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LARISSA FAVARETTO GALUPPO

EFEITOS ANTITUMORAIS E HEMATOLÓGICOS *IN VIVO* DO COMPOSTO MESOIÔNICO SIDNONA 1

CURITIBA

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EFEITOS ANTITUMORAIS E HEMATOLÓGICOS *IN VIVO* DO COMPOSTO MESOIÔNICO SIDNONA 1

Dissertação apresentada ao Programa de Pós-Graduação em Farmacologia da Universidade Federal do Paraná como requisito parcial para obtenção do título de Mestre em Farmacologia.

Orientadora: Profª. Dra. Alexandra Acco

CURITIBA

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Ministério da Educação
UNIVERSIDADE FEDERAL DO PARANÁ
Setor de Ciências Biológicas
Programa de Pós-Graduação em Farmacologia

PARECER

A Comissão Examinadora da Dissertação de Mestrado intitulada "EFEITOS ANTITUMURAIS E HEMATOLÓGICOS *IN VIVO* DO COMPOSTO MESOIÔNICO SIDNONA 1", de autoria da pós-graduanda LARISSA FAVARETTO GALUPPO, sob orientação da Prof.ª Dr.ª Alexandra Acco e composta por: Prof.ª Dr.ª Alexandra Acco (Presidente - Farmacologia - UFPR); Prof.ª Dr.ª Juliana Geremias Chichorro (Farmacologia – UFPR) e Prof.ª Dr.ª Sílvia Maria Suter Correia Cadena (Bioquímica – UFPR), reuniu-se e, de acordo com o Regimento Interno do Programa de Pós-Graduação em Farmacologia, a pós-graduanda foi Aprovada. Para a devida publicação o trabalho deverá sofrer as modificações sugeridas, que serão conferidas por sua orientadora. Em Curitiba, 26 de março de 2015.

Prof.ª Dr.ª Alexandra Acco (Presidente - Farmacologia - UFPR)

Prof.ª Dr.ª Juliana Geremias Chichorro (Farmacologia – UFPR)

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ATA DO JULGAMENTO DA 107ª DEFESA DE DISSERTAÇÃO DE MESTRADO

Ao vigésimo sexto dia do mês de março do ano de dois mil e quinze, às nove horas, no Auditório do Departamento de Farmacologia, Anexo I, Setor de Ciências Biológicas da Universidade Federal do Paraná, reuniu-se a Comissão Examinadora da Dissertação de Mestrado de autoria da pós-graduanda **LARISSA FAVARETTO GALUPO**, intitulada " EFEITOS ANTITUMURAIS E HEMATOLÓGICOS *IN VIVO* DO COMPOSTO MESOIÔNICO SIDNONA 1", sob orientação da Prof.ª Dr.ª Alexandra Acco e composta por: Prof.ª Dr.ª Alexandra Acco (Presidente - Farmacologia - UFPR); Prof.ª Dr.ª Juliana Geremias Chichorro (Farmacologia – UFPR) e Prof.ª Dr.ª Sílvia Maria Suter Correia Cadena (Bioquímica – UFPR). A Banca Examinadora iniciou os trabalhos. A candidata teve quarenta e cinco minutos para expor oralmente seu trabalho, sendo em seguida arguida durante trinta minutos por cada um dos membros da Banca e tendo trinta minutos para responder a cada uma das arguições. No final da sessão, a Comissão Examinadora emitiu o seguinte parecer: Aprovada. Para a publicação, o trabalho deverá sofrer as modificações sugeridas, que serão conferidas por sua orientadora. Nada mais havendo a tratar, a Presidente deu por encerrada a sessão, da qual foi lavrada a presente ata, que será assinada pela Presidente e pelos demais Membros da Comissão Examinadora em Curitiba, 26 de março de 2015.

Prof.ª Dr.ª Alexandra Acco (Presidente - Farmacologia - UFPR)

Prof.ª Dr.ª Juliana Geremias Chichorro (Farmacologia – UFPR)

Prof.ª Dr.ª Sílvia Maria Suter Correia Cadena (Bioquímica – UFPR)

NOTA EXPLICATIVA

Esta dissertação é apresentada em formato alternativo – artigo para publicação – de acordo com as normas do Programa de Pós-Graduação em Farmacologia da Universidade Federal do Paraná, na qual consta uma revisão de literatura, objetivos do trabalho e artigo científico abordando os experimentos realizados, resultados e discussão, bem como conclusão. O artigo foi formatado conforme as normas propostas por periódicos de circulação internacional.

Dedico este trabalho aos meus amados pais Jucelino e Eluci, por todo apoio, dedicação, amor e carinho. Vocês são meu grande exemplo, minha base, minha vida.

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Agradeço primeiramente a Deus, por todas as bênçãos e conquistas que Ele tem me proporcionado, devo a Ele tudo de bom que tem acontecido na minha vida. Aos meus pais, por tudo o que fizeram e fazem por mim, o meu amor por vocês é incondicional. À minha amada família, especialmente à minha avó Hermínia, exemplo de fé, força e amor. Às grandes amizades que fiz durante essa jornada, em especial à Carol Gomes, Graci e Fran, obrigado por terem tornado os meus dias mais suaves, felizes e divertidos, vocês sempre vão estar no meu coração. Aos colegas de laboratório por toda ajuda prestada e principalmente por terem me recebido de braços abertos, fazendo com que eu me sentisse em casa. Às meninas do Laboratório de Dor, Re e Carol, foi um prazer trabalhar junto com vocês, muito obrigada por toda ajuda e por essa parceria que foi tão produtiva e agradável. À minha orientadora, melhor dizendo a minha “mãe científica”, primeiramente por ter acreditado em mim, por ter me estendido á mão quando mais precisei, por todos os seus ensinamentos, pelos seus cuidados e mimos, nem mil palavras seriam suficientes para expressar a gratidão e admiração que sinto por ti, tenho certeza de que foi Deus quem a colocou no meu caminho. À minha grande amiga Fran, por ter sido meu porto seguro, por toda a sua paciência e incentivo, pelos seus ensinamentos, conselhos, que tanto contribuíram para o meu crescimento pessoal e profissional. A todos vocês muito obrigada por tudo.

“De alguma forma, eu não posso acreditar que existam alturas que não podem ser escaladas por um homem que conhece os segredos de fazer sonhos se tornarem realidade. Este segredo especial, parece-me, pode ser resumido em quatro “c”s. Eles são curiosidade, confiança, coragem e constância, e o maior de todos é a confiança. Quando você acredita em uma coisa, acredite 100% de forma implícita e inquestionável”.

Walt Disney

RESUMO

A incidência do câncer vem aumentando drasticamente, no mundo todo. O principal tratamento para este tipo de enfermidade é a quimioterapia, porém a citotoxicidade dos agentes quimioterápicos é muito alta, o que acaba gerando vários efeitos colaterais para o paciente em tratamento. Tendo em vista as limitações da quimioterapia e o aumento da incidência dessa doença, ressalta-se a importância do estudo de novas substâncias com ação antineoplásica, porém menos citotóxicas e com menos efeitos colaterais, proporcionando uma melhor qualidade de vida ao paciente. O objetivo deste trabalho foi determinar a atividade antitumoral do composto mesoiônico Sidnona 1 (Syd-1) no modelo tumor Walker-256 em ratos, avaliando o crescimento tumoral e o seu possível mecanismo de indução de morte celular (apoptose, estresse oxidativo e participação em vias de inflamação). Para isto foram utilizados ratos *Wistar*, machos, com peso variando de 180 g a 220 g. Para a indução do tumor foram utilizadas células neoplásicas da linhagem Walker-256, que foram inoculadas por via subcutânea em uma concentração de $1x10^7$ células, no membro pélvico direito dos animais. No dia seguinte à inoculação iniciou-se o tratamento por via oral (gavagem), com o composto Syd-1 na dose de 75 $mg \bullet kg^{-1}$ ou com veículo (Tween 20% e salina) para os grupos: 1) Basal (animais saudáveis, que receberam apenas veículo), 2) Basal-Syd (animais saudáveis, que receberam tratamento com Syd-1), 3) Tumor (animais com tumor, que receberam veículo) e 4) Tumor-Syd (animais com tumor, que receberam tratamento com Syd-1). Após 12 dias de tratamento os animais foram anestesiados para coleta de sangue, em seguida foi realizada a eutanásia para coleta dos demais tecidos. Fígado, tumor, baço e o plasma foram recolhidos e congelados para análises posteriores. Além disso, o volume e peso tumoral foram avaliados. O composto promoveu uma significativa supressão tumoral nos animais do grupo Tumor-Syd, quando comparados com o grupo Tumor. Além disso, houve uma diminuição dos níveis de GSH no tecido tumoral do grupo que recebeu o tratamento com Syd-1, indicando uma possível ação pró-oxidante no tumor, o que pode ser um mecanismo importante para auxiliar no combate à progressão tumoral. As análises bioquímicas demonstraram que o tratamento com a Syd-1 tende a amenizar alguns danos causados pela síndrome da caquexia. Não foi observada ação anti-inflamatória pelo tratamento com o composto. Ao se investigar as vias apoptóticas observou-se que no grupo Tumor-Syd houve um aumento da expressão das proteínas pró-apoptóticas Bax e p53 e diminuição da anti-apoptótica Bcl-2, indicando que o mecanismo de ação da Syd-1 ocorre pela modulação da apoptose. Nos animais tradados com Syd-1 (grupos Basal-Syd e Tumor-Syd) também houve uma considerável esplenomegalia quando comparados aos demais grupos, o que explica as alterações hematológicas observadas, como aumento de VCM, HCM e RDW. Análises histológicas demonstraram presença considerável de ferro no baço dos animais tratados com Syd-1, levando à hipótese de que a esplenomegalia ocorre por uma extensa hemólise induzida pelo composto. Em conclusão, foi demonstrada a significativa ação antitumoral do composto Syd-1 frente ao modelo de carcinossarcoma Walker-256, através de mecanismos que envolvem ativação de vias apoptóticas, porém os seus efeitos colaterais, como os hematológicos e a esplenomegalia, devem ser melhor elucidados.

Palavras-chave: Câncer, Walker-256, Syd-1, Apoptose, Esplenomegalia.

ABSTRACT

The incidence of cancer is increasing dramatically worldwide. The main treatment for this type of disease is chemotherapy, but the cytotoxicity of chemotherapeutic agents is very high, which generates several side effects for the patient in treatment. Given the limitations of chemotherapy and the increased incidence of this disease, it has been important the study of new substances with antineoplastic action, lower cytotoxic and fewer side effects, providing a better quality of life for the patient. The objective of this study was to determine the antitumor activity of the mesoionic compound Sydnone 1 (Syd-1) using the Walker-256 tumor model in rats, assessing tumor growth and its possible induction of cell death mechanism (apoptosis, oxidative stress and participation in the inflammatory process). Male Wistar rats, weighing between 180 g to 220 g, were used in the experiments. For tumor induction $1x10^7$ neoplastic cells of Walker-256 strain were inoculated subcutaneously in the right leg of animals. In the day after inoculation the treatment started orally (gavage) with Syd-1 at a dose of 75 $mg•kg^{-1}$ or vehicle (20% Tween and saline) to groups: 1) Basal (healthy animals receiving vehicle), 2) Basal-Syd (healthy animals that received Syd-1), 3) Tumor (tumor-bearing animals that received saline) and 4) Tumor-Syd (tumor-bearing animals treated with Syd-1). After 12 days of treatment the animals were anesthetized for blood collection, and then the animals were euthanized, allowing the collection of other tissues. Liver, tumor and blood were collected and frozen for later analysis. In addition, tumor volume and tumor weight were evaluated along the treatment. The compound produced significant tumor suppression in animals of the Tumor-Syd group compared with the Tumor group. Furthermore, there was a decrease in GSH levels in tumor tissue of the treated group, indicating a possible pro-oxidant action of Syd-1 in the tumor. This can be an important mechanism against cancer progression. Biochemical analyzes showned that treatment with Syd-1 tended to alleviate some damage caused by cachexia syndrome, inversely Syd-1 did not present anti-inflammatory effects. The Tumor-Syd group presented increased gene expression of the pro-apoptotic proteins Bax and p53, and decrease of the anti-apoptotic protein Bcl-2 in tumor tissue. These data elucidated the possible Syd-1 mechanism of action, which is the modulation of apoptosis. In animals treated with Syd-1 (Basal-Syd and Tumor-Syd), there was a considerable splenomegaly, which correlates with hematological changes, such as increased MCV, MCH and RDW. Further histology showed in the spleen of the animals receiving Syd-1a significant presence of iron. Thus, the splenomegaly could be a consequence of the hemolysis induced by the compound. In conclusion, this study demonstrates a significant antitumoral action of Syd-1 against the Walker-256 carcinosarcoma and elucidates its mechanism of action, which involves the activation of apoptotic pathways. However, the hematological side effects and the splenomegaly should be better elucidated.

Keywords: Cancer, Walker-256, Syd-1, Apoptosis, Splenomegaly.

LISTA DE FIGURAS

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0.01 when compared with Tumor group. °$p < 0.05$ when compared with Tumor-Syd group.

FIGURE 3: Weight of liver (A) spleen (B), kidney (C) and lung (D) in healthy (Basal) and tumor-bearing (Tumor) rats that were treated with vehicle or sydnone 1 (Syd-1) orally for 12 days. Tumor, tumor group that received only vehicle (n = 14); Tumor-Syd, tumor group that received Syd-1 treatment (75 mg•kg^{-1}, n = 10); Basal, healthy animals that received vehicle (n = 12); Basal-Syd, healthy animals that received Syd-1 (75 mg•kg^{-1}, n = 5). The results were expressed as mean ± SEM. Statistical comparisons were performed by the one-way ANOVA followed by the Bonferroni test. ### $p < 0.001$ compared to Tumor group. *** $p < 0.001$ compared to Basal group.

FIGURE 4: Histological parameters, stained with hematoxylin/eosin or prussian blue, in splenic tissue of rats after 12 days of treatment with vehicle and Syd-1. Basal (A, B), Basal-Syd (C, D), Tumor (E, F) and Tumor-Syd (G, H). Panel E notices the presence of the marginal zone hyperplasia when compared to the panel A, indicated by white line. In panel G and C is possible to observe the presence of macrophages with brown pigments dispersed in the red pulp, indicated by the white arrow. The red arrow in panel D and H points to the significant presence of blue dye when compared to the other groups, indicating expressive presence of iron in the red pulp of the spleen of animals treated with Syd-1. Legend: Basal, healthy animals; Basal-Syd, healthy animals, that received the compound Syd (75 mg•kg^{-1}); Tumor, tumor group that received only vehicle and Tumor-Syd tumor group that received treatment with Syd-1 (75 mg•kg^{-1}). Scale bar = 20 μm and 10 μm.

FIGURE 5: Histological parameters evaluated in the hepatic tissue of rats after 12 days of treatment with vehicle or Syd-1. Basal (A, B), Basal-Syd (C, D) Tumor (E, F), and Tumor-Syd (G, H). The presence of a lymphocytic infiltrate in panel B, D and F was observed as well as the presence of steatosis in panel H. Tumor, tumor group that received only vehicle; Tumor-Syd, tumor group that received Syd-1 treatment (75 mg•kg^{-1}); Basal, healthy animals that received vehicle; Basal-Syd, healthy animals that received Syd-1 (75 mg•kg^{-1}). Scale bar = 20 μm and 10 μm.

FIGURE 6: Histological parameters assessed in tumor tissue of rats after 12 days of treatment with vehicle or Syd- 1. Tumor (A, B) and Tumor-Syd (C, D). It can be seen in panel B the presence of viable tumor tissue, indicated by the black arrow, and necrosis indicated by the orange arrow. In the group receiving Syd-1 (panel D) is possible to observe the presence of apoptotic bodies, indicated by the black arrow. Tumor, tumor group that received only vehicle; Tumor-Syd, tumor group that received Syd-1

treatment (75 mg•kg^{-1}); Basal, healthy animals that received vehicle; Basal-Syd, healthy animals that received Syd-1 (75 mg•kg^{-1}). Scale bar = 20 μm and 10 μm.

FIGURE 7: Gene expression of proteins p53 (A), Bax (B) and Bcl-2 protein (C) in tumor tissue of animals treated with vehicle (Tumor group) or Sydnone 1 (Tumor-Syd). Tumor, tumor group that received only vehicle (n = 5); Tumor-Syd, tumor group that received Syd-1 treatment (75 mg•kg^{-1}; n = 5). The results were expressed as mean ± SEM. Statistical comparisons were performed by the Student's t-test. * $p < 0.05$, ** $p < 0.01$.

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TABLE 1: Plasmatic parameters in healthy and tumor-bearing rats that were treated with vehicle or sydnone 1 (Syd-1; 75 mg•kg^{-1}) orally for 12 days. The results were expressed as means ± SEM. Statistical comparisons were performed using one-way ANOVA followed by Bonferroni test. Tumor, tumor group that received only vehicle (n = 14); Tumor-Syd, tumor group that received Syd-1 treatment (75 mg•kg^{-1}, n = 10); Basal, healthy animals that received vehicle (n = 12); Basal-Syd, healthy animals that received Syd-1 (75 mg•kg^{-1}, n = 5). * $p < 0.05$ compared to Basal group. ° $p < 0.05$ compared to Basal-Syd group.

TABLE 2: Hematological parameters in healthy and tumor-bearing rats that were treated with vehicle or sydnone 1 (Syd-1; 75 mg•kg^{-1}) orally for 12 days. The results were expressed as means ± SEM. Statistical comparisons were performed using one-way ANOVA followed by Bonferroni test. Tumor, tumor group that received only vehicle (n = 14); Tumor-Syd, tumor group that received Syd-1 treatment (75 mg•kg^{-1}, n = 10); Basal, healthy animals that received vehicle (n = 12); Basal-Syd, healthy animals that received Syd-1 (75 mg•kg^{-1}, n = 5). * $p < 0.05$ compared to Basal group. # $p < 0.05$ compared to Tumor group. ° $p < 0.05$ compared to Basal-Syd group. Ø $p < 0.05$ compared to Tumor-Syd group.

TABLE 3: Histological parameters of the spleen in healthy rats that were treated with vehicle (Basal group) or sydnone-1 (Basal-Syd group) and tumor-bearing rats that were treated with vehicle (Tumor group) or sydnone 1 (Tumor-Syd group) orally for 12 days. Groups: Tumor, tumor group that received only vehicle; Tumor-Syd, tumor group that received Syd-1 treatment (75 mg•kg^{-1}); Basal, healthy animals that received vehicle; Basal-Syd, healthy animals that received Syd-1 (75 mg•kg^{-1}). Scores: (--) negative, (+) mild, (++) moderate and (+++) pronounced.

TABLE 4: Histological parameters of the liver in healthy rats that were treated with vehicle (Basal group) or sydnone-1 (Basal-Syd group) and tumor-bearing rats that were treated with vehicle (Tumor group) or sydnone 1 (Tumor-Syd group) orally for 12 days. Groups: Tumor, tumor group that received only vehicle; Tumor-Syd, tumor group that received Syd-1 treatment (75 mg•kg^{-1}); Basal, healthy animals that received vehicle; Basal-Syd, healthy animals that received Syd-1 (75 mg•kg-1). Scores: (--) negative, mild (+) and moderate (++).

TABLE 5: Histological parameters and Huvos index of tumors in rats that were treated with vehicle (Tumor group) or sydnone 1 (Tumor-Syd group) orally for 12 days. Groups: Tumor, tumor group that received only vehicle; Tumor-Syd, tumor group that received Syd-1 treatment (75 mg•kg^{-1}); Basal, healthy animals that received vehicle; Basal-Syd, healthy animals that received Syd-1 (75 mg•kg-1). Scores: (--) negative, mild (+), moderate (++) and pronounced (+++).

LISTA DE ABREVIATURAS

APAF-1: Fator ativador de apoptose 1

ATP: Trifosfato de adenosina

B10MCII: Histiocitoma fibroso

Bax: Bcl-2 associada à proteína x

Bcl-2: *B-cell lymphoma 2*

Bid: *BH3- interacting domain death agonist*

CAT: Catalase

COX: Ciclooxigenase

CRH: Hormônio liberador de corticotrofina

DNA: Ácido desoxirribonucleico

EROS: Espécies reativas de oxigênio

GADD45: Growth Arrest and DNA damage inducible

GPx: Glutationa peroxidase

GSH: Glutationa reduzida

GST: Gulationa-S-transferase

H_2O_2: Peróxido de hidrogênio

IFN-γ: Interferon gama

IL-12: Interleucina 12

IL-1β: Intereleucina 1 beta

IL-6: Interleucina 6

L1210: Células leucêmicas

NF-κB: Fator nuclear Kappa B

$O2^{\bullet-}$: Ânion superóxido

$OH^{\bullet}$: Radical hidroxila

p21: Proteína 21

p53: Proteína 53

PG: Prostaglandina

SOD: Superóxido dismutase

STAT3: Signal transducer and activator of transcription 3

Syd-1: 3-[4-cloro-3-nitrofenil]-1,2,3-oxadiazólio-5-olato

Syd-2: 3-[4-pirrolidino-3-nitrofenil]-1,2,3-oxadiazólio-5-olato

Syd-3: 3-[4-piperidino-3-nitrofenil]-1,2,3-oxadiazólio-5-olato

Syd-4: 3-[4-morfolino-3-nitrofenil]-1,2,3-oxadiazólio-5-olato

TFAA: Anidrido trifluoroacético

TGF-β1: Fator de transformação do crescimento β1

TNF-α: Fator de necrose tumoral alfa

LISTA DE ABREVIATURAS – ARTIGO CIENTÍFICO

ALT: Alanine aminotransferase

AST: Aspartate aminotransferase

ATC: Trichloroacetic acid

Bax: *Bcl-2-associated X protein*

Bcl-2: *B-cell lymphoma 2*

CAT: Catalase

CDNB: 1-chloro-2,4-dinitrobenzene

DTNB: (5,5-dithiobis-(2-nitrobenzoic acid))

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

GSH: Reduced glutathione

GST: Glutathione-S-transferase

HE: Hematoxylin and eosin

IL-6: Interleukin 6

LPO: Lipid peroxidation

MeOH: Methanol

MPO: Mieloperoxidase

NAG: N-acethyl-β-D-glucosaminidase

NO: Nitric oxide

p53: Protein 53

RNA: Ribonucleic acid

ROS: Reative oxygen species

SOD: Superoxide Dismutase

Syd-1: Sydnone 1

TNF-α: Tumor necrosis factor-alpha

SUMÁRIO

1. REVISÃO DE LITERATURA

1.1 Câncer: Incidência e principais causas da doença

Define-se como câncer o conjunto de mais de 100 doenças que têm em comum o crescimento desordenado (maligno) de células que invadem os tecidos e órgãos. Dividindo-se rapidamente, estas células tendem a ser muito agressivas e incontroláveis, determinando a formação de tumores/neoplasias malignas (Inca, 2011).

A incidência do câncer vem crescendo anualmente, no Brasil para os anos de 2014/2015 a estimativa é de 576 mil novos casos de câncer. Já, em nível mundial, para 2030 a estimativa é 27 milhões de novos casos com 17 milhões de mortes por câncer (Facina, 2014; Inca, 2014; Rodrigues e Amaral, 2012). A principal causa do aumento na incidência do câncer no mundo é o envelhecimento populacional (Petito; Gutiérrez, 2008). Em 1980 a expectativa de vida da população brasileira era de 62 anos, já para 2020 a expectativa é de 76 anos (Springer *et al.*, 2010). Outros fatores que também influenciam para o aumento de pessoas diagnosticadas com câncer é a exposição a agentes cancerígenos, como fumaça de cigarro, álcool, sedentarismo, dieta rica em lipídeos e exposição à radiação solar sem proteção (Hwang, 2008).

1.2 Estágios do desenvolvimento tumoral e seus mediadores

Tanto nos estudos pré-clínicos como nos clínicos, têm se observado a natureza complexa do desenvolvimento e progressão do câncer (O'Connor *et al*., 2014). O avanço nas pesquisas tem proporcionado uma visão valiosa sobre os mecanismos moleculares e celulares da tumorigênese, entretanto o mecanismo pelo qual as neoplasias conseguem ser resistentes ao tratamento ainda permanece indefinido (Dean *et al.,* 2005).

O processo da instalação da doença é dividido em três etapas: iniciação, promoção e progressão. A iniciação envolve ligação e dano direto ao DNA, realizado através de agentes carcinógenos. A promoção leva à pré-malignidade, através da expansão clonal das células iniciadas/ modificadas. A progressão ocorre devido a mecanismos genéticos, sendo caracterizada por ser o período entre pré-malignidade e câncer, nesta etapa as células alteradas desenvolvem modificações irreversíveis, resultando na proliferação descontrolada de células cancerosas, adquirindo a capacidade de invadir tecidos secundários (metástase), disseminando a doença para os demais órgãos. Tanto a iniciação, promoção, quanto a progressão são processos irreversíveis (Tsao *et al*., 2004; Borchers *et al*., 2004).

Todos os tumores dependem de suportes estrutural, nutricional, vascular e em alguns casos, humoral, que são supridos pelo próprio organismo, de modo a se comportarem como “parasitas”, depletando as reservas do hospedeiro (Curi, 2000). Alguns tumores podem competir com o hospedeiro por nutrientes, provocando alterações metabólicas como anorexia, astenia, anemia, saciedade prematura e perda de peso. Essas alterações, associadas ao desenvolvimento do tumor podem induzir à debilidade do organismo e levar o hospedeiro a desenvolver um quadro denominado de caquexia. A estimativa é que cerca de 20 a 70% dos pacientes com câncer apresentem este quadro (Tisdale, 2001). Estima-se que em cerca de 20% dos casos a caquexia pode ser a causa direta de óbito do paciente (Holmes, 2009).

A síndrome caquética é um estado em que o metabolismo basal está aumentado de maneira persistente, mas não é compensado pelo aumento da ingesta calórico-proteica, levando à diminuição de funções físicas e psicológicas (Rodríguez *et al.,* 2010; Blum *et al.,* 2011). Sua fisiopatologia compreende uma série de mecanismos metabólicos complexos, vinculados diretamente com a relação tumor-hospedeiro (Rodriguez *et al.,* 2010). Os mecanismos dependentes desta relação causam drásticas alterações endócrinas e metabólicas na caquexia, que compreendem alterações dos fatores tumorais, gerados pelo próprio tumor e fatores humorais, gerados como resposta biológica do hospedeiro à presença do tumor. As consequências metabólicas e endócrinas da caquexia são: aumento da gliconeogênese hepática, diminuição na captação muscular de glicose, hiperlipidemia, aumento da lipólise, aumento da síntese proteica hepática, aumento da degradação proteica muscular, aumento de hormônios contrarreguladores (como cortisol e catecolaminas), ativação linfocitária, liberação de catecolaminas e fatores inflamatórios de resposta rápida (Rodriguez *et al.,* 2010). Citocinas como a interleucina (IL)-6, IL-1β e fator de necrose tumoral alfa (TNF-α) estão envolvidas no desenvolvimento desse tipo de anorexia, possivelmente pela sua interação com os centros controladores da fome no hipotálamo. Elas atuam estimulando a liberação de substâncias supressoras do apetite, fazendo com que aumente a concentração do hormônio liberador de corticotrofina (CRH) e diminua a concentração dos hormônios estimuladores do apetite, como neuropeptídeo-Y (Argilés *et al.,* 2005; Dunlop; Campbell, 2000).

Sabe-se que a inflamação é uma resposta imune que ocorre quando a homeostase do organismo é perturbada. Processos inflamatórios crônicos afetam todas as fases do desenvolvimento tumoral (Elinav *et al*., 2013). Dentre os agentes envolvidos na inflamação relacionada ao câncer destacam-se fatores de transcrição como o fator nuclear Kappa B (NF- κB) e o transdutor de sinal e ativador de

transcrição 3 (STAT3), bem como algumas citocinas inflamatórias primárias, IL-1β, IL-6 e TNF-α (Karin, 2006; Langowski *et al.,* 2006).

O NF-κB tem um papel fundamental no desenvolvimento e progressão do câncer, pois ele pode regular angiogênese, invasão tumoral e controlar a capacidade de células pré-neoplásicas e malignas a resistirem aos mecanismos de vigilância tumoral baseados em apoptose (Karin, 2006). A STAT3 é amplamente implicada na tumorigênese de vários tecidos e está intimamente ligada a processos inflamatórios em cânceres de cólon, fígado, estômago, pulmão e pâncreas (Fukuda *et al.,* 2011; Gao *et al.,* 2007). A ativação da STAT3 é realizada pela IL-6 e TNF-α (Gao *et al.,* 2007; Park *et al.,* 2010). Em relação ao seu mecanismo, a STAT3 promove a proliferação celular por regulação da expressão de ciclina D1, ciclina D2 e ciclina 3, responsáveis por controlar o ciclo celular e aumentar a expressão de proteínas anti-apoptóticas como Bcl-2 (Bollrath *et al.,* 2009; Grivennikov *et al.,* 2009; Yu *et al.,* 2009). Estes dados sugerem que um processo inflamatório acompanhado por ativação oncogênica pode levar a uma amplificação da cascata de sinalização NF-κB e IL-6-STAT3, o que resulta em iniciação ou progressão do câncer, proporcionando assim um exemplo genuíno do efeito que a sinalização de células imunológicas possui sobre o comportamento proliferativo de células neoplásicas (Elinav *et al.,* 2013).

Segundo as estimativas cerca de 20% dos cânceres humanos são atribuídos a doenças inflamatórias, pois quando células inflamatórias são ativadas induzem à produção excessiva de espécies reativas de oxigênio (EROS) (Mantovani *et al*., 2004). As EROS são produzidas como resultado de fatores do metabolismo celular e ambientais, tais como poluentes atmosféricos ou fumaça de cigarro. São espécies altamente reativas que podem danificar as estruturas celulares, tais como hidratos de carbono, ácidos nucleicos, lípideos e proteínas, bem como podem alterar as suas funções, levando a uma cascata de oxidação e redução, também chamada de estresse oxidativo. Em condições patológicas, o estresse oxidativo pode causar alterações funcionais em células saudáveis e disfunção orgânica clinicamente detectável. Os principais radicais livres formados no corpo humano são: radical hidroxila ($OH^{\bullet}$) e ânion superóxido ($O_2^{\bullet -}$) (Birben *et al.,* 2012; Weijl *et al.,* 1997).

Mecanismos que podem levar ao estresse oxidativo no paciente com câncer são o metabolismo energético alterado, devido à caquexia, vômito e náuseas, impedindo uma nutrição normal (Mantovani *et al*., 2004) e o uso de antineoplásicos, que são capazes de produzir grande quantidade de EROS (Weijl *et al.,* 1997). Para protegerem-se as células possuem sistemas de defesas antioxidantes, que incluem antioxidantes enzimáticos e não enzimáticos. Dentre as enzimas estão a catalase (CAT), uma hemeproteína citoplasmática que catalisa a redução do H_2O_2 em H_2O e O_2

(Ferreira; Matsubara, 1997). A superóxido dismutase (SOD), que em seres eucariontes existe em duas formas, a SOD-cobre-zinco presente principalmente no citosol e a SOD-manganês presente principalmente na mitocôndria, tem como função catalisar a dismutação do radical superóxido em H_2O_2 e O_2, na presença do próton H^+ (Acharya *et al.,* 1991; Fridovich, 1997). Glutationa peroxidase (GPx), assim como a CAT a GPx, também atua catalisando a redução do H_2O_2 (Shan *et al.,* 1990; Ferreira; Matsubara, 1997), enquanto a glutationa-S-transferase (GST) tem como função inativar metabólitos secundários, tais como aldeídos insaturados, epóxidos e hidroperóxidos (Birben *et al.,* 2012). Além de defesas antioxidantes enzimáticas existem também os sistemas de defesa não-enzimáticos, como vitaminas, flavonoides da dieta e glutationa reduzida (GSH). O GSH protege as células contra danos oxidativos, elimina produtos da peroxidação lipídica e repara os danos oxidativos, sendo o antioxidante não-enzimático mais importante do organismo (Bayr, 2005; Birben *et al.,* 2012; Shan *et al.,* 1990).

Vários estudos têm apontado papéis contraditórios das EROS na tumorigênese, visto que, por causar mutações no DNA e instabilidade genômica, elas desempenham um papel importante no desenvolvimento e progressão do tumor, por outro lado, altos níveis de EROS também podem ser tóxicos para as células neoplásicas, podendo induzir a morte celular. Para equilibrar o estresse oxidativo as células cancerosas aumentam a sua capacidade antioxidante, o que sugere que níveis elevados de EROS possuem potencial suficiente para bloquear a tumorigênese. Isto torna a terapia pró-oxidante no câncer uma interessante área de estudo (Glasauer; Chandel, 2014).

1.3 Apoptose

A apoptose, também conhecida como morte celular programada, é um processo fundamental na manutenção do desenvolvimento dos seres vivos, pois elimina células defeituosas e supérfluas. É um processo ativo, caracterizado pelo gasto de ATP, no qual, devido à ativação de proteases endógenas, a célula tem seu citoesqueleto comprometido, provocando um colapso na sua estrutura, ocasionando retração da célula, perda da aderência com a matriz extracelular e células vizinhas, condensação da cromatina, fragmentação do DNA e formação de corpos apoptóticos. Existem várias moléculas envolvidas no controle de ativação das vias apoptóticas, entre elas destacam-se as caspases e proteínas anti e pró-apoptóticas (Goldar *et al.,* 2015; Grivicich *et al.,* 2007; Parolin; Reason, 2001).

Todas as vias da apoptose (Fig. 1) convergem na ativação das caspases, pertencentes à família das cisteínas proteases, estas, reconhecem e clivam substratos

que possuam resíduos de aspartato (Nicholson; Thornberry, 1997; Youle; Strasser, 2008; Hu *et al.,* 2013). Pelo menos 14 membros dessa família já foram identificados e estão presentes em processos inflamatórios e de apoptose. A partir do momento em que são ativadas as caspases iniciadoras (caspases 8 e 9) catalisam a ativação de outros membros da família, caspases efetoras (casapases 3, 6 e 7), gerando uma amplificação da cascata proteolítica (Boatright; Salvesen, 2003; Tschopp *et al.,* 2003; Hu *et al.,* 2013).

Um número crescente de genes tem sido identificado como capazes de influenciar o processo de apoptose. Dentre eles destacam-se as proteínas da família *B-cell lymphoma* 2 (Bcl-2) (Kvansakul; Hinds, 2015; Parolin; Reason, 2001). As proteínas da família Bcl-2 são fundamentais para a regulação das vias intrínsecas/mitocondriais da apoptose, podendo exercer desde inibição à ativação das vias apoptóticas. Entre as principais proteínas anti-apoptóticas encontram-se Bcl-2, Bcl-xl e Bcl-W, já dentre as principais proteínas pró-apoptóticas destacam-se a Bax, Bid e Bak (Youle; Strasser, 2008; Anilkumar; Prehn, 2014). Muitos membros dessa família residem na membrana externa das mitocôndrias, no envoltório nuclear e no retículo endoplasmático. Nas mitocôndrias três funções têm sido atribuídas a essas proteínas: dimerização, formação de poro ou atividade do canal iônico e ligação com outras proteínas (Kaplowitz, 2000).

Na presença de sinais de estresse intracelular ocorre a translocação de proteínas pró-apoptóticas, como a Bax, por exemplo, do citosol para a mitocôndria. A translocação das proteínas pró-apoptóticas para a mitocôndria resulta na liberação do citrocomo-c, que se encontra no espaço existente entre a membrana mitocondrial externa e interna, para o citosol. Em seguida, o citocromo-c forma um complexo com o fator ativador da apoptose 1 (Apaf-1), ativando a caspase 9 (enzima chave no controle da apoptose intrínseca) que, por sua vez, ativa caspases efetoras, levando à morte celular (Parolin; Reason, 2001; Kaplowitz, 2000; Tschopp *et al.,* 2003; Hu *et al.,* 2013).

A ativação das caspases pode ser desencadeada também via “receptores da morte” ou via extrínseca. A maioria dos receptores da morte identificados, fazem parte da superfamília de receptores para o fator de necrose tumoral (TNF). Dentre esses, o mais estudado e caracterizado é o receptor Fas (Faubion; Gores, 1999). Quando o ligante-Fas se liga ao receptor Fas, as moléculas individuais do receptor se trimerizam formando um agregado de domínios da morte, permitindo que as mesmas se liguem a uma proteína adaptadora presente no citosol, chamada de domínio da morte associada ao Fas ("Fas-associated death domain", FADD). Esse complexo se liga com a pro-caspase-8, ocorrendo uma clivagem proteolítica que ativa a caspase-3 (caspase efetora) (Parolin; Reason, 2001; Goldar *et al.,* 2015).

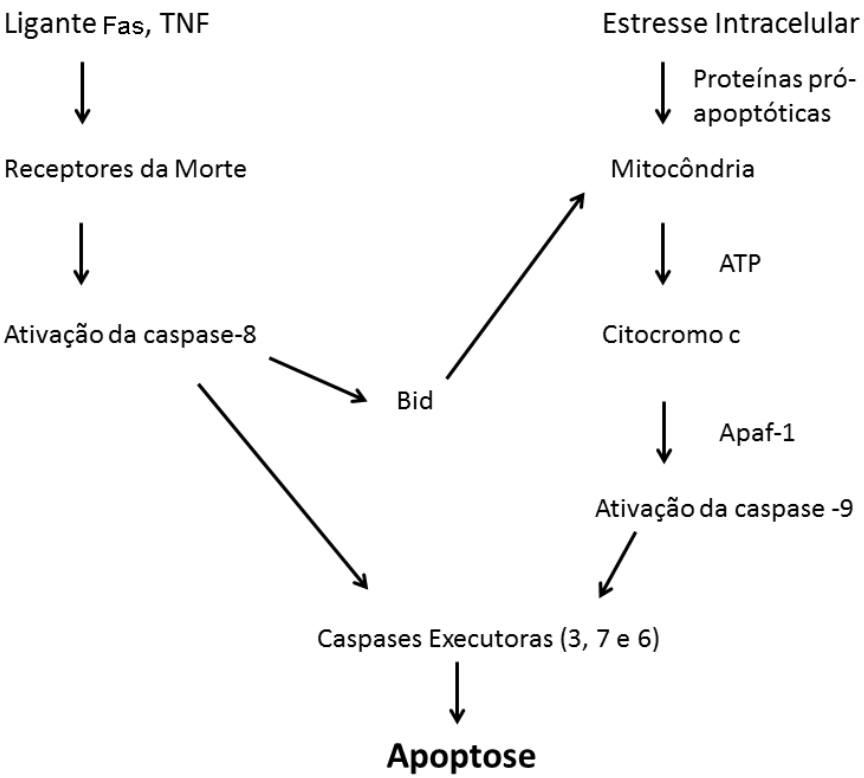

Figura 1. Ativação das vias apoptóticas por receptores da morte e disfunção mitocondrial. Legenda: TNF: Fator de necrose tumoral, Bid: BH3-interacting domain agonist, ATP: Trifosfato de adenosina, Apaf-1: Fator ativador de apoptose 1. Adaptado de Kaplowitz, 2000.

Outro gene de extrema importância diretamente ligado à apoptose é o p53. Ele é uma proteína que se liga ao DNA, regula a transcrição de diversos genes e tem um papel crítico na interrupção do ciclo celular e na indução da apoptose após dano ao DNA. Por essa razão tem sido chamado de “guardião do genoma”. Porém, em cerca de 60% das neoplasias, têm-se observado mutação do p53. Em sua forma mutada ele torna-se incapaz de controlar a proliferação celular, bem como realizar reparos no DNA, resultando em células geneticamente instáveis. Em condições normais os níveis de p53 são rapidamente elevados em resposta ao DNA danificado, levando ao aumento da transcrição dos genes alvo do p53, como p21 e GADD45, que trabalham na interrupção do ciclo celular e no reparo do DNA. A proteína p53 possui meia-vida curta, em média 20 minutos, e após o reparo do DNA é rapidamente degradada. Se o reparo for insatisfatório o p53 conduzirá a célula à morte por ativação da Bax, componente chave da cascata da apoptose. Torna-se evidente então que a perda

funcional do gene p53 pode ter consequências devastadoras para a manutenção da integridade do genoma. Sem o p53, os danos mutagênicos ao DNA passam despercebidos, a célula passa por divisão celular e a mutação se fixa no genoma (Cavalcanti *et al.,* 2002; Mcgavin; Zachary, 2009).

1.4 Quimioterapia

Apesar dos avanços médicos e cirúrgicos, o índice de pessoas que vão a óbito devido ao câncer aumenta a cada ano. A abordagem usual do tratamento para esta doença é a quimioterapia (Dunn *et al.,* 2010). A quimioterapia é um tratamento sistêmico, com agentes citotóxicos, que possui grande impacto sobre a divisão das células tumorais (Sawada *et al.,* 2009). No entanto, este tipo de tratamento é extremamente tóxico e seus efeitos são inespecíficos, pois atinge tanto tecidos malignos como normais (Dunn *et al.,* 2010).

O tratamento quimioterápico é dividido em cinco classes distintas:

- Quimioterapia prévia, neoadjuvante ou citorredutora: Indicada para tumores avançados, no intuito de melhorar o prognóstico do paciente;
- Quimioterapia adjuvante ou profilática: Indicada após o procedimento cirúrgico, quando não há mais focos de neoplasia maligna no paciente;
- Quimioterapia curativa: Tem a finalidade de curar pacientes com neoplasias malignas, representando o principal tratamento;
- Quimioterapia para controle temporário da doença: Indicada para o tratamento de tumores sólidos, avançados ou recidivados, ou neoplasias hematopoiéticas de evolução crônica. Tem a finalidade de aumentar a sobrevida do paciente, porém sem possibilidade de cura.
- Quimioterapia paliativa: Indicada para evitar o aparecimento de sinais e sintomas que comprometam a capacidade funcional do paciente, mas não repercute, obrigatoriamente, na sua sobrevida (Inca, 2011).

Os efeitos adversos da quimioterapia podem ser divididos em dois grupos: agudos, quando se iniciam em minutos após a administração dos agentes antineoplásicos e persistem por alguns dias; e tardios, quando aparecem semanas ou meses após a administração dos mesmos. Náuseas, vômitos, diarreia, estomatite, xerostomia, anemia, leucopenia, granulocitopenia, trombocitopenia, alopecia e neurotoxicidade são alguns dos efeitos adversos observados em pacientes que estão recebendo quimioterapia (Roque; Forones, 2006, Sawada *et al.,* 2009; Inca, 2014).

Nesse contexto, ressalta-se a importância das pesquisas de novos agentes que tenham como alvo apenas células tumorais, de modo a melhorar a especificidade, diminuir a toxicidade e melhorar a qualidade de vida do paciente com câncer.

1.5 Compostos Mesoiônicos

Segundo Ollis e Ramsden (1976), são classificados como compostos mesoiônicos os compostos heterocíclicos com cinco, ou eventualmente seis membros, que não podem ser representados satisfatoriamente por qualquer estrutura covalente e que possuem um sexteto de elétrons em associação com os átomos que constituem o anel. Possuem uma carga positiva no anel heterocíclico contrabalançada por carga negativa no heteroátomo, ou vice-versa. Conforme a definição citada acima a Figura 2 ilustra a estrutura geral de compostos mesoiônicos.

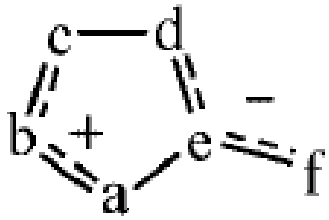

Figura 2. Representação genérica para compostos mesoiônicos.
Fonte: Athayde – Filho *et al.,* 2000.

As atividades biológicas dos compostos mesoiônicos são atribuídas ao seu caráter aromático, planar, seu tamanho relativamente pequeno, à variação da densidade dos elétrons em torno do anel, à possibilidade de selecionar diferentes padrões de densidade de elétrons por diferentes sistemas mesoiônicos e por possuírem uma rede de carga de elétrons neutra. Devido a essas características é que os compostos mesoiônicos são capazes de atravessar membranas celulares e interagir com alvos biológicos com afinidades distintas (Kier; Roche, 1967; Haider *et al.,* 2015).

Já foram relatadas diversas atividades biológicas dos compostos mesoiônicos entre elas destacam-se: atividade antimicrobiana, anti-inflamatória, analgésica, antitumoral, sequestradora de radicais livres e antidiabética (Prashant *et al.,* 2005;

Shreenivas*;* Vasantakumar, 2010*;* Deshpande; Pai, 2010; Satyanarayana; Rao*,* 1995*;* Grynberg *et al.,* 1992*;* Gireesh *et al.,* 2011). Os compostos mesoiônicos são divididos em classes de acordo com a constituição do anel mesoiônico, sendo as principais: compostos do sistema 1,2,3-oxadiazólio-5-olato denominados de sidnonas, sidinoniminas, oxatriazois, 1,3,4-tiadiazois e isosidinonas, dentre os quais as sidnonas foram os compostos mais estudados (Kawase *et al.,* 2009; Baker; Ollis, 1957).

1.6 Sidnonas

O nome “Sidnona” foi dado a esta classe porque foi estudada pela primeira vez na Universidade de Sidney, pelos pesquisadores Earl e Mackney em 1935 (Baker; Ollis, 1957). As sidnonas são solúveis em uma variedade de solventes orgânicos como hexano e éter de petróleo e geralmente não são solúveis em água, a menos que possuam em sua estrutura um grupo funcional polar (Chandrasekhar; Nanjan, 2012).

Para avaliar a toxicidade de alguns subtipos de sidnonas denominadas 4-[1-oxo-3-(X)-2propenil]-3-(4-clorofenil), onde X é igual: fenil (Syd-3 a), 2-furil (Syd-3 b), 3, 4, 5-trimetoxifenil (Syd-3 d), 4-nitrofenil (Syd-3 e), 4-N,N-dimetilaminofenil (Syd-3 f), 2-nitrofenil (Syd-3 g), 4-hidroxi-3-metoxifenil (Syd-3 h) e 2-hidroxi-3-quinolinil (Syd-3 i), Deshpande e Pai (2010) administraram doses crescentes dos subtipos do Syd-3 (250, 500, 750 e 1000 mg/kg) por via oral em ratos, que foram observados durante 3 horas no intuito de avaliar o comportamento e possíveis alterações neurológicas; enquanto a letalidade foi observada após 24 horas da administração. Não houve nenhuma morte neste período, mas na dose de 1000 mg/kg foram observadas alterações comportamentais, como estado de alerta, resposta ao toque e inquietação.

Um grande número de sidnonas vem sendo sintetizado devido às suas propriedades biológicas. Dentre elas destacam-se: antitumoral, anti-inflamatória, antibacteriana, anti-helmíntica, analgésica e sequestradora de radicais livres (Greco *et al.,* 1962; Grynberg *et al.*, 1992; Satyanarayana; Rao, 1995; Moustafa *et al.,* 2004; Kalluraya *et al.,* 2001; Bizetto *et al.,* 2012; Gozzi *et al.,* 2013). O potencial valor das sidnonas (Fig. 3) como substâncias biologicamente ativas encontra-se no seu caráter aromático e planar, tamanho relativamente pequeno, e variação de densidade dos elétrons em torno do anel. Acredita-se que a sua estrutura de ressonância promova significativas interações com moléculas biológicas (Chandrasekhar; Nanjan, 2012). As sidnonas também tem sua importância como precursoras de muitas moléculas bioativas, pois a partir das mesmas podem-se obter vários compostos imidazol-sidnonil substituídos, que são de grande interesse devido às suas características químicas e bioquímicas (Shih *et al.,* 2007).

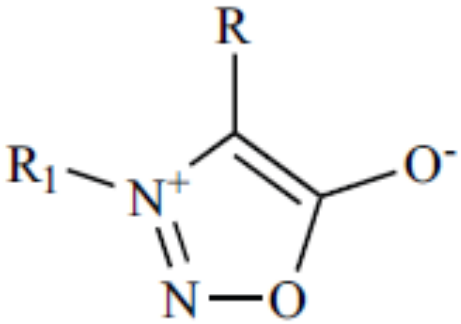

Figura 3. Estrutura química geral dos compostos mesoiônicos do sistema 1,2,3-oxadiazólio-5-olato, também conhecidos como Sidnonas.
Fonte: Chandrasekhar; Nanjan, 2012.

Em 1992 Grynberg e colaboradores estudaram os efeitos das aril sidnonas sobre tumores murinos. Os compostos, (3-[4-X-3-nitrofenil]-1,2,3-oxadiazólio-5-olatos), onde X = Cl (Syd-1), pirrolidino (Syd-2), piperidino (Syd-3) ou morfolino (Syd-4), foram sintetizados para avaliar a sobrevida de camundongos portadores de sarcoma 180, carcinoma de Ehrlich, histiocitoma fibroso (B10MCII) e leucemia (L1210). Os compostos Syd-1 e Syd-2 promoveram um aumento na sobrevida dos animais, bem como diminuição da progressão dos três tipos de tumor, porém os mecanismos pelos quais a supressão tumoral ocorreu não foram elucidados. O Syd-2 também foi efetivo contra células L1210, enquanto os compostos Syd-3 e Syd-4 não apresentaram atividade antitumoral. Posteriormente Dunkley e Thoman (2003), sintetizaram e avaliaram a atividade antitumoral de seis compostos análogos aos utilizados por Grynberg *et al.,* (1992), o X do composto *N*-(4'-X-3'-nitrofenil) sidnonas, foi substituído por: Cl (3a), F (3b), Dietilamino (4a), Azetidino (4b), Indolino (4c) e Isoindolino (4c). A atividade antitumoral desses compostos foi testada utilizando linhagens de células de câncer de mama (MCF7), pulmão (NCl-H460) e de sistema nervoso central (SF-268). Ao final do experimento observaram que tanto os composto 3a, referente a Syd-1 como o composto 3b, derivado da 4'-fluorsidnona, foram efetivos contra pelo menos uma das linhagens celulares testadas, enquanto que os demais compostos 4 a-d não apresentaram atividade antitumoral.

Dando continuidade nas pesquisas sobre a ação antitumoral do Syd-1 (Fig. 4), Halila *et al.,* (2007) demonstraram *in vitro* que o composto atua sobre o metabolismo mitocondrial, pois apresenta um efeito inibitório do transporte de elétrons da cadeia

respiratória, indicando que o mecanismo de ação do composto está relacionado com a alteração da respiração mitocondrial.

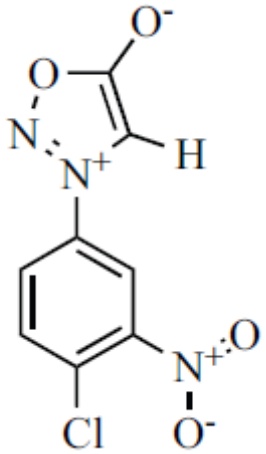

Figura 4. Representação da estrutura química do composto (3-[4-cloro-3-nitrofenil]-1,2,3-oxadiazólio-5-olato), conhecido como sidnona 1 ou Syd-1.
Fonte: Chandrasekhar; Nanjan, 2012.

Apesar destes dados, os relatos do uso de Syd-1 em modelos de neoplasia *in vivo* são escassos na literatura e o mecanismo pela qual a supressão tumoral ocorre não foi totalmente elucidado. Tendo em vista os dados citados acima, este trabalho objetivou avaliar a atividade antitumoral do composto Syd-1 frente ao modelo de carcinossarcoma Walker-256, bem como esclarecer o seu possível mecanismo de ação.

1.7 Tumor Walker–256

Em 12 de fevereiro de 1928, o Dr. George Walker, da Johns Hopkins University (EUA), observou uma massa do tamanho de uma noz-pecan, na porção inferior do abdômen de uma rata grávida. Durante o período de lactação essa massa regrediu a ponto de quase desaparecer, porém após o desmame ela voltou a crescer aumentando seu tamanho com grande rapidez. Foi realizada eutanásia na rata e a massa foi removida. A descrição do Dr. Walker para esse tumor foi de que seu tamanho era similar a um ovo pequeno, suave, aparentemente originado a partir da mama do animal (Earle, 1935).

Este tumor, descrito como um carcinossarcoma, foi utilizado em muitos estudos por ser facilmente transplantável, ser espécie-específico para ratos e de crescimento rápido em um curto período de tempo após a implantação. Com pouco tempo após a

inoculação, os animais apresentam perda de peso, anorexia e mudanças no catabolismo de proteína, carboidratos e lipídeos. Passados 14 dias após a implantação das células neoplásicas, a massa tumoral já pode representar uma fração significativa do peso corporal do animal, depois deste período o índice de mortalidade aumenta consideravelmente (Acco *et al.,* 2012). O câncer associado à síndrome da caquexia é uma importante característica clínica reproduzida pelo tumor Walker-256, e contribui para a incapacidade física, diminuição da ingestão de alimentos, perda de peso, mudanças no catabolismo de proteínas, carboidratos e lipídeos, e morte do hospedeiro (Tidale, 2001; Vicentino *et al.,* 2002; Togni *et al.,* 2003). Alguns fatores que podem explicar o comportamento invasivo e destrutivo, ocasionando o crescimento do tumor e a síndrome da caquexia, característicos do tumor Walker-256, são o aumento da expressão da enzima ciclooxigenase (COX-2), cujos principais produtos são as prostaglandinas (PG) E_2 e D_2. Além de ter sua expressão aumentada pelas células neoplásicas, a PGE_2 também pode ser liberada pelas células de Kupffer de ratos portadores de Walker-256, este mecanismo pode ser mediado pela epinefrina. Embora as PGE_2 e PGD_2 tenham ações antiproliferativas e imunossupressoras no câncer, elas são transformadas enzimaticamente em PGA_2 e PGJ_2, respectivamente, por exposição ao plasma, soro ou soluções contendo albumina. Tanto a PGA_2 quanto PGJ_2 são encontradas em quantidades consideráveis, no tecido imune de ratos portadores de Walker-256, aumentando a imunossupressão e proliferação tumoral neste modelo animal (Acco *et al.,* 2012; Seyberth *et al.,* 1980; Seelaender *et al.,* 1999; Kikawa *et al.,* 1984; Homem de Bittencourt; Curi, 2001).

Já foram relatados em estudos com Walker-256 a estimulação de diversos mediadores inflamatórios envolvidos na síndrome da caquexia, como IL-1β, IL-6, IFN-γ e TNF-α. Além disso, o fator de transformação do crescimento β1 (TGF-β1), citocina antiinflamatória que desempenha papel fundamental na hematopoiese, está relacionado com metástase e progressão tumoral. Vido *et al.,*(2000) demonstraram que a produção TGF-β1 aumenta no sétimo dia após inoculação das células Walker-256, sugerindo que este é um período crítico do crescimento tumoral (Acco *et al.,* 2012; Vido *et al.,* 2000).

Tendo em vista de que o crescimento do tumor Walker-256 é favorecido pela oxidação dos tecidos circundantes, este modelo também pode ser utilizado para avaliar o estresse oxidativo, causado pelo câncer. Já foi relatado o envolvimento de espécies reativas de nitrogênio e oxigênio durante o desenvolvimento desse modelo tumoral, que podem ser derivadas da ativação crônica de citocinas e de alterações do metabolismo energético. Em adição a este fator, os animais portadores de tumor podem apresentar mudanças na atividade oxidante e nas enzimas antioxidantes, o

que pode levar ao estresse oxidativo (Acco *et al.,* 2012; Mantovani *et al.,* 2004; Yano *et al.,* 2008).

Duas variantes de Walker-256 foram descritas, denominadas A e AR. A variante A é mais agressiva, pois possui uma taxa maior de crescimento e pode induzir efeitos sistêmicos, tais como: anorexia, baixos níveis de hemoglobina e retenção de sódio e água, seguida de perda de peso e morte. A linhagem regressiva AR foi obtida após várias inoculações de células tumorais Walker-256 na cavidade intraperitoneal de ratos. Acredita-se que a agressividade da variante A está relacionada à modulação por citocinas, principalmente uma menor expressão de interferon gamma (IFN-γ), facilitando o crescimento tumoral, enquanto a IL-12 parece estar expressa nas duas variantes A e AR (Perroud *et al.,* 2006; Cavalcanti *et al.,* 2003).

Devido às características bem conhecidas e facilidade de manutenção das células Walker-256, é que o presente trabalho utilizou este modelo de neoplasia para avaliar a atividade antineoplásica do composto Syd-1.

2. OBJETIVOS

2.1 Objetivos Gerais

Avaliar a atividade antitumoral do composto mesoiônico Sidnona 1 e seu possível mecanismo de ação, frente ao modelo de carcinossarcoma Walker-256.

2.2 Objetivos Específicos

- Avaliar o crescimento tumoral em ratos tratados e não tratados com Syd-1;
- Acompanhar o peso ponderal dos animais;
- Realizar análises de estresse oxidativo e avaliar suas implicações no desenvolvimento tumoral e no metabolismo hepático;
- Avaliar a toxicidade aguda da Syd-1;
- Avaliar a ação da Syd-1 em vias envolvidas na apoptose celular através da detecção de genes pró-apoptóticos e anti-apoptóticos;
- Avaliar o efeito do tratamento com Syd-1 na resposta inflamatória tumoral;
- Avaliar se o tratamento com Syd-1 diante do tumor altera parâmetros hematológicos e bioquímicos do plasma.

3. ARTIGO CIENTÍFICO

Sydnone 1: a mesoionic compound with antitumoral and hematological effects in rats bearing the Walker-256 tumor

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Running title: Syd-1 effects in Walker-256 tumor

Abstract

The present study evaluated the antitumor activity of the mesoionic compound sydnone 1 (Syd-1) against Walker-256 carcinosarcoma. Tumor cells were subcutaneously inoculated in the hind limb in male Wistar rats. The animals were orally treated for 12 days with Syd-1 (75 mg•kg^{-1}) or vehicle. At the end of treatment, considerable decreases in tumor volume (54%) and tumor weight (41%) were observed in treated animals. Samples of these tumors presented increases in apoptotic bodies and pro-apoptotic protein expression and a decrease in reduced glutathione levels. However, significant splenomegaly was evident in animals that received Syd-1, most likely attributable to the induction of hemolysis.

Keywords: Mesoionic compound, antineoplastic, splenomegaly, Sydnone 1, carcinosarcoma, tumor Walker-256.

3.1 Introduction

Cancer denotes the uncontrolled (i.e., malignant) growth of cells that have the ability to invade tissues and organs (McGavin and Zachary, 2009). By dividing rapidly, tumor cells tend to be very aggressive, determining the formation of tumors and malignancies that are responsible for ~14.6% of all human deaths (Pavlopoulou *et al.,* 2014). The estimated prevalence of cancer cases has increased annually. According to the World Health Organization for the year 2030 are expected about 27 million cases of cancer worldwide (Rodrigues and Amaral, 2012).

Chemotherapy is the treatment of choice for several kinds of cancer. However, chemotherapeutic drugs are highly cytotoxic and have a low therapeutic index, several side effects, and low selectivity for tumor cells. These characteristics restrict their use (Chen and Hu, 2009). Thus, the need for new agents with antitumor activity with fewer side effects has arisen to improve the quality of life of cancer patients who undergo treatment.

Mesoionic compounds belong to a group of heterocyclic compounds that are noted for their possible therapeutic actions (Athayde-Filho *et al.*, 2000). Their biological activities are related to their chemical structures, represented by betainic and planar characteristics, relatively small molecular size, and variations in positive electron density in the ring and negative charge density that extends to the exocyclic side chain, which afford amphiphilic properties (Athayde-Filho *et al.*, 2000). Of the mesoionic compounds, 1,2,3-oxadiazole-5-olates, also called sydnones, has been the most extensively studied (Baker and Ollis, 1957). Sydnones have numerous pharmacological activities, including antibacterial, antiinflammatory, analgesic, and antitumor effects (Moustafa *et al.,* 2004; Bizetto *et al.,* 2012; Kalluraya *et al.,* 2000; Grynberg *et al.,* 1992). In addition to the pharmacological activities that have been attributed to these compounds, sydnones also have great importance because they act as precursors of bioactive molecules (Shih *et al*., 2007).

The antitumor activity of sydnone 1 (Syd-1) has been reported in mice with Ehrlich carcinoma, sarcoma 180, and fibrous histiocytoma (B10MCII), but no specific mechanism of action has been elucidated (Grynberg *et al.,* 1992). The antitumor activity of Syd-1 may be related to alterations in energy metabolism in mitochondria (Halila *et al.,* 2007). Data on the *in vivo* use of Syd-1 against tumors are scarce. The present study investigated the possible antineoplastic, antioxidant, antiinflammatory, and toxicological effects of Syd-1 in rats with Walker-256 tumors. Walker-256 is a fast-growing carcinosarcoma, widely used because it is species-specific for rat, easily transplantable and is considerate an appropriate model for the study of cachexia syndrome and oxidative state induced by cancer (Acco *et al.,* 2012).

3.2 Material and Methods

3.2.1 Material

The mesoionic compound Syd-1 was synthesized in the Department of Chemistry, Federal Rural University of Rio de Janeiro. ^{1}H and ^{13}C NMR, and mass spectroscopy (Grynberg *et al.,* 1992) confirmed the compound structure.

3.2.2 Animals

Male rats (*Ratus norvegicus*, Wistar strain), weighing 180-220 g, were obtained from the vivarium of Federal University of Parana (Curitiba, Brazil) and used in the experiments. The animals were maintained under controlled conditions at 22°C with a 12 h/12 h light/dark cycle and free access to food and water. All of the protocols were performed in accordance with ethics in animal experimentation and approved by the local committee (no. 765).

3.2.3 Tumor Walker-256 inoculation and treatment of groups

Walker-256 cells were maintained by weekly passages in rats that were intraperitoneally inoculated with 1×10^7 cells/animal in 1 ml of phosphate-buffered saline (PBS; 16.5 mM phosphate, 137 mM NaCl, and 2.7 mM KCl) (Vicentino *et al.,* 2002). After 4-5 intraperitoneal passages and verifying their viability by the trypan blue exclusion method in a Neubauer chamber, carcinosarcoma cells were injected subcutaneously (1×10^7 cells per rat in 0.4 ml of PBS) in the right pelvic limb. Oral (gavage) treatment began the day after tumor cell inoculation and once per day thereafter. The following groups were formed: (*i*) Basal (non-tumor-bearing rats that received vehicle [20% Tween + saline], *n* = 12), (*ii*) Basal-Syd (non-tumor-bearing rats that received Syd-1, *n* = 5), (*iii*) Tumor (tumor-bearing rats that received vehicle [20% Tween + Saline], *n* = 14), and (*iv*) Tumor-Syd (tumor-bearing rats that received Syd-1,

n = 10). The dose of Syd-1 (75 mg•kg^{-1}) was based on Grynberg *et al.* (1992). After 12 days of treatment, the animals were anesthetized with 80 mg•kg^{-1} ketamine (Quetamina, Vetnil Industry and Trade of Veterinary Products LTDA, Louveira, Brazil) and 10 mg•kg^{-1} xylazine (Syntec of Brazil LTDA, Cotia, Brazil) by unique intraperitoneal injection. Blood was collected from the inferior cava vein for hematologic and plasma biochemical analyses. The liver was subsequently harvested. The animals were then euthanized by puncture of the diaphragm, and the tumor and other organs were harvested and weighed. Immediately after collection of the tumor, all of the biophysical parameters were measured as described in section 3.2.4 below. The tissue samples were stored at -80°C, and plasma was stored at -20°C for further analyses.

3.2.4 Mensuration of biophysical parameters

The tumor volume was assessed daily and calculated according to Mizuno *et al.* (1999) as V(cm^3) = $4\pi/3 \bullet a^2 \bullet (b/2)$, where *a* is the smallest tumor diameter and *b* is the largest tumor diameter (in centimeters). The inhibitory effect on the tumor was calculated using the following formula: Tumor suppression (%) = (1 – T/C), where *T* is the average tumor volume in the treated group, and *C* is the average tumor volume in the control group. The tumor weight was also recorded at the end of treatment. During the experiment, the animals' body weights were recorded every 3 days.

3.2.5 Biochemical and hematological assays

Plasma samples were obtained after blood centrifugation at 4000 rotations per minute (rpm) for 5 min. The samples were used to determine urea, creatinine, glucose, total protein, albumin, globulin, alkaline phosphatase, alanine aminotransferase (ALT), and aspartate aminotransferase (AST) levels using commercial kits (Labtest Diagnostica, Lagoa Santa, Brazil) and a Mindray BS-200 automated device. Total blood was also used to generate complete hemograms using a BC2800-Vet automated device.

3.2.6 Oxidative stress parameters

To determine the influence of Syd-1 on oxidative stress in tumor and hepatic tissue, the following parameters were measured.

3.2.6.1 Catalase (CAT)

Samples of the tumor and liver (0.3 g of tissue) were homogenized in potassium phosphate buffer (pH 6.5) and then centrifuged at 9700 rpm for 20 min at 4°C in a 1:10 dilution. The supernatant was collected and another 1:10 dilution was performed. The activity of catalase was quantified according to Aebi (1984). The reaction was performed using hydrogen peroxide (Tris EDTA buffer [pH 8.0], MilliQ water, and 30% hydrogen peroxide). Readings were performed in a 96-well microplate at 240 nm. Enzymatic activity was expressed as mmol•min^{-1}•mg of $protein^{-1}$.

3.2.6.2 Superoxide Dismutase (SOD)

Samples of the tumor and liver were homogenized in potassium phosphate buffer (pH 6.5) and then centrifuged at 9700 rpm for 20 min at 4°C in a 1:10 dilution. The supernatant was collected and another 1:10 dilution was performed. Superoxide dismutase (SOD) activity was measured according to the ability of SOD in the tissue to inhibit the autoxidation of pyrogallol reagent (Gao *et al*., 1998). To this end, 60 µl of each sample was added to 1327.5 µl of Tris-EDTA (0.4 M, pH 8.9), vortexed, and added to 75 µl of a solution that contained 15 mM pyrogallol. After 30 min incubation at ambient temperature, the reaction was stopped with 37.5 µl of 1N HCl solution. Readings were performed in a 96-well microplate reader at 440 nm. The amount of enzyme that inhibited the reaction by 50% (IC_{50}) was defined as one unit of SOD, and the enzymatic activity of SOD was expressed in U SOD•mg of $protein^{-1}$.

3.2.6.3 Glutathione-S-transferase (GST)

This analysis was performed only in the liver because tumor tissue does not express significant amounts of glutathione-*S*-transferase (GST). Samples of the liver were homogenized in potassium phosphate buffer (pH 6.5) and then centrifuged at 9700 rpm for 20 min at 4°C in a 1:10 dilution. The supernatant was collected and a 1:30 dilution was performed. Glutathione-*S*-transferase catalyzes the conjugation of the substrate 1-chloro-2,4-dinitrobenzene (CDNB) to reduced glutathione (GSH), forming a thioether that can be monitored by increasing absorbance according to the method of Habig *et al.* (1974). Thus, 200 µl of reagent solution that contained 3 mM CDNB (diluted in ethanol PA) and 3 mM GSH (diluted in potassium phosphate buffer) was added to 100 µl of the supernatant. The linear increase in absorbance was measured in a 96-well microplate at a wavelength of 340 nm, and enzymatic activity was expressed as mmol•minute^{-1}•mg of protein^{-1}.

3.2.6.4 Quantification of proteins

Samples of the tumor and liver were homogenized in potassium phosphate buffer (pH 6.5) and then centrifuged at 9700 rpm for 20 min at 4°C in a 1:10 dilution. The supernatant was collected and another 1:10 dilution was performed. The quantification of tissue proteins (liver and tumor) was performed according to Bradford (1976). Readings were performed in a 96-well microplate reader. In each well, 10 µl of the sample was added and reacted with 250 µl of the Bradford solution. Readings were performed in a 96-well microplate at 595 nm.

3.2.6.5 Reduced glutathione (GSH)

Reduced glutathione levels were measured according to the method described for Sedlak and Lindsay (1968). Samples of the tumor and liver were homogenized in potassium phosphate buffer (pH 6.5) in a 1:10 dilution. Afterward, 100 µl of the homogenate was separated and mixed with 80 µl of 12.5% trichloroacetic acid (ATC). The samples were then centrifuged at 6000 rpm for 15 min at 4°C. In a 96-well microplate, 20 µl of the supernatant was added and mixed with 280 µl of Tris HCl and 5 µl of 5,5-dithiobis-(2-nitrobenzoic acid) (DTNB) in methanol. The absorbance of the reaction solution was measured at 415 nm in a 96-well microplate reader using GSH as the external standard. The results were expressed as mg GSH•g of tissue^{-1}.

3.2.6.6 Determination of rate of the lipid peroxidation (LPO)

The rate of lipid peroxidation (LPO) was measured according to the FOX method or using xylenol orange as described by Jiang *et al.* (1991). This method quantifies the formation of hydroperoxides during LPO. Samples of the tumor and liver were homogenized in potassium phosphate buffered (pH 6.5) and then centrifuged at 9700 rpm for 20 min at 4ºC in a 1:10 dilution. The supernatant was resuspended in methanol (1:1) and again centrifuged at 5000 x g for 5 min at 4ºC. Afterward, 100 µl of the supernatant was added to 900 µl of FOX-2 solution. The samples were then incubated for 30 min at room temperature and analyzed spectrophotometrically in a 96-well microplate at 560 nm. The results were expressed as mmol hydroperoxides•mg of protein^{-1}.

3.2.7 Inflammatory parameters in tumor

3.2.7.1 Mieloperoxidase (MPO)

The amount of neutrophils in the tumor samples was measured by assaying myeloperoxidase (MPO) activity according to the method described by Bradley *et al.* (1982). Tumor samples (200 mg) were homogenized in 0.1% saline Triton-X and

centrifuged at 10000 x g at 4°C for 30 min. In a 96-well microplate, 30 µl of the supernatant was added to 200 µl of a 0.017% hydrogen peroxide solution in 80 mM PBS. The reaction was initiated by adding 20 µl of TMB (18.4 mM tetramethylbenzidine in dimethylformamide). The plate was then incubated for 3 min at 37°C and put on ice after incubation. The reaction was stopped with 30 µl of sodium acetate (1.46 M, pH = 3.0). The linear increase in absorbance was measured at a wavelength of 620 nm, and enzymatic activity was expressed as optical density (OD)•mg of tissue^{-1}.

3.2.7.2 N-acethyl-β-D-glucosaminidase (NAG)

The infiltration of mononuclear cells into the tumor was quantified by measuring the levels of the lysosomal enzyme *N*-acetyl-β-D-glucosaminidase (NAG) that is present in high levels in activated macrophages (Lloret and Moreno, 1995). Samples of the tumor were initially processed as described in 3.2.7.1 section. From the supernatant, 100 µl was transferred in duplicate to a 96-well microplate with 100 µl of p-nitrophenyl-2-acetamide-β-D-glucopyranoside (2.24 mM) in citrate buffer (39 mM, pH 4.5). The samples were incubated for 60 min at 37°C. The reaction was interrupted with the addition of 100 µl of glycine buffer (200 mM, pH 10.4). The linear increase in absorbance was measured at a wavelength of 405 nm, and enzymatic activity was expressed as mmol•mg of tissue^{-1}.

3.2.7.3 Tumor necrosis factor-alpha (TNF-α)

Two hundred micrograms of tumor samples were homogeinized in Drabkin reagent and centrifuged at 10000 x g for 45 min. The supernatants were filtered through a 25-µm pore membrane. TNF-α in the supernatant was measured using the enzyme-linked immunosorbent assay (ELISA) method according to the manufacturer's instructions

(R&D System, Minneapolis, USA). Briefly, 100 µl of the samples were added in duplicate to a 96-well plate coated with a specific murine monoclonal antibody against the cytokine, followed by the addition of a second horseradish peroxidase-conjugated polyclonal against the cytokine. After washing the plate, a substrate solution (50 µl of 1:1 hydrogen peroxide and tetramethylbenzidine 10 mg•ml^{-1} in DMSO) was added to the wells. The color development was interrupted by adding 50 µl of 2 M sulphuric acid. The absorbance of the reaction solution was measured at 450 nm in a 96-well microplate reader using recombinant rat TNF-α as external standard. The results were expressed as picograms•mg of tissue^{-1}.

3.2.7.4 Nitric oxide (NO)

The Griess reaction was described by Green *et al.* (1982) and infers the amount of nitric oxide (NO) in tissue. The filtered supernatant (100 µl) of tumor samples in Drabkin was added in duplicate to a 96-well microplate followed by the addition of 100 µl of reaction solution (100 µl of a 1:1 sulfanilamide and naphthylenediamine in 2.5% phosphoric acid). A standard curve was built with linear concentration of sodium nitrate. Absorbance was measured at a wavelength of 540 nm, and the results were expressed as µmol nitrite•mg of tissue^{-1}.

3.2.8 Toxicological assays

As toxicological parameters, the liver, spleen, kidneys, and lungs were weighed to determine whether Syd-1 can macroscopically modify (increase or decrease) these organs.

3.2.9 Liver, Tumor and Spleen Histology

The tissues were quickly harvested after euthanasia, stored in 10% buffered formalin for fixation, and stained with hematoxylin and eosin (HE) and Prussian blue for the spleen. The slices were analyzed using an optical microscope (Leica DM2500) to evaluate cellular changes, cell death, and cell necrosis that resulted from the tumor and treatment. The histological alterations of the liver and spleen were scored as (--) negative, (+) mild, moderate (++) and (+++) pronounced. The histology of the tumor was characterized as degrees of necrosis using the Huvos index (Huvos *et al.*, 1977). Grade I indicates 10-50% necrosis in the examined area, Grade II indicates > 50% necrosis, Grade III indicates > 90% necrosis, and Grade IV indicates 100% necrosis.

3.2.10 Gene Expression

To extract RNA, tumor tissue samples were homogenized in TRIzol reagent (Life Technologies, Carlsbad, California, USA). Complementary DNA was prepared from 500 ng RNA, in a 20 µl reaction volume containing 0,5 mM deoxyribonucleotide triphosphate, 1 µM oligodT, 10 U RNAsin (Promega, Madison, WI, USA), and a high-capacity polymerase enzyme kit (Life Technologies, Carlsbad, California, USA). The negative control consisted of adding all the products needed for cDNA synthesis except the reverse transcriptase.

Quantitative polymerase chain reaction (qPCR) was performed using a Step One Plus thermocycler (Life Technologies, Carlsbad, CA, USA) with the 1x Syber Green PCR Master Mix Kit (Applied Biosystems, by Life Technologies, Carlsbad, CA, USA). The primers were prepared (Invitrogen, Breda, The Netherlands) with the following sequences: internal control (600 nM) GAPDH (forward: 5'-AAGGACCCCTTCATTGAC-3'; reverse: 5'-TCCACGACATACTCAGCAC-3'), and the apoptotic markers (200 nM) Bcl-2 (forward: 5'-GACTGAGTACCTGAACCGGC-3'; reverse: 5'-AGTTCCACAAAGGCATCCAG-3'), Bax (forward: 5'-AAACTGGTGCTCAAGGCCC-3';

reverse: 5’-GGGTCGCGAAGTAGGAAAGG-3’), and p53 (forward: 5’-AGCGACTACAGTTAGGGGGTA-3’; reverse: 5’-ACAGTTATCCAGTCTTCAGGGG-3’).

3.2.11 Statistical Analysis

All values are presented as mean ± standard error of mean (SEM). Differences between averages were determined by one-way analysis of variance (ANOVA) followed by the Bonferroni *post hoc* test, or two-way ANOVA followed by the Bonferroni test when comparing body weight and tumor volume. For comparisons between two groups, Student’s *t*-test was used. All of the analyses were performed using Prism 5.0 software for Windows (GraphPad, San Diego, CA, USA). Differences were considered significant at $p < 0.05$.

3.3 Results

3.3.1 Measurement of biophysical parameters

Tumor volume was measured daily from day 6 (when the tumor became palpable) until day 13. The group that received Syd-1 treatment presented a 54% reduction of tumor volume (Fig. 1A) and 41% reduction of tumor weight (Fig. 1B) compared with the control group. No significant difference in body weight was observed between groups (Fig. 1C).

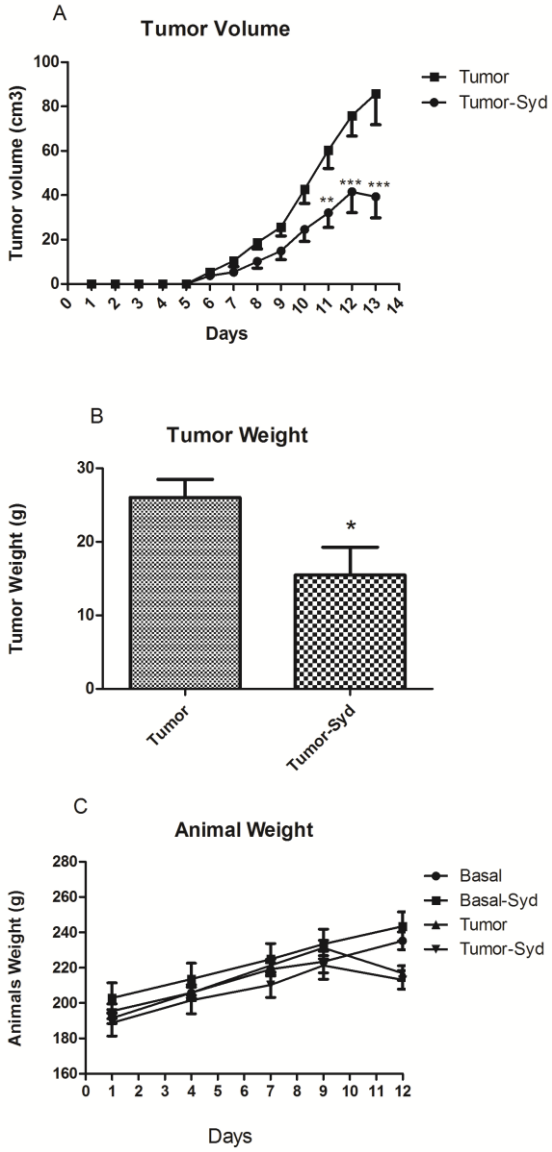

Figure 1. Tumor volume (A), tumor weight (B), and body weight (C) in healthy (Basal) and tumor-bearing (Tumor) rats that were treated with vehicle or sydnone 1 (Syd-1) orally for 12 days. Tumor, tumor group that received only vehicle (n = 14); Tumor-Syd, tumor group that received Syd-1 treatment (75 mg•kg^{-1}, n = 10); Basal, healthy animals that received vehicle (n = 12); Basal-Syd, healthy animals that received Syd-1 (75 mg•kg^{-1}, n = 5). The results were expressed as mean ± SEM. Statistical comparisons were performed using two-way ANOVA for (A) and (C) or Student's t-test for (B). *p < 0.05, **p < 0.01, ***p < 0.001.

3.3.2 Biochemical and hematological assays

3.3.2.1 Biochemical assays

To assess the influence of the tumor or treatment on metabolism and organ function, several biochemical parameters were analyzed. Decreases in glucose and

amylase levels were observed in both tumor groups. Decreases in alkaline phosphatase activity, total protein, and albumin levels were also observed, with an increase in AST levels in the Tumor group (Table 1).

Table 1. Plasmatic parameters in healthy and tumor-bearing rats that were treated with vehicle or sydnone 1 (Syd-1; 75 mg•kg^{-1}) orally for 12 days.

Parameter	Experimental group			
	Basal	**Basal-Syd**	**Tumor**	**Tumor-Syd**
Amylase (U/L)	700.6 ± 36.96	794.4 ± 24.69	397.6 ± 44.5*°	479.0 ± 20.80*°
Glucose (mg/dl)	126.4 ± 5.64	106.7 ± 13.72	85.41 ± 6.30*	90.71 ± 9.53*
Urea (mg/dl)	56.38 ± 1.64	58.46 ± 2.90	57.58 ± 1.47	56.20 ± 1.99
Creatinine (mg/dl)	0.70 ± 0.02	0.74 ± 0.02	0.66 ± 0.02	0.60 ± 0.07
Total protein (g/dl)	6.05 ± 0.13	6.46 ± 0.02	5.62 ± 0.09 °	5.96 ± 0.14
Albumin (g/dl)	3.39 ± 0.08	3.64 ± 0.02	3.15 ± 0.04*°	3.33 ± 0.05
Globulin (g/dl)	2.66 ± 0.06	2.82 ± 0.03	2.47 ± 0.07	2.68 ± 0.08
Alkaline phosphatase (U/L)	205.0 ± 18.81	220.6 ± 11.54	149.3 ± 9.39*°	161.3 ± 7.61
AST (U/L)	109.3 ± 11.55	92.9 ± 5.38	219.7 ± 23.60*°	158.4 ± 19.40
ALT (U/L)	57.08 ± 6.454	57.76 ± 6.405	46.80 ± 2.12	43.19 ± 3.50

The results were expressed as means ± SEM. Statistical comparisons were performed using one-way ANOVA followed by Bonferroni test. Tumor, tumor group that received only vehicle (n = 14); Tumor-Syd, tumor group that received Syd-1 treatment (75 mg•kg^{-1}, n = 10); Basal, healthy animals that received vehicle (n = 12); Basal-Syd, healthy animals that received Syd-1 (75 mg•kg^{-1}, n = 5). * $p < 0.05$ compared to Basal group. ° $p < 0.05$ compared to Basal-Syd group.

3.3.2.2 Hematological assays

Hematologic parameters (Table 2) revealed decreased platelet and pro-calcitonin in Tumor group. Both groups that were treated with Syd-1 exhibited increases in mean corpuscular volume, mean corpuscular hemoglobin, red cell distribution width and a decrease in erythrocytes compared with the basal and control groups. In the Tumor-

Syd group, decreases in hemoglobin, hematocrit and mean corpuscular hemoglobin concentration were observed.

Table 2. Hematological parameters in healthy and tumor-bearing rats that were treated with vehicle or sydnone 1 (Syd-1; 75 mg•kg^{-1}) orally for 12 days.

Parameter	Experimental group			
	Basal	**Basal-Syd**	**Tumor**	**Tumor-Syd**
Erythrocytes (x 10^6/µl)	8.74 ± 0.09	6.10 ± 0.22 *#	8.51 ± 0.14	6.27 ± 0.48*#
Hemoglobin (g/dl)	14.11 ± 0.19	13.40 ± 0.08	13.48 ± 0.26	12.41 ± 0.57*
Hematocrit (%)	44.18 ± 0.66	43.58 ± 0.51	41.59 ± 0.79	40.35 ± 1.27*
Mean corpuscular volume (fl)	50.55 ± 0.44	71.90 ± 2.88*#	48.73 ± 0.29	67.55 ± 3.51*$^{\#}$
Mean corpuscular hemoglobin (pg)	16.16 ± 0.08	22.04 ± 0.66*#	15.70 ± 0.11	20.49 ± 0.79*$^{\#}$
Mean corpuscular hemoglobin concentration (g/dl)	32.06 ± 0.24	30.70 ± 0.35	32.23 ± 0.14	30.58 ± 0.52*$^{\#}$
Red cell distribution width (%)	11.89 ± 0.45	15.70 ± 0.30*#	12.35 ± 0.38	17.24 ± 0.67*$^{\#}$
Platelets (× 10³/µl)	493.2 ± 33.23	469.2 ± 22.86	271.6 ± 25.14*°^{Ø}	405.8 ± 47.74
Mean platelet volume (fl)	6.71 ± 0.14	6.74 ± 0.12	6.50 ± 0.09	6.68 ± 0.11
Platelet distribution index (%)	15.62 ± 0.14	15.70 ± 0.08	15.29 ± 0.07	15.68 ± 0.13
Pro-calcitonin (%)	0.33 ± 0.02	0.31 ± 0.02	0.17 ± 0.016*°^{Ø}	0.29 ± 0.025

The results were expressed as means ± SEM. Statistical comparisons were performed using one-way ANOVA followed by Bonferroni test. Tumor, tumor group that received only vehicle (n = 14); Tumor-Syd, tumor group that received Syd-1 treatment (75 mg•kg^{-1}, n = 10); Basal, healthy animals that received vehicle (n = 12); Basal-Syd, healthy animals that received Syd-1 (75 mg•kg^{-1}, n = 5). * p < 0.05 compared to Basal group. $^{\#}$ p < 0.05 compared to Tumor group. ° p < 0.05 compared to Basal-Syd group. ^{Ø} p < 0.05 compared to Tumor-Syd group.

3.3.3 Oxidative stress parameters

Considering that oxidative stress is a consequence of the presence of tumors, enzymatic and non-enzymatic parameters of oxidative stress were assayed in tumor and liver tissue. Treatment with Syd-1 decreased the levels of GSH in tumor tissue by

approximately 51% (Fig. 2A). In contrast, Syd-1 increased the activity of GSH in hepatic tissue by approximately 29% in the Basal group but had no effect in tumor-bearing rats (Fig. 2B). With regard to the other oxidative stress parameters, such as GST, catalase, SOD, and LPO, no significant differences were observed in any of the analyzed tissues (data not shown).

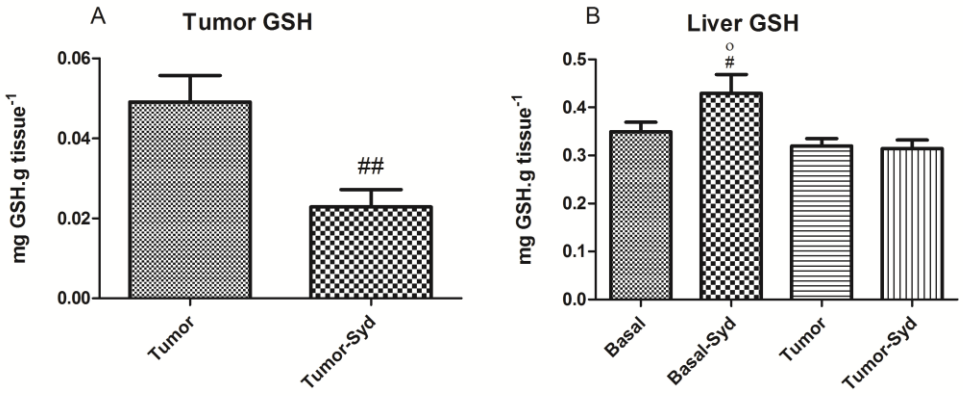

Figure 2. Parameters of oxidative stress in healthy (Basal) and tumor-bearing (Tumor) rats that were treated with vehicle or sydnone 1 (Syd-1) orally for 12 days: tumor GSH (A) and liver GSH (B). Tumor, tumor group that received only vehicle (n = 14); Tumor-Syd, tumor group that received Syd-1 treatment (75 mg•kg^{-1}, n = 10); Basal, healthy animals that received vehicle (n = 12); Basal-Syd, healthy animals that received Syd-1 (75 mg•kg^{-1}, n = 5). The results were expressed as mean ± SEM. Statistical comparisons of liver were performed by the one-way ANOVA followed by the Bonferroni test, while Student's t-test was used for analysis of tumor. # $p < 0.05$, ## $p < 0.01$ when compared with Tumor group. °$p < 0.05$ when compared with Tumor-Syd group.

3.3.4 Inflammatory parameters

Inflammation is a part of tumor development, and the influence of Syd-1 on inflammatory parameters was assessed. The enzymes MPO and NAG, NO, and the cytokine tumor necrosis factor α (TNF-α) did not present differences in tumor tissue between groups (data not shown). Thus, no antiinflammatory activity of Syd-1 was observed.

3.3.5 Toxicological assays

No changes in the weight of the liver (Fig. 3A), kidneys (Fig. 3C), or lungs (Fig. 3D) were observed. However, significant splenomegaly was observed in both groups that received Syd-1 (Fig. 3B), which increased by approximately 250% compared with the Basal group. This unexpected organ alteration led us to further investigate the possible mechanisms that caused splenomegaly.

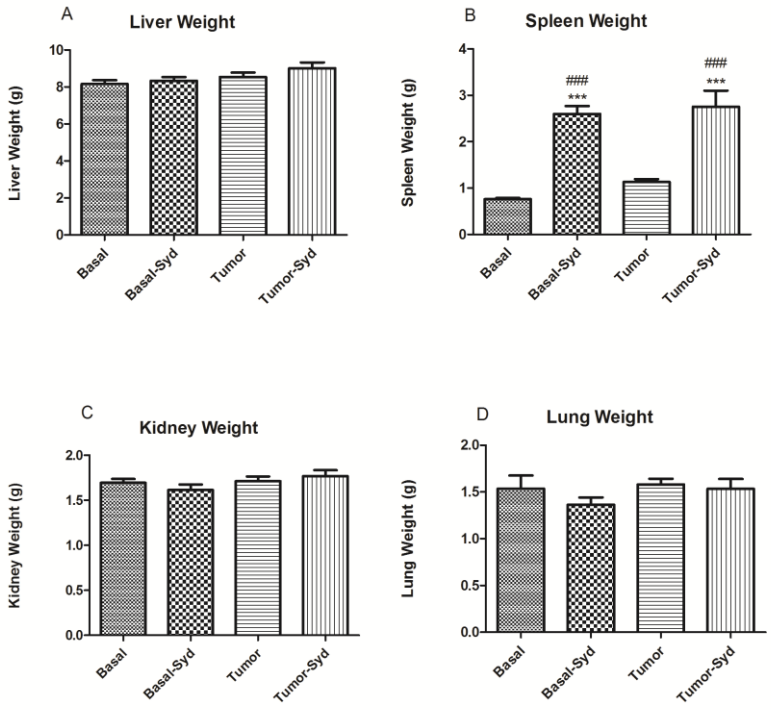

Figure 3. Weight of liver (A) spleen (B), kidney (C) and lung (D) in healthy (Basal) and tumor-bearing (Tumor) rats that were treated with vehicle or sydnone 1 (Syd-1) orally for 12 days. Tumor, tumor group that received only vehicle (n = 14); Tumor-Syd, tumor group that received Syd-1 treatment (75 mg•kg^{-1}, n = 10); Basal, healthy animals that received vehicle (n = 12); Basal-Syd, healthy animals that received Syd-1 (75 mg•kg^{-1}, n = 5). The results were expressed as mean ± SEM. Statistical comparisons were performed by the one-way ANOVA followed by the Bonferroni test. ### p < 0.001 compared to Tumor group. *** p < 0.001 compared to Basal group.

3.3.6 Spleen, liver, and tumor Histology

3.3.6.1 Spleen histology

Spleen histology was performed to better understand the splenomegaly that was observed macroscopically. In the Tumor group, hyperplasia of the splenic marginal zone was detected, including the presence of macrophages with brown pigmentation in

their cytoplasm in the red pulp (Fig. 4E, Table 3). However, the Tumor-Syd group also exhibited hyperplasia of the marginal zone, and the presence of macrophages was considerably greater than in the Tumor group (Fig. 4G). Macrophages with brown pigmentation were also observed in large quantities in the Basal-Syd group (Fig. 4C). To determine whether the pigment was iron, indicating possible hemolysis in the spleen, Prussian blue staining was performed. The results were positive, with a considerably higher level of macrophages with iron in the cytoplasm in the groups that were treated with Syd-1 compared with both the Tumor and Basal groups (Fig. 4B, D, F, H). No histological changes in the spleen were observed in the Basal group compared with the other groups (Fig. 4A, B).

Table 3. Histological parameters of the spleen in healthy rats that were treated with vehicle (Basal group) or sydnone-1 (Basal-Syd group) and tumor-bearing rats that were treated with vehicle (Tumor group) or sydnone 1 (Tumor-Syd group) orally for 12 days.

Optical Microscopy of the Spleen	**Basal**	**Basal-Syd**	**Tumor**	**Tumor-Syd**
White pulp: hyperplasia of marginal zone	--	--	+++	++
Red pulp: presence of iron	+	+++	+	+++

Groups: Tumor, tumor group that received only vehicle; Tumor-Syd, tumor group that received Syd-1 treatment (75 mg•kg^{-1}); Basal, healthy animals that received vehicle; Basal-Syd, healthy animals that received Syd-1 (75 mg•kg^{-1}). Scores: (--) negative, (+) mild, (++) moderate and (+++) pronounced

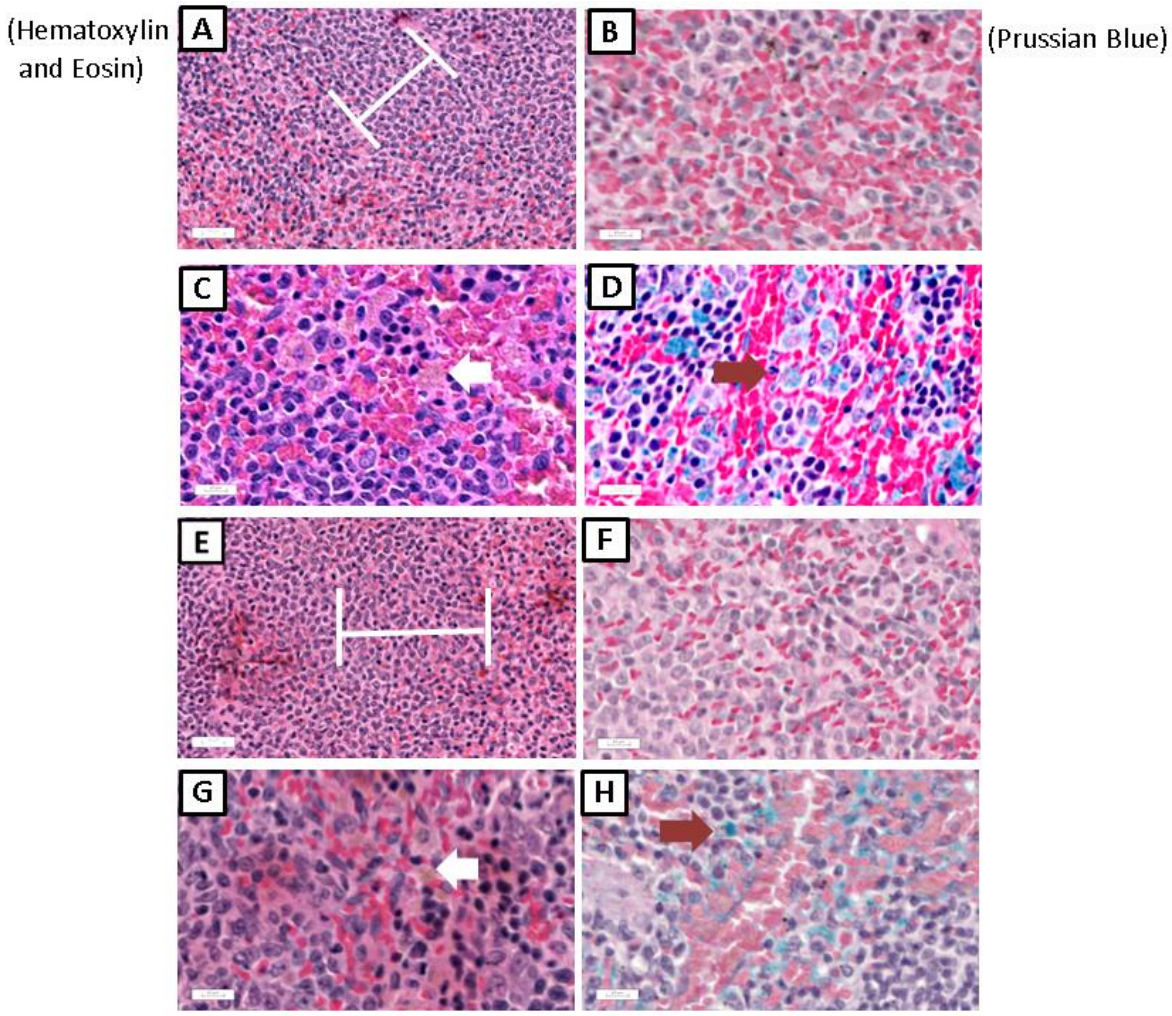

Figure 4. Histological parameters, stained with hematoxylin/eosin or prussian blue, in splenic tissue of rats after 12 days of treatment with vehicle and Syd-1. Basal (A, B), Basal-Syd (C, D), Tumor (E, F) and Tumor-Syd (G, H). Panel E notices the presence of the marginal zone hyperplasia when compared to the panel A, indicated by white line. In panel C and G is possible to observe the presence of macrophages with brown pigments dispersed in the red pulp, indicated by the white arrow. The red arrow in panel D and H points to the significant presence of blue dye when compared to the other groups, indicating expressive presence of iron in the red pulp of the spleen of animals treated with Syd-1. Legend: Basal, healthy animals; Basal-Syd, healthy animals, that received the compound Syd (75 mg•kg^{-1}); Tumor, tumor group that received only vehicle and Tumor-Syd tumor group that received treatment with Syd-1 (75 mg•kg^{-1}). Scale bar = 20 µm and 10 µm.

3.3.6.2 Liver histology

No significant histological findings were observed in the liver in the Basal group or Basal-Syd group (Table 4). In the Tumor group, a greater intensity of lymphocytic infiltration with lobular necrosis was found (Fig. 5E, F), including portal lymphocytic infiltration and hyperplasia of Kupffer cells, which resembled the other groups. In the

Tumor-Syd-1 group, these alterations were also observed in small amounts (Fig. 5G, H). Thus, these findings do not characterize Syd-1 as a hepatotoxic compound.

Table 4. Histological parameters of the liver in healthy rats that were treated with vehicle (Basal group) or sydnone-1 (Basal-Syd group) and tumor-bearing rats that were treated with vehicle (Tumor group) or sydnone 1 (Tumor-Syd group) orally for 12 days.

Optical Microscopy of the Liver	**Basal**	**Basal-Syd**	**Tumor**	**Tumor-Syd**
Lobular lymphocytic infiltrate with necrosis	--	--	+	+
Lobular lymphocytic infiltrate without necrosis	+	+	--	+
Portal lymphocytic infiltrate	+	--	++	--
Kupffer cell hyperplasia	+	+	+	+
Periportal steatosis	--	--	--	+

Groups: Tumor, tumor group that received only vehicle; Tumor-Syd, tumor group that received Syd-1 treatment (75 $mg \bullet kg^{-1}$); Basal, healthy animals that received vehicle; Basal-Syd, healthy animals that received Syd-1 (75 $mg \bullet kg^{-1}$). Scores: (--) negative, mild (+) and moderate (++).

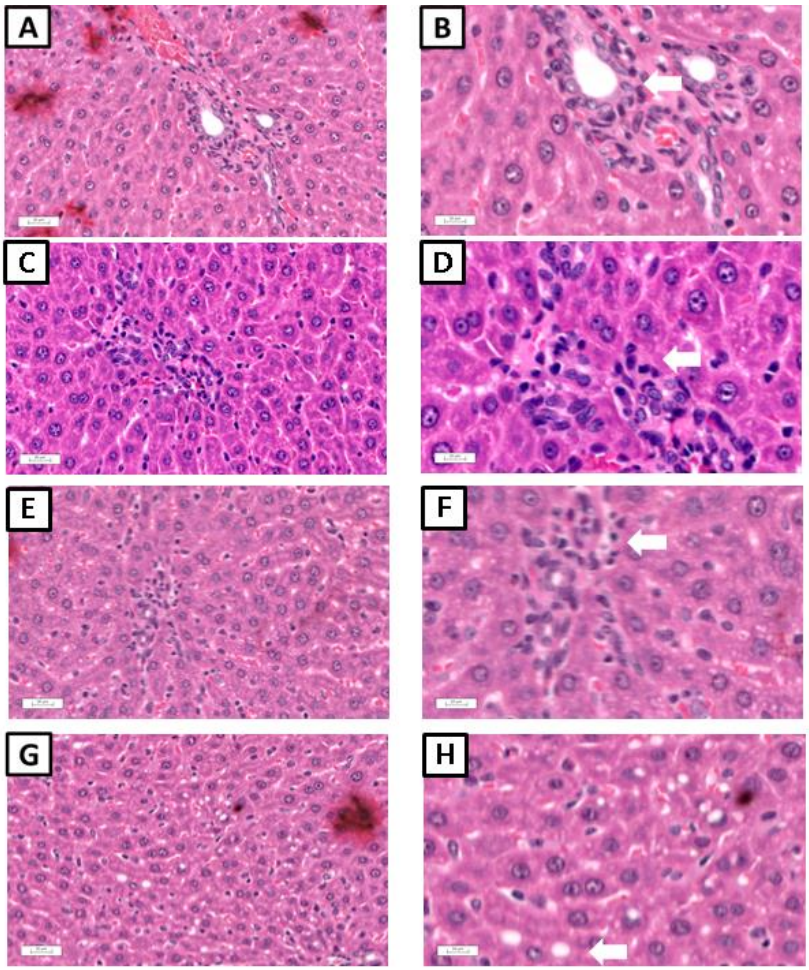

Figure 5. Histological parameters evaluated in the hepatic tissue of rats after 12 days of treatment with vehicle or Syd-1. Basal (A, B), Basal-Syd (C, D) Tumor (E, F), and Tumor-Syd (G, H). The presence of a lymphocytic infiltrate in panel B, D and F was observed as well as the presence of steatosis in panel H. Tumor, tumor group that received only vehicle; Tumor-Syd, tumor group that received Syd-1 treatment (75 mg•kg^{-1}); Basal, healthy animals that received vehicle; Basal-Syd, healthy animals that received Syd-1 (75 mg•kg^{-1}). Scale bar = 20 μm and 10 μm.

3.3.6.3 Tumor histology

The Tumor group exhibited more coagulative necrosis than apoptosis (Fig. 6A, B). The Huvos index (grades I, II, and III) remained similar to the group that was treated with Syd-1. In tumor samples from animals that received Syd-1, coagulative necrosis and apoptotic foci were observed. Additionally, in some slices, apoptosis was more intense than necrosis (Fig. 6C, D).

Table 5. Histological parameters and Huvos index of tumors in rats that were treated with vehicle (Tumor group) or sydnone 1 (Tumor-Syd group) orally for 12 days.

Optical Microscopy of the Tumor	Tumor	Tumor-Syd
Apoptosis	+	++
Grade I (10-50% coagulative necrosis)	+	+
Grade II (> 50% coagulative necrosis)	++	+
Grade III (> 90% coagulative necrosis)	++	++
Grade IV (100% coagulative necrosis)	--	--

Groups: Tumor, tumor group that received only vehicle; Tumor-Syd, tumor group that received Syd-1 treatment (75 mg•kg^{-1}); Basal, healthy animals that received vehicle; Basal-Syd, healthy animals that received Syd-1 (75 mg•kg^{-1}). Scores: (--) negative, mild (+), moderate (++) and pronounced (+++).

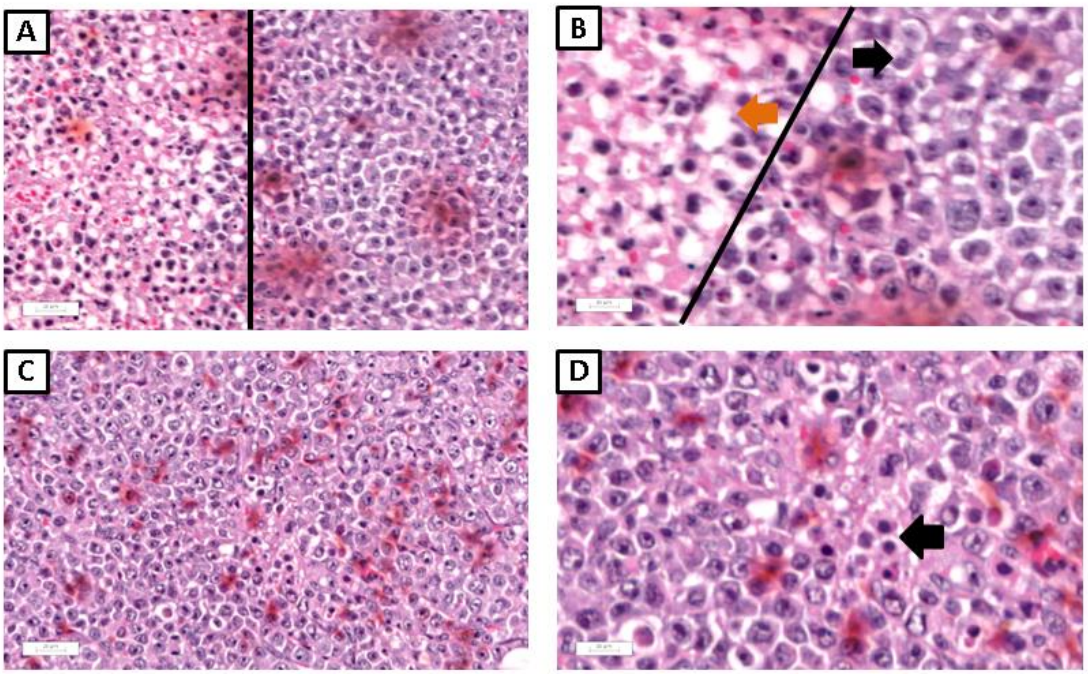

Figure 6. Histological parameters assessed in tumor tissue of rats after 12 days of treatment with vehicle or Syd. Tumor (A, B) and Tumor-Syd (C, D). It can be seen in panel B the presence of viable tumor tissue, indicated by the black arrow, and necrosis indicated by the orange arrow. In the group receiving Syd-1 (panel D) is possible to observe the presence of apoptotic bodies, indicated by the black arrow. Tumor, tumor group that received only vehicle; Tumor-Syd, tumor group that received Syd-1 treatment (75 mg•kg^{-1}); Basal, healthy animals that received vehicle; Basal-Syd, healthy animals that received Syd-1 (75 mg•kg^{-1}). Scale bar = 20 µm and 10 µm.

3.3.7 Gene expression

In the group that was treated with Syd-1, considerable increases in the gene expression of pro-apoptotic proteins, such as Bax and p53, were observed, with a decrease in the gene expression of the anti-apoptotic protein Bcl-2 compared with the control group (Fig. 7). These results indicate that Syd-1 modulated the apoptotic cascade.

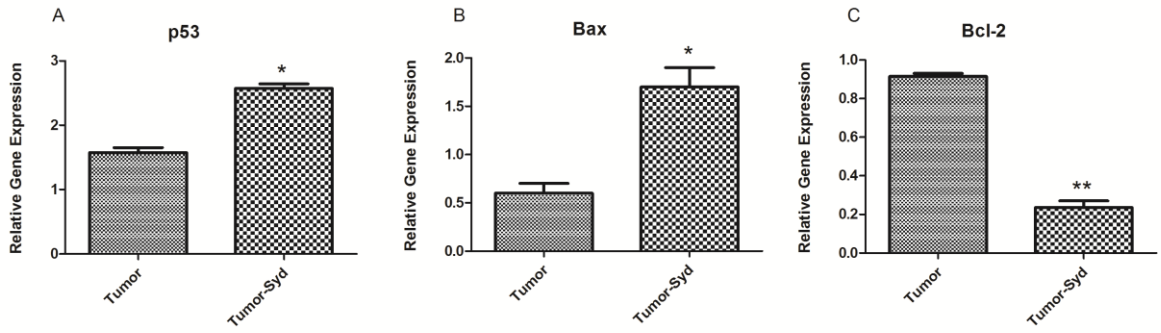

Figure 7. Gene expression of proteins p53 (A), Bax (B) and Bcl-2 protein (C) in tumor tissue of animals treated with vehicle (Tumor group) or Sydnone 1 (Tumor-Syd). Tumor, tumor group that received only vehicle (n = 5); Tumor-Syd, tumor group that received Syd-1 treatment (75 mg•kg^{-1}; n = 5). The results were expressed as mean ± SEM. Statistical comparisons were performed by the Student's t-test. * p < 0.05, ** p < 0.01.

3.4 Discussion

The present study demonstrated the antineoplasic activity of the mesoionic compound Syd-1 against Walker-256 carcinosarcoma. The antitumor activity of Syd-1 was reflected by a 54% reduction of tumor growth. These data corroborate the findings of Grynberg *et al*. (1992), who reported the antitumor activity of Syd-1 in mice with Ehrlich carcinoma, sarcoma 180, and fibrous histiocytoma (B10MCII). In this study, however, higher intraperitoneal doses of Syd-1 (112 and 220 mg•kg^{-1}) were used, in comparison with the dose applied in the present study (75 mg•kg^{-1}).

Cachexia syndrome is a characteristic of this tumor model, which contributes to physical disability and mortality (Tisdale, 2001). According to Acco *et al.* (2012), significant metabolic changes in the liver can be observed in cachexia syndrome. Both the Tumor and Tumor-Syd groups exhibited low levels of plasma glucose and amylase. A high rate of glucose consumption is a feature of such solid tumors as Walker-256 (Acco *et al.,* 2012). Additionally, Syd-1 was previously reported to affect metabolic function related to energy provision in hepatocytes culture, decreasing their viability (Brandt *et al.*, 2014). In the Tumor group, AST increased and alkaline phosphatase, total protein, and albumin decreased. However, the group that was treated with Syd-1 (Tumor-Syd) exhibited a tendency toward recovery of these alterations that were caused by cachexia. Additionally, our data no showed indications of hepatotoxicity, which was confirmed by the absence of liver damage in the histological evaluation, as previously observed in the studies with hepatocyte culture that received Syd-1 (Brandt *et al*., 2014).

Inflammation is a key innate immune response when body homeostasis is disturbed. Chronic inflammatory processes affect all stages of tumor development and therapy (Elinav *et al.,* 2013). Using *in vitro* assays, Bizetto *et al.* (2012) reported the possible antiinflammatory activity of Syd-1, reflected by the inhibition of interleukin-6, a decrease in the phagocytic activity of macrophages, and a decrease in NO production. Tests that evaluate the antiinflammatory action of Syd-1 have only been performed *in vitro*, which may explain the absence of this activity in the present study that used an *in vivo* model that has more confounding factors. Other hypothesis for the absence of antiinflammatory effect of Syd-1 in our study could be the timing for the tumor harvesting. After 12 days of tumor development the main sources of enzymes NAG (mononuclear cells) and MPO (neutrophils) could be less present.

Mallur *et al.* (2007) previously reported the ability of sydnones to scavenge free radicals *in vitro*. Later on Gozzi *et al.* (2013), investigating the role of Syd-1 in oxidative stress, observed that it has the ability to inhibit the iron-induced lipoperoxidation in the

isolated mitochondria of rats, as well as it has ability to sequester superoxide radicals. This authors also observed that Syd-1 did not promote significant alterations to the activity of SOD and CAT. In our *in vivo* studies, a dual effect of Syd-1 on GSH was observed. First, hepatic GSH levels increased in the Basal-Syd group compared with both tumor-bearing groups (Fig. 2B). Alterations in energy metabolism may be a factor that influences the oxidative stress that is observed in tumors (Mantovani *et al.,* 2004), leading to a decrease in the activity of such antioxidant agents as GSH. Second, Syd-1 treatment decreased GSH levels in tumor tissue (Fig. 2A). This dual activity may be an important mechanism of action of Syd-1 in tumor suppression. According to Badraoui *et al.* (2009), the antioxidant capacity of cancer cells can favor tumor development. High levels of reactive oxygen species can also be toxic to cancer cells and potentially induce cell death (Glasauer *et al*., 2014).

Halila *et al*. (2007) conducted *in vitro* experiments and showed that Syd-1 impaired mitochondrial function that is related to energy production by inhibiting electron transport. This action at the mitochondrial level may be related to the tumor-suppressing effect reported by Grynberg *et al*. (1992) and the present study. Disruptions of mitochondrial function can induce caspase activation, which can lead cells to undergo apoptosis (Parolin and Reason, 2001). The histological findings in tumor tissue indicated the higher presence of apoptotic bodies that were induced by Syd-1 treatment.

The apoptotic action of Syd-1 was confirmed by gene expression. Increases in the expression of both pro-apoptotic p53 and Bax and a decrease in the expression of anti-apoptotic Bcl-2 were observed in tumors in animals that were treated with Syd-1. These data strongly suggest that the mechanism of action of Syd-1 involves the modulation of apoptosis, leading to the death of tumor cells. According to Youle and Strasser (2008), Bcl-2 family proteins are essential for the regulation of intrinsic or mitochondrial pathways of apoptosis. In the presence of intracellular signals of stress, the translocation of pro-apoptotic proteins, such as Bax, from the cytosol to

mitochondria occurs. This translocation results in the release of cytochrome-c into the cytosol, which forms a complex with factor activator of apoptosis 1 (Apaf-1), which activates caspases that subsequently trigger apoptosis (Parolin and Reason, 2001; Kaplowitz, 2000; Tschopp *et al.,* 2003). Syd-1 also increased p53 gene expression. p53 is directly linked to apoptosis, which occurs when mutations in DNA are present. p53 blocks the cell cycle or activates apoptotic pathways; thus, the mutated cell does not undergo cell division. Recent studies have demonstrated that p53 also promotes cell metabolism regulation, including mitochondrial oxidative phosphorylation, pentose phosphate pathway, glycolysis, fatty acid synthesis and oxidation. The maintenance of the metabolic homeostasis contributes to the role of p53 in tumor suppression. Furthermore, p53 activation in different cancer cell lines causes accumulation of ROS, causing the oxidative stress, which contributes to the induction of apoptosis (Cavalcanti *et al.,* 2002; McGavin and Zachary, 2009, Liu *et al.,* 2015, Sablina *et al.,* 2005). Therefore the GSH depletion caused by Syd-1 along with the increase in p53 gene expression observed in animals that received treatment with the compound may be acting as complementary mechanisms of this drug. By increasing the expression of p53, Syd-1 could increase ROS in tumor tissue, triggering cell death and promoting its antitumor effects. Because their functions to maintain homeostasis of the organism, the p53 has been called the “guardian of the genome” (McGavin and Zachary, 2009).

The toxicological analysis showed that only the spleen exhibited changes (Fig. 3B). In some types of cancer, splenomegaly is common (Pozo *et al*., 2009). However, variations in the weight and size of the spleen were more pronounced in animals that were treated with Syd-1 compared with the Tumor group, indicating that Syd-1 is also involved in the induction of splenomegaly. This effect may explain the hematologic abnormalities (Table 2) and histological changes (Fig. 4) in animals that received Syd-1. The histological findings indicated hyperplasia of the marginal zone in the Tumor and Tumor-Syd groups. Splenomegaly was likely caused by possible hemolysis that was induced by Syd-1, corroborating the significant presence of iron that was observed in

the red pulp of the spleen. Extravascular hemolysis causes the release of large quantities of erythrocyte corpuscles, which are stored in the red pulp of the spleen and contribute to splenomegaly (Asha, 2004). According to Petroianu (2001), erythrocytes can also be damaged by trauma, antibodies, drugs, organic and functional disorders, and toxins.

In conclusion, the present study demonstrated the antitumor activity of Syd-1 against Walker-256 carcinosarcoma. Its mechanism of action is linked to the activation of apoptotic pathways that lead to tumor cell death, probably by increasing ROS and by p53 activation. The antitumor action promoted by Syd-1 is relevant since it become a promising agent for cancer treatment. Further investigations should be performed to better understand the side effects of Syd-1, including splenomegaly and hematological alterations that were likely caused by hemolysis.

3.5 Acknowledgments

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3.6 Declaration of Interest

There was no conflict of interest. The authors LFG, FARL, GGM and LMCK received financial support from CAPE

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4. CONSIDERAÇÕES FINAIS

O presente estudo confirmou a atividade antineoplásica do composto mesoiônico Syd-1 frente ao modelo de carcinossarcoma Walker-256, pois foi capaz de promover diminuição do volume e peso tumoral. O efeito antineoplásico está relacionado com a modulação das vias apoptóticas, visto que ele aumentou a expressão dos genes pró-apoptóticos p53 e Bax e diminuiu do anti-apoptótico Bcl-2.

Alterações esplênicas e hematológicas foram observadas nos grupos que receberam Syd-1, indicando que provavelmente o composto induza extensa hemólise, acarretando em uma considerável esplenomegalia. Porém, os mecanismos pelos quais estas alterações ocorrem devem ser melhor elucidados.

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