis	>256	64	128	>256	>256	64	128	<0.25
ATCC 51299	(>481)	(62.5)	(120)	(>1740)	(>3246)	(410)	(710)	(<0.85)
E. casseliflavus	>256	32	16	>256	>256	16	32	<0.25
ATCC 700327	(>481)	(31.25)	(15)	(>1740)	(>3246)	(102.5)	(177.5)	(<0.85)
M. luteus	>256	256	32	>256	>256	64	128	<0.25
ATCC 7468	(>481)	(250)	(30)	(>1740)	(>3246)	(410)	(710)	(<0.85)
Gram-negative								
E. coli	>256	>256	128	>256	>256	32	128	<0.25
ATCC 25922	(>481)	(>250)	(120)	(>1740)	(>3246)	(205)	(710)	(<0.85)
K. pneumoniae	>256	>256	128	>256	>256	32	128	0.5
ATCC 700603	(>481)	(>250)	(120)	(>1740)	(>3246)	(205)	(710)	(1.7)
S. typhimurium	>256	>256	128	>256	>256	32	128	<0.25
ATCC 14028	(>481)	(>250)	(120)	(>1740)	(>3246)	(205)	(710)	(<0.85)
P. aeruginosa	>256	128	64	>256	>256	64	64	2
ATCC 27853	(>481)	(125)	(60)	(>1740)	(>3246)	(410)	(355)	(6.8)
OXA-23-producing	>256	64	64	>256	>256	16	64	<0.25
A. baumannii	(>481)	(62.5)	(60)	(>1740)	(>3246)	(102.5)	(355)	(<0.85)

[a] TGC = tigeciclyne

Supplementary Information

Dokument: Unbenannt (VarioMICRO) vom: --.--.-- (modifiziert)

Messung vom 25.04..2012
varioMICRO CHNS
serial number: 15081001

Text-Ausdruck

Nr.	Gewicht [mg]	Name	N [%]	C [%]	H [%]	S [%]	N-Fläche	C-Fläche	H-Fläche	S-Fläche	N-Faktor	C-Faktor	H-Faktor	S-Faktor
16	2.7240	BK-CS1L	28.81	57.20	3.593	0.000	26 084	37 198	5 928	0	1.0179	1.0068	1.0856	1.0000
17	2.0710	BK-CS1L	28.85	57.21	3.816	0.048	19 864	28 273	4 675	10	1.0179	1.0068	1.0856	1.0000
18	2.7100	BK-CS1Cu-Ple	20.75	57.36	3.125	0.025	18 688	37 109	5 048	7	1.0179	1.0068	1.0856	1.0000
19	2.3290	BK-CS1Cu-Ple	20.94	57.95	3.365	0.068	16 206	32 214	4 631	16	1.0179	1.0068	1.0856	1.0000
20	3.0030	BK-CS1Cu-bip	22.04	56.14	3.355	0.000	22 002	40 250	6 123	0	1.0179	1.0068	1.0856	1.0000
21	1.8720	BK-CS1Cu-bip	22.02	56.22	3.618	0.042	13 683	25 111	3 935	8	1.0179	1.0068	1.0856	1.0000
22	1.8930	BK-CS1Cu-py	21.27	53.56	4.180	0.000	13 360	24 188	4 681	0	1.0179	1.0068	1.0856	1.0000
23	2.3200	BK-CS1Cu-py	21.17	53.44	4.048	0.000	16 315	29 588	5 663	0	1.0179	1.0068	1.0856	1.0000

Name: eassuperuser, Zugriff: VarioMICRO superuser 03.05.2012 15:29:27

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Elementar Analysensysteme GmbH

Seite 1 (von 1)

Fig. S1 CHNS Elemental Analysis of **HL** and complexes (**1-3**).

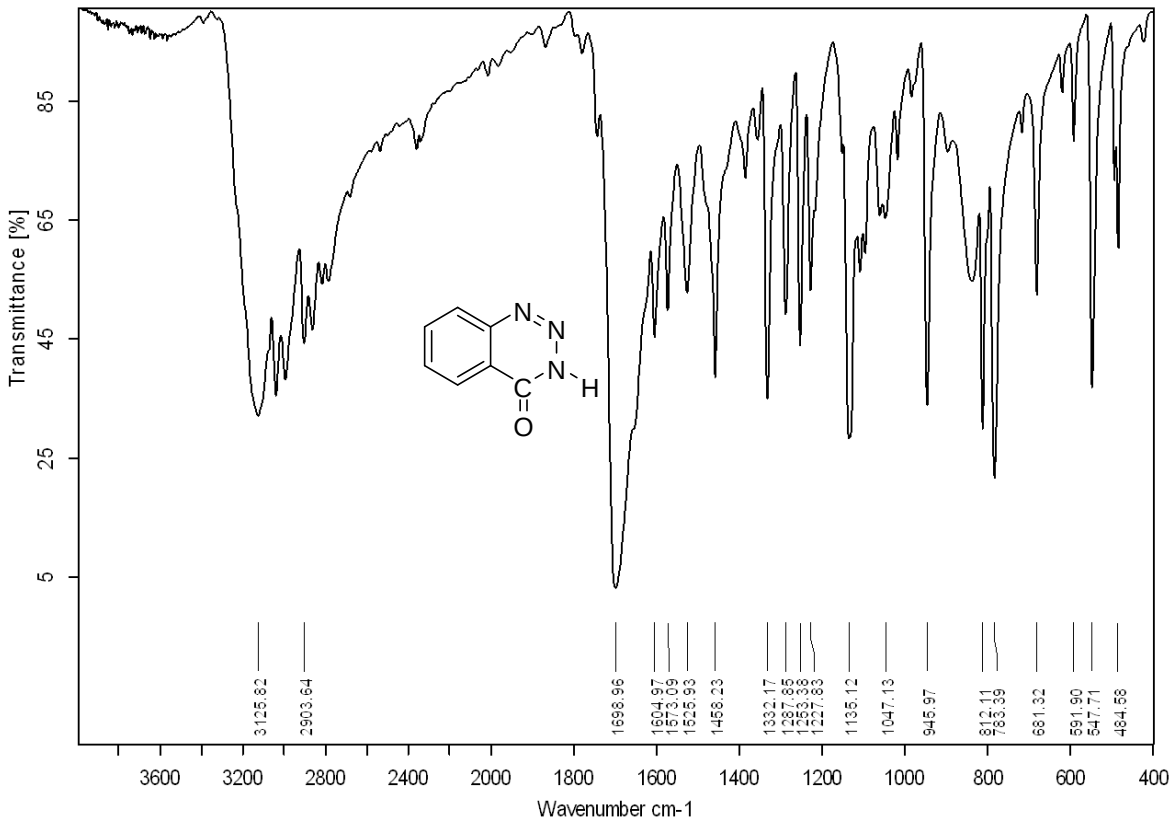

Fig. S2 Infrared spectra (KBr) of **HL**.

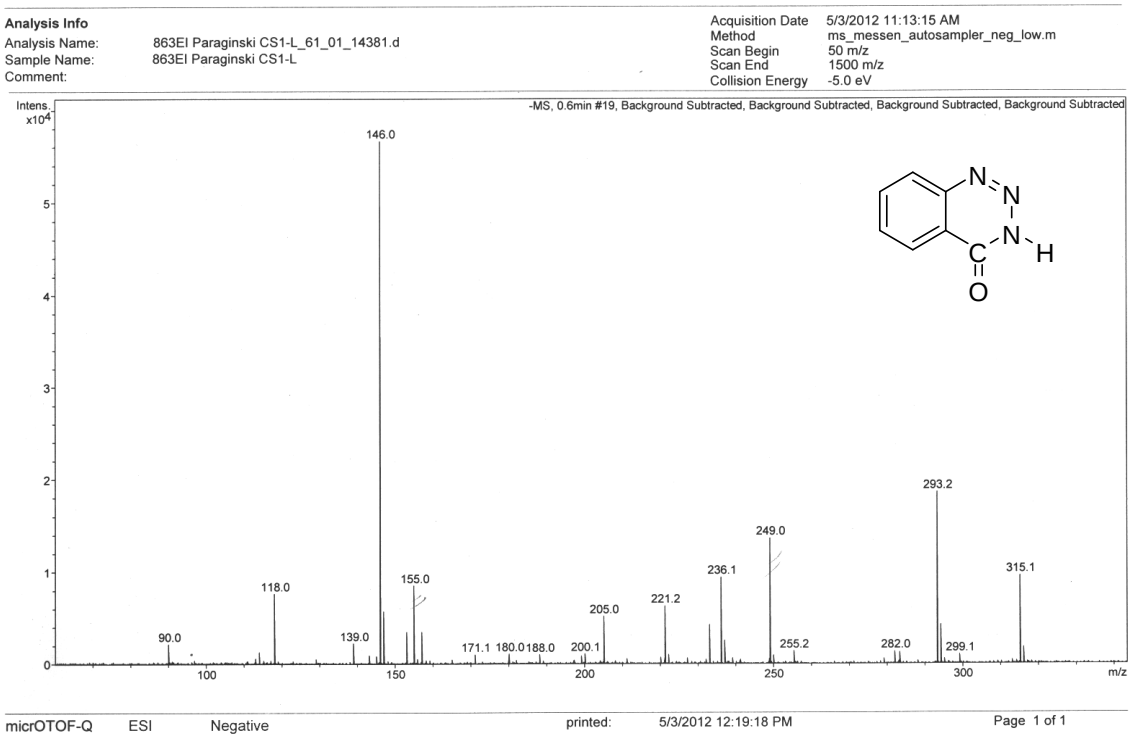

Fig. S3 Mass spectra (ESI-TOF negative mode) of **HL**

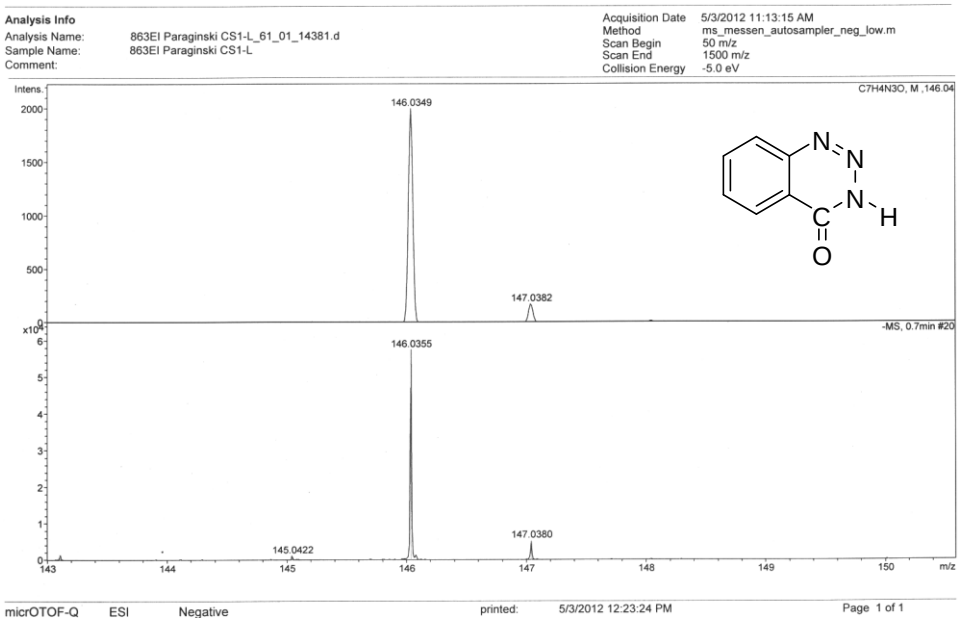

Fig. S4 Mass spectra (ESI-TOF negative mode) of **HL**. The region where the ion localized strongly is magnified [147,0, M]$^+$ and [146,0, M-H]$^+$.

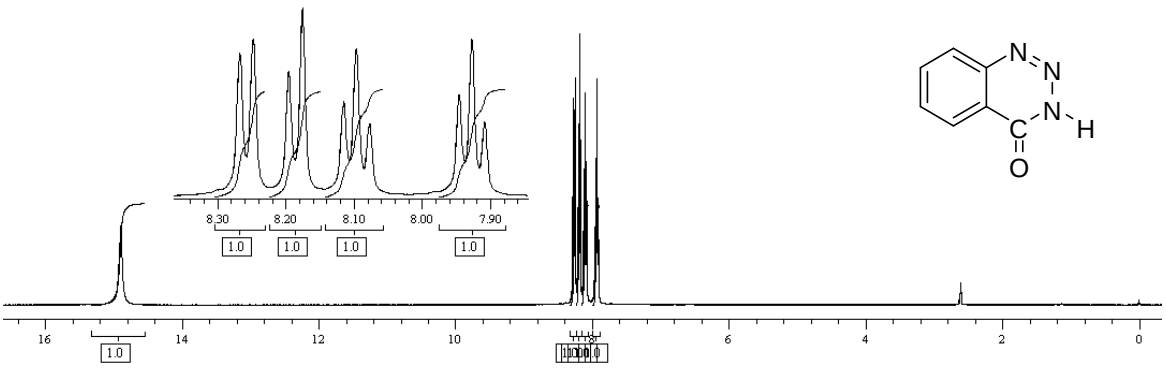

Fig. S5 ^{1}H NMR spectra of HL (DMSO-d_6/TMS, 400MHz).

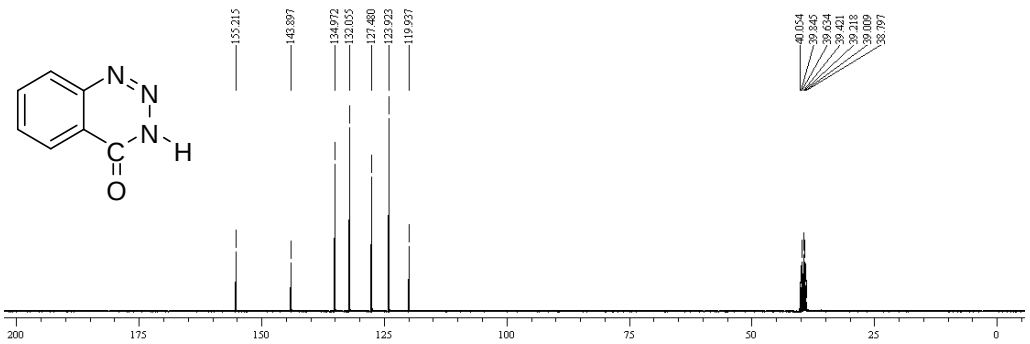

Fig. S6 ^{13}C NMR spectra of HL (DMSO-d_6, 100MHz).

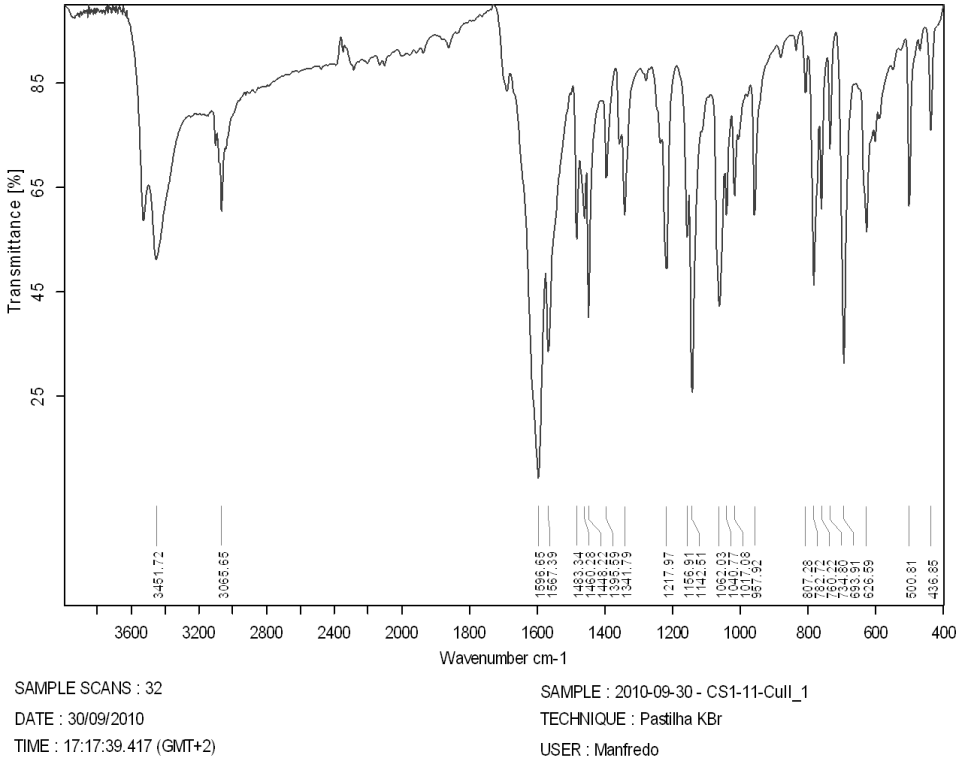

Fig. S7 Infrared spectra (KBr) of $[(L)_2(Py)_2(OH_2)Cu(II)]$ (**1**).

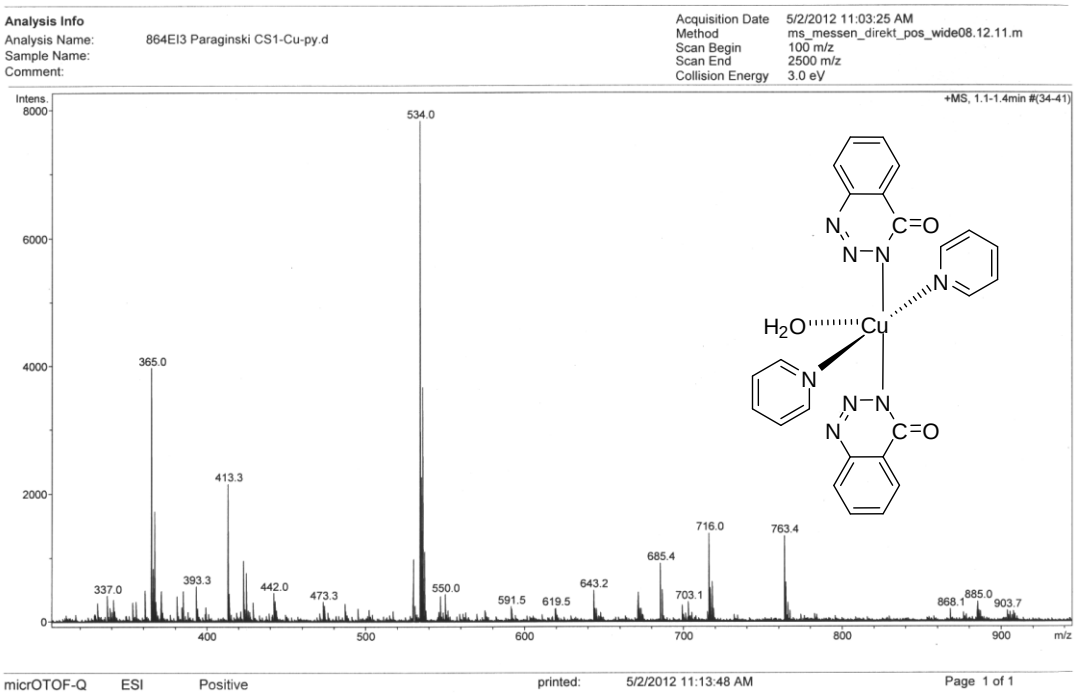

Fig. S8 Mass spectra (ESI-TOF positive mode) of $[(L)_2(Py)_2(OH_2)Cu(II)]$ (**1**).

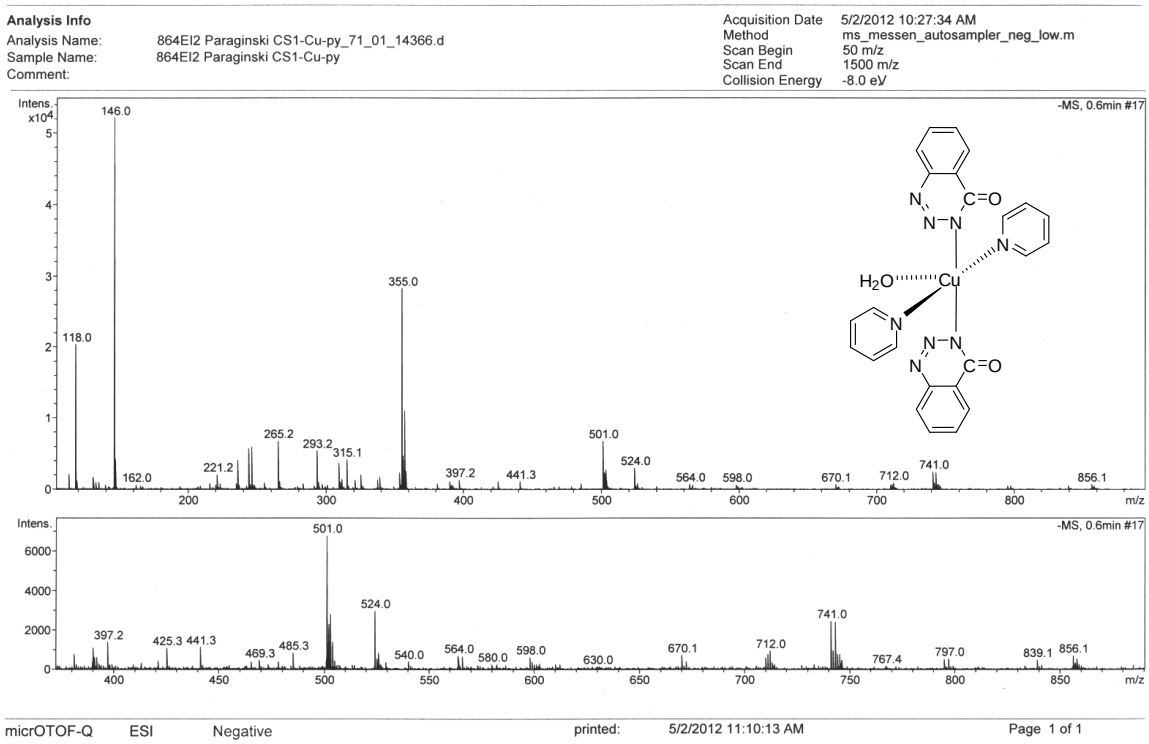

Fig. S9 Mass spectra (ESI-TOF positive mode) of $[(L)_2(Py)_2(OH_2)Cu(II)]$ (**1**).

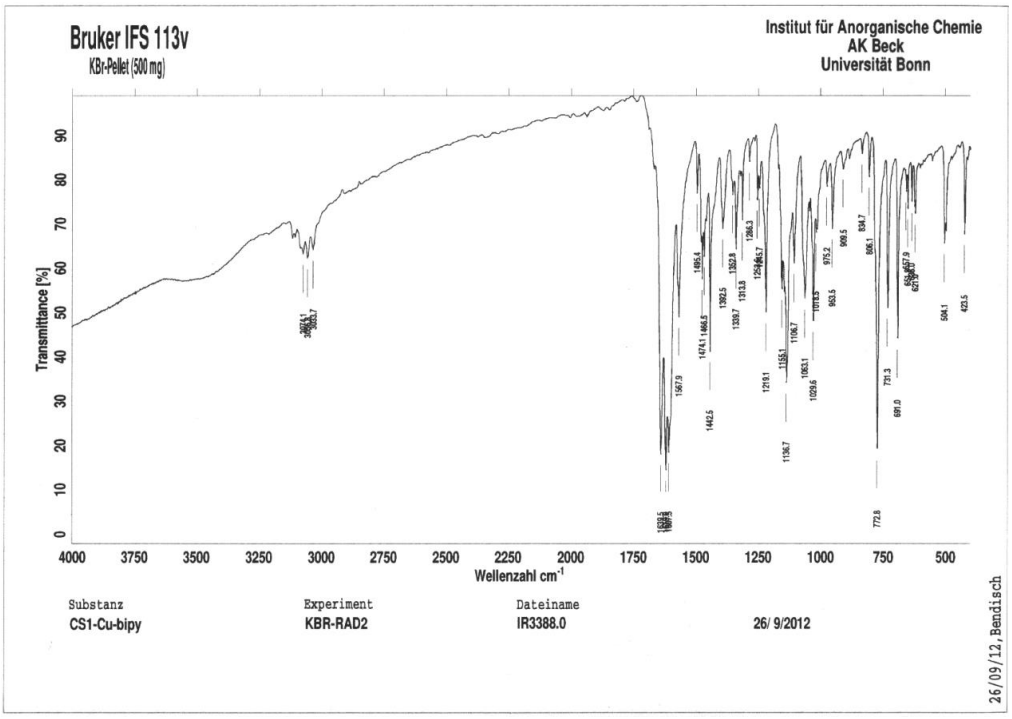

Fig. S10 Infrared spectra (KBr) of $[(bipy)_2(L)_4Cu(II)_2]$ (**2**).

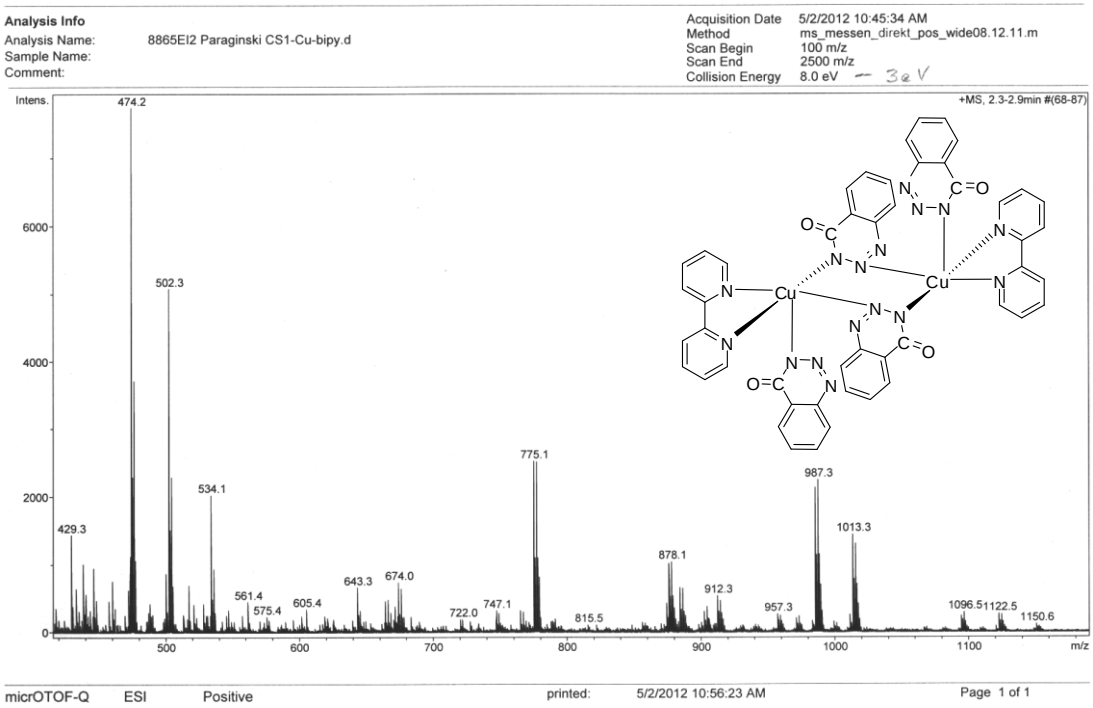

Fig. S11 Mass spectra (ESI-TOF positive mode) of $[(bipy)_2(L)_4Cu(II)_2]$ (**2**).

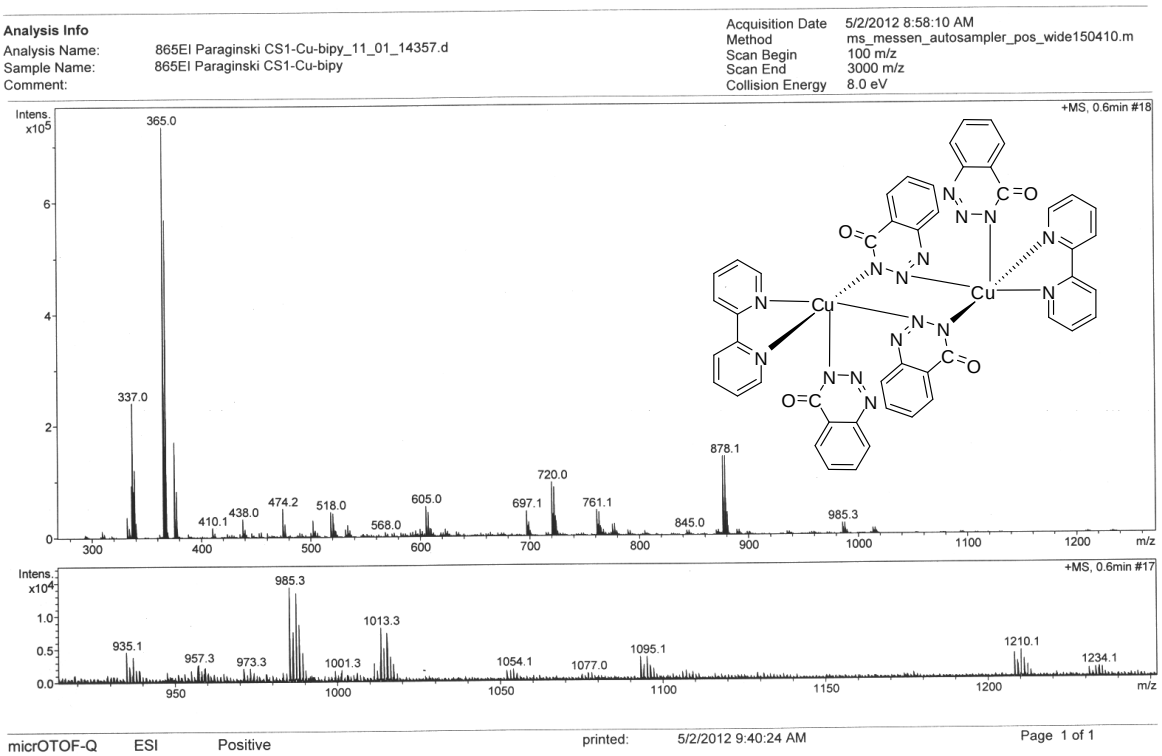

Fig. S12 Mass spectra (ESI-TOF positive mode) of $[(bipy)_2(L)_4Cu(II)_2]$ (**2**).

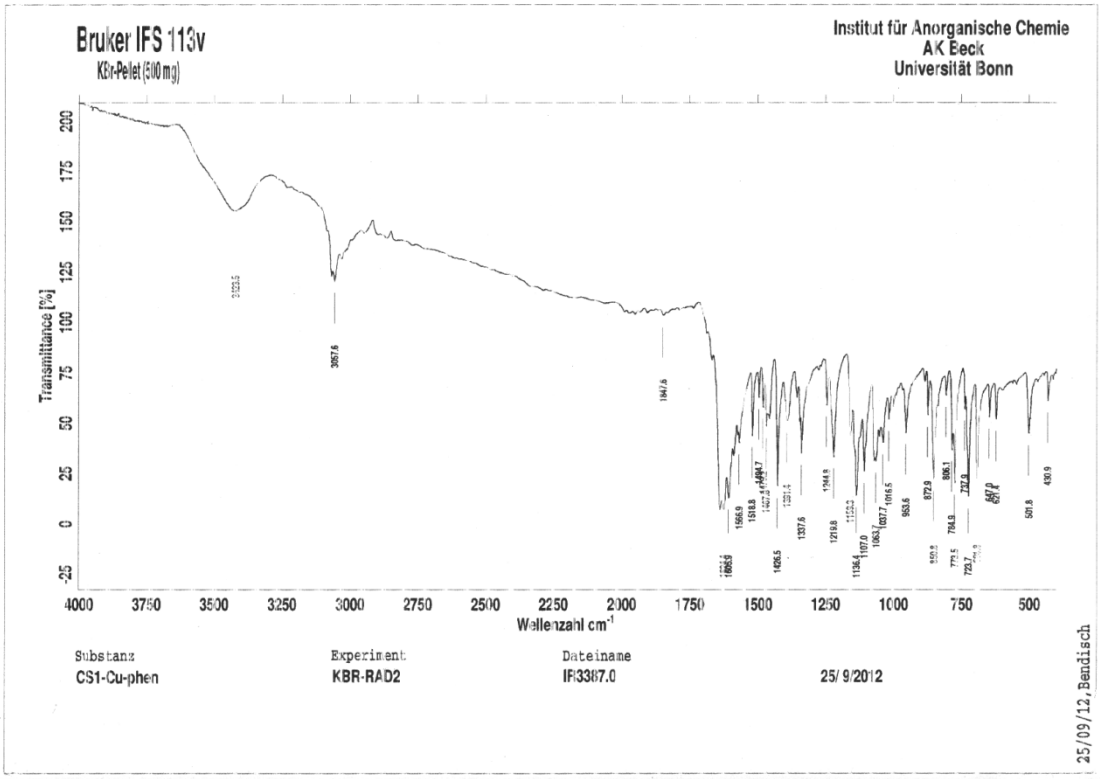

Fig. S13 Infrared spectra (KBr) of $[(Phen)_2(L)_4Cu(II)_2]$ (**3**).

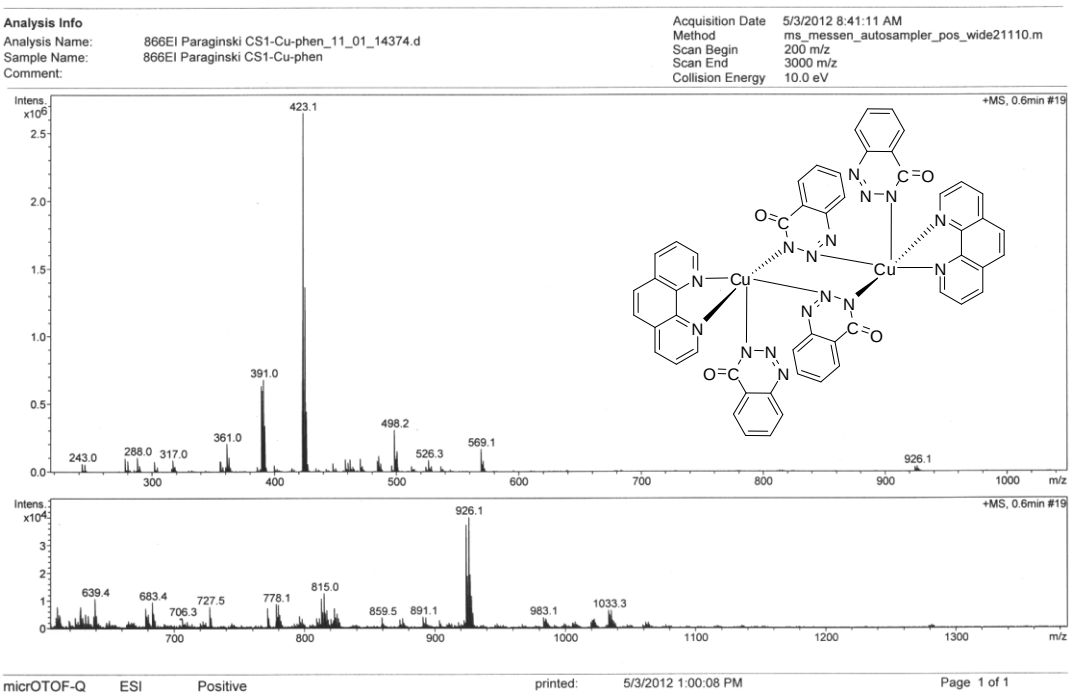

Fig. S14 Mass spectra (ESI-TOF positive mode) of $[(Phen)_2(L)_4Cu(II)_2]$ (**3**).

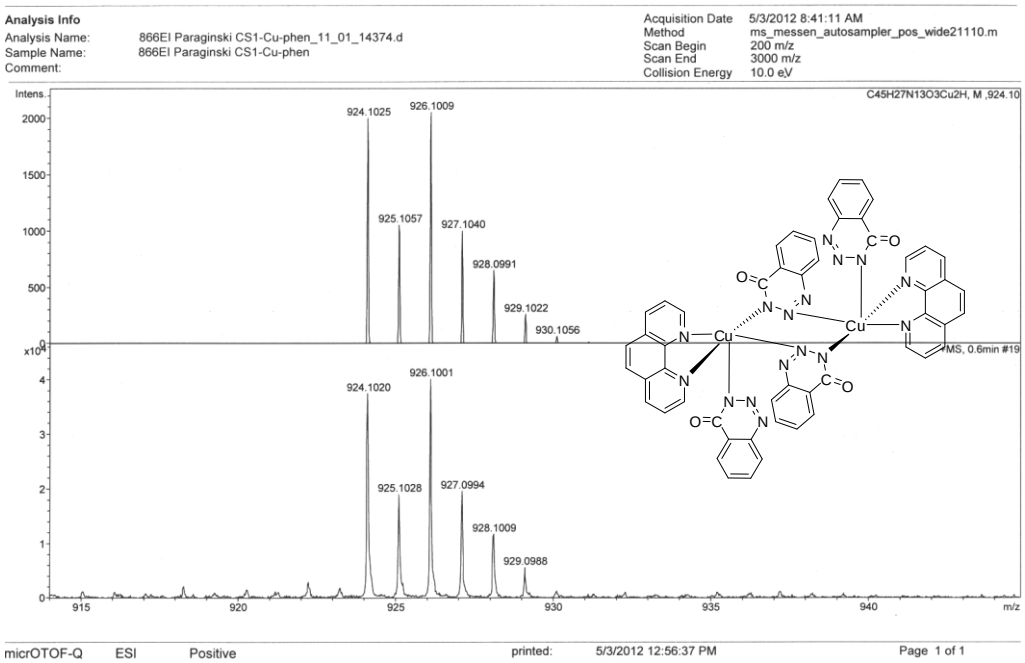

Fig. S15 Mass spectra (ESI-TOF positive mode) of $[(Phen)_2(L)_4Cu(II)_2]$ (**3**).

4 DISCUSSÃO

Esta seção apresenta uma visão geral e uma breve discussão dos principais resultados obtidos nos estudos de atividade biológica para o complexo de Au(I) e para os três complexos de Cu(II). Ressalta-se que em relação aos efeitos antitumorais *in vitro* dos compostos, alguns termos foram empregados para qualificar a sua citotoxicidade considerando os valores de CI_{50}. Assim, os compostos foram reportados como inativos (quando $CI_{50} > 100$ µM) ou com atividade citotóxica moderada (quando $CI_{50} = 10 - 100$ µM), significativa (quando $CI_{50} < 10$ µM) e potente (quando $CI_{50} < 5$ µM).

Dessa forma, a partir dos resultados apresentados na seção 3 (**PUBLICAÇÕES CIENTÍFICAS**), pode-se inferir que tanto o complexo triazenido de Au(I), $[(L)Au(PPh_3)]$, quanto dois complexos dinucleares de Cu(II), $[(bipy)_2(L)_4Cu(II)_2]$ e $[(Phen)_2(L)_4Cu(II)_2]$, demostraram promissora atividade biológica *in vitro*. No entanto, o complexo mononuclear de Cu(II), $[(L)_2(Py)_2(OH_2)Cu(II)]$, mostrou-se inativo frente a células tumorais e bacterianas.

Notavelmente, $[(L)Au(PPh_3)]$ foi muito mais seletivo sobre células tumorais do que os complexos de cobre(II), visto que, quando testado frente a células primárias derivadas de paciente não oncológico este complexo não foi capaz de inibir o crescimento celular, exibindo um valor de CI_{50} muito maior do que 100 µM.

Sobre as células primárias derivadas de paciente com Síndrome mielodisplásica $[(L)Au(PPh_3)]$ apresentou um efeito citotóxico significativo ($CI_{50} = 7{,}72$ µM), podendo representar uma molécula promissora a ser investigada para o tratamento desse tipo de neoplasia. Já em células primárias derivadas de pacientes diagnosticados com leucemia aguda ou crônica a atividade deste complexo foi moderada, uma vez que, os valores de CI_{50} obtidos foram maiores do que 10 µM. Interessantemente, a menor atividade do ligante triazenido e da trifenilfosfina Au(I) livres indica que as propriedades antitumorais de $[(L)Au(PPh_3)]$ podem estar relacionadas a um efeito sinérgico do íon metálico e dos ligantes coordenados, especialmente do grupo trifenilfosfano.

Estudos envolvendo relações estrutura-atividade de compostos de Au(I) mostraram que os complexos coordenados a grupos fosfinas terciárias, como trifenilfosfina, são mais ativos do que compostos semelhantes sem substituintes fosfina. Dessa forma, as propriedades lipofílicas deste tipo de ligante foram relacionadas a maior atividade dos complexos, visto que, podem favorecer a permeabilidade através da membrana celular (SCHEFFLER; YOU; OTT, 2010).

Em relação a atividade dos complexos dinucleares de Cu(II) em células primárias obtidas de pacientes, foi possível observar que o complexo $[(Phen)_2(L)_4Cu(II)_2]$ apresentou efeito citotóxico significativo frente a células de pacientes com LMC (CI_{50} = 7,76 μM) e Síndrome mieloproliferativa (CI_{50} = 7,01 μM). Já o complexo $[(bipy)_2(L)_4Cu(II)_2]$ apresentou atividade moderada nestas mesmas amostras. Quando ambos os complexos foram testados em modelos de leucemia aguda e de LLC em crise blástica mostraram-se claramente inativos.

Até o início da última década, a principal forma de tratamento para pacientes com LMC era o transplante de medula óssea, com taxas de sobrevida muitos baixas. O advento do mensilato de imatinibe (Glivec®), um agente inibidor da proteína tirosina quinase, fez com que houvesse uma revolução no tratamento da LMC, sendo na grande maioria dos casos, a terapia preferencial para esta patologia. Entretanto, apesar do sucesso deste agente antineoplásico, alguns pacientes não respondem de forma satisfatória ao tratamento, por intolerância ou desenvolvimento de resistência (HAMERSCHLAK, 2012). Estudos realizados entre 2001 e 2005, envolvendo compostos triazenos e linhagens celulares tumorais de LMC, evidenciaram que a inibição da tirosina quinase pode representar um dos mecanismos da ação antitumoral de compostos dessa classe (MATHESON et al., 2001; MATHESON et al., 2004; KATSOULAS et al., 2005).

Vários estudos têm relatado a potencial atividade citotóxica de compostos triazenos frente a células neoplásicas de pacientes com LMA (TURRIZIANI et al., 2006; CAPORASO et al., 2007; SEITER et al., 2009; DOMINGUES et al., 2010; BONMASSAR et al., 2013). Contudo, neste estudo os complexos contendo ligantes triazenido e benzotriazenido-ona não apresentaram valores de CI_{50} relevantes quando incubados com células de pacientes com leucemia aguda, sugerindo que os mesmos podem não representar abordagens terapêuticas promissoras para este tipo de neoplasia.

No que se refere à citotoxicidade dos complexos de Cu(II) frente as linhagens tumorais, pode-se verificar que novamemente os complexos dinucleares apresentaram promissoras propriedades antitumorais. Os ligantes livres foram inativos nas linhagens celulares investigadas, indicando que a atividade dos complexos dinucleares pode estar relacionada ao sinergismo dos íons Cu(II) com os ligantes planares coordenados.

Notadamente, o complexo $[(Phen)_2(L)_4Cu(II)_2]$ destacou-se pela sua potente atividade citotóxica (CI_{50} = 4,37 μM) frente a linhagem de melanoma murino (B16F10) e pela significativa citotoxicidade (CI_{50} = 6,16 μM) em células de câncer de mama (MCF-7). Apesar disso, mostrou-se inativo na linhagem leucêmica (K562), sendo que esta falta de atividade pode ser atribuída à heterogeneidade e à resistência tumoral, uma vez que esta linhagem foi

estabelecida de paciente com LMC em crise blástica (HEHLMANN, 2012; BURRELL et al., 2013). Considerando os resultados referentes a seletividade dos complexos dinucleares de Cu(II), é possível verificar que ambos apresentam seletividade baixa, mostrando-se citotóxicos frente a linhagem celular não tumoral (VERO).

Um resultado interessante pode ser observado quando da comparação das propriedades antiproliferativas dos complexos $[(Phen)_2(L)_4Cu(II)_2]$ e $[(bipy)_2(L)_4Cu(II)_2]$ as do metalofármaco Cisplatina. É digno de nota que estes complexos apresentam maior efeito citotóxico nas linhagens celulares testadas, sendo que o complexo $[(Phen)_2(L)_4Cu(II)_2]$ exibiu potencial antitumoral até 16 vezes maior do que a Cisplatina. Além disso, tanto a Cisplatina quanto os complexos dinucleares de Cu(II) foram citotóxicos frente a células normais. Em conjunto, esses dados revelam que tais complexos, especialmente $[(Phen)_2(L)_4Cu(II)_2]$, podem representar candidatos promissores para a terapia do câncer.

Em relação a atividade antibacteriana, o complexo triazenido de Au(I) demonstrou potente efeito inibitório sobre o crescimento de cepas Gram positivas. Já os complexos dinucleares de Cu(II) apresentam efeitos antibacterianos mais discretos, sendo também mais ativos frente a cepas Gram positivas.

5 CONCLUSÃO

As considerações finais, abaixo relacionadas, estão em consonância com os objetivos almejados e os resultados obtidos.

- Atividade antiproliferativa em células primárias derivadas de pacientes.

a) [(L)Au(PPh_3)] apresenta citotoxicidade significativa frente a células obtidas de paciente com Síndrome mielodisplásica (CI_{50} = 7,72 μM), sendo moderadamente ativo em células leucêmicas;

b) [$(Phen)_2(L)_4Cu(II)_2$] demonstra efeito citotóxico significativo frente a células de pacientes com LMC (CI_{50} = 7,76 μM) e Síndrome mieloproliferativa (CI_{50} = 7,01 μM), porém, é inativo em amostras de leucemia aguda e de LLC em crise blástica.

- Citotoxicidade dos complexos de Cu(II) frente as linhagens tumorais.

a) A atividade citotóxica de [$(Phen)_2(L)_4Cu(II)_2$] é muito maior do que a de [$(bipy)_2(L)_4Cu(II)_2$];

b) [$(Phen)_2(L)_4Cu(II)_2$] é potencialmente citotóxico para as linhagens de melanoma (B16F10) (CI_{50} = 4,37 μM) e de câncer de mama (MCF-7) (CI_{50} = 6,16 μM), porém é inativo na linhagem leucêmica (K562);

c) As propriedades citotóxicas deste complexo podem estar relacionada ao sinergismo dos íons Cu(II) com os ligantes planares phen coordenados.

- Seletividade dos complexos.

a) O complexo de Au(I) é amplamente seletivo, mostrando-se citotóxico apenas para células tumorais;

b) Os complexos dinucleares de Cu(II) apresentam seletividade baixa, mostrando-se citotóxicos frente a linhagem celular não tumoral (VERO).

- Comparação com a atividade do metalofármaco Cisplatina.

a) [$(Phen)_2(L)_4Cu(II)_2$] e [$(bipy)_2(L)_4Cu(II)_2$] apresentam maior efeito citotóxico nas linhagens celulares testadas do que Cisplatina;

- Atividade antibacteriana.

a) [(L)Au(PPh_3)], [$(Phen)_2(L)_4Cu(II)_2$] e [$(bipy)_2(L)_4Cu(II)_2$] demonstram potente atividade antibacteriana, principalmente em cepas Gram positivas.

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