Mariana de Oliveira Klein

AVALIAÇÃO DA PRESENÇA DE ANTINEOPLÁSICOS E SEUS METABÓLITOS EM EFLUENTES NO MUNICÍPIO DE BARRETOS, SP, E SUAS INTERAÇÕES ECOLÓGICAS E GENOTÓXICAS

"Tese apresentada ao Programa de Pós-Graduação da Fundação Pio XII – Hospital de Câncer de Barretos para obtenção do Título de Doutor em Ciências da Saúde"

Área de Concentração: Oncologia

Orientador: Prof. Dr. Henrique César Santejo Silveira

Barretos, SP
2021

SUPORTE À PESQUISA POR AGÊNCIA DE FOMENTO

Este trabalho recebeu apoio do Ministério Público do Trabalho (Federal).

Esta tese foi elaborada e está apresentada de acordo com as normas da Pós-Graduação do Hospital de Câncer de Barretos – Fundação Pio XII, baseando-se no Regimento do Programa de Pós-Graduação em Oncologia e no Manual de Apresentação de Dissertações e Teses do Hospital de Câncer de Barretos. Os pesquisadores declaram ainda que este trabalho foi realizado em concordância com o Código de Boas Práticas Científicas (FAPESP), não havendo nada em seu conteúdo que possa ser considerado como plágio, fabricação ou falsificação de dados. As opiniões, hipóteses e conclusões ou recomendações expressas neste material são de responsabilidade dos autores e não necessariamente refletem a visão da Fundação Pio XII – Hospital de Câncer de Barretos.

Embora o Núcleo de Apoio ao Pesquisador do Hospital de Câncer de Barretos tenha realizado as análises estatísticas e orientado sua interpretação, a descrição da metodologia estatística, a apresentação dos resultados e suas conclusões são de inteira responsabilidade dos pesquisadores envolvidos.

Os pesquisadores declaram não ter qualquer conflito de interesse relacionado a este estudo.

AGRADECIMENTOS

Ao Prof. Dr. Henrique César Santejo Silvera, pela oportunidade e confiança em mim depositada para realização deste trabalho.

Agradeço a todos os colaboradores da Fundação PIO XII, colegas do grupo de pesquisa, colaboradores do SAAE, colegas da Unifeb os quais, sem o auxílio e apoio, a realização deste trabalho não teria sido possível, como dito por Clarice Lispector: *"Quem caminha sozinho pode até chegar mais rápido, mas aquele que vai acompanhado, com certeza vai mais longe."*

E pôr fim aos meus familiares, em especial ao meu esposo, Otavio Klein. Uma tese de doutorado nunca será realizada sem o suporte daqueles que estão mais próximos, e que entendem e suportam nossas ausências em alguns momentos. Obrigada!

SUMÁRIO

LISTA DE TABELAS

LISTA DE FIGURAS

LISTA DE ABREVIATURAS

2-DOH-DiF	2´-deoxi-2´,2´-difluorouridina
3-NH_2-F	Alfa-fluoro-beta-alanina
5-FU	5-Fluorouracil
AC	Acroleína
ANVISA	Agência Nacional de Vigilância Sanitária
ATC	Classificação Terapêutica Anatômica (do inglês *Anatomical Therapeutic Classification)*
CDDP	Cisplatina
CG-EM	Cromatografia Gasosa acoplada a Espectrometria de Massas
CL	Concentração Letal
CLAE-EM/EM	Cromatografia Líquida de Alta Eficiência acoplada a Espectrometria de Massas
CLAE-FLD	Cromatografia Líquida de Alta Eficiência com Detecção por Fluorescência
CONAMA	Conselho Nacional do Meio Ambiente
CP	Ciclofosfamida
dFdUDP	Gemcitabina difosfato
dFdUTP	Gemcitabina trifosfato
DAU	Daunorrubicina
DNA	Ácido desoxirribonucleico (do inglês *Desoxyribonucleic Acid*)
DOX	Doxorrubicina
EC50	Concentração Efetiva (do inglês *Effective Concentration*)
ECHA	Agência Europeia de Substâncias Químicas (do inglês *European Chemicals Agency*)
EMA	Agência Europeia de Medicamentos (do inglês *European Medicines Agency*)
EPA	Agência de Proteção Ambiental Americana (do inglês *Environmental Protection Agency*)
EPI	Epirrubiana
ET	Etoposide
ETE	Estação de Tratamento de Efluentes

FDA	Agência Americana de Controle de Alimentos e Medicamentos (do inglês *Food and Drug Administration*)
GEM	Gemcitabina
GHS	Sistema Mundial Harmonizado de Classificação e Rotulagem de Produtos Químicos (do inglês *Globally Harmonized System of Classification and Labelling of Chemicals*)
HCB	Hospital de Câncer de Barretos
HepG2	Linhagem de hepatocarcinoma humano
HPBL	Linfócitos de Sangue Periférico Humano (do inglês *Human Peripheral Blood Lymphocytes*)
HPLC-MS/MS	Cromatografia Líquida de Alta Eficiência acoplada a Espectrometria de Massas (do inglês *High Performance Liquid Chromatography tandem Mass Spectrometry*)
IC50	Metade da concentração inibitória máxima (do inglês *Half Maximal Inhibitory Concentration*)
IF	Ifosfamida
IDN	Índice de Divisão Nuclear
IM	Imatinibe
IRI	Irinotecano
LD	Limite de detecção
LOEC	Menor Concentração de Efeito Observado (do inglês *Lowest Observed-Effect Concentration)*
LOQ	Limite de quantificação (do inglês *Limit of Quantification*)
MN	Micronúcleo
MTS	3 - (4,5-dimethyl-2-yl) -5 - (3-carboxymethoxyphenyl) -2 - (4-sulfophenyl)-2H-tetrazolium
MTX	Metotrexato
NDI	Índice de Divisão Nuclear (do inglês *Nuclear Division Index*)
NOEC	Concentração sem efeito observado (do inglês *No Observed Effect Concentration*)

OCDE	Organização para a Cooperação e Desenvolvimento Econômico (do inglês *Organisation for Economic Cooperation and Development*)
PAC	Paclitaxicel
PITC	Isotiocianato de fenil (do inglês *Phenyl Isothiocyanate*)
RNA	Ácido ribonucleico (do inglês *Ribonucleic Acid*)
RRBP	Proteínas de ligação ao RNA ribossômico (do inglês *Ribosomal RNA Binding Protein*)
SCE	Troca de cromátide irmã (do inglês *Sister Chromatid Exchange*)
SP	São Paulo
SPE	Fase sólida de extração (do inglês *Solid Phase Extraction*)
SUS	Sistema Único de Saúde
TMX	Tamoxifeno
UmuC	Umu Chromotest
WWTP	Estação de Tratamento de Efluentes (do inglês *Wastewater Treatment Plant*)
ZFL	Células de fígado de *zebrafish* (do inglês *Zebrafish Liver Cell*)

LISTA DE SÍMBOLOS

“	Aspas
/	Barra
-	Menos
>	Maior
<	Menor
-	Menos
µ	Micro
( )	Parênteses
%	Porcentagem
I	Um
II	Dois
III	Três
IV	Quatro
XII	Doze

ESBOÇO DA TESE

A tese de doutorado aqui apresentada foi elaborada no modelo baseado em trabalho publicado.

Para esta modalidade de apresentação, as seções “Introdução” e “Discussão” são sucintas e tem o objetivo de situar e atualizar o leitor sobre o assunto. Além disso, as seções “Material e Métodos” e “Resultados” foram substituídas pelo arquivo do artigo publicado e do artigo a ser publicado.

RESUMO

Oliveira Klein M. Avaliação da presença de antineoplásicos e seus metabólitos em efluentes no município de Barretos, SP, e suas interações ecológicas e genotóxicas. **Tese (Doutorado)**. Barretos: Hospital de Câncer de Barretos; 2021.

Com o crescente aumento dos casos de câncer no Brasil e no mundo, há, também uma maior demanda no consumo de drogas antineoplásicas administradas na quimioterapia. Estudos em diversos países têm demonstrado que estas drogas, devido às suas características recalcitrantes, estão presentes em efluentes hospitalares e persistem mesmo após passarem por tratamento em estações de tratamento de efluentes (ETE's). Os antineoplásicos estão classificados entre os fármacos mais tóxicos, levantando preocupações a respeito dos seus efeitos adversos aos organismos, entre eles citotoxicidade e genotoxicidade. No Brasil, as pesquisas são incipientes em relação a presença de drogas em efluentes hospitalares e municipais, assim como estudos que avaliem os danos destes compostos aos organismos.
Diante disso, no presente estudo, quantificamos a presença dos antineoplásicos 5-fluorouracil (5-FU), gemcitabina (GEM), ciclofosfamida (CP), e dos metabólitos alfa-fluoro-beta-alanina (3-NH_2-F) e 2´-deoxi-2´,2´-difluorouridina (2-DOH-DiF) no efluente do Hospital de Câncer de Barretos (HCB) e na entrada e saída dos efluentes da ETE IV do Município de Barretos, SP, por meio das ferramentas analíticas de cromatografia líquida de alta eficiência acoplada a espectrometria de massas (CLAE-EM/EM). Para avaliação ecotoxicológica realizamos análise de toxicidade aguda dos compostos com peixe *zebrafish* (*Danio rerio*), com análise histológica das brânquias e fígado dos peixes expostos aos compostos em mistura. A avaliação da citotoxicidade e genotoxicidade dessas drogas, foi realizada em células de hepatocarcinoma humano (HepG2), pelo ensaio de viabilidade celular (MTS) e teste de micronúcleo, respectivamente. A presença de CP, GEM e ambos os metabólitos (3-NH_2-F e 2-DOH-DiF) foram avaliadas nas águas residuais do hospital e no efluente da ETE, antes do tratamento, em quantidades entre 0,11 ng/mL a 116 ng/mL. GEM, 2-DOH-DiF e CP, foram detectados após tratamento em ETE, sendo 2-DOH-DiF com valor abaixo do limite de quantificação (<LOQ), acima de 1,4 ng/mL, e CP com valor <LOQ, acima de 0,3 ng/mL. A GEM foi quantificada em 0,42 ng/mL. Somente o 5-FU não foi detectado em nenhum ponto e em nenhuma campanha de coleta. Quanto a avaliação de ecotoxicidade aguda em *zebrafish*, para os compostos CP,

GEM, 5-FU e 3-NH_2-F a concentração letal (CL) foi superior a 118 mg/L, ou seja, considerado praticamente não tóxico. Para acroleína (AC), a concentração letal foi abaixo de 0,1 mg/L, considerada extremamente tóxica. O metabólito 2-DOH-DiF não foi avaliado. A exposição do *zebrafish* à mistura das drogas em quantidades detectadas nos efluentes ocasionou alterações histológicas, nas brânquias foram observados aneurismas e edema e no fígado ocorreram pontos de necrose. A redução na viabilidade celular foi observada para GEM em concentrações acima de 0,1 ng/mL (IC50 de 25,26 ng/mL), para 2-DOH-DiF em concentrações acima de 1 ng/mL (IC50 de 83,65 ng/mL) e para 5 -FU em concentrações acima de 1 ng/mL (IC50 de 18,07 ng/mL). Para CP e 3-NH_2-F, nenhuma redução na viabilidade celular foi verificada nas concentrações avaliadas. Quanto aos danos genotóxicos, foi possível observar a formação de pontes nucleoplasmáticas diferentemente do CN, para GEM e CP em concentrações acima de 0,001 ng/mL e para o 3-NH_2-F, em concentrações acima de 50 ng/mL. Os outros compostos, 2-DOH-DiF e 5-FU, não mostraram formação de pontes nucleoplasmáticas significativamente diferente do CN nas concentrações avaliadas. Micronúcleos e brotamento nuclear foram observados em número significativamente aumentado para CP, GEM, 2-DOH-DiF e 3-NH_2-F em concentrações acima de 0,001 ng/mL, enquanto para 5-FU os mesmos danos foram observados em concentrações acima de 0,01 ng/mL. Os resultados demonstram que três (5-FU, GEM e 2-DOH-DiF) dos cinco compostos avaliados são citotóxicos para HepG2, diminuindo sua viabilidade celular em concentrações presentes no meio ambiente. 5-FU e GEM também alteraram o índice de divisão nuclear. Quanto à genotoxicidade, todos os compostos aumentaram o número de micronúcleos e brotamento nuclear. CP e 3-NH_2-F também causaram aumento no número de pontes nucleoplasmáticas. Os dados apontam a presença das drogas nos efluentes, inclusive após tratamento em ETE, e seus danos ecotoxicológicos como mistura a *zebrafish*, assim como danos citotóxicos e genotóxicos dos compostos a HepG2 em quantidades presentes nos efluentes. Destacamos a importância de estudos que avaliem desde a detecção de drogas no meio ambiente até seus efeitos citotóxicos e genotóxicos em diferentes linhagens celulares e organismos, contribuindo para o conhecimento e auxiliando na implementação de políticas públicas que minimizem possíveis contaminações ambientais.

PALAVRAS-CHAVE: antineoplásicos, genotoxicidade, citotoxicidade, HepG2

ABSTRACT

Oliveira Klein M. Analysis of the presence of antineoplastic agents and their metabolites in effluents from the city of Barretos, SP, as well as their ecological and genotoxic interactions. **Thesis (Doctorate)**. Barretos: Barretos Cancer Hospital; 2021.

As the number of cancer cases continues to increase in Brazil and around the world, the demand for the use of antineoplastic drugs administered in chemotherapy has also increased. Studies carried out in several different countries have shown that these drugs are often present in hospital effluents, due to their recalcitrant characteristics, and may continue to be present even after undergoing treatment in effluent treatment plants (ETPs). Antineoplastics are ranked among the most toxic drugs, raising concerns about their adverse effects to organisms, including their cyto-genotoxicity. In Brazil, the studies regarding the presence of drugs in hospital and municipal effluents are incipient, as well as studies assessing the harmful effects of these compounds on organisms. Hence, in this present study, we have quantified the presence of the antineoplastics 5-fluorouracil (5-FU), gemcitabine (GEM), cyclophosphamide (CP), and the metabolites alpha-fluoro-beta-alanine (3-NH_2-F) and 2 ´-deoxy-2´,2´-difluorouridine (2-DOH-DiF) in the effluent from the Barretos Cancer Hospital (HCB) and the inlet and outlet effluents from the ETP IV of the Municipality of Barretos, SP, by means of high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) analysis. Acute toxicity analysis of the compounds was performed using Zebrafish (Danio rerio) to assess ecotoxicity, including histological analysis of the fish gills and liver exposed to the compounds in mixture. The cytotoxicity and genotoxicity evaluation of these drugs was conducted on human hepatocarcinoma (HepG2) cells, via cell viability assay (MTS) and micronucleus test, respectively. The presence of CP, GEM and both metabolites (3-NH_2-F and 2-DOH-DiF) were evaluated in the hospital wastewater and ETP effluent, before treatment, in amounts ranging from 0.11 ng/mL to 116 ng/ml. GEM, 2-DOH-DiF and CP, were detected in ETP post-treatment, with 2-DOH-DiF at a value below the limit of quantification (<LOQ), above 1.4 ng/mL, and CP at a value <LOQ, above 0.3 ng/ml. The GEM was quantified at 0.42 ng/ml. 5-FU was the only compound not detected at any site nor sampling campaigns. With respect to the acute ecotoxicity evaluation in zebrafish, the lethal concentration (LC) for

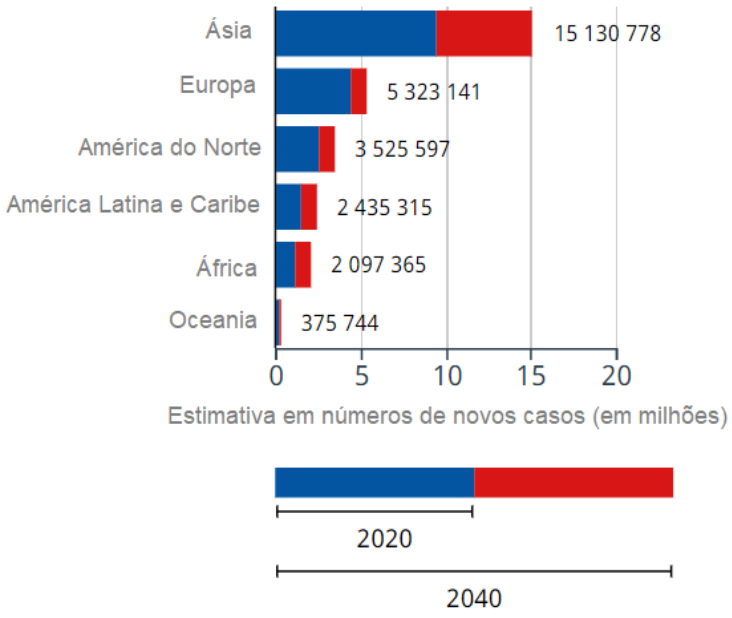
the compounds CP, GEM, 5-FU and 3-NH_2-F was higher than 118 mg/L, in other words, considered practically non-toxic. For acrolein (AC), the lethal concentration was below 0.1 mg/L, which is considered extremely toxic. The metabolite 2-DOH-DiF was not evaluated. Zebrafish exposed to the mixture of drugs in quantities detected in the effluents resulted in histological changes, aneurysms and edema were observed in the gills, and necrosis was observed in the liver. Reduction in cell viability was observed for GEM at concentrations above 0.1 ng/mL (IC50 of 25.26 ng/mL), for 2-DOH-DiF at concentrations above 1 ng/mL (IC50 of 83.65 ng/ml) and for 5-FU at concentrations above 1 ng/ml (IC50 of 18.07 ng/ml). For CP and 3-NH_2-F, no reduction in cell viability was observed for the concentrations evaluated. As for genotoxic damage, nucleoplasmic bridge formation was observed differently from CN for GEM and CP at concentrations above 0.001 ng/mL and for 3-NH_2-F at concentrations above 50 ng/mL. The other compounds, 2-DOH-DiF and 5-FU, did not show nucleoplasmic bridge formation significantly different from CN at the concentrations evaluated. Micronuclei and nuclear budding were observed in significantly increased numbers for CP, GEM, 2-DOH-DiF and 3-NH_2-F at concentrations above 0.001 ng/mL, while for 5-FU the same damage was observed at concentrations above 0 .01 ng/ml. The results demonstrate that three (5-FU, GEM and 2-DOH-DiF) of the five compounds evaluated are cytotoxic to HepG2, reducing its cell viability at concentrations present in the environment. 5-FU and GEM also altered the nuclear division index. Regarding genotoxicity, an increase in the number of micronuclei and nuclear budding was observed in all compounds. An increase in the number of nucleoplasmic bridges was also caused by CP and 3-NH_2-F. The data indicates the presence of drugs in effluents, even after treatment in ETPs, as well as their ecotoxicological damage to zebrafish as a mixture and the cytotoxic and genotoxic damage of the compounds to HepG2 in amounts present in the effluents. We highlight the importance of such studies that range from the assessment of drug detection in the environment to their cytotoxic and genotoxic effects on various cell lines and organisms, thereby contributing to understanding and assisting in the implementation of public policies that minimize possible environmental contamination.

KEYWORDS: antineoplastics, genotoxicity, cytotoxicity, HepG2.

1. INTRODUÇÃO

O câncer é o principal problema de saúde pública no mundo, estando entre as quatro principais causas de morte prematura (antes dos 70 anos de idade) na maioria dos países [4]. O aumento na incidência e mortalidade ocorre em parte pelo envelhecimento e crescimento populacional, como também pela mudança na distribuição e na prevalência dos fatores de risco de câncer, especialmente aos associados ao desenvolvimento socioeconômico [4].

Globalmente, estima-se um aumento de 19,3 milhões de casos em 2020 para 28,9 milhões de casos em 2040 [1] (Figura 1). No Brasil, para o triênio 2020-2022, 1,8 milhões de novos casos de câncer são esperados [4], estima-se que até os 75 anos de idade, um em cada cinco brasileiros desenvolva algum tipo de câncer [5].



	Total
2020	19 292 789
2040	28 887 940

Figura 1. Gráfico representativo com a estimativa de novos casos de câncer em nível global em 2040. Adaptado de Ferlay et al. [1], base de dados IARC (Agência Internacional de Pesquisa em Câncer, do inglês *International Agency for Research on Cancer*).

A quimioterapia é uma terapia citotóxica, que mata ou inibe o crescimento de células cancerosas, e é indicada como tratamento na maior parte dos diagnósticos de câncer. A maioria das drogas quimioterápicas causam danos ao ácido desoxirribonucleico (DNA, do inglês *Desoxyribonucleic Acid*) ou cessam a replicação dos cromossomos por meio de interrupção na divisão celular, levando a célula à apoptose [6].

Existem diferentes indicações para a realização de quimioterapia, a saber (I) a neoadjuvante realizada antes da cirurgia, com a intenção de reduzir o tumor; (II) a adjuvante realizada após a cirurgia, adotada como complemento à ressecção cirúrgica; (III) a quimioterapia de consolidação, indicada para neoplasias hematológicas para a remissão total da doença; e (IV) a quimioterapia de manutenção empregada no tratamento das leucemias, após as etapas de indução e consolidação, mantendo o paciente em remissão da doença [7].

As drogas antineoplásicas são classificadas pelo sistema de Classificação Terapêutica Anatômica (do inglês *Anatomical Therapeutic Classification* – ATC), baseado na estrutura química e nas propriedades terapêuticas das mesmas, nas seguintes categorias [8]:

L01A - agentes alquilantes;

L01B - antimetabólitos;

L01C - plantas alcalóides e outros produtos naturais;

L01D - antibióticos citotóxicos e substâncias similares relacionadas;

L01X - agentes antineoplásicos com outro modo de ação; e

L01XY - mistura de diferentes antineoplásicos.

Os antineoplásicos possuem diferentes formas de ação, de acordo com a sua classificação. Alguns compostos antineoplásicos interagem diretamente no ciclo celular, em etapas distintas, conforme elucidado na Figura 2 [2].

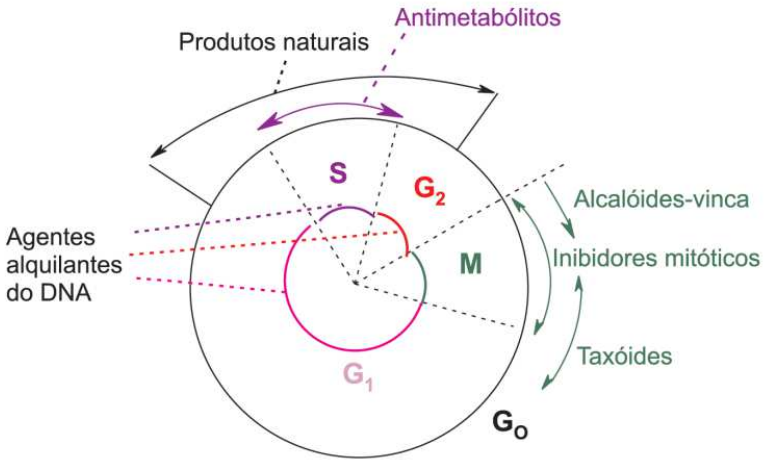


Figura 2. Atividade de grupos antineoplásicos que interagem com o DNA, de acordo com a fase do ciclo celular [2].

1.1. Drogas antineoplásicas e meio ambiente

Os antineoplásicos são substâncias recalcitrantes, também conhecidas como persistentes, ou seja, que não apresentam biodegradação sob determinadas condições impostas pelo meio ambiente, o que torna sua presença mais elevada em efluentes [9].

Estes medicamentos possuem ação não-seletiva, isto é, atingem todas as células em replicação, apresentando, por isso, potencial carcinogênico e mutagênico [10, 11]. Devido à ação como disruptor endócrino, supõe-se que os antineoplásicos provoquem danos à vida humana e animal mesmo em baixas doses [12]. A hipótese é que, devido ao seu modo de ação, praticamente todos os organismos eucarióticos são vulneráveis aos danos causados por estes compostos [12, 13].

Os antineoplásicos não são inteiramente metabolizados pelo organismo humano e são excretados em sua forma inalterada e como metabólito, através da urina e das fezes de pacientes em tratamento para os efluentes. Devido à sua baixa biodegradabilidade, permanecem no sistema de tratamento de esgoto e no meio ambiente, mesmo após o tratamento em estações de tratamento de águas residuais [14]. A presença desses compostos farmacêuticos ativos varia em diferentes ambientes naturais e estes podem interagir com outros poluentes presentes nos efluentes e se tornarem mais tóxicos para os seres vivos [15].

Os primeiros estudos a avaliarem a presença de antineoplásicos em ambientes aquáticos foram realizados nos anos 80, por Aherne et al.[16] e Richardson & Bowron [17], ambos avaliando a presença de metotrexato (MTX), seguido por investigações de Aherne et al. nos anos 90 [18], tendo o assunto retomado com maior força nos anos 2010.

Uma parcela dos estudos existentes se restringe à avaliação da presença dos antineoplásicos em efluentes hospitalares e municipais, que podem ser detectados na ordem de ng/L a µg/L [9, 16, 19-35], outros avaliam efeitos toxicológicos e genotóxicos das drogas em determinadas espécies [28, 36-42] e outros avaliam novas tecnologias para tratamento de efluentes contaminados com antineoplásicos, como nanofiltração, ozonização, degradação fotoquímica e tratamento por eletrocoagulação [23, 25, 43].

Em recente revisão, Li et al. [3] verificaram que dos 100 medicamentos antineoplásicos aprovados pela FDA (Agência Americana de Controle de Alimentos e Medicamentos, do inglês *Food and Drug Administration*) para uso nos Estados Unidos, 33 foram detectados em ecossistemas aquáticos. Dentre eles, 26 são classificados como agentes citotóxicos com vários

modos de ação (agentes alquilantes, antimetabólitos, antibióticos anticâncer, entre outros) e 07 possuem alvos endócrinos (antiestrógenos, antiandrógenos e inibidores de aromatase). Ao avaliarem a realização de pesquisas que buscam detectar antineoplásicos em efluentes nos ecossistemas aquáticos, incluindo água, sedimentos e biota, verificaram que as mesmas se limitam a 15 países (Figura 3).

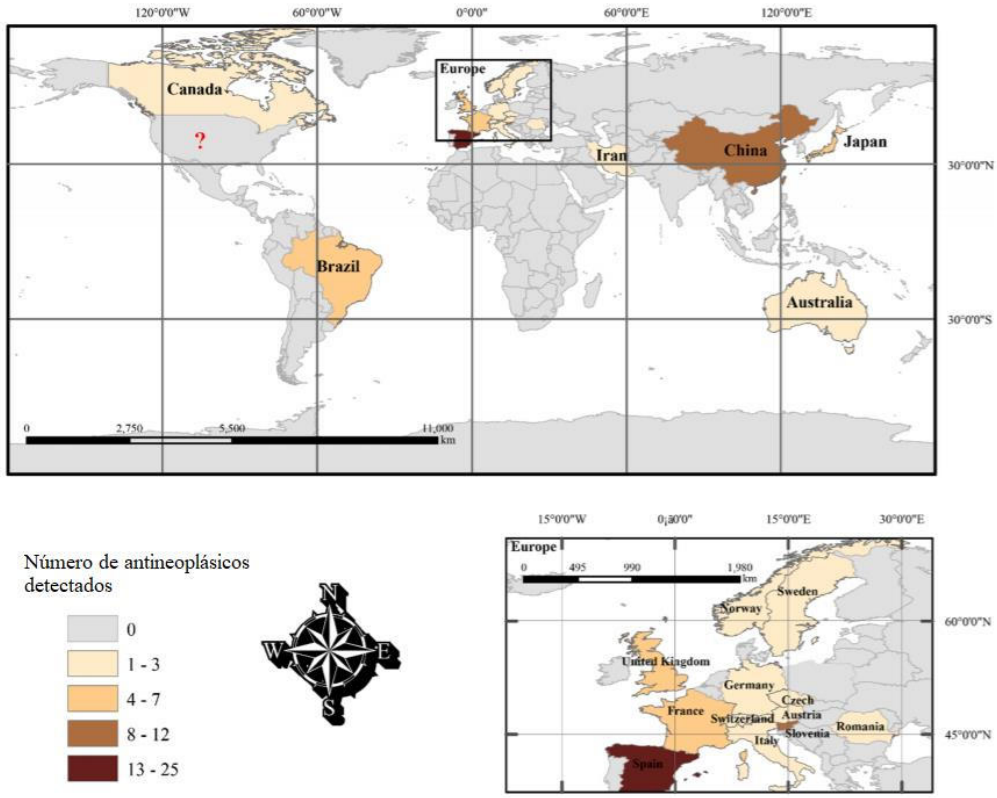


Figura 3. Mapa representando a quantidade de drogas antineoplásicas detectadas globalmente, por país. Figura adaptada de Li et al. [3].

Nassour et al. [44] ao realizarem uma revisão sistemática com 75 publicações, constataram que os antineoplásicos mais estudados foram a CP (39 publicações), tamoxifeno (TMX) (30 publicações), ifosfamida (IF) (29 publicações) e MTX (17 publicações).

A CP e a IF estão entre os antineoplásicos altamente detectados em efluentes, em concentrações que variam de ng/L [22, 45] a µg/L [26, 27, 34, 46]. Muitos estudos concluem que, mesmo após tratamento destes efluentes em estações de tratamento apropriadas, a remoção total dos antineoplásicos não ocorre [10, 47-49].

Estudo realizado em 2018 em Santa Maria, no Rio Grande do Sul [11], avaliaram por meio de cromatografia líquida de alta eficiência com detecção por fluorescência (CLAE-FLD),

seguido de processo de microfiltragem com SPE (fase sólida de extração, do inglês *solid phase extraction*), a presença de drogas antineoplásicas em efluente hospitalar nas seguintes concentrações: irinotecano (IRI) (< limite de detecção (LD) – 3.40 ng/mL), doxorrubicina (DOX) (< LD – 4.69 ng/mL), epirrubiana (EPI) (< LD – 6.22 ng/mL) e daunorrubicina (DAU)(< LD – 3.69 ng/mL).

No Brasil, os resíduos de antineoplásicos são classificados pelas Resoluções do Conselho Nacional de Meio Ambiente - CONAMA Nº 358/2005 [50] e da Agência Nacional de Vigilância Sanitária - ANVISA Nº 222/2018 [51] como Grupo B – resíduos químicos, e seus efluentes contaminados com estas drogas devem receber o tratamento de acordo com a sua periculosidade. A Resolução ANVISA Nº 222/2018 estabelece em seu artigo 63º que as excretas de pacientes tratados com quimioterápicos antineoplásicos podem ser lançadas em rede coletora de esgotos sanitários, conectada à estação de tratamento, desde que atendam às normas e diretrizes da concessionária do sistema de coleta e tratamento de esgotos sanitários ou lançadas diretamente em corpos hídricos após tratamento próprio no serviço de saúde.

Porém, as resoluções do CONAMA e da ANVISA não estabelecem os parâmetros a serem atingidos, tendo em vista que, mesmo após tratamento em ETE, resíduos de antineoplásicos podem estar presentes nestes efluentes [10, 47-49]. O grau de remoção de diferentes produtos farmacêuticos em ETE's é altamente variável, dependendo do tipo de fármaco, suas propriedades físico-químicas e a eficiência de remoção da tecnologia da ETE [52]. Existem grandes discrepâncias nas eficiências de remoção de produtos farmacêuticos em ETE's entre os países, e mesmo entre ETE's dentro do mesmo país [53]. Nenhuma técnica foi encontrada para remover todos os poluentes relevantes das águas residuais [52].

A Resolução do CONAMA Nº 430/2011, que no Brasil dispõe sobre as condições e padrões de lançamento de efluentes, complementa e altera a Resolução CONAMA Nº 357/2005, e restringe seu controle a metais pesados, óleos e graxas, e compostos químicos como xileno e tolueno.

Quanto aos padrões de potabilidade da água para consumo humano, definidos pela Portaria do Ministério da Saúde Nº 2.914, publicada em 12 de dezembro de 2011, bem como pela Portaria do Ministério da Saúde Nº 888, de 04 de maio de 2021, há valores definidos para diversas classes de produtos químicos, agrotóxicos, cianotoxinas e desinfetantes, sem citar

fármacos. Outro agravante relacionado ao tratamento de efluentes está que, no Brasil e também em outros países subdesenvolvidos e em desenvolvimento, cerca de 40% dos efluentes municipais não recebem qualquer tipo de tratamento [54].

Ao ampliarmos a avaliação para nível mundial, já existem algumas diretrizes que buscam normatizar a emissão de efluentes contaminados com antineoplásicos. A EMA (Agência Europeia de Medicamentos, do inglês *European Medicines Agency*) e a FDA desenvolveram diretrizes para monitorar a ocorrência de drogas antineoplásicas em diferentes ecossistemas aquáticos com base na saúde e no meio ambiente e estudos de avaliação de risco ambiental, com valores definidos pela EMA e FDA de 0,01 mg/L (10 ng/mL) e 1 mg/L (1.000 ng/mL), respectivamente [55].

A OCDE (Organização para a Cooperação e Desenvolvimento Econômico, do inglês *Organisation for Economic Cooperation and Development*) também desenvolveu novas diretrizes para a gestão de poluentes farmacêuticos perigosos em efluentes. No documento, além da caracterização da detecção de fármacos em efluentes e impactos negativos na saúde da população e ao meio ambiente, traz exemplos de boas práticas em países como Alemanha, França, Reino Unido e comunidade européia como um todo, recomendando aos governos dos países do mundo inteiro que iniciem uma abordagem coletiva do ciclo de vida dos fármacos, por meio da colaboração entre diferentes departamentos governamentais, autoridades locais e partes interessadas [52].

1.2. Toxicidade e genotoxicidade dos antineoplásicos

Em paralelo às pesquisas que detectam a presença de drogas antineoplásicas em efluentes hospitalares e ambientes aquáticos, diversos autores buscam avaliar a toxicidade e genotoxicidade destes compostos [40, 56].

Misík et al. [40] descreveram resultados das avaliações de risco relacionadas à segurança ambiental, baseadas nos dados gerados em projeto da União Européia intitulado "*Cytothreat*". 5-FU, cisplatina (CDDP), imatinibe (IM) e etoposide (ET), drogas antineoplásicas amplamente utilizadas com diferentes mecanismos de ação terapêutica, foram avaliadas em relação a ecotoxicidade em organismos bioindicadores (cianobactérias, algas, plantas, rotíferos, crustáceos, peixes (*zebrafish*) e também células de hepatocarcinoma humana (HepG2) e células de fígado de peixe *zebrafish* (ZFL). As espécies de crustáceos foram as mais

sensíveis aos experimentos de toxicidade aguda. Li et al.[3] em artigo de revisão com 09 antineoplásicos, 5-FU, CDDP, IM, ET, IF, gemcitabina (GEM), capecitabina (CAP), doxorrubicina (DOX) e tamoxifeno (TMX), apontaram os mesmos resultados.

No tratamento crônico para análise dos efeitos de inibição reprodutiva, os efeitos mais pronunciados foram detectados na ordem de ng/L com IM, seguido por CDDP e 5-FU [40].

Grzesiuk et al. [57] demonstraram que a exposição à CP e a CDDP em concentrações detectadas no ambiente afetam significativamente as espécies de pequenos crustáceos *Daphnia magna* em parâmetros como sobrevivência, número de ovos, taxa de crescimento e taxa de crescimento populacional. Os autores também avaliaram o perfil do proteoma, que revelou que tanto CP quanto CDDP modificam a expressão de algumas proteínas envolvidas no metabolismo da *D. magna*.

No estudo de Gajski et al. [58], os antineoplásicos 5-FU, ET e CDDP demonstraram citotoxicidade por ensaio de viabilidade celular à células HepG2, ZFL e em linfócitos de sangue periférico humano (do inglês *Human Peripheral Blood Lymphocytes - HPBL*). Nesse mesmo estudo, avaliaram o dano genotóxico de 5-FU por ensaio cometa e micronúcleo (MN), observando danos do DNA por ensaio cometa apenas em ZFL em concentrações acima de 100 ng/mL, e por ensaio de micronúcleo o dano do DNA foi observado apenas para HepG2, em concentrações acima de 100 ng/mL.

Indução da formação de MN foi observada em plantas do gênero *Tradescantia*, para 5-FU, CDDP, ET e IM. Os autores também observaram que a presença de IM aumenta as atividades genotóxicas dos antineoplásicos 5-FU, CDDP e ET [41].

O 5-FU é uma das drogas antineoplásicas com maior número de estudos de citotoxicidade e genotoxicidade. Lutterbeck et al. [39] observaram alterações cromossômicas e formação de MN em células da planta *Allium cepa* expostas a droga em concentração de 10.000 ng/mL. Kovacs et al. [59] observaram formação de MN em eritrócitos de *zebrafish* expostos a concentrações entre 0,01 ng/mL e 100 ng/mL, com aumento na formação de MN de acordo com o aumento da concentração. Com o ensaio cometa, os danos foram avaliados em ZFL nas concentrações entre 1,0 ng/mL e 100 ng/mL, e em eritrócitos na concentração 1,0 ng/mL. Em mexilhões de água doce *Unio pictorum* e *Unio tumidus*, 5-FU induziu quebras de fita de DNA avaliado por ensaio cometa em concentrações acima de 52 ng/mL [60].

CP foi capaz de induzir estresse oxidativo no mexilhão marinho *Mytilus galloprovincialis* sob uma exposição de 14 dias a 1 ng/mL, juntamente com o dano oxidativo, aumento na prevalência de danos ao DNA e redução da viabilidade celular. Resultados não replicáveis as células humanas HELA (células endoteliais, do inglês *human transformed endothelial*) e RPE (células do epitélio pigmentar da retina, do inglês *immortalized retinal pigment epithelium*) [36]. Linfócitos humanos tratados com CP em concentrações acima de 160 ng/mL apresentaram alterações cromossômicas estruturais, SCE's (troca de cromátide irmã, do *inglês sister chromatid exchange*) e frequência de MN aumentada [61, 62].

A literatura é escassa quanto a dados de citotoxicidade e genotoxicidade de GEM e seu metabólito 2-DOH-DiF.

Em 2010, Zounkova et al. [47] ao avaliarem a ecotoxicidade aguda da GEM em testes de imobilização e reprodução *D. magna* e testes de inibição de crescimento com a alga *Desmodesmus subspicatus* e bactérias *Pseudomonas putida*, encontraram os respectivos valores de EC50 (concentração efetiva) de 110.000 ng/mL, 45.000 ng/mL e 100.000 ng/mL, quantidades muito superiores aos valores do fármaco detectados nos efluentes e aos valores de citotoxicidade no HepG2 deste estudo.

Estudos recentes também têm revelado que as misturas das drogas antineoplásicas e seus metabólitos tem potencial genotóxico aumentado se comparado às drogas isoladas [37, 63-65]. Porém poucos dados estão disponíveis e dizem respeito a possíveis efeitos sinérgicos e antagônicos nas misturas dos diferentes medicamentos antineoplásicos [40].

1.3. Local e drogas alvo do estudo

Após análise do consumo de antineoplásicos por quantidade (mg) no Hospital de Câncer de Barretos (HCB) nos anos 2015, 2016 e 2017, por meio de dados obtidos junto ao Departamento de Farmácia, assim como análise do consumo nacional por meio do SUS (Sistema Único de Saúde), foi possível definir as seguintes drogas alvo do estudo: 5-FU, GEM, CP e seus principais metabólitos (Tabela 1).

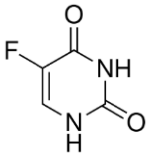
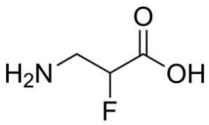
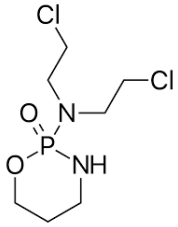
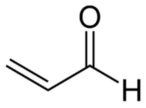
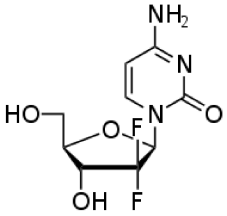
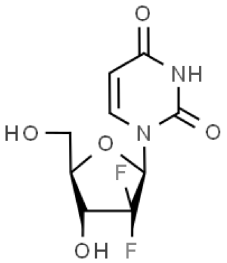
O 5-FU foi introduzido no tratamento de tumores sólidos em 1957 [66]. Esta droga é administrada principalmente no tratamento de câncer de reto, estômago, cólon, pâncreas, glândula tireóide e mama [66]. Por ser análogo a pirimidina, o 5-FU age como antimetabólito ao uracil. A ação destes compostos ocorre devido a seus produtos de anabolismo, que provocam interferência na síntese de DNA, bloqueando a conversão de ácido desoxiuridílico em ácido

timidílico pela enzima *timidilato sintetase* [67]. O 5-FU também interfere na atividade de proteínas de ligação ao RNA ribossômico (RRBP, do inglês *ribosomal RNA binding protein*) [10]. O 5-FU é caracterizado como fármaco persistente. Desta forma, parte da medicação é excretada inalteradamente (entre 4 – 20%) nos efluentes [10] e, mesmo após tratamento nas ETE's, pode estar presente no meio ambiente, interferindo no ecossistema aquático [68]. Os metabólitos do 5-FU são excretados por via respiratória na forma de dióxido de carbono e na urina como uréia, alfa-fluoro-beta-alanina, ácido alfa-fluoro-beta-guanidopropiona e ácido alfa-fluoro-beta-ureidoproprionico, tendo sido escolhido, para análise neste estudo, o metabólito alfa-fluoro-beta-alanina (3-NH_2-F).

A CP, também amplamente utilizada no HCB e no SUS, é um agente alquilante com propriedades imunossupressoras. Este agente é utilizado há mais de 50 anos em vários tratamentos contra o câncer, tais como mama, linfomas não Hodgkin e estômago [69, 70]. A CP trata-se de uma pró-droga, que necessita de transformações metabólicas para atuar nas neoplasias [71, 72]. Estas transformações formam uma série de metabólitos citotóxicos [73], sendo que os metabólitos excretados via urina constituem-se basicamente de acroleína (AC) [74] e mostarda fosforamida, bem como de 10-20% da CP em sua forma inalterada [75, 76]. A AC é conhecida por seu potencial tóxico e classificada pela agência de proteção ambiental americana (EPA, do inglês *Environmental Protection Agency*) como um possível agente carcinogênico [77], sendo escolhida como metabólito para análise neste estudo.

A GEM, segunda droga antineoplásica mais consumida em mg no HCB, aplicada principalmente no tratamento de câncer de mama, ovário, bexiga, pâncreas, brônquios e pulmão, é um antimetabólito do grupo das pirimidinas, com capacidade de inibir a síntese do DNA, por meio da inibição da DNA *polimerase* e do ribonucleotídeo *transferase* [78]. A atuação desta droga ocorre na fase S do ciclo celular [78] (Figura 2). A GEM necessita de quebras enzimáticas para ação, é fosforilada intracelularmente pela *cinase deoxicitidina* para gemcitabina monofosfato, que é posteriormente fosforilada para os metabólitos ativos gemcitabina difosfato (dFdUDP) e gemcitabina trifosfato (dFdUTP). A gemcitabina difosfato inibe a síntese de DNA, inibindo o ribonucleotídeo *redutase*, e a gemcitabina trifosfato é incorporada no DNA inibindo a DNA polimerase [79]. Cerca de 10% da GEM é excretada inalterada, juntamente com seu metabólito, 2´-deoxi-2´,2´-difluorouridina (2-DOH-DiF) [79], que é alvo deste estudo.

Tabela 1. Caracterização das moléculas avaliadas, por nome, abreviação, fórmula molecular, estrutura molecular, número CAS (divisão da Sociedade Americana de Química, do inglês *Chemical Abstracts Service*) , peso molecular e porcentagem de excreção, após metabolização.

Nome, abreviatura e fórmula molecular	Estrutura molecular	CAS	Peso molecular (g/mol)	Excreção
5-Fluorouracil (5-FU) $C_4H_3FN_2O_2$		51-21-8	130.07	2 – 20% [80]
Alfa-fluoro-beta-alanina (3-NH_2-F) $C_3H_6FNO_2$		3821-81-6	107.08	2 – 20% [80]
Ciclofosfamida (CP) $C_7H_{15}Cl_2N_2O_2P$		50-18-0	261.08	10 - 20% [81]
Acroleína (AC) CH_2CHCHO		107-02-8	56.06	Informações imprecisas [82]
Gemcitabina (GEM) $C_9H_{11}F_2N_3O_4$		95058-81-4	263.19	10% [79]
2´-deoxi-2´,2´-difluorouridina (2-DOH-DiF) $C_9H_{10}F_2N_2O_5$		114248-23-6	264.19	10% [79]

A área de estudo compreende o município de Barretos, localizado no interior do estado de São Paulo com cerca de 112 mil habitantes [83], conhecido internacionalmente pela presença do Hospital de Câncer de Barretos (HCB), um dos maiores e mais sofisticados centros de tratamento oncológico da América Latina, com cerca de 11.000 novos casos de câncer por ano, 100% cobertos pelo Sistema Único de Saúde (SUS) [84]. O HCB recebe diariamente cerca de 200 pacientes no Centro de Terapia Infusional, para realização de terapia medicamentosa para o tratamento do câncer.

O município de Barretos possui atualmente sete estações de tratamento de esgoto em atividade. A água residuária do HCB é lançada juntamente com dejetos dos efluentes domésticos e industriais dos bairros próximos na ETE IV. Esta é a única estação de tratamento de lodo ativado existente no município de Barretos. Neste sistema de tratamento, a água retorna aos mananciais.

2. JUSTIFICATIVA

A presença de micropoluentes de difícil degradação, que afetam a qualidade da água e com potencial de causar danos à saúde e ao meio ambiente, representa um dos tópicos mais relevantes para mudanças de políticas públicas.

No Brasil, a legislação vigente permite o descarte de antineoplásicos apenas com base no tratamento dos efluentes em ETE's municipais, sem fixar parâmetros para as emissões.

Além disso, o impacto do descarte de drogas como os antineoplásicos e seus metabólitos nos efluentes hospitalares no Brasil é pouco explorado. O município de Barretos, SP, por possuir o maior hospital especializado no tratamento de combate ao câncer do Brasil com alto consumo diário de antineoplásicos, pode ser considerado local estratégico para o estudo da presença destes compostos e seus metabólitos em efluentes.

Tendo em vista que dentre os antineoplásicos alvo deste estudo, apenas o 5-FU foi analisado no Brasil e que, entre os metabólitos que serão abordados, nenhum teve sua presença no meio ambiente avaliada, e que, devido as características citotóxicas e genotóxicas destes compostos, os mesmos podem interferir no ecossistema e gerar impactos ao meio ambiente, a realização desta caracterização, desde a detecção das drogas até avaliação ambiental por meio de ecotoxicidade, citotoxicidade e genotoxicidade, torna-se de extrema importância a nível nacional e internacional, possibilitando que novas políticas públicas sejam criadas.

3. OBJETIVOS

Avaliar a presença dos antineoplásicos 5-FU, GEM, CP e seus principais metabólitos no efluente do HCB, e, com isso, verificar sua ecotoxicidade, citotoxicidade e genotoxicidade.

Objetivos específicos

- Avaliar a presença dos antineoplásicos 5-FU, GEM, CP, e dos metabólitos 3-NH_2-F, 2-DOH-DiF e AC no efluente do HCB, bem como na entrada e saída dos efluentes da ETE IV do Município de Barretos, SP;
- Realizar análise de toxicidade aguda e risco ambiental dos antineoplásicos 5-FU, GEM, CP, e dos metabólitos 3-NH_2-F, 2-DOH-DiF e AC com *zebrafish* (*Danio rerio*);
- Avaliar, de acordo com as concentrações obtidas dos antineoplásicos e seus metabólitos no efluente, a citotoxicidade pelo ensaio de MTS e a genotoxicidade por ensaio micronúcleo em linhagem celular de fígado humano (HepG2); e
- Fornecer dados científicos aos órgãos ambientais que contribuam com políticas públicas futuras relacionadas ao tema.

4. ARTIGOS

Objetivos contemplados:

- Avaliação da presença dos antineoplásicos 5-FU, GEM, CP, e dos metabólitos 3-NH_2-F e 2-DOH-DiF no efluente do HCB, bem como na entrada e saída dos efluentes da ETE IV do Município de Barretos, SP;
- Análise de toxicidade aguda e risco ambiental dos antineoplásicos 5-FU, GEM, CP, e dos metabólitos 3-NH_2-F e 2-DOH-DiF com *zebrafish* (*Danio rerio*); e
- Fornecimento de dados científicos aos órgãos ambientais que contribuam com políticas públicas futuras relacionadas ao tema.

Os resultados presentes nesta seção foram publicados na revista *Environmental Pollution* (Fator de impacto 2021: 8.071):

4.1. Detection of anti-cancer drugs and metabolites in the effluents from a large Brazilian cancer hospital and an evaluation of ecotoxicology

de Oliveira Klein M, Serrano SV, Santos-Neto Á, da Cruz C, Brunetti IA, Lebre D, Gimenez MP, Reis RM, Silveira HCS.
Environ Pollut. 2021 Jan 1;268(Pt A):115857. doi: 10.1016/j.envpol.2020.115857.

Environmental Pollution 268 (2021) 115857

Contents lists available at ScienceDirect

Environmental Pollution

journal homepage: www.elsevier.com/locate/envpol

Detection of anti-cancer drugs and metabolites in the effluents from a large Brazilian cancer hospital and an evaluation of ecotoxicology☆

Mariana de Oliveira Klein [a], Sergio V. Serrano [a, b], Álvaro Santos-Neto [c], Claudinei da Cruz [d], Isabella Alves Brunetti [d], Daniel Lebre [e], Maíse Pastore Gimenez [e], Rui M. Reis [a, f, g], Henrique C.S. Silveira [a, h, *]

[a] Molecular Oncology Research Center, Barretos Cancer Hospital, Barretos, São Paulo, 14784-400, Brazil
[b] Barretos School of Health Sciences-FACISB, Barretos, São Paulo, 14785-002, Brazil
[c] São Carlos Institute of Chemistry, University of São Paulo, São Carlos, São Paulo, Brazil
[d] University Center of the Barretos Educational Foundation (UNIFEB), Barretos, São Paulo, Brazil
[e] Center for Applied Mass Spectrometry, Sao Paulo, Brazil
[f] Life and Health Sciences Research Institute (ICVS), Medical School, University of Minho, Braga, Portugal
[g] ICVS/3B's-PT Government Associate Laboratory, Braga/Guimarães, Portugal
[h] University of Cuiabá, Cuiabá, Mato Grosso, Brazil

ARTICLE INFO

Article history:
Received 18 May 2020
Received in revised form
5 September 2020
Accepted 13 October 2020
Available online 16 October 2020

Keywords:
Anti-cancer drugs
Hospital wastewater
Wastewater treatment plant
Liquid chromatography-mass spectrometry
Acute toxicity

ABSTRACT

The use of chemotherapy agents has been growing worldwide, due to the increase number of cancer cases. In several countries, mainly in Europe countries, these drugs have been detected in hospitals and municipal wastewaters. In Brazil this issue is poorly explored. The main goal of this study was to assess the presence of three anti-cancer drugs, 5-fluorouracil (5-FU), gemcitabine (GEM) and cyclophosphamide (CP), and two metabolites, alpha-fluoro-beta-alanine (3-NH_2-F) and 2′-deoxy-2′,2′-difluorouridine (2-DOH-DiF), in effluents from a large cancer hospital, in the municipal wastewater treatment plant (WWTP) influent and effluent, and also to evaluate toxicity of the mixtures of these compounds by ecotoxicological testing in zebrafish. The sample collections were performed in Barretos Cancer Hospital of the large cancer center in Brazil. After each collection, the samples were filtered for subsequent Liquid Chromatography Mass Spectrometry analysis. The presence of CP, GEM, and both metabolites (3-NH_2-F and 2-DOH-DiF) were detected in the hospital wastewater and the WWTP influent. Three drugs, GEM, 2-DOH-DiF and CP, were detected in the WWTP effluent. Two drugs were detected below the limit of quantification, 2-DOH-DiF: <LOQ (above 1400 ng L^{-1}) and CP: <LOQ (above 300 ng L^{-1}), and GEM was quantified at 420 ng L^{-1}. Furthermore, 2-DOH-DiF (116,000 ng L^{-1}) was detected at the highest level in the hospital wastewater. There were no zebrafish deaths at any of the concentrations of the compounds used. However, we observed histological changes, including aneurysms and edema in the gills and areas of necrosis of the liver. In summary, we found higher concentrations of CP, GEM and both metabolites (3-NH_2-F and 2-DOH-DiF) were detected for the first time. There is currently no legislation regarding the discharge of anti-cancer drugs in effluents in Brazil. This study is first to focus on effluents from specific treatments from a large cancer hospital located in small city in Brazil.

1. Introduction

There are an estimated 14.1 million new cases of cancer each year in the world, with an annual incidence projected to rise to 21.4 million new patients by 2032. (Ferlay et al., 2015). With this increasing rate of new cases of cancer, there will be more extensive use of chemotherapy agents. These drugs are highly cytotoxic and intercalate into DNA, causing damage, especially to cell replication and signalling or to the inhibition of cell proliferation (Bialk-Bielinska et al., 2017). Therefore, there is a growing concern about environmental contamination with anti-cancer drug residues.

☆ This paper has been recommended for acceptance by Da Chen.

* Corresponding author. Barretos Cancer Hospital - Molecular Oncology Research Center, Barretos, 14784-400, São Paulo, Brazil.
E-mail address: henriquecssilveira@gmail.com (H.C.S. Silveira).

https://doi.org/10.1016/j.envpol.2020.115857
0269-7491/

These drugs have non-selective action; they affect all replicating cells, and thus have carcinogenic, mutagenic, and teratogenic potential (Brunton et al., 2011; Souza et al., 2018). Since some of these drugs also act as endocrine disruptors, they are believed to cause damage to humans and animals even at low doses (Mullot et al., 2010). It is hypothesized that, due to their mode of action, basically, all eukaryotic organisms are prone to damage caused by these compounds (Bialk-Bielinska et al., 2017; Mullot et al., 2010).

Anti-cancer or antineoplastic drugs are not entirely broken down by human metabolism and are passed into the effluents through the urine and faeces of cancer patients at different levels. Chemotherapeutic contamination of effluents may be of the order of nanograms to micrograms (Isidori et al., 2016; Mater et al., 2014), and due to their low biodegradability, they remain in the sewage treatment system and the environment even after treatment in wastewater treatment plants (Llewellyn et al., 2011).

Several studies performed in different countries, but predominantly in Europe, indicate the presence of anti-cancer drugs in wastewater effluent discharged from hospitals and wastewater treatment plants (WWTP) at concentrations in the order of magnitude of ng L^{-1} to $\mu g L^{-1}$ (Aherne et al., 1990; Castiglioni et al., 2005; Cesen et al., 2015; Ferrando-Climent et al., 2014; Ferrando-Climent et al., 2013; Ferre-Aracil et al., 2016; Franquet-Griell et al., 2015; Gómez-Canela et al., 2012; Gouveia et al., 2020; Isidori et al., 2016; Kosjek et al., 2013; Kovalova et al., 2009; Kümmerer et al., 2016; Mahnik et al., 2007; Mullot et al., 2009; Negreira et al., 2013; Santana-Viera et al., 2019; Santos et al., 2018; Yin et al., 2010). On the other hand, the recent study realized by Nassour et al. (2020) which conducted a systematic review with 75 publications, found that the 4 most studied anticancer agents in WWTP were cyclophosphamide (39 publications), tamoxifen (30 publications), ifosfamide (29 publications) and methotrexate (17 publications) with concentrations measured ranging between 0.01 and 86,200 ng L^{-1}, possibly because they are the most commonly used anticancer drugs in the corresponding countries.

The target drugs of this study were cyclophosphamide (CP), 5-fluorouracil (5-FU) and gemcitabine (GEM) as well as two metabolites, alpha-fluoro-beta-alanine (3-NH_2-F), a 5-FU metabolite, and 2′-deoxy-2′,2′-difluorouridine (2-DOH-DiF), a gemcitabine metabolite. These drugs were selected due to their percentage of excretion and use in Brazil and other countries as published in past studies (Kasel et al., 2004; Micromedex, 2005; Weigel, 1999). Therefore, this study was performed in a large cancer center in Latin America studying the selected the anticancer drugs based on the quantity of consumption in this Institution. The Barretos Cancer Hospital is located in the city of Barretos (São Paulo state, Brazil), was founded in 1962 to attend oncological patients from the local region and other rural areas of the State of São Paulo (Palmero et al., 2016). Currently, the hospital is recognized as one of the largest and most sophisticated oncological treatment centers in Latin America, with approximately 4000 medical visits per day, with an around 120 beds, that receives approximately 200 patients daily for chemotherapy treatment. Many patients come from different regions of the country, totalizing about 11,000 new cases per year with all cancer treatment costs being covered entirely by the Brazilian Public Healthcare System (SUS) (Palmero et al., 2016). The main types of cancers treated in the hospital, based on the year 2018, correspond to non-melanoma skin (22.8%), tumors of the digestive system (15.5%), urological tumors (13.6%), tumors of breast (11.6%), gynecological tumors (10%) and head and neck tumors (8.3%), and the most used drugs in order of consumption, in mg, were 5-fluorouracil, gemcitabine, ifosfamide, cyclophosphamide, carboplatin, paclitaxicel, asparaginase and transtuzumab.

CP is used for the treatment of brain and bone cancer, leukemia and other autoimmune diseases (Colvin, 1999). It is an alkylating agent that must be metabolized in the liver by the P450 cytochrome complex enzymes, releasing metabolites such as non-nitrogen mustard, which intercalates into DNA (Chan et al., 1994; Colvin, 1999). CP is considered a persistent compound and is already found in municipal effluents and rivers (Kiffmeyer et al., 1998; Kümmerer et al., 2000). 5-FU and GEM have similar modes of action, with pyrimidine base structures, which affect the S phase of the cell cycle and inhibit enzymes that are crucial for DNA replication (Xie, 2012). GEM is used to treat breast, pancreatic, ovarian and lung cancer (Toschi et al., 2005). 5-FU is administered in cases of colorectal and breast cancer, as well as aerodigestive tract cancers (Longley et al., 2003).

Some studies have evaluated the toxicity and genotoxicity of these compounds, such as the drugs as CP and 5-FU. Araujo et al. (2019) observed morphological changes in the intestinal-condition parameters and in the interocular distance, as well as mutagenic effects on *L. catesbeianus* tadpoles with administration of quantities between 0.2 $\mu g L^{-1}$ and 123.5 $\mu g L^{-1}$ (environmental concentrations) of CP and 5-FU. Misik et al. (2019) evaluated and described the results of risk assessments concerning their environmental safety, which are based on data generated in the framework of a coordinated European Union project ("Cytothreat"). This study concluded that the most sensitive species in experiments concerning acute toxic and long term effects were in general crustacea (daphnids), and after chronic treatment, the most pronounced effects were detected with imatinibe (IM) followed by cisplatin (CDDP) and 5-FU. Grzesiuk et al. (2019) demonstrated that exposure to CP at concentrations registered in the environment significantly affect Daphnia species in measured parameters as survivorship, number of eggs, growth rate, and population growth rate. All of these studies emphasize that anti-cancer effluent, even at low concentrations, can affect aquatic biota.

These evaluations of toxicity and genotoxicity have typically studied the effects of each drug separately, with few studies evaluating their combined effects as environmental contaminants. For this type of analysis, Zebrafish (*D. rerio*) has been found to be a safe and attractive model for use in bioassays due to its small size, genetics, breeding capacities, and especially its molecular and physiological similarity to humans (Chakravarthy et al., 2014) also for analysis with anti-cancer drugs (Gajski et al., 2016; Karas et al., 2019; Kovacs et al., 2016; Misik et al., 2019; Novak et al., 2017) and cancer development studies (Farooq et al., 2019; Yen et al., 2014).

Furthermore, cytostatic drugs as ifosfamide (IF), CP and their metabolites were quantified in hospital wastewater and municipal wastewater treatment plant (WWTP) in a hospital specialized in the treatment of cancer in Slovenia, Europe (Cesen et al., 2016a). The drugs levels detected were 2690, 47.0, 13,200, 2100 and 178 ng L^{-1} for CP, IF, carboxy-CP, N-decl-CP and keto-CP, respectively, while in influent and effluent samples concentrations were below LOQs.

In Europe antineoplastic drugs are the only pharmaceutical products explicitly classified as "hazardous" under the Waste Framework Directive of the European Commission (Directive, 2008). However, in Brazil and most low to middle-income countries, less than 40% of the municipal wastewater is treated (SNIS, 2018). Moreover, the Brazilian regulatory laws that refer to the discharge of effluents do not establish precise limits for anti-cancer or any other drugs (ALESP, 1976; ANVISA, 2018; CONAMA, 2011). There have been very few previous ecotoxicological risk assessments performed in Brazil. Recently, Souza et al. (2018) used high-performance liquid chromatography with fluorescence detection (HPLC-FLD) to evaluate the presence of anti-cancer drugs in a general hospital wastewater at the following concentrations:

irinotecan (<limit of detection (LOD) of 3.40 μg L^{-1}), doxorubicin (<LOD of 4.69 μg L^{-1}), epirubicin (<LOD of 6.22 μg L^{-1}) and daunorubicin (<LOD of 3.69 μg L^{-1}). In this way, we believe that this kind of study is extremely important for a deeper understanding of the presence and real impacts of these drugs and their interactions on ecosystems.

During the treatment in Barretos Cancer Hospital patients come of different Brazilian regions, usually reside in the same neighbourhood as the hospital and since, much of the excretion of the anti-cancer drugs and metabolites occurs when the patients are no longer at the hospital. The city of Barretos currently has seven active wastewater treatment plants. The wastewater from the Barretos Cancer Hospital is discharged along with the wastewater from the domestic effluents industrial waste from parts of the city close to wastewater treatment plant (WWTP). This facility is the only active sludge treatment plant in Barretos city. In this treatment system, the water returns to the environment Ribeirão das Pitangueiras stream.

Against this background, with a lack of clear guidelines in current legislation in Brazil we undertook evaluation of effluent from the largest cancer treatment center in Latin America. Also, it is known that the anticancer drugs were not removed from all the effluents using conventional technologies at the WWTP's. Moreover, the environmental impact of chemotherapy residues in the effluent treatment systems in Brazil is unknown. Therefore, the main goal of this study was to assess the presence of three most commonly used anti-cancer drugs in the Barretos Cancer Hospital, 5-FU, GEM and CP, and two metabolites, 3-NH_2-F and 2-DOH-DiF, during a six-month collection period of wastewater from Cancer Hospital, the influents and effluents of the municipal wastewater treatment plant (WWTP) and also the acute toxicity assessment of the mixtures of these compounds by ecotoxicological testing using zebrafish.

2. Materials and methods

2.1. Standards, reagents, and handling of the anti-cancer drugs

Formic acid, triethylamine, phenyl isothiocyanate (PITC) and all of the standards (drugs and metabolites) were acquired from Sigma-Aldrich® (Missouri, USA). Acetonitrile (ACN) and n-hexane were acquired from Tedia® (Ohio, USA), and methanol (MeOH) was acquired from J.T. Baker® (New Jersey, USA). The ultrapure water was obtained using a Millipore Milli-Q Integral 3 system. Further information on the drugs and metabolites as well as the CAS numbers are described in the Supplementary material of the supplementary material.

The anti-cancer drugs and their metabolites are toxic substances that require special caution when handling them. All the stock solutions were prepared in safe environments in laminar flow cabinets and with proper safety equipment. All the material contaminated with the drugs was disposed of as hazardous waste to receive proper treatment.

2.2. Preparation of analytical standard stock solutions

The solutions were prepared separately for each analytical standard. Approximately 1.00 mg (99%) of the analytical standard was weighed in a 2 mL eppendorf amber flask. Then, 1000 μL (or the volume required to reach the desired final concentration) of 50% ACN/50% H_2O (v/v) solution was added. The solutions were stored in a refrigerator at 3 °C, reaching a final concentration of 1.00 mg mL^{-1} (1000 μg mL^{-1}).

2.3. Study sites and sample collection

The study area was the city of Barretos (São Paulo state) in Brazil, with approximately 120,000 inhabitants (IBGE, 2016). The economy of the city is mostly composed of agriculture (primarily, by cane sugar, oranges and soybeans), trade and services (IBGE, 2016).

The baseline levels of contamination used for this study were similar to those of Fernando-Climent et al., (2014), who evaluated WWTP effluent in Girona (96.000 habitants), Italy a small city which is the central referral hospital for patients at that part of Italy. The collection points of the effluent samples were determined according to other studies (Cesen et al., 2015; Ferrando-Climent et al., 2014; Isidori et al., 2016) and comprised sampling the hospital wastewater as well as the WWTP IV influent and effluent.

The collection samplings were performed in two periods: (i) dry season (July to September) and (ii) rainy season (November to January). In total there were six collection campaigns. In each campaign, effluent samples were collected in two days, one sample per collection day, per collection point (Hospital wastewater, WWTP influent and WWTP effluent), totaling twelve samples per collection point (Fig. 1). Wastewater samples, 500 mL from each sampling point were collected in sterilized glass bottles, immediately (less than 30 min) transported to the laboratory in a cooler box, for subsequent filtration using a PES (polyethersulfone) 0.22 μM filter from Sarstedt®, placed in an 50 mL amber glass flask, stored in cold chamber at 5 °C for three days and analysed by Liquid Chromatography Mass Spectrometry(LC-MS/MS).

2.4. Analytical method

For the method development and sample analysis two LC-MS/MSsystems available were used: (1) HPLC system (quaternary pump, degasser) with an autoinjector model series 1100 from Agilent Technologies® coupled with a triple quadrupole mass spectrometer (MS/MS) model API 2000™ (Sciex, Canada); and (2) HPLC system (binary pump, degasser) with an autoinjector model series 1260 from Agilent Technologies® coupled with a hybrid triple quadrupole linear ion trap mass spectrometer (MS/MS) model 3200 QTRAP™ (Sciex, Canada). The compounds were ionized by electrospray (ESI) and analysed in the MS/MS system by Multiple Reaction Monitoring (MRM) mode, that allowed their quantitation in hospital wastewater samples. The results were analysed by the Analyst software version 1.4.2 and 1.5.1 (Sciex, Canada).

The hospital effluent samples were prepared in two different procedures: (1) For the analysis of the CP, GEM and 2-DOH-DiF compounds the strategy used was the sample filtration using an 0,45 μm pore membrane and direct injection into the LC-MS/MS; and (2) To increase the selectivity by improving the retention time of the molecules, the analytical method for the compounds 3-NH_2-F and 5-FU in the hospital effluent samples included the derivatization with the agent phenyl isothiocyanate (PITC) before LC-MS/MS analysis (Kwanyuen and Burton, 2010; Zheng et al., 2015).

2.4.1. Sample preparation for reaction with PITC

The PITC solution (phenyl isothiocyanate, d = 1.132 g mL^{-1}) was prepared at the concentration of 0.1 mol L^{-1} in ACN by diluting 12 μL of PITC with 988 μL of ACN (final vol. of 1.0 mL), followed by agitation (Shi et al., 2013).

The triethylamine solution (d = 0.73 g mL^{-1}) was prepared at the concentration of 1.0 mol L^{-1} in ACN by diluting 139 μL of triethylamine with 861 μL of ACN (final vol. of 1 mL). For sample preparation, 100 μL of the hospital wastewater sample (filtered)

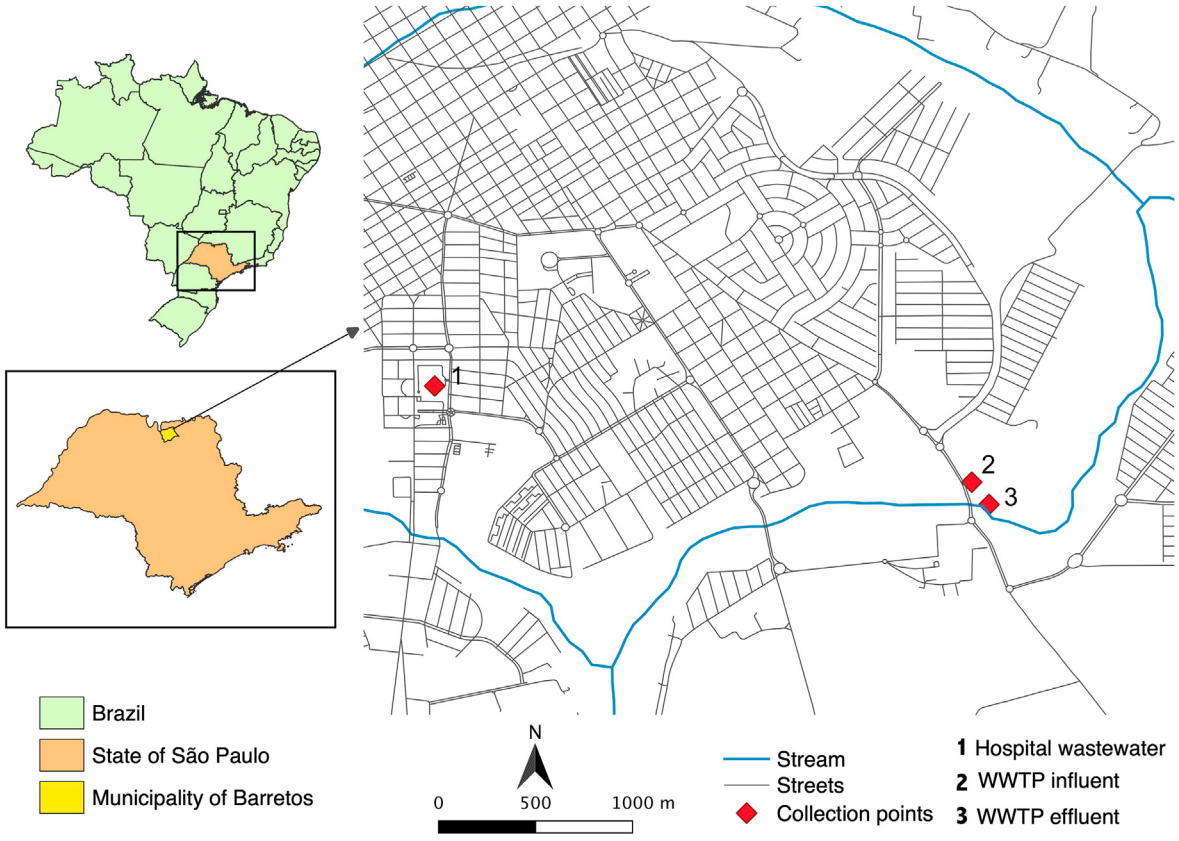


Fig. 1. Identification map of the effluent collection points in the municipality of Barretos, São Paulo State, Brazil.

was aliquoted in a 1.5 mL eppendorf tube and 50 μL of PITC solution and 50 μL of the triethylamine solution were added, followed by vortexing for 10 s and finally incubation for 20 min at 40 °C under agitation (350 rpm). The sample was removed from the tube, and 200 μL of n-hexane (clean-up) was added, followed by vortexing for 30 s. Twenty microliters of the aqueous phase was removed and transferred to the vial or well (96-well injection plate) containing 180 μL of H_2O for injection into the LC–MS/MS system.

2.4.2. LC-MS/MS analysis

The chromatographic separation of the compounds was performed in a 3 μm ultra-aqueous 150 × 2.1 mm reverse-phase column from Restek® (room temperature) by applying a flow rate of 200 μL min^{-1} for the following mobile phases: A, aqueous solution containing 0.1% of formic acid and B, 50% ACN/50% ultrapure water (v/v) solution containing 0.1% formic acid. The elution gradient was as follows: 0.00 min, 10% B until 0.50 min; at 0.51 min, switch to 100% B until 9 min; and at 9.01 min switch back to 10% B until the end of the 14 min chromatographic run. The injection volume of the sample was 5 μL.

The samples were analysed using the ESI-MS/MS system using two acquisition methods, the positive mode (monitored compounds 3-NH_2-F, CP, and GEM) and negative mode (monitored compounds 5-FU and 2-DOH-DiF as well as the reaction products with PITC of 3-NH_2-F and 5-FU). For the positive mode, the following ionisation conditions were optimised: capillary voltage (+)5200 V; curtain gas 20 a.u. (arbitrary units); temperature 350 °C; heater gas 55 a.u.; and nebuliser gas 50 a.u. For the negative mode, the ionisation conditions were optimised as described above, except for the applied voltage, which was (−) 4200 V. The MS/MS system was operated in the multiple reactions monitoring (MRM) mode, selecting the precursor ion in the first quadrupole and two product ions (one for quantification and another for qualitative confirmation) in the third quadrupole, which were fragmented in the second quadrupole, for each analysed compound.

The limits of detection (LODs) and quantification (LOQs) were estimated for the analysed compounds, as described in Table 1. They were obtained by spiking a blank domestic sewage wastewater at 250 ng mL^{-1} and extrapolating the obtained signal-to-noise ratio to 3 and 10, respectively, for LOD and LOQ. In order to have a simple method to estimate the concentration of the analytes in the samples, a single point calibration was used for quantification by spiking 250 ng mL^{-1} of the standards into a sample previously tested as blank. Since a simple filtration was employed for the preparation, a recovery close to 100% is expected and, therefore, matrix effect is estimated equal to process efficiency (Table 1) and measured as the relation of the analyte spiked in blank sample matrix and the analyte spiked in water (Matuszewski et al., 2003).

2.5. Ecotoxicology and histology test

For the acute toxicity tests adult specimens of Zebrafish (*Danio rerio*) weighing between 1.50 and 2.0 g were acclimated in a bioassay room in 100 L aquariums with continuous aeration system promoted by air pumps, with temperature at 25.0 ± 2 °C, with a 12-h photoperiod of light, and fed freely, once a day, with commercial ration (28% crude protein) for ten days. After acclimatization, part of the fish batch was submitted to the sensitivity test with the substance reference potassium chloride (KCl) (ABNT, 2016) with five concentrations 0.52; 0.91; 1.60; 2.81; 4.92 g L^{-1}), with one control, with three replicates and three fish per replica. The total

Table 1
Limits of detection (LOD) and quantification (LOQ) for the analytes and their respective electrospray ionisation modes of analysis.

Substance	Abbreviation	MRM Transition (*m/z*)[a]	ESI Mode	Process efficiency[b]	Analysis 3200 QTRAP[c]		Analysis API 2000[d]	
					LOD[e] (ng L^{-1})	LOQ[f] (ng L^{-1})	LOD[e] (ng L^{-1})	LOQ[f] (ng L^{-1})
5-Fluorouracil	5-FU	128.9 > 42.0	Negative	40%	2650	8840	4000	13,400
5-Fluorouracil + PITC	5-FU + PITC	261.9 > 110	Negative	8%	13,000	43,500	20,100	67,000
Alpha-fluoro-beta-alanine	3-NH_2-F	108 > 90	Positive	−95,5%	5700	19,000	53,600	179,000
Alpha-fluoro-beta-alanine + PITC	3-NH_2-F + PITC	240.9 > 106	Negative	−4%	1600	5340	660	2200
Cyclophosphamide	CP	261 > 140	Positive	6%	300	1000	1520	5080
Gemcitabine	GEM	264 > 112	Positive	−5%	600	2000	30	100
2′-Deoxy-2′,2′-difluorouridine	2-DOH-DiF	263 > 220	Negative	43%	1400	4720	1130	3780

[a] Showed an MRM quantitation transition.
[b] Matuszewski et al., (2003).
[c] Analysis using 3200 QTRAP − Nov/2017, Dez/2017 e Jan/2018.
[d] Analysis using API 2000−Jul/2017, Aug/2017 e Sep/2018.
[e] LOD − Calculated by Signal/Noise = 3.
[f] LOQ − Calculated by Signal/Noise = 10.

number of animals per concentration was 9 animals to meet the recommendation of the maximum density of 1.0 g L^{-1} of fish for exposure to compounds. The 50% lethal concentration (LC50; 48 h) of sodium chloride for zebrafish was 1.46 g L^{-1}, with a 95% confidence interval between 1.07 and 1.98 g L^{-1}, and correlation between mortality response and concentration was $R^2 = 0.94$.

In the static acute toxicity tests (LC50; 48 h) of the isolated drugs (CP, 5-FU and GEM) the fish were exposed to concentrations of 0.0, 0.1, 1.0, 3.4, 11.6, 36.4, and 118.0 mg L^{-1}, with three replicates and three fish in each replica, totaling n = 9. Test glass aquariums with a capacity of 3.0 L of test solution were used, maintaining the maximum density of 1.0 g of L^{-1} fish as recommended by Aquatic ecotoxicology - Acute toxicity - Test method with fish (Cyprinidae) (ABNT, 2016). For the performance of the acute toxicity test, the initial water quality was temperature 25.0 ± 2.0 °C, pH 7.5 and hardness of 45.0 ± 2.0 mg $CaCO_3$ L^{-1}, >4.0 mg L^{-1} of dissolved oxygen (ABNT, 2016).

Then, a definitive acute toxicity test, for further histology test, was performed at the following concentrations: 0.1 mg L^{-1} of mixture 1 (0.0259 mg GEM + 0.116 mg 2-DOH-DiF + 0.020 mg CP and 0.0135 mg 3- NH 2 −F); 1.0 mg L^{-1} of mixture 2 (0.0518 mg GEM + 0.232 mg 2-DOH-DiF + 0.040 mg CP and 0.027 mg 3-NH2-F); and 3.4 mg L^{-1} of mixture 3 (0.0777 mg GEM + 0.348 mg 2-DOH-DiF + 0.060 mg CP and 0.0405 mg 3-NH2-F) with three replicates and three fish per replica, totaling n = 9. The choice of concentrations used in zebrafish exposure was based on the maximum concentration of each compound detected in the wastewater in this study. Then we perform the tests with the maximum concentrations of each detected compound (mixture 1), and this value doubled (mixture 2) and tripled (mixture 3). Test glass aquariums with a capacity of 3.0 L of test solution were used, maintaining the maximum density of 1.0 g of L^{-1} fish as recommended by Aquatic ecotoxicology - Acute toxicity - Test method with fish (Cyprinidae) (ABNT, 2016). The use of mixed concentrations of drugs based on the concentrations obtained in the residue analysis aims to simulate the possible environmental exposures that may occur for zebrafish. The cytoxicity and genotoxicity in liver cells of Zebrafish (*D. rerio*) of mixtures of 5-fluorouracil (5-FU) and cisplatin (CDDP) based on the maximum concentration present in the effluent of a hospital oncology ward (Novak et al., 2017).

The LC50; 48 h estimate was performed with Trimmed Spearman Karber software (Hamilton et al., 1977). During the definitive test (0, 24 and 48 h) the following water quality variables were monitored: pH, dissolved oxygen (mg L^{-1}), temperature (°C) and electrical water conductivity (μS cm^{-1}).

After testing for acute zebrafish drug toxicity, the gills and liver were removed and immersed in buffered formaldehyde fixative solution (0.1 M phosphate buffer; pH 7.2) for 24 h. After fixation, the fragments were dehydrated, diaphanized and included in Histosec® (Merck). Next, a manual microtome microtomy was performed, obtaining 5.0 μm thick sections, which were stained with Hematoxylin-Eosin and PAS (Schiff Periodic Acid) (Behmer et al., 1976).

2.6. Data analysis

The statistical analyses and preparation of graphs were performed using the SPSS software from *Windows*® version 21.0. The quantitative data obtained in the tests were described using measures such as the mean, standard deviation, minimum and maximum. The concentrations of drugs and metabolites in the effluents were evaluated in regard to the distribution of the variables according to each collection point and at the different months, in addition to percentage calculations.

3. Results

3.1. Detection of compounds at environment

The limits of detection (LOD) and quantification (LOQ) were determined for each compound, which are described in Table 1. Four of the five target compounds were detected in the analyses; only 5-FU was not detected due to the inherent difficulties of detecting the low molecular weight of this compound for establishing LOD and LOQ limits.

GEM, CP, 3-NH_2-F, and 2-DOH-DiF were detected in the hospital wastewater and in the WWTP influent, and three drugs (GEM, 2-DOH-DiF, CP) were detected in the effluents treated by the wastewater treatment plant located approximately 5 km from the initial discharge point, only GEM was quantified at 420 ng L^{-1} (Table 2) (Figs. 1 and 2).

In comparison to the anti-cancer drugs, the metabolites were detected in more samples from the hospital wastewater and WWTP influent. The 5-FU metabolite (3-NH_2-F) was detected in 5 of the 12 collections from the hospital wastewater and in 2 of the 12 collections from the WWTP influent. The GEM metabolite (2-DOH-DiF) was detected in 6 of the 12 samples of hospital wastewater, 2 of the 12 samples of the WWTP influent and 1 of the 12 samples of WWTP effluent. Therefore the 3-NH_2-F was not detected in the WWTP effluent (Table 2) (Fig. 3).

Regarding the drugs identified, CP was detected in 5 of the 12 samples of hospital wastewater, in 4 of the 12 samples of WWTP

Table 2
Anti-cancer drug and metabolite concentrations determined in the wastewater samples.

Sample identification			Concentrations of the drugs (ngL[1])				
Local	Season	Date and time	CP	5-FU	3-NH₂-F	GEM	2-DOH-DiF
Hospital wastewater	Dry[a]	7/19/2017−2 p.m.	<LOD	<LOD	<LOD	**570**	**13,080**
		7/20/2017−2 p.m.	<LOD	<LOD	<LOD	**<LOQ**	**14,500**
		September 8, 2017−2 p.m.	<LOD	<LOD	**3020**	**160**	<LOD
		October 8, 2017−2 p.m.	<LOD	<LOD	**12,100**	<LOD	**116,000**
		9/13/2017−2 p.m.	<LOD	<LOD	<LOD	<LOD	<LOD
		9/14/2017−2 p.m.	**20,700**	<LOD	<LOD	<LOD	**7490**
	Rainy[b]	August 11, 2017−2 p.m.	**<LOQ**	<LOD	**11,100**	<LOD	<LOD
		September 11, 2017−2 p.m.	<LOD	<LOD	<LOD	<LOD	<LOD
		June 12, 2017−2 p.m.	**29,100**	<LOD	**18,200**	<LOD	**6800**
		July 12, 2017−2 p.m.	<LOD	<LOD	<LOD	**25,900**	**74,200**
		1/24/2018−2 p.m.	<LOD	<LOD	<LOD	<LOD	<LOD
		1/25/2018−2 p.m.	**<LOQ**	<LOD	**<LOQ**	<LOD	<LOD
WWTP influent	Dry	7/19/2017−2 p.m.	**<LOQ**	<LOD	<LOD	**750**	<LOD
		7/20/2017−2 p.m.	<LOD	<LOD	<LOD	**<LOQ**	<LOD
		September 8, 2017−2 p.m.	<LOD	<LOD	<LOD	<LOD	<LOD
		October 8, 2017−2 p.m.	<LOD	<LOD	<LOD	**110**	**5400**
		9/13/2017−2 p.m.	<LOD	<LOD	<LOD	**<LOQ**	<LOD
		9/14/2017−2 p.m.	<LOD	<LOD	<LOD	<LOD	<LOD
	Rainy	August 11, 2017−2 p.m.	<LOD	<LOD	<LOD	<LOD	<LOD
		September 11, 2017−2 p.m.	<LOD	<LOD	<LOD	<LOD	<LOD
		June 12, 2017−2 p.m.	**<LOQ**	<LOD	<LOD	<LOD	**<LOQ**
		July 12, 2017−2 p.m.	<LOD	<LOD	**<LOQ**	<LOD	<LOD
		1/24/2018−2 p.m.	**<LOQ**	<LOD	**13,500**	<LOD	<LOD
		1/25/2018−2 p.m.	**<LOQ**	<LOD	<LOD	<LOD	<LOD
WWTP effluent	Dry	7/20/2017−4:15 p.m.	<LOD	<LOD	<LOD	**420**	<LOD
		7/21/2017−4:15 p.m.	<LOD	<LOD	<LOD	<LOD	<LOD
		October 8, 2017−4:15 p.m.	<LOD	<LOD	<LOD	<LOD	<LOD
		November 8, 2017−4:15 p.m.	<LOD	<LOD	<LOD	<LOD	<LOD
		9/14/2017−4:15 p.m.	<LOD	<LOD	<LOD	**<LOQ**	<LOD
		9/15/2017−4:15 p.m.	<LOD	<LOD	<LOD	<LOD	<LOD
	Rainy	September 11, 2017−4:15 p.m.	**<LOQ**	<LOD	<LOD	<LOD	<LOD
		October 11, 2017−4:15 p.m.	**<LOQ**	<LOD	<LOD	<LOD	<LOD
		July 12, 2017−4:15 p.m.	**<LOQ**	<LOD	<LOD	<LOD	<LOD
		August 12, 2017−4:15 p.m.	**<LOQ**	<LOD	<LOD	<LOD	<LOD
		1/25/2018−4:15 p.m.	**<LOQ**	<LOD	<LOD	<LOD	<LOD
		1/26/2018−4:15 p.m.	**<LOQ**	<LOD	<LOD	<LOD	**<LOQ**

WWTP: wastewater treatment plant.
[a] All campaigns carried out in the months of July, August and September 2017 were analysed by the equipment Analysis API 2000. The specifics LODs and LOQs were demonstrated in Table 1.
[b] All campaigns carried out in the months of November, December 2017 and January 2018 were analysed by the equipment 3200 QTRAP. The specifics LODs and LOQs were demonstrated in Table 1.

influent and in 6 of the 12 samples of WWTP effluent, although it was not quantified due the values were below the LOQ. GEM was detected in 4 of the 12 samples of hospital wastewater, 4 of the 12 samples of WWTP influent and 2 of the 12 samples of WWTP effluent, with one quantified and another below the LOQ (Table 2) (Fig. 3).

The only drug that was not detected in any of the six samplings was 5-FU, however, its metabolite (3-NH_2-F) was found in the hospital wastewater (<LOQ to 18,200 ng L^{-1}) and in the wastewater treatment plant influent (<LOQ to 13,500 ng L^{-1}) (Table 2) (Fig. 2).

The highest levels detected in the hospital wastewater corresponded to the GEM metabolite (2-DOH-DiF), with a concentration of 116,000 ng L^{-1} (Table 2) (Fig. 2). No significant differences were observed between the presence of the drugs in the hospital wastewater, WWTP influent and WWTP effluent between the rainy and dry seasons. Overall, the drug levels when detected in this study were higher in comparison to other studies performed in different countries as can be observed in Table 3.

3.2. Acute toxicity and histological of zebrafish (D. rerio)

In the acute toxicity tests of the isolated drugs (LC50; 48 h) there was no mortality at any concentration evaluated, thus the 50% lethal concentration of CP, 5-FU, and GEM were considered > 118.0 mg L^{-1} for *Zebrafish*. Exposure for 48 h to CP caused agitation in animals at concentrations of 3.4, 11.6, and 11.6 mg L^{-1} and lethargy in animals exposed to 118.0 mg L^{-1}. Exposure to 5-FU, and GEM did not cause any signs of toxicity in fish at any concentration.

In the evaluation of the acute effects of the drugs, the gills of the control (0.0 mg L^{-1}) and of the mixture 1 in the concentration of 0.1 mg L^{-1} (0.0259 mg GEM + 0.116 mg 2-DOH-DiF + 0.020 mg CP and 0.0135 mg 3-NH2-F) of zebrafish (*D. rerio*) were analysed. The structure of the gill arches is composed of cartilaginous tissue and dense connective cartilaginous tissue organized to support the blood vessels and the mucous lining the epithelial cells. From the gill arches both the primary lamellae and the secondary lamellae contain internal blood vessels lined with mucous cells, chloride cells, and single-layered cells. The interlamellar spaces presented the same general organization and cell types (Fig. 4).

The acute toxicity evaluation for mixture 2 at the concentration 1.0 mg L^{-1} (0.0518 mg GEM + 0.232 mg 2-DOH-DiF + 0.040 mg CP and 0.027 mg 3-NH2-F) showed cell hyperplasia coating of the secondary lamellae and interlamellar spaces and disorganization of the supporting structure of the secondary lamellae (Fig. 4).

For mixture 3 (Fig. 4), the 3.4 mg L^{-1} concentration of 0.0777 mg GEM + 0.348 mg 2-DOH-DiF + 0.060 mg CP and 0.0405 mg 3-NH2-F, in addition to the effects described for mixture 2, secondary

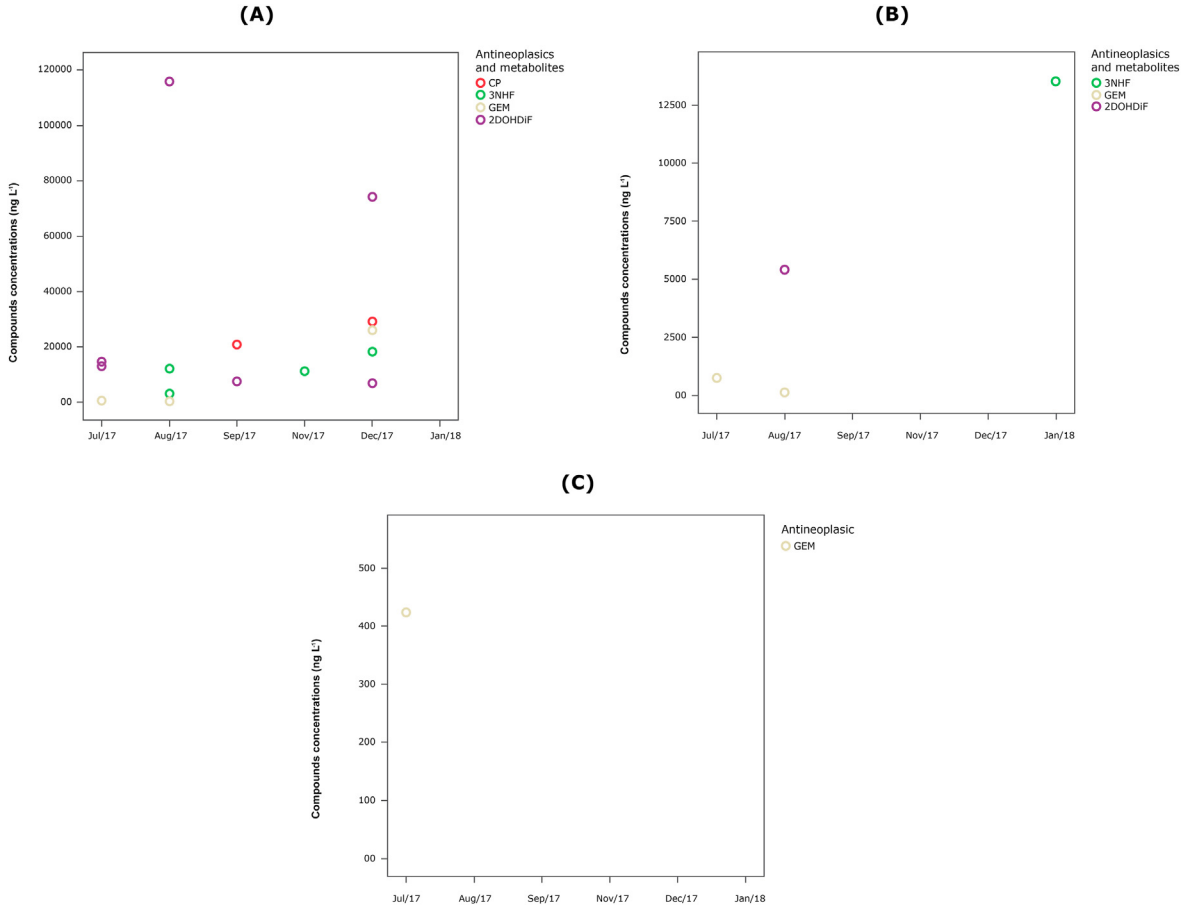


Fig. 2. Presence of anti-cancer drugs and metabolites in effluents by sampling and collection point. Identification of colours: red: cyclophosphamide; green: alpha-fluoro-beta−alanine; yellow: gemcitabine; and purple: 2′-deoxy-2′,2′-difluorouridine. A = concentration per collection point and per compound in the hospital wastewater; B = concentration per collection point and per compound in the WWTP effluent; and C = concentrations per collection point and per compound in the WWTP influent. For some collections, repeated samplings are shown because two collections were made.

lamella fusion, lamellar aneurysm and blood stasis, and subepithelial edema were also apparent (Fig. 4).

In the analysis of the zebrafish liver, in the control (0.0 mg L^{-1}) there was cordonal organization of hepatocytes, with irregular sinusoid capillaries intermingled with hepatocytes. These cells were rounded with a central nucleus. After exposure to mixtures 1 and 2 blood stasis in the sinusoid capillaries and in some regions of cordonal organization was observed. In mixture 3, in addition to the changes described above, some pyknotic nuclei, nuclei degeneration, loss of cytoplasm, necrosis points were also present in some regions of the liver (Fig. 4).

4. Discussion

The use of chemotherapy agents has been growing worldwide, due to the increased number of cancer cases. In several countries, mainly in Europe countries, these drugs have been detected in hospitals and municipal wastewaters. In the world there are deficiencies in current legislation about regulation of effluents treatments. In Brazil these issues are poorly explored. Furthermore, the cytotoxic properties and the carcinogenic, mutagenic, and teratogenic potential of residues of anti-cancer drugs in effluents from medical facilities is a reason for concern for public and environmental control agencies (Brunton et al., 2011; McKnight, 2003). This study was performed in the large cancer center treatment in Latin America, located in small city inside the State of São Paulo. We evaluated the presence of three anti-cancer drugs, 5-FU, GEM and CP, and two metabolites, 3-NH_2-F and 2-DOH-DiF, in cancer hospital effluents, in the WWTP influent and effluent, and also assessed the toxicity of the mixtures of these compounds by ecotoxicological testing in zebrafish.

Drugs and metabolites are excreted in the urine and faeces of cancer patients at different levels and have different administration regimens in the hospitals, which are factors that affect the amounts of the drugs detected in the effluents (Isidori et al., 2016; Kasel et al., 2004; Micromedex, 2005; Weigel, 1999).The continuous discharge of these persistent substances into the environment, even at low concentrations, can lead to their accumulation at levels considered toxic (Leblanc, 2004). In Brazil, where only approximately 40% of effluents are treated (SNIS, 2018), and there is no legislation regarding the discharge of anti-cancer drugs in effluents, the toxicity levels are concerning. Furthermore, it is relevant to emphasize that after chemotherapy infusion, many patients return to their cities of origin and excrete drug residues in locations without any wastewater treatment, a common problem in Brazil. It is also important to highlight that even with the application of municipal wastewater treatment the drugs persist in Barretos because standard treatment is only biological and mechanical, and the process does not effectively remove chemical compounds such as chemotherapeutic agents.

Also, the Barretos Cancer Hospital performs an average of 200 daily chemotherapy infusions and their discharge into a low

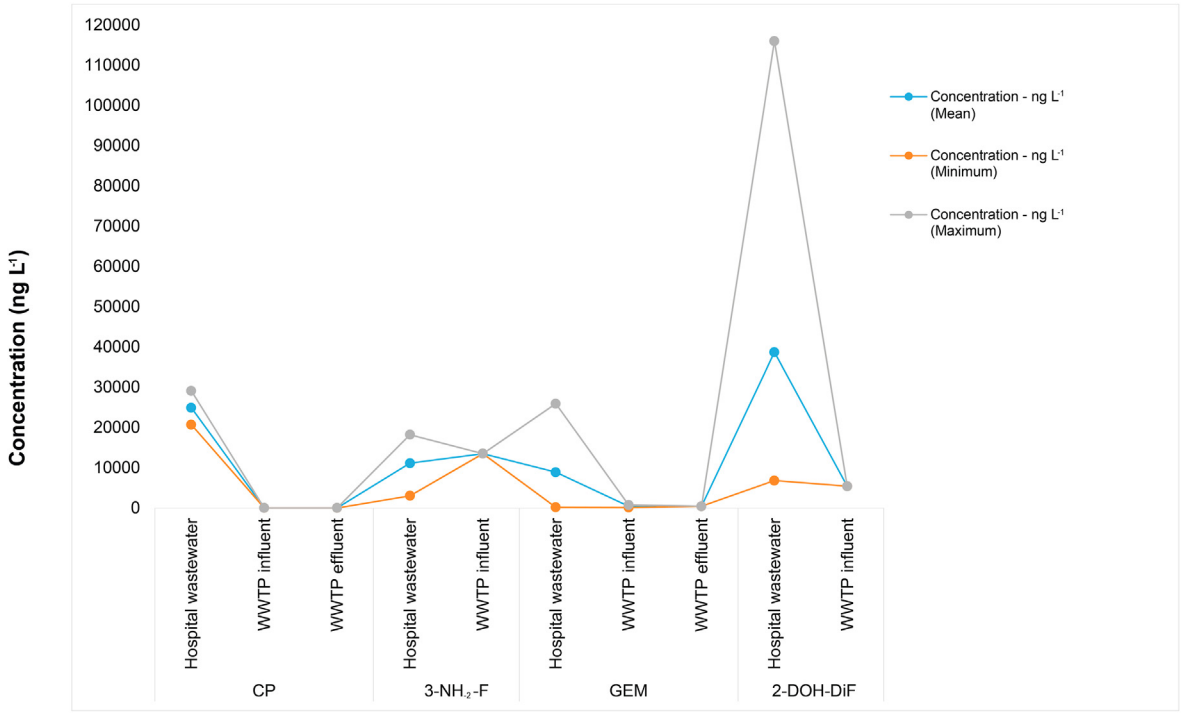


Fig. 3. Average (blue line), minimum (orange line) and maximum (grey line) concentrations of each compost detected in the effluents, per point of collection. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

capacity wastewater treatment system (the city only has 120 thousand permanent residents) with a comparatively low dilution rate, could explain the relatively high levels of these compounds. Especially when compared to other studies performed in larger centers from European countries, China (Yin et al., 2010) and Canada (Garcia-Ac et al., 2009; Rabii et al., 2014) with larger capacity treatment systems (Table 3). In addition, these cited studies were evaluating effluents from general hospitals with a much smaller proportion of patients who were undergoing chemotherapy. It is probable that drug levels we detected in the effluents were considerably higher, because the sampled hospital area receives only cancer patients. Also, the evaluation was derived from a large cancer hospital based in a small town, so that drug levels in effluent are expected to be more concentrated than those in general hospitals with other services.

Regarding individual drugs there was also some variation to levels observed in other studies. For CP the maximum value found in the hospital wastewater in this study was 29,100 ng L^{-1}, while in a study performed by (Cesen et al., 2015), a value of 22,000 ng L^{-1} was found, and the other studies reported values below 2000 ng L^{-1} (Ferrando-Climent et al., 2014; Ferre-Aracil et al., 2016; Isidori et al., 2016; Negreira et al., 2013; Thomas et al., 2007; Yin et al., 2010). Furthermore, considering the presence of CP in the WWTP influents in this study, in July (2017) above the detection limit of 1520 ng L^{-1} and below the limit of quantification of 5080 ng L^{-1}, in December (2017) and January (2018) above of detection limit of 300 ng L^{-1} and below the limit of quantification of 1000 ng L^{-1}, this concentrations are higher than the levels found studies by Negreira et al. (2013) and Santana-Rodriguez (2019). With regard to the presence of CP after treatment in the WWTP, we detected in all campaigns in November (2017), December (2017) and January (2018), with the detection limit of 300 ng L^{-1} and below the limit of quantification of 1000 ng L^{-1}, which is higher than the levels of 25 ng L^{-1} found by Ferrando-Climent et al., (2014) in Spain (2014), 21 ng L^{-1} found by Rabii et al., (2014) (2014) in Canada and by another studies in Europe (Castiglioni et al., 2005; Cesen et al., 2015; Isidori et al., 2016; Llewellyn et al., 2011; Santana-Viera et al., 2019; Thomas et al., 2007). When evaluated the toxicity and genotoxicity studies, Bialk-Bielinska et al. (2017) evaluated the acute toxicity towards selected aquatic organisms, bacteria *Vibrio fischeri*, algae *Raphidocelis subcapitata*, crustaceans *Daphnia magna* and duckweed *Lemna minor* and although CF and IF can be classified as non-toxic to all the investigated organisms, their EC50 values are higher than 100 mg L. Other studies in different organisms (bacteria, algea, cyanobacteria, invertebrates, polychaetes and plants) have also demonstrated ecotoxicological data of CP (EC50/LC50) above 100 mg L^{-1} (Cesen et al., 2016a; Fonseca et al., 2018; Grung et al., 2008; Sanderson et al., 2003; Zounkova et al., 2007). These studies corroborate with our acute toxicity tests performed on zebrafish, when the isolated drugs there was no mortality at any concentration evaluated.

Grzesiuk et al. (2019) rated how the exposure to CP and cisplatin (CDDP), at detected in environment concentrations, influence proteome profile, life history and population parameters of naturally setting surface waters *Daphnia pulex* and *Daphnia pulicaria*, concluding that, the individual growth rate was affected only by CP and exclusively in the case of *D. pulicaria*, decreased number of eggs was observed in both exposures and the proteome profile revealed that tested anti-cancer modified expression of some proteins involved in Daphnia metabolism, demonstrating that CP and CDDP in low concentrations detected in environment effect freshwater organisms. In our results in this study the exposure for 48 h to isolate CP caused agitation in animals at concentrations of in low concentrations and lethargy in animals exposed to high concentrations.

The literature is poor in data concerning GEM in effluents, as well as in toxicity and genotoxicity studies. Our study detected levels of 25,900 ng L^{-1} in the hospital wastewater, which are considerably higher than the values found in previous reports. Isidori and collaborators in 2016 (Isidori et al., 2016), did not

Table 3
Comparison between the values detected in our study with those found by other authors in ng/L^{-1} (average ± SD or minimum to maximum detection) of analysed anti-neoplastic drugs and metabolites in hospital wastewater, before treatment in wastewater treatment plants (WWTP influent) and after treatment in wastewater treatment plants (WWTP effluent).

Substance	Point of collection	Concentration (ng L^1)	Reference
5-FU	Hospital wastewater	**<LOD**[a]	**Our study, 2018 (Brazil)**
		2.1 ± 0.3	Isidori et al. (2016) (Spain)
		6.9 ± 1	Isidori et al. (2016) (Slovenia)
		35 to 92	Kosjek et al. (2013) (Slovenia)
		90 to 4000	Mullot et al. (2009) (France)
		<LOQ[b] to 123,500	Mahnik et al. (2007) (Austria)
		20	Mahnik, 2004 (Austria)
	WWTP influent	**<LOD**	**Our study, 2018 (Brazil)**
		3.5 ± 0.5	Isidori et al. (2016) (Spain)
		3.1 ± 0.4	Isidori et al. (2016) (Slovenia)
		4.7 to 14	Kosjek et al. (2013) (Slovenia)
	WWTP effluent	**<LOD**	**Our study, 2018 (Brazil)**
		<LOQ	Isidori et al. (2016) (Spain)
		<LOD	Isidori et al. (2016) (Slovenia)
		<LOD	Kosjek et al. (2013) (Slovenia)
CP	Hospital wastewater	**<LOD to 29,100**	**Our study, 2018 (Brazil)**
		1218	Santana-Rodriguez, 2019 (Spain)
		114 to 1187	Ferre-Aracil et al. (2016) (Spain)
		32 ± 1	Isidori et al. (2016) (Spain)
		1080 ± 200	Isidori et al. (2016) (Slovenia)
		0.4 to 22,000	Cesen et al. (2015) (Slovenia)
		43	Ferrando-Climent et al. (2014) (Spain)
		5.9 to 100	Negreira, 2014 (Spain)
		<2 to 2000	Yin, 2009 (China)
		<2	Thomas et al. (2007) (Norway)
	WWTP influent	**<LOD to < LOQ**	**Our study, 2018 (Brazil)**
		<LOD to 80	Gouveia et al. (2020) (Portugal)
		6 ± 2.5	Isidori et al. (2016) (Spain)
		27 ± 7	Isidori et al. (2016) (Slovenia)
		<LOD to 27	Cesen et al. (2015) (Slovenia)
		8 to 26	Ferrando-Climent et al. (2014) (Spain)
		17 to 22	Rabii et al. (2014) (Canada)
		<LOD to 38.8	Negreira, 2014 (Spain)
		9	Garcia, 2009 (Canada)
		<2	Thomas et al. (2007) (Norway)
	WWTP effluent	**<LOD to < LOQ**	**Our study, 2018 (Brazil)**
		<LOD to 45	Gouveia et al. (2020) (Portugal)
		55.94 to 91.25	Santana-Rodriguez, 2019 (Spain)
		<LOD	Isidori et al. (2016) (Spain)
		17 ± 5	Isidori et al. (2016) (Slovenia)
		17	Cesen et al. (2015) (Slovenia)
		7 to 25	Ferrando-Climent et al. (2014) (Spain)
		18 to 21	Rabii et al. (2014) (Canada)
		0.19 to 3.7	Llewellyn et al. (2011) (United Kingdom)
		<2	Thomas et al. (2007) (Norway)
		0.6	Castiglioni et al. (2005) (Italian)
GEM	Hospital wastewater	**<LOD to 25,900**	**Our study, 2018 (Brazil)**
		<LOD	Isidori et al. (2016) (Spain)
		<LOD	Isidori et al. (2016) (Slovenia)
	WWTP influent	**<LOD to 750**	**Our study, 2018 (Brazil)**
		<LOD	Isidori et al. (2016) (Spain)
		61 ± 1	Isidori et al. (2016) (Slovenia)
		<LOD	Negreira, 2014 (Spain)
	WWTP effluent	**<LOD to 420**	**Our study, 2018 (Brazil)**
		<LOD	Isidori et al. (2016) (Spain)
		<LOD	Isidori et al. (2016) (Slovenia)
		<LOD	Negreira, 2014 (Spain)

WWTP: wastewater treatment plant.
[a] LOD: limit of detection.
[b] LOQ: limit of quantification.

detected GEM in the effluent from a Hospital in Slovenia and was only detected in the WWTP influent, at a level of 61 ng L^{-1}. This value is 12-fold lower then we observed in our study (750 ng L^{-1}). Additionally, we reported a level of 420 ng L^{-1} in the treatment plant effluent in June.

We observed the predominance of the metabolites 3-NH_2-F and 2-DOH-DiF in the hospital and municipal effluents. Additionally, the compound detected at the highest concentration (116,000 ng L^{-1}) was the gemcitabine metabolite 2-DOH-DiF in the hospital wastewater, in the literature the metabolite concentrations used are often more than twice as high as for GEM, and its clearance is approximately one tenth that of the parent molecule (Benyumov et al., 2011), likely reason why the metabolite is found in higher concentrations than GEM. Benyumov et al. (2011), when evaluated teratogenic effects in zebrafish eggs, while 2-DOH-DiF has shown no evidence of teratogenicity, the screens showed that gemcitabine

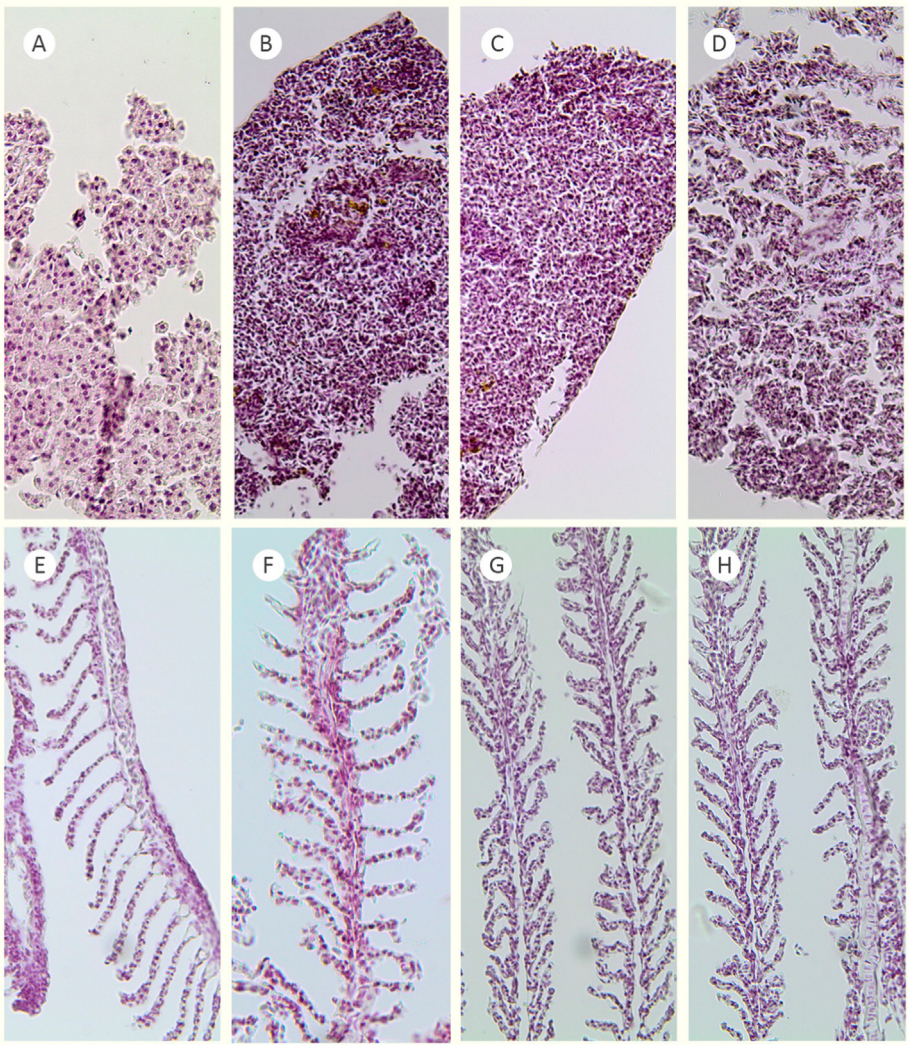


Fig. 4. Acute toxicity test in Zebrafish. A, B, C and D: immunohistochemistry from zebrafish liver. Control, mixture 1, mixture 2 and mixture 3, respectively. E, F, G and H: immunohistochemistry from zebrafish gills. Control, mixture 1, mixture 2 and mixture 3, respectively.

is a highly potent teratogenic agent. Gemcitabine treatment (>25 μM) was associated with congenital malformations, which became gradually apparent in 100% of the treated eggs and decreased the survival rate at hatching 95–100%. 2-DOH-DiF is also classified by the European Chemicals Agency (ECHA) in the GHS Hazard Statements as a "danger", causing genotoxic damage to the germinative cells, being toxic for the aquatic life and causing irritation when in contact with the skin and mucosa ((ECHA), 2018). Importantly, this is the first report that detected 2-DOH-DiF, a metabolite from gemcitabine, widely utilized in oncology in Brazil. In the acute toxicity tests of the isolated drugs the exposure to 5-FU, and GEM did not cause any signs of toxicity in fish at any concentration.

5-FU was the only drug not detected, which may be explained by the high detection and quantification limits, given that other authors detected concentrations of 2.1 ng L^{-1} to 123 ng L^{-1} (Isidori et al., 2016; Kosjek et al., 2013; Mahnik et al., 2007; Mullot et al., 2009), values below of detection and quantification limits were defined in this study.

Nevertheless, it is also important to highlight that most of the 5-FU is eliminated by the organism through the respiratory tract, approximately 90% as carbon dioxide (Micromedex, 2005), a factor that could decrease its renal excretion, which is the second excretion route (Micromedex, 2005). The derived metabolite is alpha-fluoro-beta-alanine, which is one of the last to be broken down by the organism, and is metabolized from 5-fluoro-5,6-dihydrouracil to alpha-fluoro-beta-alanine. Approximately 80% of this compound is catabolized and excreted in urine (Hardman and Limbird, 2001; Turci et al., 2003), which could explain its detection in the effluents even without 5-FU detection.

Many different factors could influence the detection levels of drugs in hospital wastewater, such as drug dose administration (amount in mg); duration of inpatient time in the hospital; and different use of wastewater in the hospital routine practices. Furthermore, factors related to the wastewater collection network of the neighbourhood or the district close to the hospital will influence the dilution of the compounds according to the coverage of the local and treatment system. In addition, the distance travelled from the hospital wastewater discharge point to the wastewater treatment plant is an important consideration. Nassour et al. (2020) conducted a systematic review of 75 publications and showed that diverse methodological approaches were adopted to measure anti-cancer drugs in the aquatic environment that could explain the significant variation in anti-cancer concentrations detected.

In this study it was possible to evaluate damage caused to gill and liver tissues in zebrafish, such as necrosis and changes in the

gills, when the animals were exposed to the drug mixtures at concentrations equivalent to the levels detected in the hospital effluent. Da Fonseca et al. (2019) described that a single compound toxicity data are not sufficient to predict the environmental risks offered by anti-cancer drugs when they occur in combination and the mixtures of anti-cancer agents can elicit different trends of biochemical effects as a result of subtle changes in their concentrations, even in low effluent concentarations of ng L^{-1}. On the other hand, in our work we followed the guideline of the Brazilian Association of Technical Standards (2016) in the adults Zebrafish assays with a time of exposure to drugs of 48 h. Recent studies have evaluated the effect of anti-cancer drugs on exposure times shorter than 96 h as recommended by other guidelines. In the study realized by Liu et al. (2019) the exposure of the snout bream fish (*Megalobrama amblycephala*) to 0.032 e 0.32, mg mL^{-1} of CP for 24 h was evaluated which caused an increase in the lactate dehydrogenase (LDH) release in the blood, interruption of the mitochondrial membrane potential and generation of reactive oxygen species (ROS), with increased caspases-9, interleukins-1β (IL-1β) and tumor necrosis alpha factor (TNF-œ), causing cytotoxicity, inflammatory response and induction of apoptosis. Kóvacs et al. (2016) evaluated the exposure of adult zebrafish to concentrations of 0.01; 1.0; and 100.0 μg L-1 of 5-fluorouracil (5-FU), for 72 h and showed there was no effect on survival, growth and reproduction, however, in the histopathological analysis of the liver, lipidosis and regressive degeneration were observed in all concentrations and hepatic atrophy at 100.0 μg L^{-1} and hypertrophy of renal haematopoietic tissue at 0.01 and 1.0 μg L^{-1} and atrophy and depletion of haematopoietic tissue and degeneration of the renal tubular epithelium. These data corroborate our ecotoxicology analysis of morphological changes and demonstration that the mixture of drugs do not kill the *Zebrafish*. Isidori et al. (2016) exposed ZFL cells to 10, 20 or 30% (v/v) of the wastewater sample contained the anti-cancer drugs 5-FU, Pt (platinum), IF (ifosfamide) and CP for 72 h and evaluated any induced DNA damage. Their results demonstrated that concentrations that were several orders higher than the levels detected in effluents induced DNA damage. Similarly, Novak et al. (2017) evaluated cytotoxic and genotoxic potential of CP and IF as individual compounds and in a complex mixture together with 5-FU and cisplatin (CDDP) in ZFL. Both compounds induced DNA strand breaks and genomic instability, however, effects were only evident at relatively high concentrations. These data are in agreement with our ecotoxicology results using zebrafish.

Despite the interesting results, the present study harbours some limitation that should be considered. Or note, pre-concentration of the samples by solid-phase extraction (SPE) was not performed. On the other hand, Cesen et al. (2016b) quantified IF and CP and their metabolites in hospital wastewater, they used SPE for clean-up and sample pre-concentration followed by chemical derivatization of compounds (silylation and acetylation reaction) before GC/MS analysis. The drugs were quantified in hospital wastewater, levels up to 2690 ng L^{-1} for CP, while in influent and effluent (WWTP) samples concentrations were below of LOQs. In our study we did not use SPE, therefore, we developed and used two analytical method based on: (1) direct water dilution of the wastewater sample and injection in optimised LC-MS/MS system; and (2) chemical derivatization using PITC reagent for the compounds 5-FU and 3-NH_2-F with available primary amino group, prior LC-MS/MS analysis (Kwanyuen and Burton, 2010). These developed methods provided reliable results with emphasis in simplicity and specificity without compromise the sensibility, using this method it was possible to detect drugs even with detection limits considered higher.

Additionally, it is also necessary to evaluate whether the quantities detected in the effluents offer potential risk to the environment or even to human health. In this context, studies that aim to measure the ecotoxicological and genotoxic effects in different plant and animal species, such as bacteria green algae (Zounkova et al., 2010), herbaceous plants (Lutterbeck et al., 2015), zebrafish liver (ZFL) cells (Gajski et al., 2016; Novak et al., 2017) and human liver (HepG2) cells (Gajski et al., 2016; Mater et al., 2014; Novak et al., 2016), have shown that they could present genotoxicity and cytotoxicity at low levels, on the order of magnitude of μgL^{-1}, and with an increased effect when combined (Mater et al., 2014). Therefore, further studies are needed to extend and validated the presence of anti-cancer drugs in hospital and municipal effluents. Samples should be evaluated using standardised techniques that are widely available for these types of detections, and evaluations of the ecotoxicological and genotoxic effects of these compounds, separate and as a mixtures should be performed. This approach would provide a solid framework to formulate new public policies.

5. Conclusions

The present study evaluated levels of anti-cancer drugs and their metabolites throughout the collection and treatment system of the Barretos Cancer Hospital wastewater and municipality effluent treatment facility. The detected values were higher than in other studies, which may be due to the characteristics of the study area that contains a large dedicated national cancer hospital is a small city. There is currently no legislation regarding the discharge of anti-cancer drugs in effluents in Brazil. At present only in some places in the word address this issue with public policy regulations. This work is first study with focus on effluents from specific treatments from a cancer hospital located in small city in Brazil.

Thus, the values found on the order of magnitude of ng L^{-1} are lower than the levels considered toxic and genotoxic in some studies. However, studies have shown that the mixture of the drugs, which is how they are found in the environment, has an increased ecotoxicological potential, emphasizing the need to evaluate the presence of anti-cancer drugs and understand their ecotoxicity and the potential genotoxic damage in the environment.

Sample credit author statement

Mariana de Oliveira Klein: Conceptualization, Methodology, Investigation; Formal analysis; Writing - original draft; Project administration; Sergio V. Serrano: Conceptualization; Álvaro Santos-Neto: Formal analysis; Writing - review & editing; Claudinei da Cruz: Investigation; Formal analysis; Writing - review & editing; Isabella Alves Brunetti: Investigation; Daniel Lebre: Investigation; Formal analysis; Writing- Reviewing and Editing; Maíse Pastore Gimenez: Investigation; Formal analysis; Rui M. Reis: Resouces; Writing - review & editing; Henrique C. S. Silveira: Conceptualization; Writing - original draft; Writing - review & editing; Supervision; Funding acquisition; Project administration

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors would like to express their gratitude to the PAIP

from Barretos Cancer Hospital and Public Ministry of Labor Campinas (Research, Prevention and Education of Occupational Cancer Project), Campinas, Brazil for their financial support. We would also like to thank staff at the Barretos Cancer Hospital from sectors such as pharmacy, library, statisticians and events, which assisted in data collection and elaboration of figures. We would like to thank Dr. Jeremy Squire by English review.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2020.115857.

References

ABNT, 2016. NBR 15088:2016. Aquatic Ecotoxicology - Acute Toxicity - Test Method with Fish (Cyprinidae). Brazilian Association of Technical Standards, Sao Paulo, SP.

Aherne, G.W., Hardcastle, A., Nield, A.H., 1990. Cytotoxic drugs and the aquatic environment: estimation of bleomycin in river and water samples. J. Pharm. Pharmacol. 42, 741–742.

ALESP, 1976. Lei Estadual nº 997/1976 - dispõe sobre o controle da poluição do meio ambiente. In: Paulo, A. (Ed.), Lei Estadual nº 997/1976 - Dispõe sobre o controle da poluição do meio ambiente. Assembléia Legislativa do Estado de São Paulo.

ANVISA, 2018. Regulamenta as boas práticas de Gerenciamento dos resíduos de Serviços de Saúde. In: Sanitária, A.N.d.V. (Ed.), 222/2018. Agência Nacional de Vigilância Sanitária.

Araujo, A., Mesak, C., Montalvao, M.F., Freitas, I.N., Chagas, T.Q., Malafaia, G., 2019. Anti-cancer drugs in aquatic environment can cause cancer: insight about mutagenicity in tadpoles. Sci. Total Environ. 650, 2284–2293.

Behmer, O., Tolosa, E., Neto, A., 1976. Manual de técnicas para histologia normal e patológica. EDART. USP.

Benyumov, A., Gurvich, V.J., Lis, L.G., Williams, B.W., Kirstein, M.N., 2011. Combinatorial pharmacologic effects of gemcitabine and its metabolite dFdU. ChemMedChem 6, 457–464.

Bialk-Bielinska, A., Mulkiewicz, E., Stokowski, M., Stolte, S., Stepnowski, P., 2017. Acute aquatic toxicity assessment of six anti-cancer drugs and one metabolite using biotest battery - biological effects and stability under test conditions. Chemosphere 189, 689–698.

Brunton, L.L., Chabner, B., Knollmann, B.C., 2011. Goodman & Gilman's the Pharmacological Basis of Therapeutics.

Castiglioni, S., Bagnati, R., Calamari, D., Fanelli, R., Zuccato, E., 2005. A multiresidue analytical method using solid-phase extraction and high-pressure liquid chromatography tandem mass spectrometry to measure pharmaceuticals of different therapeutic classes in urban wastewaters. J. Chromatogr. A 1092, 206–215.

Cesen, M., Kosjek, T., Laimou-Geraniou, M., Kompare, B., Sirok, B., Lambropolou, D., Heath, E., 2015. Occurrence of cyclophosphamide and ifosfamide in aqueous environment and their removal by biological and abiotic wastewater treatment processes. Sci. Total Environ. 527–528, 465–473.

Cesen, M., Elersek, T., Novak, M., Zegura, B., Kosjek, T., Filipic, M., Heath, E., 2016a. Ecotoxicity and genotoxicity of cyclophosphamide, ifosfamide, their metabolites/transformation products and their mixtures. Environ. Pollut. 210, 192–201.

Cesen, M., Kosjek, T., Busetti, F., Kompare, B., Heath, E., 2016b. Human metabolites and transformation products of cyclophosphamide and ifosfamide: analysis, occurrence and formation during abiotic treatments. Environ. Sci. Pollut. Res. Int. 23, 11209–11223.

Chakravarthy, S., Sadagopan, S., Nair, A., Sukumaran, S.K., 2014. Zebrafish as an in vivo high-throughput model for genotoxicity. Zebrafish 11, 154–166.

Chan, K.K., Hong, P.S., Tutsch, K., Trump, D.L., 1994. Clinical pharmacokinetics of cyclophosphamide and metabolites with and without SR-2508. Canc. Res. 54, 6421–6429.

Colvin, O., 1999. An overview of cyclophosphamide development and clinical applications. Curr. Pharmaceut. Des. 5, 555–560.

CONAMA, 2011. Resolução CONAMA nº 430/2011. In: CONAMA), M. (Ed.), Ministério do Meio Ambiente - Conselho Nacional de Meio Ambiente (CONAMA) (Brasil).

da Fonseca, T.G., Abessa, D.M.S., Bebianno, M.J., 2019. Effects of mixtures of anticancer drugs in the benthic polychaete Nereis diversicolor. Environ. Pollut. 252, 1180–1192.

Directive, E., 2008. Directive 2008/98/EC of the European Parliament and of the Council of 19 November 2008 on Waste and Repealing Certain Directives, p. 312.

(ECHA), E.C.A., 2018. Notified classification and labelling from ECHA's C&L Inventory. Name: 2',2'-Difluoro-2'-deoxyuridine. In: ECHA), E.C.A. (Ed.), European Chemicals Agency (ECHA. https://echa.europa.eu/information-on-chemicals/cl-inventory-database/-/discli/details/241829.

Farooq, M., Sharma, A., Almarhoon, Z., Al-Dhfyan, A., El-Faham, A., Taha, N.A., Wadaan, M.A., Beatriz, G., Albericio, F.J.B.c., 2019. Design and Synthesis of Mono-And Di-pyrazolyl-s-triazine Derivatives, Their Anticancer Profile in Human Cancer Cell Lines, and in Vivo Toxicity in Zebrafish Embryos, vol. 87, pp. 457–464.

Ferlay, J., Soerjomataram, I., Dikshit, R., Eser, S., Mathers, C., Rebelo, M., Parkin, D.M., Forman, D., Bray, F., 2015. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. Int. J. Canc. 136.

Ferrando-Climent, L., Rodriguez-Mozaz, S., Barceló, D., 2013. Development of a UPLC-MS/MS method for the determination of ten anticancer drugs in hospital and urban wastewaters, and its application for the screening of human metabolites assisted by information-dependent acquisition tool (IDA) in sewage samples. Anal. Bioanal. Chem. 405, 5937–5952.

Ferrando-Climent, L., Rodriguez-Mozaz, S., Barcelo, D., 2014. Incidence of anticancer drugs in an aquatic urban system: from hospital effluents through urban wastewater to natural environment. Environ. Pollut. 193, 216–223.

Ferre-Aracil, J., Valcárcel, Y., Negreira, N., de Alda, M.L., Barceló, D., Cardona, S., Navarro-Laboulais, J., 2016. Ozonation of hospital raw wastewaters for cytostatic compounds removal. Kinetic modelling and economic assessment of the process. Sci. Total Environ. 556, 70–79.

Fonseca, T.G., Auguste, M., Ribeiro, F., Cardoso, C., Mestre, N.C., Abessa, D.M.S., Bebianno, M.J., 2018. Environmental relevant levels of the cytotoxic drug cyclophosphamide produce harmful effects in the polychaete Nereis diversicolor. Sci. Total Environ. 636, 798–809.

Franquet-Griell, H., Gómez-Canela, C., Ventura, F., Lacorte, S., 2015. Predicting concentrations of cytostatic drugs in sewage effluents and surface waters of Catalonia (NE Spain). Environ. Res. 138, 161–172.

Gajski, G., Geric, M., Zegura, B., Novak, M., Nunic, J., Bajrektarevic, D., Garaj-Vrhovac, V., Filipic, M., 2016. Genotoxic potential of selected cytostatic drugs in human and zebrafish cells. Environ. Sci. Pollut. Res. Int. 23, 14739–14750.

Garcia-Ac, A., Segura, P.A., Gagnon, C., Sauve, S., 2009. Determination of bezafibrate, methotrexate, cyclophosphamide, orlistat and enalapril in waste and surface waters using on-line solid-phase extraction liquid chromatography coupled to polarity-switching electrospray tandem mass spectrometry. J. Environ. Monit. 11, 830–838.

Gómez-Canela, C., Cortés-Francisco, N., Oliva, X., Pujol, C., Ventura, F., Lacorte, S., Caixach, J., 2012. Occurrence of cyclophosphamide and epirubicin in wastewaters by direct injection analysis–liquid chromatography–high-resolution mass spectrometry. Environ. Sci. Pollut. Control Ser. 19, 3210–3218.

Gouveia, T.I.A., Silva, A.M.T., Ribeiro, A.R., Alves, A., Santos, M.S.F., 2020. Liquid-liquid extraction as a simple tool to quickly quantify fourteen cytostatics in urban wastewaters and access their impact in aquatic biota. Sci. Total Environ. 740, 139995.

Grung, M., Kallqvist, T., Sakshaug, S., Skurtveit, S., Thomas, K.V., 2008. Environmental assessment of Norwegian priority pharmaceuticals based on the EMEA guideline. Ecotoxicol. Environ. Saf. 71, 328–340.

Grzesiuk, M., Bednarska, A., Mielecki, D., Garbicz, D., Marcinkowski, M., Pilzys, T., Malinowska, A., Swiderska, B., Grzesiuk, E., 2019. Anticancer agents found in environment affect Daphnia at population, individual and molecular levels. Aquat. Toxicol. 215, 105288.

Hamilton, M.A., Russo, R.C., Thurston, R.V.J.E.S., Technology, 1977. Trimmed Spearman-Karber method for estimating median lethal concentrations in toxicity bioassays, 11, 714–719.

Hardman, J.G., Limbird, L.E., 2001. Goodman & Gilman's: the Pharmacological Basis of Therapeutics, Goodman & Gilman's: the Pharmacological Basis of Therapeutics.

IBGE, 2016. Estimativa Populacional 2016. Instituto Brasileiro de Geografia e Estatística (IBGE).

Isidori, M., Lavorgna, M., Russo, C., Kundi, M., Zegura, B., Novak, M., Filipic, M., Misik, M., Knasmueller, S., de Alda, M.L., Barcelo, D., Zonja, B., Cesen, M., Scancar, J., Kosjek, T., Heath, E., 2016. Chemical and toxicological characterisation of anticancer drugs in hospital and municipal wastewaters from Slovenia and Spain. Environ. Pollut. 219, 275–287.

Karas, B.F., Corte-Real, L., Doherty, C.L., Valente, A., Cooper, K.R., Buckley, B.T., 2019. A novel screening method for transition metal-based anticancer compounds using zebrafish embryo-larval assay and inductively coupled plasma-mass spectrometry analysis. J. Appl. Toxicol. 39, 1173–1180.

Kasel, D., Jetter, A., Harlfinger, S., Gebhardt, W., Fuhr, U., 2004. Quantification of cyclophosphamide and its metabolites in urine using liquid chromatography/tandem mass spectrometry. Rapid Commun. Mass Spectrom. 18, 1472–1478.

Kiffmeyer, T., Götze, H.-J., Jursch, M., Lüders, U., 1998. Trace enrichment, chromatographic separation and biodegradation of cytostatic compounds in surface water. Fresen. J. Anal. Chem. 361, 185–191.

Kosjek, T., Perko, S., Žigon, D., Heath, E., 2013. Fluorouracil in the environment: analysis, occurrence, degradation and transformation. J. Chromatogr. A 1290, 62–72.

Kovacs, R., Bakos, K., Urbanyi, B., Kovesi, J., Gazsi, G., Csepeli, A., Appl, A.J., Bencsik, D., Csenki, Z., Horvath, A., 2016. Acute and sub-chronic toxicity of four cytostatic drugs in zebrafish. Environ. Sci. Pollut. Res. Int. 23, 14718–14729.

Kovalova, L., McArdell, C.S., Hollender, J., 2009. Challenge of high polarity and low concentrations in analysis of cytostatics and metabolites in wastewater by hydrophilic interaction chromatography/tandem mass spectrometry. J. Chromatogr. A 1216, 1100–1108.

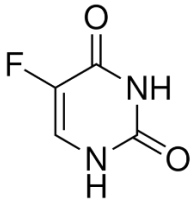
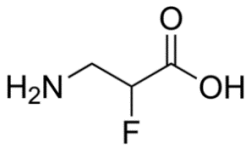
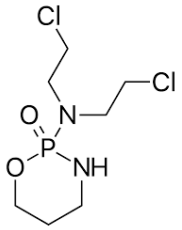
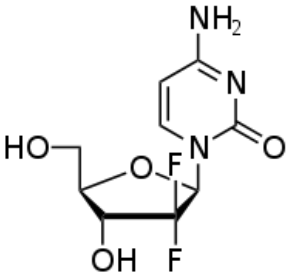
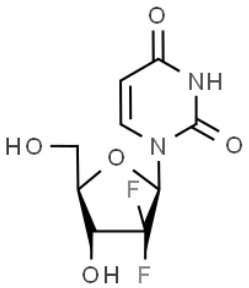
Kümmerer, K., Al-Ahmad, A., Bertram, B., Wießler, M., 2000. Biodegradability of antineoplastic compounds in screening tests: influence of glucosidation and of stereochemistry. Chemosphere 40, 767–773.

Kümmerer, K., Haiß, A., Schuster, A., Hein, A., Ebert, I., 2016. Antineoplastic compounds in the environment—substances of special concern. Environ. Sci. Pollut. Control Ser. 23, 14791–14804.

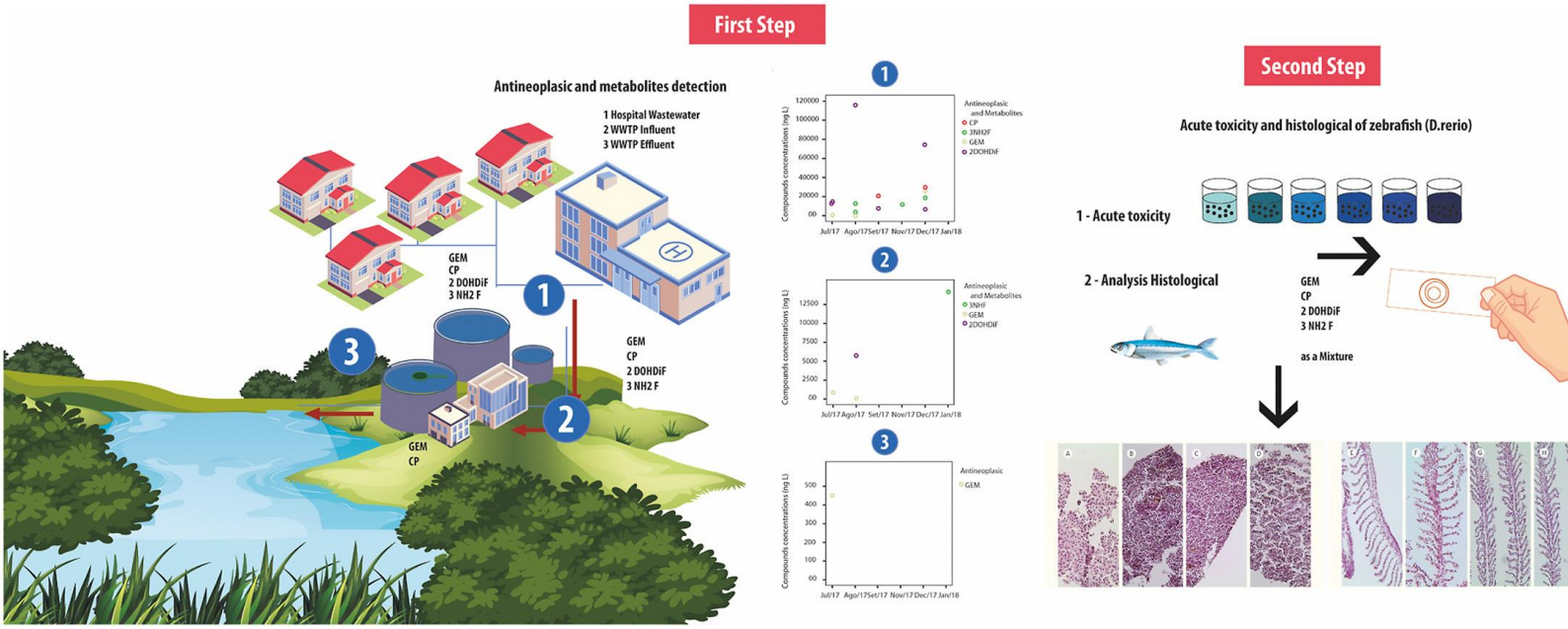
Kwanyuen, P., Burton, J.W., 2010. A modified amino acid analysis using PITC derivatization for soybeans with accurate determination of cysteine and half-cystine. J. Am. Oil Chem. Soc. 87, 127–132.
Leblanc, G.A., 2004. Acute Toxicity. A Textbook of Modern Toxicology, third ed., pp. 213–224
Llewellyn, N., Lloyd, P., Jurgens, M.D., Johnson, A.C., 2011. Determination of cyclophosphamide and ifosfamide in sewage effluent by stable isotope-dilution liquid chromatography-tandem mass spectrometry. J. Chromatogr. A 1218, 8519–8528.
Longley, D.B., Harkin, D.P., Johnston, P.G., 2003. 5-fluorouracil: mechanisms of action and clinical strategies. Nat. Rev. Canc. 3, 330–338.
Lutterbeck, C.A., Kern, D.I., Machado, E.L., Kummerer, K., 2015. Evaluation of the toxic effects of four anti-cancer drugs in plant bioassays and its potency for screening in the context of waste water reuse for irrigation. Chemosphere 135, 403–410.
Mahnik, S., Lenz, K., Weissenbacher, N., Mader, R., Fuerhacker, M., 2007. Fate of 5-fluorouracil, doxorubicin, epirubicin, and daunorubicin in hospital wastewater and their elimination by activated sludge and treatment in a membrane-bioreactor system. Chemosphere 66, 30–37.
Mater, N., Geret, F., Castillo, L., Faucet-Marquis, V., Albasi, C., Pfohl-Leszkowicz, A., 2014. In vitro tests aiding ecological risk assessment of ciprofloxacin, tamoxifen and cyclophosphamide in range of concentrations released in hospital wastewater and surface water. Environ. Int. 63, 191–200.
Matuszewski, B., Constanzer, M., Chavez-Eng, C.J.A.c., 2003. Strategies for the assessment of matrix effect in quantitative bioanalytical methods based on HPLC– MS/MS, 75, 3019–3030.
McKnight, J.A., 2003. Principles of chemotherapy. Clin. Tech. Small Anim. Pract. 18, 67–72.
Micromedex, T., 2005. Drug Information for the Health Care Professional, vol. 1, pp. 2569–2572.
Misik, M., Filipic, M., Nersesyan, A., Kundi, M., Isidori, M., Knasmueller, S., 2019. Environmental risk assessment of widely used anticancer drugs (5-fluorouracil, cisplatin, etoposide, imatinib mesylate). Water Res. 164, 114953.
Mullot, J.-U., Karolak, S., Fontova, A., Huart, B., Levi, Y., 2009. Development and validation of a sensitive and selective method using GC/MS-MS for quantification of 5-fluorouracil in hospital wastewater. Anal. Bioanal. Chem. 394, 2203–2212.
Mullot, J.-U., Karolak, S., Fontova, A., Levi, Y., 2010. Modeling of hospital wastewater pollution by pharmaceuticals: first results of Mediflux study carried out in three French hospitals. Water Sci. Technol. 62, 2912–2919.
Nassour, C., Barton, S.J., Nabhani-Gebara, S., Saab, Y., Barker, J., 2020. Occurrence of anticancer drugs in the aquatic environment: a systematic review. Environ. Sci. Pollut. Res. Int. 27, 1339–1347.
Negreira, N., de Alda, M.L., Barceló, D., 2013. On-line solid phase extraction–liquid chromatography–tandem mass spectrometry for the determination of 17 cytostatics and metabolites in waste, surface and ground water samples. J. Chromatogr. A 1280, 64–74.
Novak, M., Zegura, B., Baebler, S., Stern, A., Rotter, A., Stare, K., Filipic, M., 2016. Influence of selected anti-cancer drugs on the induction of DNA double-strand breaks and changes in gene expression in human hepatoma HepG2 cells. Environ. Sci. Pollut. Res. Int. 23, 14751–14761.
Novak, M., Zegura, B., Modic, B., Heath, E., Filipic, M., 2017. Cytotoxicity and genotoxicity of anticancer drug residues and their mixtures in experimental model with zebrafish liver cells. Sci. Total Environ. 601–602, 293–300.
Palmero, E.I., Galvao, H.C., Fernandes, G.C., Paula, A.E., Oliveira, J.C., Souza, C.P., Andrade, C.E., Romagnolo, L.G., Volc, S., M, C.N., Sabato, C., Grasel, R., Mauad, E., Reis, R.M., Michelli, R.A., 2016. Oncogenetics service and the Brazilian public health system: the experience of a reference Cancer Hospital. Genet. Mol. Biol. 39, 168–177.
Rabii, F.W., Segura, P.A., Fayad, P.B., Sauve, S., 2014. Determination of six chemotherapeutic agents in municipal wastewater using online solid-phase extraction coupled to liquid chromatography-tandem mass spectrometry. Sci. Total Environ. 487, 792–800.
Sanderson, H., Johnson, D.J., Wilson, C.J., Brain, R.A., Solomon, K.R., 2003. Probabilistic hazard assessment of environmentally occurring pharmaceuticals toxicity to fish, daphnids and algae by ECOSAR screening. Toxicol. Lett. 144, 383–395.
Santana-Viera, S., Hernandez-Arencibia, P., Sosa-Ferrera, Z., Santana-Rodriguez, J.J., 2019. Simultaneous and systematic analysis of cytostatic drugs in wastewater samples by ultra-high performance liquid chromatography tandem mass spectrometry. J Chromatogr B Analyt Technol Biomed Life Sci 1110–1111, 124–132.
Santos, M.S.F., Franquet-Griell, H., Alves, A., Lacorte, S., 2018. Development of an analytical methodology for the analysis of priority cytostatics in water. Sci. Total Environ. 645, 1264–1272.
Shi, Z., Li, H., Li, Z., Hu, J., Zhang, H.J.I.P., 2013. Pre-column derivatization RP-HPLC determination of amino acids in Asparagi radix before and after heating process, 5, 351–356.
SNIS, S., 2018. Diagnóstico dos Serviços de Água e Esgotos – 2016. In: Ambiental. Ministério das Cidades - Secretaria Nacional de Saneamento Ambiental, Brasília. M.d.C.-S.N.d.S. (Ed.).
Souza, D.M., Reichert, J.F., Martins, A.F., 2018. A simultaneous determination of anticancer drugs in hospital effluent by DLLME HPLC-FLD, together with a risk assessment. Chemosphere 201, 178–188.
Thomas, K.V., Dye, C., Schlabach, M., Langford, K.H., 2007. Source to sink tracking of selected human pharmaceuticals from two Oslo city hospitals and a wastewater treatment works. J. Environ. Monit. 9, 1410–1418.
Toschi, A., Capone, M., Ortolani, M., Calvani, P., Lupi, S., Castellani, C., 2005. Temperature dependence of the optical spectral weight in the cuprates: role of electron correlations. Phys. Rev. Lett. 95, 097002.
Turci, R., Sottani, C., Spagnoli, G., Minoia, C., 2003. Biological and environmental monitoring of hospital personnel exposed to antineoplastic agents: a review of analytical methods. J Chromatogr B Analyt Technol Biomed Life Sci 789, 169–209.
Weigel, J.A., 1999. Process for making gemcitabine hydrochloride. Google Patents.
Xie, H., 2012. Occurrence, Ecotoxicology, and Treatment of Anticancer Agents as Water Contaminants, vol. 2, pp. 1–11.
Yen, J., White, R.M., Stemple, D., development, 2014. Zebrafish models of cancer: progress and future challenges, 24, 38–45.
Yin, J., Shao, B., Zhang, J., Li, K., 2010. A preliminary study on the occurrence of cytostatic drugs in hospital effluents in Beijing, China. Bull. Environ. Contam. Toxicol. 84, 39.
Zheng, G., Jin, W., Fan, P., Feng, X., Bai, Y., Tao, T., Yu, L., 2015. A novel method for detecting amino acids derivatized with phenyl isothiocyanate by high-performance liquid chromatography–electrospray ionization mass spectrometry, 392, 1–6.
Zounkova, R., Odraska, P., Dolezalova, L., Hilscherova, K., Marsalek, B., Blaha, L., 2007. Ecotoxicity and genotoxicity assessment of cytostatic pharmaceuticals. Environ. Toxicol. Chem. 26, 2208–2214.
Zounkova, R., Kovalova, L., Blaha, L., Dott, W., 2010. Ecotoxicity and genotoxicity assessment of cytotoxic antineoplastic drugs and their metabolites. Chemosphere 81, 253–260.

Supplemental 1

Table 1. Characterization of the analyzed molecules with name, abbreviation, molecular form, structures, CAS numbers, molecular weight (MW) and average percentage of the drug excreted (excretion).

Name, abbreviation and molecular form	Structure	CAS	MW (g/mol)	Excretion
5-Fluorouracil (5-FU) $C_4H_3FN_2O_2$		51-21-8	130.07	2 – 20% (Micromedex, 2005)
Alpha-fluoro-beta-alanine (3-NH_2-F) $C_3H_6FNO_2$		3821-81-6	107.08	2 – 20% (Micromedex, 2005)
Cyclophosphamide (CP) $C_7H_{15}CI_2N_2O_2P$		50-18-0	261.08	10 - 20% (Kasel D *et al*., 2004)
Gemcitabine (GEM) $C_9H_{11}F_2N_3O_4$		95058-81-4	263.19	10% (Weigel JA, 1999)
2'-Deoxy-2',2'-difluorouridine (2-DOH-DiF) $C_9H_{10}F_2N_2O_5$		114248-23-6	264.19	10% (Weigel JA, 1999)

Graphic Abstract



Objetivos contemplados:

- Avaliação, de acordo com as concentrações obtidas dos antineoplásicos e seus metabólitos no efluente, a citotoxicidade pelo ensaio de MTS e a genotoxicidade por ensaio micronúcleo em linhagem celular de fígado humano (HepG2); e
- Fornecimento de dados científicos aos órgãos ambientais que contribuam com políticas públicas futuras relacionadas ao tema.

Os resultados presentes nesta seção estão em fase final de revisão e serão submetidos para publicação:

4.2. Environmental risks assessment of antineoplastic drugs and metabolites by cytotoxicity and genotoxicity assays

Authors: Mariana de Oliveira Klein [1], Luiza Flavia Veiga Francisco[1], Izabela Natália Faria Gomes[1], Sergio V. Serrano [1, 2], Rui M. Reis [1,3,4] and Henrique C. S. Silveira [1,5].

Affiliations:

[1] Molecular Oncology Research Center, Barretos Cancer Hospital, Barretos, São Paulo, 14784-400, Brazil.

[2] Barretos School of Health Sciences-FACISB, Barretos, São Paulo 14785-002, Brazil

[3] Life and Health Sciences Research Institute (ICVS), Medical School, University of Minho, Braga, Portugal.

[4] ICVS/3B's-PT Government Associate Laboratory, Braga/Guimarães, Portugal.

[5] University of Cuiabá, Cuiabá, Mato Grosso, Brazil.

TYPE: FULL PAPER

ENVIRONMENTAL RISKS ASSESSMENT OF ANTINEOPLASTIC DRUGS AND METABOLITES BY CYTOTOXICITY AND GENOTOXICITY ASSAYS

Authors: Mariana de Oliveira Klein[1*], Luiza Flavia Veiga Francisco[1*], Izabela Natália Faria Gomes[1], Sergio V. Serrano [1, 2], Rui M. Reis [1,3,4] and Henrique C. S. Silveira [1,5].

Affiliations:

[1] Molecular Oncology Research Center, Barretos Cancer Hospital, Barretos, São Paulo, 14784-400, Brazil.

[2] Barretos School of Health Sciences-FACISB, Barretos, São Paulo 14785-002, Brazil

[3] Life and Health Sciences Research Institute (ICVS), Medical School, University of Minho, Braga, Portugal.

[4] ICVS/3B's-PT Government Associate Laboratory, Braga/Guimarães, Portugal.

[5] University of Cuiabá, Cuiabá, Mato Grosso, Brazil.

[*] The first two authors contributed equally to this work.

HIGHLIGHTS

- GEM reduces cell viability of HepG2 at concentrations above 0.1 ng/mL
- 2-DOH-DiF and 5-FU reduce cell viability of HepG2 at concentrations above 1 ng/mL
- MNi in HepG2 were observed for CP, GEM, 2-DOH-DiF and 3-NH_2-F at concentrations above 0.001 ng/mL
- MNi and NBDUs were observed for 5-FU at concentrations above 0.01 ng/mL.

Corresponding author: Henrique C. S. Silveira
E-mail address: henriquecssilveira@gmail.com
Barretos Cancer Hospital - Molecular Oncology Research Center, Barretos, 14784-400, São Paulo, Brazil.

ABSTRACT

Antineoplastic agents are among the most toxic drugs. Their release in aquatic ecosystems has been reported, raising concerns about their adverse effects, including cytotoxicity and genotoxicity, on the organisms exposed. However, studies regarding its effects are scarce, especially in relation to their metabolites. In this study was performed cytotoxicity analysis through the MTS assay and genotoxicity with micronucleus evaluation in HepG2 cell line for the drugs 5-fluorouracil (5-FU) and its metabolite alpha-fluoro-beta-alanine (3-NH_2-F), gemcitabine (GEM) and its metabolite 2'-deoxy-2',2'-difluorouridine (2-DOH-DiF) and cyclophosphamide (CP). The drugs concentrations used in this study were previously found in effluents of a large cancer hospital located in Brazil. The results showed the reduction in cell viability for GEM at concentrations above 0.1 ng/mL (IC_{50} of 25.26 ng/mL), for 2-DOH-DiF and 5-FU at concentrations above 1 ng/mL, with IC_{50} of 83.65 ng/ml and 18.07 ng/mL, respectively. For CP and 3-NH_2-F, no reduction in cell viability was evaluated at the concentrations evaluated. In genotoxic evaluation by cytokinesis block micronucleus (CBMN) the damage was observed in formation of nucleoplasmic bridges (NPBs) for CP at concentrations above 0.001 ng/mL and for the 3-NH_2-F, at concentrations above 50 ng/mL. Micronuclei (MNi) and nuclear buds (NBDUs) were observed at a significantly increased frequency for CP, GEM, 2-DOH-DiF and 3-NH_2-F at concentrations above 0.001 ng/mL, while for 5-FU the same damage was observed at concentrations above 0.01 ng/mL. The results showed that compounds evaluated at concentrations present in the environment induced cytotoxic and genotoxic to HepG2 cells. These data highlight the potential cytotoxic and genotoxic effects on different cell lines and organisms, of antineoplastic agents, and help in the to implementation of public policies that minimize possible environmental contamination.

Keywords: Antineoplastic drugs, metabolites, cytotoxicity, genotoxicity, HepG2, micronucleus

Funding sources supporting the work

This work was supported by Barretos Cancer Hospital, and Public Ministry of Labor Campinas (Research, Prevention and Education of Occupational Cancer Project), Campinas, Brazil. LVF was recipient of a CAPES PhD fellowship.

1. INTRODUCTION

Cancer is a leading cause of death worldwide, and it is estimated an increase from 19.3 million cases in 2020 to 28.9 million cases in 2040 (Ferlay et al., 2020). Obviously, environmental pollution, the unhealthy lifestyle of population and increasing longevity have contributed to the increase in the incidence of cancer (Pilleron et al., 2019). Furthermore, the use the anticancer drugs globally have increased, principally in countries that there is the availability of this compounds (Aitken et al., 2019). In addition, several developed countries have specialized large cancers centers and are restricted the laws to discard these drugs (de Oliveira Klein et al., 2021; Isidori et al., 2016). Also, the use of anticancer drugs in development countries will continue to expand, what increase the risk of anticancer drugs from becoming a global environmental pollution issue (Li et al., 2021).

The presence of antineoplastics in hospital effluents has been detected since the 1980s (Aherne et al., 1985). Yet, only in 2000s, this topic gained greater intensity with the assessment of the presence of drugs, not only in hospital effluents, but also in municipal effluents, including effluent treatment stations. Several anticancer drugs were detected in the order of ng/L to µg/L (Azuma et al., 2019; Cesen et al., 2015; de Oliveira Klein et al., 2021; Ferrando-Climent et al., 2014; Ferre-Aracil et al., 2016; Franquet-Griell et al., 2015; Gouveia et al., 2020; Isidori et al., 2016; Kosjek et al., 2013; Kundi et al., 2016; Mahnik et al., 2007; Santana-Viera et al., 2019; Santos et al., 2018). Furthermore, several studies showed that, after treating effluents contaminated with antineoplastic agents in treatment plants, not occur the total removal of these compounds (Brunton et al., 2011; Seira et al., 2013; Zounkova et al., 2010; Zounkova et al., 2007). A recent review pointed out that, of the 100 antineoplastics approved for use by the US Food and Drug Administration (FDA), 33 were detected in effluents, and of these, 26 are classified as cytotoxic (Li et al., 2021). On the other hand, there is a growing number of studies seeking alternatives for the treatment of effluents contaminated with antineoplastic agents, such as nanofiltration, ozonation, photochemical degradation and electrocoagulation (EC) treatment (Cristóvão et al., 2019; Ferre-Aracil et al., 2016; Franquet-Griell et al., 2017; Zaied et al., 2020).

We recently reported the presence the anticancer drugs cyclophosphamide (CP), gemcitabine (GEM) and its metabolite 2'-deoxy-2',2'-difluorouridine (2-DOH-DiF) and the metabolite of 5-FU, alpha-fluoro-beta-alanine (3-NH_2-F) in hospital and municipal effluents. Thus, we obtained the detection of GEM, 2-DOH-DiF and CP even after treatment in ETE, with values in the order of ng/ml (de Oliveira Klein et al., 2021). Our

study was carried out in Barretos Cancer Hospital (HCB), one of the largest and most innovative cancer hospital in Brazil and Latin America (de Oliveira Klein et al., 2021).

Antineoplastics are recalcitrant substances, also known as persistent, that is, they do not biodegrade in the environment, which makes their presence higher in effluents and in the environment (Mullot et al., 2009). In addition, these compounds cause damage in animals and plants (Li et al., 2021; Misik et al., 2019; Misik et al., 2016) and some studies characterize the action of this drugs in human cell lines, such as human hepatocarcinoma cells (HepG2), human peripheral blood lymphocytes (HPBL), cells derived from a cervical cancer (HeLa) and retinal pigment epithelial cells (RPE) (Fernandes et al., 2020; Gajski et al., 2016; Gajski et al., 2018). As for drug metabolites, studies that assess their damage are scarce.

Since these drugs present in effluents can cause damage to organisms exposed to them, it is extremely important to carry out studies that assess their cytotoxic and genotoxic effects. It is important to emphasize that currently, there is no legislation regarding the release of antineoplastic agents in effluents in Brazil (ALESP, 1976; ANVISA, 2018; CONAMA, 2011). At present, only a few parts of the world address this issue with public policy regulations (Directive, 2008; OECD, 2019; Russo et al., 2020). Thus, in this study we evaluated the cytotoxic and genotoxic effects of the antineoplastics 5-FU, GEM, CP and the metabolites 2-DOH-DiF and 3-NH_2-F through the assay of MTS and micronucleus test in HepG2 cells.

2. MATERIALS AND METHODS

2.1. Concentrations of drugs used in trials

The concentration of drugs CP, GEM and 5-FU and metabolites 2-DOH-DiF and 3-NH_2-F were values chosen based on environmental detection in effluents as described by Oliveira Klein et al. (2021).

For the MTS assay the seven concentrations of all compounds (0.001, 0.01, 0.1, 1.0, 10, 25, 50 ng/mL) were based on the results of our previous study (de Oliveira Klein et al., 2021) in which we verified levels of this drugs within of the range of 0.11-116 ng/mL in effluents in Barretos city, Brazil.

For the CBMN assay the concentrations of GEM, CP, 5-FU, 2-DOH-DiF and 3-NH_2-F used in this study were based also on the concentrations detected in effluents by de Oliveira Klein et al. (2021) as the IC_{50} values previously obtained in the MTS assay, therefore, the concentrations were different for each compound. For CP and 3-NH_2-F were

0.001, 1.0 and 50 ng/mL. For 5-FU were 0.01, 0.1 and 1.0 ng/mL. For GEM were 0.001, 0.01 and 0.1 ng/mL. For 2-DOH-DiF were 0.001, 1.0 and 25 ng/mL.

2.2. Chemicals

DMEM (Dulbecco's modified Eagle's medium) and Fetal Bovine Serum, HEPES (4.5 mM), sodium bicarbonate (170 mM), penicillin (100 IU/mL) and streptomycin (100 mg/mL), as well DMSO, mitomycin C, cytochalasin-B, Giemsa and all of the standards (drugs and metabolites) were acquired from Sigma-Aldrich® (Missouri, USA). Trypan blue were acquired from Invitrogen® (Massachusetts, EUA). Methanol and acetic acid (3:1) were acquired from Merck® (Darmstadt, Alemanha). The ultrapure water was obtained using a Millipore Milli-Q Integral 3 system.

2.3. Preparation of analytical standard stock solutions

The solutions were prepared separately for each analytical standard. Approximately 1 mg (99%) of the analytical standard was weighed and add in 1000 µL (or the volume required) of 50% DMSO solution was added to reach the desired final concentration of 1000 µg/mL.

2.4. Cell line

HepG2 cell line was obtained from Rio de Janeiro Cell Bank (BCRJ). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM 1X, high glucose; Gibco, Invitrogen®, Grand Island, NY) supplemented with 10% Fetal Bovine Serum (FBS, Sigma-Aldrich®) and 1% penicillin/streptomycin solution (P/S), at 37 °C and 5% CO_2. To conduct the assays, cell viability was previously analysed the trypan blue exclusion technique, using Countess® Automated Cell Counter (Life Technologies®, CA, USA). Absence of mycoplasma contamination was assessed by PCR. For the execution of the PCR the primers GPO3 (5'GGG AGC AAA CAGG AT TAG ATA CCC T3') and MGSO (TGC ACC ATCTGT CAC TCT GTT AAC CTC3') were used that amplify a part of the 16S unit gene of any species of the genus *mycoplasma* .For the PCR reaction, 1µL of the DNA of the extracted samples was used, 1.25 mM of each triphosphate deoxynucleotide (dNTP), 10 µM of each primer in solution containing 10 mM Tris-HCl pH 8.0, KCl 50 mM and 50 mM MgCl 2 to a final volume of 25 µL. Reaction conditions were: initial denaturation at 95 °C for 5 min, 30 cycles at 95 °C for 30 sec, primer annealing at 55 °C for 30 sec and extension at 72 °C for 50 sec, and the last step at 72 °C for 7 min for final

extension The amplified products were applied in 1.5% agarose gel, submitted to electrophoresis and stained with GelRed (Biotium) for visualization in transilluminator.

2.5. Cell viability assay

The Cell Viability Assay in Aqueous Solution (Cell Titer 96 Aqueous One Solution Cell Proliferation Assay - Promega®), a colorimetric method to determine the number of viable cells in the proliferation or cytotoxicity assays, was used as described by the manufacturer as reported and by Silva-Oliveira et al. (2016). Cell Titer 96 ® contains the tetrazolium compound [3 - (4,5-dimethyl-2-yl) -5 - (3-carboxymethoxyphenyl) -2 - (4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] and an electron coupling reagent (phenazine ethosulfate: PES).

Briefly, cells were seeded in 96-well culture plates in a density of $5x10^3$ cells/well and allowed to adhere for 24 h before drugs additions at 37 °C and 5% CO_2. After incubation, cells were treated with concentrations of CP, GEM, 5-FU, 2-DOH-DiF and 3-NH_2-F (0.001, 0.01, 0.1, 1.0, 10, 25, 50 ng/mL) and DMSO (negative control) in DMEM medium supplemented with 0.5% FBS for 72 hs at 37 °C and 5% CO_2. After incubation, the MTS reagent was added to each well and the cells were incubated at 37ºC for a period of 4 hours. Absorbance was measured in an ELISA plate reader (Varioskan Flash-Thermo Scientific®) at 490 nm. Absorbance values of the negative control were considered as 100% of cell viability, and the results were expressed as a percentage of viable cells. Three independent experiments were carried out in triplicate and means ± standard deviations were calculated. The mean inhibition concentration (IC_{50}) values were calculated from dose response curve.

2.6. Cytokinesis-block micronucleus (CBMN) assay

The CBMN assay is a comprehensive method for measuring DNA damage and cytotoxicity. DNA damage occurs specifically in binucleated cells (BN) and includes micronuclei (MNi), which can be presented due to: a) chromosome breakage or loss of an entire chromosome; b) nucleoplasmic bridges (NPBs), DNA repair and/or final telomere fusions; as well as by c) nuclear buttons (NBUDs), amplified DNA deletion biomarker or DNA repair complexes (Fenech, 2007).

The CBMN assay was performed as described by Fenech et al. (2007) and with the recommendations from OECD test guideline Nº 487 (2016). HepG2 were exposed to non-cytotoxic concentrations (determined by MTS assay) of the drugs for 24 h at 37 °C in an

atmosphere of 5 % CO2. For this, cells were seeded in 12-well culture plates in a density of $5x10^5$ cells/well and allowed to adhere for 24 h before drugs additions at 37 °C and 5% CO_2. Afterwards, HepG2 were treated with CP (0.001, 1 and 50 ng/mL), GEM (0.001, 0.01 and 0.1 ng/mL), 5-FU (0.01, 0.1 and 1 ng/mL), 2-DOH-DiF (0.001, 1 and 25 ng/mL) and 3-NH_2-F (0.001, 1 and 50 ng/mL), negative control (DMSO) and positive control (mitomycin C, 2 ug/mL) diluted in DMEM medium supplemented with 0.5% FBS for 24 hs at 37°C and 5% CO_2. Compounds concentrations were based on the values detected by de Oliveira Klein et al. (2021) as well as the IC_{50} values determined in the MTS analysis.

After the treatment period, cells were incubated with complete culture medium containing cytochalasin-B (6 µg/mL) for 24 h. Afterwards, the cells were harvested, treated with hypotonic solution (0.075 M KCl), fixed with methanol and acetic acid (3:1), stained with 5% Giemsa, placed to dry at room temperature and subsequently analysed. Three independent experiments were performed in triplicate. Thus, a total of 3.000 binucleated cells (500 binucleated cells for slide) were scored for each concentration for experiment.

Three thousand cells (500 cells for slide) were counted for each concentration for experiment to determine the nuclear division index (NDI) using the following formula: NDI = [M1 + 2(M2) + 3(M3) + 4(M4)]/N, where M1-M4 indicates the number of cells with 1–4 nuclei and N is the number of cells assayed. MNi, NPBs, and NBUDs, was determined following the standard criteria reported by Fenech (2007).

2.7. Statistical analysis

For the statistical analysis all tests were evaluated using the R software®. The calculation of IC_{50} values was by non-linear regression using Graph Pad Prism 8® (Graph Pad Software, La Jolla, USA), and all other analysis were performed using the same softwares. The data obtained were tested to verify normality using the Shapiro-Wilk test. The cell viability assay and cytokinesis-block micronucleus assay as a result were non-parametric data. After this evaluation, the data were subjected to Kruskal Wallis followed by the Dunn test. NDI were parametric data with calculations through One-way Analysis of Variance (ANOVA), test followed by the Tukey test. The differences between the treatments and the NC were considered significant at $p < 0.05$ for all tests.

3. RESULTS

3.1. *Analysis of cell viability assay*

The half maximal inhibitory concentration (IC_{50}) represents the concentration of a drugs required for a 50% inhibition of growth *in vitro*, a reduction in cell viability was observed for GEM at concentrations above 0.1 ng/mL, with IC_{50} of 25.26 ng/mL, for its metabolite 2-DOH-DiF at concentrations above 1 ng/mL, with IC_{50} of 83 .65 ng/ml, and for 5-FU at concentrations above 1 ng/ml, with IC_{50} of 18.07 ng/mL (Table 1 and Figure 1). For CP and the metabolite 3-NH_2-F, not was observed the reduction in cell viability at the concentrations evaluated, and it the IC_{50} values were not determined. The results show that the concentrations of GEM, 2-DOH-DiF and 5-FU present in the effluents cause a decrease in cell viability (Table 1 and Figure 1).

Table 1. Identification of the IC_{50} for cyclophosphamide (CP), gemcitabine (GEM), 2'-deoxy-2',2'-difluorouridine (2-DOH-DiF), 5-fluorouracil (5-FU) and alpha-fluoro-beta-alanine (3-NH_2-F) for human hepatocarcinoma cells (HepG2) after 72h of exposure.

Compound	IC_{50} with standard deviation (ng/mL)	Values detected in effluents (ng/mL)[b]
CP	N. D. [a]	> LD[c] to 29.10
GEM	26.25 (15.26 – 44.75)	> LD to 25.90
Met GEM (2-DOH-DiF)	83.65 (53.47 – 144.10)	> LD to 116.00
5-FU	18.07 (12.70 – 25.43)	> LD
Met 5-FU (3-NH_2-F)	N. D.	> LD to 18.20

[a] N.D. – not determined
[b] Values detected in according with de Oliveira Klein et al. 2021
[c] LD - Limit of detection

3.2. Determination of Nuclear Division Index (NDI)

The mitotic activity and/or cytostasis was evaluated by determination of NDI. The results of NDI experiments with HepG2 are show in the Figure 1. The NDI was significantly decrease at concentrations above 1 ng/mL for 5-FU, above 0.1 for GEM and above 25 ng/mL for 2-DOH-DiF concentrations in relation to NC (Figure 1). However, to other compounds, CP and for the 3-NH_2-F, no significant statistical differences were observed.

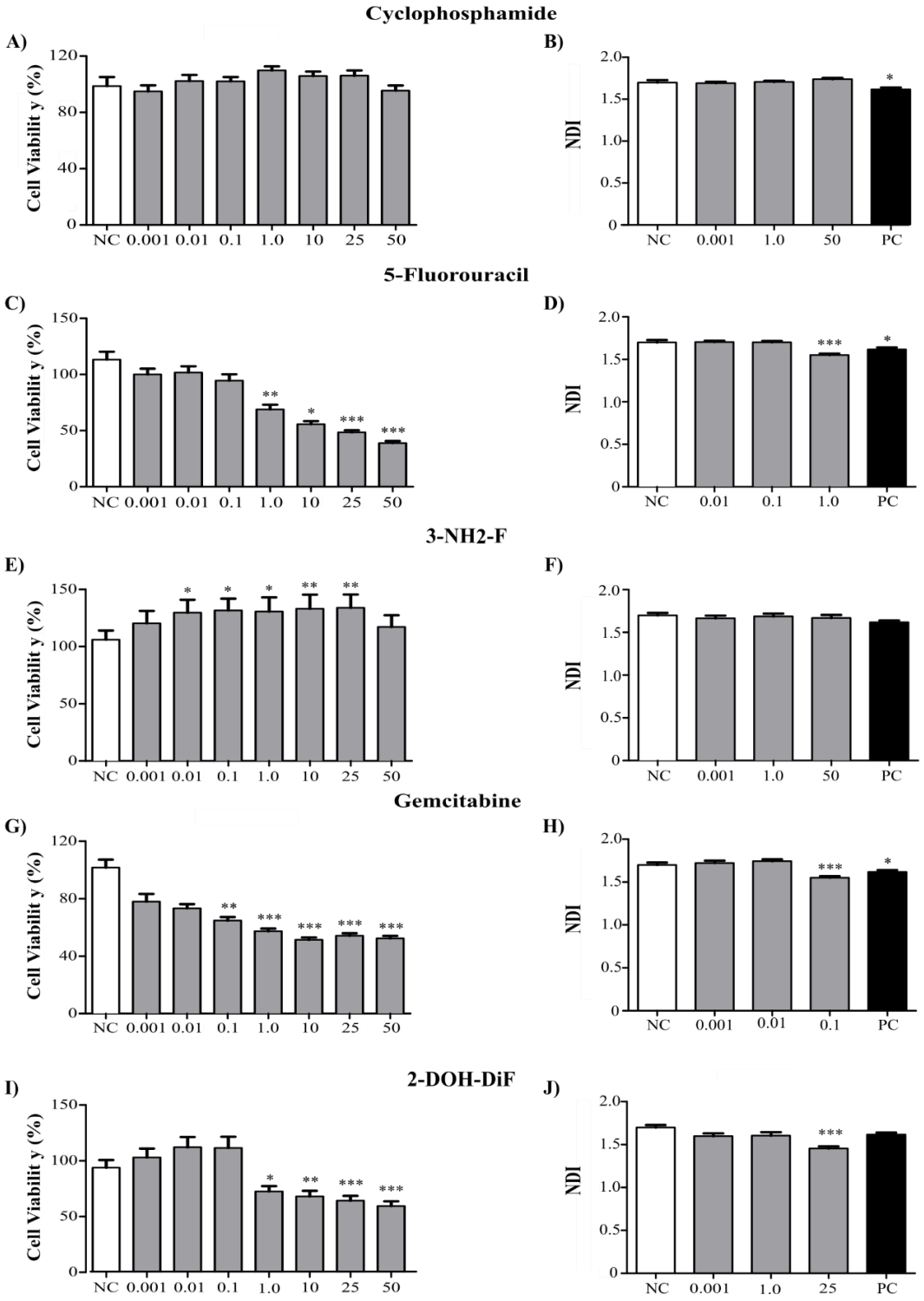


Figure 1. Cell viability and nuclear division index (NDI) of HepG2 treated with CP (A, B), 5-FU (C, D), 3-NH_2-F (E, F), GEM (G, H) and 2-DOH-DiF (I, J) for 72 and 24 h respectively. All data are expressed as means ± standard deviation of three independent experiments performed in triplicates. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ when compared to negative control by Kruskal Wallis followed by Dunn for cell viability test and ANOVA test, followed by the Tukey test for NDI test.

3.3. Evaluation of Cytokinesis-block micronucleus (CBMN) assay

The parameters of NBUDs, NPBs, and MNi were analysed to assess the genotoxic effects that the drugs can cause in HepG2 cell line.

When the cells were treated with 5-FU did not observe significant increase in the number of NPBs in the concentrations evaluated (Figure 2A). However, the number of NBDUs and MNi increased significantly ($p<0.001$) at all concentrations of 5-FU when compared to that of the NC (Figure 2B-C). When the treatment with 3-NH_2-F (5-FU metabolite) was evaluated, the cells significantly increased ($p<0.001$) the number of NPBs at the concentration of 50 ng/mL (Figure 2D). Similar results of NBUD and MNi were observed for 5-FU, its metabolite 3-NH_2-F also significantly increased these two parameters at all concentrations evaluated when compared to CN (Figure 2E-F).

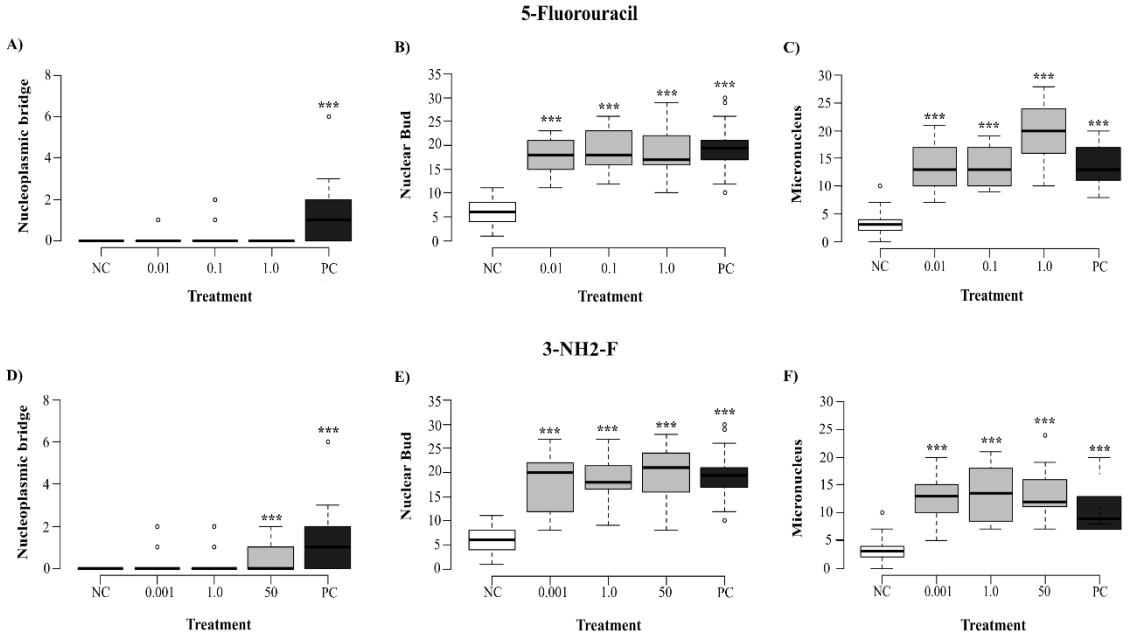


Figure 2. Genotoxicity in HepG2 treated with 5-FU (A, B and C), and 3-NH_2-F (D, E and F) at concentrations present in the effluents for 24 h. Data are expressed as means ± standard deviation of three independent experiments performed in triplicates. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ when compared to negative control by Kruskal Wallis followed by Dunn test.

For the parameters NPBs (Figure 3A), NBUDs (Figure 3B) and MNi (Figure 3C), the cells treated with GEM, showed significant increase in the number of these parameters at all concentrations analysed when compared to that in the control. It was observed that the lowest GEM concentration (0.001 ng/mL) tested was able to cause genotoxic effects in

these cells. To metabolites 2-DOH-DiF, there was not significant increase in the number of NPBs in the concentrations evaluated (Figures 3D). However, for NBUDs (Figure 3E) and MNi (Figure 3F), the 2-DOH-DiF caused a significant increase ($p<0.001$) in these parameters in relation to the CN in all concentrations assessed, similar to what was observed for the GEM.

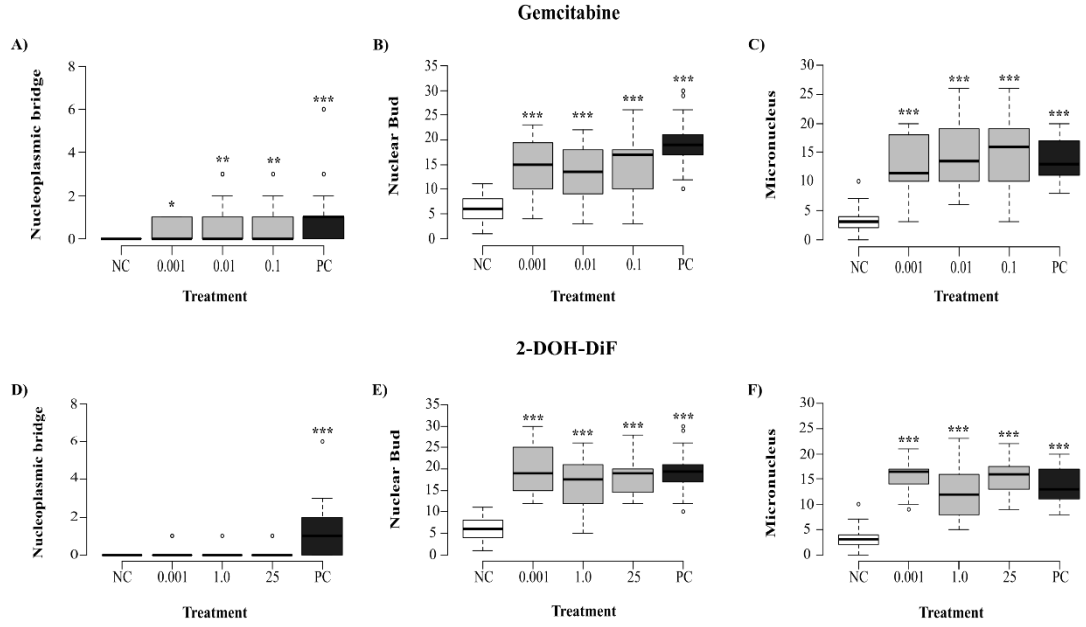


Figure 3. Genotoxicity in HepG2 treated with GEM (A, B and C), and the metabolite 2-DOH-DiF (D, E and F) at concentrations present in the effluents for 24 h. Data are expressed as means ± standard deviation of three independent experiments performed in triplicates. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ when compared to negative control by Kruskal Wallis followed by Dunn test.

In relation to genotoxic effects caused by CP, the number of NPBs (Figure 4A), NBDU (Figure 4B) and MNi (Figure 4C) increased significantly ($p<0.001$) for all concentrations assessed after 24h of treatment compared to that of the NC. The results of CBMN assay showed that all compounds present in the effluents present genotoxicity to HepG2.

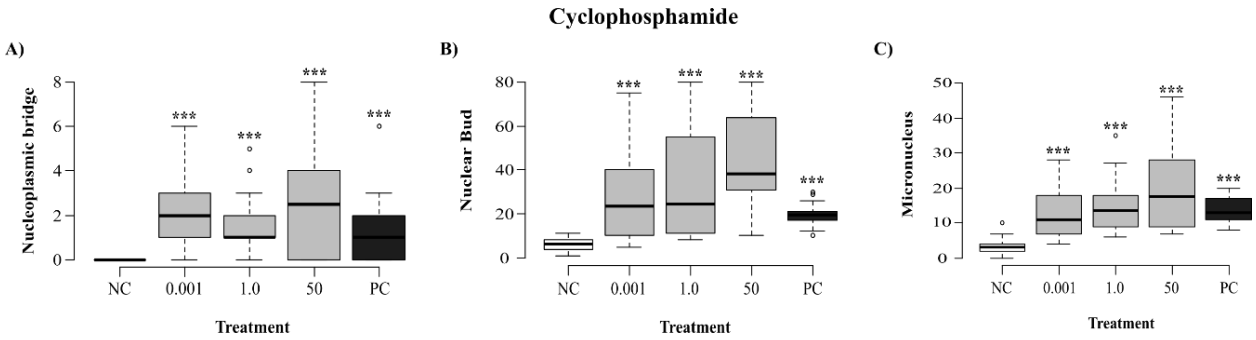


Figure 4. Genotoxicity in HepG2 treated with cyclophosphamide (A, B and C), at concentrations present in the effluents for 24 h. Data are expressed as means ± standard deviation of three independent experiments performed in triplicates. * p <0.05, ** p<0.01, *** p<0.001 when compared to negative control by Kruskal Wallis followed by Dunn test.

4. DISCUSSION

In our study, we evaluated the cytotocity and genotoxicity of the antineoplastics 5-FU, GEM, CP and the metabolites 2-DOH-DiF 3-NH_2-F in HepGe2 cell line. The MTS assay was used to evaluate the cytotoxicity and CBMN assay was performed to evaluate the NDI, MNi and other morphologies alterations in HepG2. HepG2 is considered a cellular model for studies involving *in vitro* toxicity and genotoxicity to identify toxicogenetic risks in humans, mainly xenobiotics (Amacher et al., 2016; Gajski et al., 2016; Guillouzo et al., 2007), performing these two tests with HepG2 cells will enable an assessment of possible damage to organisms exposed to concentrations of antineoplastics and metabolites present in the effluents. We found that the values that cause decreased cell viability and altered mitotic activity and/or cytostasis (NDI) for GEM and its metabolite 2-DOH-DiF are within the concentrations detected in effluents, in influents before treatment and after treatment in WWTPs (de Oliveira Klein et al., 2021; Kundi et al., 2016).

When evaluating the genotoxicity data for GEM and 2-DOH-DiF, it was possible to verify for both that at concentrations above 0.001 ng/mL there was a significant increase in MNi and NBDUs, and only for GEM there was a significant increase in NPBs at concentrations above of 0.001 ng/mL. In the literature is scarce regarding cytotoxicity and genotoxicity data of GEM and its metabolite 2-DOH-DiF. In 2010, Zounkova et. al. (Zounkova et al., 2010) when evaluating the ecotoxicity of GEM in the acute phase in

immobilization and reproduction tests with crustacean *Daphnia magna* and growth inhibition tests with *Desmodesmus subspicatus* alga and *Pseudomonas putida* bacteria, they found the respective EC_{50} (effective concentration) values of 110,000 ng/mL, 45,000 ng/mL and 100,000 ng/mL, quantities much higher than the drug values detected in effluents and the cytotoxicity values in HepG2 of this study. Thus, given the scarcity of studies, it is important to highlight the need for research of evaluation these damages, since GEM is consumed on a large scale worldwide, with an excretion rate around 10% (Weigel, 1999), as well as its metabolite, excreted and detected in effluents (de Oliveira Klein et al., 2021). As GEM and its metabolite 2-DOH-DiF, the 5-FU showed a decrease in cell viability at concentrations present in the effluents, and a significant increase in NBDUs and formation of MNi when compared to NC at concentrations present in the effluents (Isidori et al., 2016; Kosjek et al., 2013; Mullot et al., 2009).

5-FU is one of the antineoplastic drugs with the highest number of cytotoxicity and genotoxicity studies. In their study Gajski et al. (2016) found IC_{50} of 5-FU for HepG2 in 72h MTT assay of 5,270 ng/mL, a value higher than IC_{50} per MTS assay evaluated in our study, of 18.07 ng/mL. In this same study, Gajski et al. (2016) evaluated genotoxic damage of 5-FU by comet and micronucleus assay in cell lines ZFL, HPBLs and HepG2, observing damage by comet assay only in ZFL at concentrations above 100 ng/mL, and by micronucleus assay the damage was observed only for HepG2, at concentrations above 100 ng/mL. In our CBMN assay, we observed an increase in the frequency of MNi to HepG2 at concentrations above 0.01 ng/mL.–Similar to our genotoxicity results for HepG2 at concentrations above 0.01 ng/L of 5-FU, Kovacs et al. (2016) observed in his study genotoxicity at this same concentration in zebrafish erythrocytes by the comet assay. Furthermore, 5-FU genotoxicity studies in different cell lines, including HepG2, as well as acute and chronic cytotoxicity assays, observed concentrations that cause damage higher than those found in our study and present in effluents (Gacic et al., 2014; Gajski et al., 2016; Lutterbeck et al., 2015). Emphasizing the importance that, despite being one of the anticancer drugs with the largest number of studies, there is still a lack of precise information that can confirm the safe amounts of it in effluents.

The 5-FU metabolite, 3-NH_2-F, did not show a decrease in cell viability in HepG2, also show no significant change in NDI. On the other hand, in the genotoxicity assay, it was possible to observe a significant increase in NDBUs and MNi at concentrations above 0.001 ng/mL, and in NPBs at concentrations above 50 ng/mL. Considering the presence these drugs in the environment, as evaluated by de Oliveira Klein et al. (2021) when the

values present in effluents can be indicate a significant increase in NBDUs and MNi in human cells HepG2.

In 2010, Zounkova et. al. when evaluating the ecotoxicity of 3-NH_2-F in the acute phase in immobilization and reproduction tests with crustacean *D. magna* and growth inhibition tests with *D. subspicatus* alga and *P. putida* bacteria, they reached respective values of EC_{50} >100,000 ng/mL, 80,000 ng/mL and 140,000 ng/mL, quantities much higher than the range evaluated in our cytotoxicity assay, which may explain that we did not identify EC_{50} in the quantities evaluated, even if in another cell model.

5-FU and GEM are antimetabolic drugs, that is, they exert their effects mainly by biochemically blocking DNA synthesis, being restricted to the S phase of the cell cycle (Peters et al., 2000) are used in cancer treatment to stop cell growth and division and prevent the progression of the replication fork by incorporating into DNA (Avendaño and Menendez, 2015), this mechanism of action may explain the change in the NDI of both, for GEM at concentrations above 0.1 ng/mL and for 5-FU above 1 ng/mL.

CP is widely used as an antineoplastic drug for the treatment of malignant lymphoma, lung and ovarian cancer. The pharmacological action of CP is its ability to alkylate the N-7 position of guanine to cause DNA crosslinking (Liu et al., 2019), affects DNA replication and leads to cell apoptosis (Chan et al., 1994; Colvin, 1999). Furthermore, CP is an alkylating agent of DNA, the alkylcarbon groups of drugs normally bind to a wide range of biological molecules, thus altering their cellular structures and functions. Specifically, aromatic nitrogen and extra-cyclic oxygen from DNA bases are targeted and generate a variety of covalent adducts (with methyl groups or complex alkyl additions) (Drabløs et al., 2004). The formation of DNA adducts can lead to a series of genotoxic events, such as: 1) a reduction in DNA synthesis, preventing the progress of replicating bifurcations; 2) formation of cross-links preventing the DNA from being separated for synthesis or transcription; and 3) chromosomal aberrations and genetic mutations in cells (Peterson, 1980). The form of action of alkylating agents, acting directly on DNA, can explain cytotoxic damage only at high concentrations and above drug concentrations in effluents (Mater et al., 2014; Yadav et al., 2020) and genotoxic damage to HepG2 at lower concentrations, above 0.001 ng/mL pointed out in our study.

GEM, mainly applied in the treatment of breast, ovarian, bladder, pancreas, bronchial and lung cancer, is an antimetabolite of the pyrimidine group, with the ability to inhibit DNA synthesis through the inhibition of DNA polymerase and of the ribonucleotide transferase (McEvoy, 2000), about 10% of GEM is excreted unchanged, together with its

metabolite, 2-DOH-DiF (Weigel, 1999). 5-FU acts as an antimetabolite to uracil, acting through its anabolism products, causing interference in DNA synthesis (Dorr and Von Hoff, 1994). The drug is mainly administered in the treatment of cancer of the rectum, stomach, colon, pancreas, thyroid gland and breast (Mahnik et al., 2004), between 2 to 20% of 5-FU is excreted as metabolites such as 3-NH_2-F (Micromedex, 2005).

Finalizing the evaluation of drugs in this study, CP did not decrease cell viability and proliferation in HepG2 cells in the range evaluated and it was not possible to determine the IC_{50} value. Mater et al. (2014), when using the same methodology for cytotoxicity assay (MTS) with HepG2 and similar range (0.01 to 10 ng/mL) they also did not determine IC_{50} values of CP. On the other hand, for genotoxic damage, CP caused a significant increase in the number of NPBs, NBDUs and MNi at concentrations presents in effluents, even after treatment in wastewater treatment plants (WWTPs) (Cesen et al., 2015; de Oliveira Klein et al., 2021; Ferrando-Climent et al., 2014; Ferre-Aracil et al., 2016; Gouveia et al., 2020; Santana-Viera et al., 2019). Furthermore, many studies demonstrated genotoxicity in different biological models as polychaete *Nereis diversicolor*, marine mussel *Mytilus galloprovincialis*, *A. cepa* cells, human cells HeLa, RPE and HBPL (Fernandes et al., 2020; Fonseca et al., 2018; Kocaman et al., 2013; Lutterbeck et al., 2015; Yuksel et al., 2017), with values higher than the concentration that demonstrated genotoxicity in our study (0.001 ng/mL).

In summary, in our study the range of concentrations used for different drugs that are environmentally found in effluents induced cytotoxicity for 5- FU, GEM and 2-DOH-DiF. On the other hand, the genotoxicity evaluated under the same conditions was induced by all drugs in the *in vitro* tests with the HepG2 cell line. Thus, we can suggest that these anticancer drugs can cause genotoxic changes in different cell types in the environment.

5. CONCLUSIONS

This study evaluated the cytotoxicity and genotoxicity of the antineoplastics CP, 5-FU and GEM and the metabolites 3-NH_2-F and 2-DOH-DiF excreted together with the drugs and present in the environment, demonstrating the damage to human cells in the concentrations at which they are found in effluents. Thus, is unknown the effects healthy in regarding the chronic exposure of compounds on organisms in the quantities that they are present in the environment. Furthermore, we may question whether these drugs and their metabolites undergo trophic transfer and can impact human health through the

consumption of contaminated food or ingestion of contaminated water. The theme should receive attention for further studies, with a wider range of organisms and cells evaluated, to confirm the need for public policies that restrict the presence of these compounds in effluents.

Study limitations

It is important to highlight that the analysis was performed *in vitro*, with controlled conditions, different from what happens in the environment. These analyses were in human hepatocarcinoma cells (HepG2), a model known to be used for genotoxicity analysis, but the tests are restricted to a single cell lineage, and for a wider spectrum environmental condition. Also, other cell lines can be evaluated, such as HPBLs that can possibly clarify the cellular effects of anticancer drugs. Also, there is other methodologies as comet assay to evaluate the genotoxicity, however do not considered in this work.

Sample CRediT author statement

Mariana de Oliveira Klein: Conceptualization, Methodology, Investigation, Writing - Original Draft. **Luiza Flavia Veiga Francisco:** Methodology, Software, Investigation, Formal analysis, Writing - Original Draft. **Izabela Natália Faria Gomes:** Methodology, Formal analysis. **Sergio V. Serrano:** Conceptualization, Resources. **Rui M. Reis:** Resources, Writing - Review & Editing. **Henrique C. S. Silveira:** Conceptualization, Methodology, Resources, Supervision, Writing - Review & Editing.

Conflict of interests

The authors have declared no conflicts of interest.

Acknowledgements

The authors would like to express their gratitude to Barretos Cancer Hospital and Public Ministry of Labor Campinas (Research, Prevention and Education of Occupational Cancer Project), Campinas, Brazil for the financial support of the study. INFG is the recipient of a FAPESP Ph.D. fellowship (2017/22305-9). We would also like to thank staff at the Barretos Cancer Hospital from sectors such as library and statisticians.

6. REFERENCES

Aherne, G. W., et al., 1985. The role of immunoassay in the analysis of microcontaminants in water samples. Ecotoxicol Environ Saf. 9**,** 79-83.

Aitken, M., et al., 2019. Global oncology trends 2019, therapeutics, clinical development and health system implications. IQVIA Institute for Human Data Science.

ALESP, Lei Estadual nº 997/1976 - Dispõe sobre o controle da poluição do meio ambiente. In: A. L. d. E. d. S. Paulo, (Ed.), Lei Estadual nº 997/1976 - Dispõe sobre o controle da poluição do meio ambiente, Vol. Lei nº 997/1976. Assembléia Legislativa do Estado de São Paulo, 1976.

Amacher, A. E., et al., 2016. Experiences of general practitioners, home care nurses, physiotherapists and seniors involved in a multidisciplinary home-based fall prevention programme: a mixed method study. BMC Health Serv Res. 16**,** 469.

ANVISA, Regulamenta as Boas Práticas de Gerenciamento dos Resíduos de Serviços de Saúde In: A. N. d. V. Sanitária, (Ed.), 222/2018. Agência Nacional de Vigilância Sanitária, 2018.

Avendaño, C., Menendez, J. C., 2015. Medicinal chemistry of anticancer drugs. Elsevier.

Azuma, T., et al., 2019. Environmental fate of pharmaceutical compounds and antimicrobial-resistant bacteria in hospital effluents, and contributions to pollutant loads in the surface waters in Japan. Sci Total Environ. 657**,** 476-484.

Brunton, L. L., et al., 2011. Goodman & Gilman's the pharmacological basis of therapeutics. McGraw-Hill Medical New York.

Cesen, M., et al., 2015. Occurrence of cyclophosphamide and ifosfamide in aqueous environment and their removal by biological and abiotic wastewater treatment processes. Sci Total Environ. 527-528**,** 465-73.

Chan, K. K., et al., 1994. Clinical pharmacokinetics of cyclophosphamide and metabolites with and without SR-2508. Cancer Res. 54**,** 6421-9.

Colvin, O. M., 1999. An overview of cyclophosphamide development and clinical applications. Curr Pharm Des. 5**,** 555-60.

CONAMA, Resolução CONAMA nº 430/2011. In: M. d. M. A.-C. N. d. M. A. (CONAMA), (Ed.), Vol. 430/2011. Ministério do Meio Ambiente - Conselho Nacional de Meio Ambiente (CONAMA), Brasil, 2011.

Cristóvão, M., et al., 2019. Treatment of anticancer drugs in hospital and wastewater effluents using nanofiltration. Separation and Purification Technology. 224**,** 273-280.

de Oliveira Klein, M., et al., 2021. Detection of anti-cancer drugs and metabolites in the effluents from a large Brazilian cancer hospital and an evaluation of ecotoxicology. Environ Pollut. 268**,** 115857.

Directive, E. J. O. J. o. t. E. U. L., 2008. Directive 2008/98/EC of the European Parliament and of the Council of 19 November 2008 on waste and repealing certain Directives. 312.

Dorr, R., Von Hoff, D., 1994. Cancer chemotherapy book. Appleton and Lange, Norwalk.

Drabløs, F., et al., 2004. Alkylation damage in DNA and RNA—repair mechanisms and medical significance. DNA repair. 3**,** 1389-1407.

Fenech, M., 2007. Cytokinesis-block micronucleus cytome assay. Nat Protoc. 2**,** 1084-104.

Ferlay, J., et al., CI5 IX: cancer incidence in five continents, volumes I to X 2014. 2020.

Fernandes, E., et al., 2020. Cytotoxic responses of the anticancer drug cyclophosphamide in the mussel Mytilus galloprovincialis and comparative sensitivity with human cells lines. Chemosphere. 261**,** 127678.

Ferrando-Climent, L., et al., 2014. Incidence of anticancer drugs in an aquatic urban system: from hospital effluents through urban wastewater to natural environment. Environ Pollut. 193**,** 216-223.

Ferre-Aracil, J., et al., 2016. Ozonation of hospital raw wastewaters for cytostatic compounds removal. Kinetic modelling and economic assessment of the process. Sci Total Environ. 556**,** 70-9.

Fonseca, T. G., et al., 2018. Environmental relevant levels of the cytotoxic drug cyclophosphamide produce harmful effects in the polychaete Nereis diversicolor. Sci Total Environ. 636**,** 798-809.

Franquet-Griell, H., et al., 2015. Predicting concentrations of cytostatic drugs in sewage effluents and surface waters of Catalonia (NE Spain). Environ Res. 138**,** 161-72.

Franquet-Griell, H., et al., 2017. Biological and photochemical degradation of cytostatic drugs under laboratory conditions. J Hazard Mater. 323**,** 319-328.

Gacic, Z., et al., 2014. The impact of in vivo and in vitro exposure to base analogue 5-FU on the level of DNA damage in haemocytes of freshwater mussels Unio pictorum and Unio tumidus. Environ Pollut. 191**,** 145-50.

Gajski, G., et al., 2016. Genotoxic potential of selected cytostatic drugs in human and zebrafish cells. Environ Sci Pollut Res Int. 23**,** 14739-50.

Gajski, G., et al., 2018. Genotoxicity assessment of a selected cytostatic drug mixture in human lymphocytes: A study based on concentrations relevant for occupational exposure. Environ Res. 161**,** 26-34.

Gouveia, T. I. A., et al., 2020. Liquid-liquid extraction as a simple tool to quickly quantify fourteen cytostatics in urban wastewaters and access their impact in aquatic biota. Sci Total Environ. 740**,** 139995.

Guillouzo, A., et al., 2007. The human hepatoma HepaRG cells: a highly differentiated model for studies of liver metabolism and toxicity of xenobiotics. Chem Biol Interact. 168**,** 66-73.

Isidori, M., et al., 2016. Chemical and toxicological characterisation of anticancer drugs in hospital and municipal wastewaters from Slovenia and Spain. Environ Pollut. 219**,** 275-287.

Kocaman, A. Y., et al., 2013. In vitro evaluation of the protective effects of 4-thujanol against mitomycin-C and cyclophosphamide-induced genotoxic damage in human peripheral lymphocytes. Toxicol Ind Health. 29**,** 23-37.

Kosjek, T., et al., 2013. Fluorouracil in the environment: analysis, occurrence, degradation and transformation. J Chromatogr A. 1290**,** 62-72.

Kovacs, R., et al., 2016. Acute and sub-chronic toxicity of four cytostatic drugs in zebrafish. Environ Sci Pollut Res Int. 23**,** 14718-29.

Kundi, M., et al., 2016. Prediction and assessment of ecogenotoxicity of antineoplastic drugs in binary mixtures. Environ Sci Pollut Res Int. 23**,** 14771-9.

Li, D., et al., 2021. Anticancer drugs in the aquatic ecosystem: Environmental occurrence, ecotoxicological effect and risk assessment. Environ Int. 153**,** 106543.

Liu, H., et al., 2019. Vitamin D Resists Cyclophosphamide-Induced Genomic and DNA Damage in CHL Cells In Vitro and in Mice In Vivo. Nutr Cancer. 71**,** 1030-1039.

Lutterbeck, C. A., et al., 2015. Evaluation of the toxic effects of four anti-cancer drugs in plant bioassays and its potency for screening in the context of waste water reuse for irrigation. Chemosphere. 135**,** 403-10.

Mahnik, S. N., et al., 2007. Fate of 5-fluorouracil, doxorubicin, epirubicin, and daunorubicin in hospital wastewater and their elimination by activated sludge and treatment in a membrane-bio-reactor system. Chemosphere. 66**,** 30-7.

Mahnik, S. N., et al., 2004. Determination of 5-fluorouracil in hospital effluents. Anal Bioanal Chem. 380**,** 31-5.
Mater, N., et al., 2014. In vitro tests aiding ecological risk assessment of ciprofloxacin, tamoxifen and cyclophosphamide in range of concentrations released in hospital wastewater and surface water. Environ Int. 63**,** 191-200.
McEvoy, G., American Hospital Formulary Service/AHFS Drug Information 2000. Bethesda: The American Society of Health-System Pharmacists. Inc, 2000.
Micromedex, T., 2005. Drug information for the health care professional. Volume. 1**,** 2569-2572.
Misik, M., et al., 2019. Environmental risk assessment of widely used anticancer drugs (5-fluorouracil, cisplatin, etoposide, imatinib mesylate). Water Res. 164**,** 114953.
Misik, M., et al., 2016. Analyses of combined effects of cytostatic drugs on micronucleus formation in the Tradescantia. Environ Sci Pollut Res Int. 23**,** 14762-70.
Mullot, J. U., et al., 2009. Development and validation of a sensitive and selective method using GC/MS-MS for quantification of 5-fluorouracil in hospital wastewater. Anal Bioanal Chem. 394**,** 2203-12.
No, O. T., 2016. 487: In vitro mammalian cell micronucleus test. OECD guidelines for the testing of chemicals, Section. 4**,** 1-26.
OECD, Pharmaceutical Residues in Freshwater : Hazards and Policy Responses. 2019.
Peters, G. J., et al., 2000. Basis for effective combination cancer chemotherapy with antimetabolites. Pharmacol Ther. 87**,** 227-53.
Peterson, A., 1980. DNA synthesis, mutagenesis, DNA damage, and cytotoxicity in cultured mammalian cells treated with alkylating agents. Cancer research. 40**,** 682-688.
Pilleron, S., et al., 2019. Global cancer incidence in older adults, 2012 and 2035: a population-based study. International journal of cancer. 144**,** 49-58.
Russo, C., et al., Toxicity of anticancer drug residues in organisms of the freshwater aquatic chain. Fate and effects of anticancer drugs in the environment. Springer, 2020, pp. 379-401.
Santana-Viera, S., et al., 2019. Simultaneous and systematic analysis of cytostatic drugs in wastewater samples by ultra-high performance liquid chromatography tandem mass spectrometry. J Chromatogr B Analyt Technol Biomed Life Sci. 1110-1111**,** 124-132.
Santos, M. S. F., et al., 2018. Development of an analytical methodology for the analysis of priority cytostatics in water. Sci Total Environ. 645**,** 1264-1272.
Seira, J., et al., 2013. Optimization of pressurized liquid extraction using a multivariate chemometric approach for the determination of anticancer drugs in sludge by ultra high performance liquid chromatography-tandem mass spectrometry. J Chromatogr A. 1283**,** 27-38.
Silva-Oliveira, R. J., et al., 2016. Cytotoxicity of allitinib, an irreversible anti-EGFR agent, in a large panel of human cancer-derived cell lines: KRAS mutation status as a predictive biomarker. Cellular Oncology. 39**,** 253-263.
Weigel, J. A., Process for making gemcitabine hydrochloride. Google Patents, 1999.
Yadav, A., et al., 2020. In Vitro Cytotoxicity Study of Cyclophosphamide, Etoposide and Paclitaxel on Monocyte Macrophage Cell Line Raw 264.7. Indian J Microbiol. 60**,** 511-517.
Yuksel, S., et al., 2017. Protective effect of thymoquinone against cyclophosphamide-induced genotoxic damage in human lymphocytes. Bratisl Lek Listy. 118**,** 208-211.

Zaied, B. K., et al., 2020. A comprehensive review on contaminants removal from pharmaceutical wastewater by electrocoagulation process. Sci Total Environ. 726**,** 138095.

Zounkova, R., et al., 2010. Ecotoxicity and genotoxicity assessment of cytotoxic antineoplastic drugs and their metabolites. Chemosphere. 81**,** 253-60.

Zounkova, R., et al., 2007. Ecotoxicity and genotoxicity assessment of cytostatic pharmaceuticals. Environ Toxicol Chem. 26**,** 2208-14.

5. RESULTADO NÃO APRESENTADO EM ARTIGO

Os resultados obtidos por meio do ensaio de toxicidade aguda em *zebrafish* com acroleína, metabólito da ciclofosfamida, selecionado para o estudo, não foram publicados e encontram-se descritos nesta seção.

A metodologia realizada é mesma descrita no artigo publicado e intitulado "*Detection of anti-cancer drugs and metabolites in the effluents from a large Brazilian cancer hospital and an evaluation of ecotoxicology*".

O ensaio de toxicidade aguda em *zebrafish* demonstrou alta toxicidade, com mortalidade total dos peixes em ensaio preliminar e posteriormente em ensaios definitivos (Tabela 2), sendo classificada como extremamente tóxica (CL < 0,1)[85], com peixes vivos apenas na concentração de 0,001 mg/L (1 ng/mL).

É importante destacar que, nas concentrações acima de 0,01 mg/L os peixes apresentaram mortalidade rápida, até 30 minutos após exposição.

Tabela 2. Teste de ecotoxicidade aguda definitivo da acroleína realizado em triplicata, com 3 peixes por aquário, de 3 litros cada.

	Identificação		**Mortalidade**	
Aquário	**Peso médio (g)[a]**	**[ ] (mg L^{-1})[b]**	**24 h**	**48 h**
controle	1,68	-	0	0
1	1,79	0,001	0	0
2	1,67	0,005	3	0
3	1,52	0,01	3	0
4	2,20	0,05	3	0
5	1,80	0,1	3	0

[a] Peso médio total dos 3 peixes no aquário (média dos pesos na triplicata).

[b] Concentração em mg/L do composto no aquário.

6. DISCUSSÃO

As propriedades citotóxicas e o potencial carcinogênico, mutagênico e teratogênico de resíduos de drogas antineoplásicas em efluentes hospitalares e municipais são motivo de preocupação para órgãos de controle público e ambiental [10, 52, 86, 87]. Com a realização deste estudo foi possível avaliar de forma amostral a presença de antineoplásicos e metabólitos em efluentes hospitalares e municipais, verificando se as quantidades detectadas e quantificadas podem causar danos ecotoxicológicos e histológicos a *zebrafish*, assim como citotoxicidade e genotoxicidade em células HepG2. Abaixo serão discutidos os resultados obtidos por antineoplásico avaliado e seu metabólito.

Gemcitabina e metabólito 2-DOH-DiF

A GEM foi quantificada em concentração de 25,9 ng/mL nos efluentes hospitalares. Isidori et al [28] em 2016 não detectaram GEM em efluente hospitalar na Eslovênia e na Espanha, detectando o mesmo apenas no efluente na entrada da ETE na Eslovênia, na quantidade de 0,061 ng/mL. Esse valor é 12 vezes menor do que observamos em nosso estudo, de 0,75 ng/mL na entrada da ETE, antes do tratamento. Além disso, relatamos um nível de 0,42 ng/mL em efluente após tratamento na ETE. Em nossa revisão na literatura, nenhum outro estudo detectou ou quantificou GEM após tratamento em ETE.

O metabólito 2-DOH-DiF foi detectado nos efluentes hospitalares e municipais, sendo o composto detectado na concentração mais elevada (116 ng/mL) no efluente hospitalar. A detecção do metabólito em concentrações mais elevadas e em um número maior de amostras se comparado à GEM pode ser explicada pelo fato de que, em torno de 5% da quantidade total de GEM administrada é excretada inalterada na urina durante as primeiras 6 horas após o tratamento e 60% da concentração administrada é excretada como metabólito em 24 horas [88]. O 2-DOH-DiF é classificado pela Agência Europeia de Substâncias Químicas (do inglês *European Chemicals Agency* - ECHA) nas Declarações de Perigo do GHS (Sistema Mundial Harmonizado de Classificação e Rotulagem de Produtos Químicos, do inglês *Globally Harmonized System of Classification and Labelling of Chemicals*) como “perigoso”, causando danos genotóxicos às células germinativas, sendo tóxico para a vida aquática e causando irritação quando em contato com a pele e mucosa [89]. É importante ressaltar que este estudo

detectou pela primeira vez o 2-DOH-DiF, metabólito da GEM, amplamente utilizada em tratamento oncológico no Brasil.

No ensaio de ecotoxicidade aguda da GEM em *zebrafish*, a concentração letal foi superior a 118 mg/L, ou seja, considerado praticamente não tóxico. Não foi realizada avaliação de toxicidade aguda de 2-DOH-DiF, devido as quantidades necessárias e custo para realização da mesma, sendo o composto avaliado apenas em mistura com os demais compostos, na avaliação histológica de *zebrafish*.

Ao avaliar a citotoxicidade pelo ensaio MTS em HepG2, verificamos que os valores que causam diminuição da viabilidade celular para GEM e seu metabólito 2-DOH-DiF estão dentro das concentrações detectadas em efluentes [90, 91]. Em relação ao índice de divisão nuclear (IDN), para ambos os compostos houve diminuição significativa da divisão celular de HepG2 em concentrações acima de 0,1 ng/mL para GEM e acima de 25 ng/mL para 2-DOH-DiF.

Em 2010, Zounkova et al.[47] ao avaliarem a ecotoxicidade da GEM na fase aguda em testes de imobilização e reprodução com *D. magna* e testes de inibição de crescimento com a alga *D. subspicatus* e bactéria *P. putida*, encontraram os respectivos valores de EC50 de 110.000 ng/mL, 45.000 ng/mL e 100.000 ng/mL, quantidades muito superiores aos valores do fármaco detectados nos efluentes e aos valores de citotoxicidade em HepG2 obtidos neste estudo.

Podemos observar que para GEM e 2-DOH-DiF os danos causados às células são similares, genotoxicamente o aumento no número de MN e de brotamento celular deram-se na mesma concentração. Quanto a citotoxicidade, GEM diminuiu a viabilidade celular em concentrações mais baixas (0,1 ng/mL comparado a 1 ng/mL para 2-DOH-DiF) e houve também formação de pontes nucleoplasmáticas, o que não foi observado para 2-DOH-DiF. Estes resultados podem ser observados devido ao modo de ação da GEM, que após a absorção pelas células malignas, a mesma é fosforilada pela desoxicitidina quinase para formar o monofosfato de GEM, que é então convertido nos compostos ativos, difosfato de GEM (dFdCDP) e trifosfato de GEM (dFdCTP), responsáveis pela ação citotóxica da droga nas células, sendo o 2-DOH-DiF metabólito inativo formado após ação de dFDCDP e dFdCTP [79, 92]. ‘

Assim, diante da escassez de estudos de GEM e seu metabólito, é importante destacar a necessidade de pesquisas que avaliem esses danos, uma vez que a GEM é consumida em

larga escala no mundo, com taxa de excreção em torno de 10% (Weigel, 1999), assim como seu metabólito, excretado e detectado em efluentes (de Oliveira Klein et al., 2021).

5-fluorouracil e metabólito 3-NH_2-F

O 5-FU foi a única droga não detectada em nosso estudo, o que pode ser explicado pelos altos limites de detecção e quantificação, visto que outros autores detectaram concentrações de 0,0021 ng/mL a 0,123 ng/mL [9, 28-30], valores abaixo dos limites de detecção e quantificação definidos neste estudo e publicados[90]. Já o metabólito 3-NH_2-F foi quantificado em efluentes hospitalares no range de 3,02 a 18,2 ng/mL, nos efluentes antes do tratamento na ETE no range entre >LOD e <LOQ até 13,5 ng/mL, não sendo detectado após tratamento na ETE.

É importante destacar que a maior parte do 5-FU é eliminada pelo organismo pelo trato respiratório, aproximadamente 90% como dióxido de carbono [80], fator que poderia diminuir sua excreção renal, que é a segunda via de excreção [80] . O metabólito derivado é alfa-fluoro-beta-alanina, que é um dos últimos a ser decomposto pelo organismo. Aproximadamente 80% desse composto é catabolizado e excretado na urina [93, 94], o que poderia explicar sua detecção nos efluentes mesmo sem a detecção do 5-FU.

Quanto aos danos citotóxicos, observamos diminuição na viabilidade celular de HepG2 exposta a concentrações acima de 1 ng/mL de 5-FU, com IC50 de 18.07 ng/mL, já para o metabólito 3-NH_2-F não foi observada diminuição na viabilidade celular.

Em 2010, Zounkova et al.[47] ao avaliarem a ecotoxicidade de 3-NH_2-F na fase aguda em testes de imobilização e reprodução com crustáceo *D. magna* e testes de inibição de crescimento com a alga *D. subspicatus* e a bactéria *P. putida*, encontraram os respectivos valores de EC50 >100.000 ng/mL , 80.000 ng/mL e 140.000 ng/mL, valores muito superiores à faixa avaliada em nosso ensaio de citotoxicidade, o que pode explicar não termos verificado diminuição na viabilidade celular, mesmo que em outro modelo.

Ao avaliarmos os dados genotóxicos obtidos pelo ensaio de MN em HepG2, observamos um aumento significativo de brotamentos nucleares e formação de MN em concentrações acima de 0,01 ng/mL para 5-FU quando comparadas ao controle negativo. Podendo concluir que valores detectados por outros estudos de 5-FU em efluentes hospitalares, no range de 0,0021 ng/mL até 0,123 ng/mL [9, 29, 66, 91] e antes de tratamento em ETE, no range de 0,0031 a 0,014 ng / mL [29, 91] causam genotoxicidade em células HepG2.

O metabólito 3-NH_2-F por sua vez, causou mais danos genotóxicos e em menores concentrações se comparado ao 5-FU e, além de aumento do número de MN e brotamentos nucleares em concentrações acima de 0,001 ng/mL, aumentou o número de pontes nucleoplasmáticas em concentrações acima de 50 ng/mL. Resultado diferente se comparado a GEM e seu metabólito, onde a droga foi mais tóxica do que o metabólito.

Em seu estudo Gajski et al. [58] encontraram IC50 de 5-FU para HepG2 em ensaio de viabilidade celular de 72h de 5.270 ng/mL, valor superior ao IC50 avaliado em nosso estudo, de 18,07 ng/mL. Nesse mesmo estudo, Gajski et al.[58] avaliaram o dano genotóxico de 5-FU por ensaio cometa e MN em linhagens celulares ZFL, HPBLs e HepG2, observando dano por ensaio cometa apenas em ZFL em concentrações acima de 100 ng/mL, e por ensaio de MN o dano foi observado apenas para HepG2, em concentrações acima de 100 ng/mL. Em nosso teste de micronúcleo, observamos um aumento do número de MN para HepG2 em concentrações acima de 0,01 ng/mL, concentração inferior ao ensaio realizado por Gajski et al [58].

Os dados da literatura enfatizam que 5-FU é detectado em efluentes em concentrações mais baixas se comparado a outras drogas antineoplásicas [9, 29, 66, 91], porém mesmo nestas concentrações foi possível avaliar danos genotóxicos a HepG2, assim como danos ocasionados por seu metabólito, enfatizando a importância na realização de estudos que busquem a detecção da droga em efluentes, assim como caracterização ecotóxica, citotóxica e genotóxica.

Ciclofosfamida e metabólito AC

Para CP o valor máximo quantificado nos efluentes hospitalares neste estudo foi de 29,10 ng/mL, enquanto no estudo realizado por Cesen et al. [23], foi quantificado valor de 22 ng/mL, sendo que outros estudos relataram valores abaixo de 2 ng/mL [19, 25, 28, 34, 95, 96].

No que se refere à presença de CP após tratamento na ETE, detectamos a mesma em todas as campanhas em novembro (2017), dezembro (2017) e janeiro (2018), com limite de detecção de 0,3 ng/mL e abaixo do limite de quantificação de 1 ng/mL, que é superior aos níveis de 0,025 ng/mL encontrados por Ferrando-Climent et al.[19] na Espanha, 0,021 ng/mL encontrado por Rabii et al.[97] no Canadá e por outros estudos na Europa [14, 22, 23, 28, 31, 95].

Não foi possível estabelecer metodologia por meio de cromatografia líquida acoplada a espectrometria de massas para avaliação da AC nos efluentes, devido ao baixo peso molecular

(56,06 g/mol), alta volatividade e assinatura irregular da quebra da molécula [98], para a realização desta análise a tecnologia de cromatografia gasosa acoplada a espectrometria de massas (CG/EM) seria a mais indicada. Devido a alta toxicidade do composto [99] foram realizados apenas os testes de ecotoxicidade aguda em *zebrafish*, com resultados confirmando os dados da literatura [99], com mortalidade total dos peixes em ensaio preliminar e posteriormente em ensaios definitivos, sendo classificada como extremamente tóxica (CL < 0,1) [85], com peixes vivos apenas na concentração de 0,001 mg/L.

No ensaio de ecotoxicidade aguda da CP em *zebrafish*, a concentração letal foi superior a 118 mg/L, ou seja, considerado praticamente não tóxico.

A CP não diminuiu a viabilidade e proliferação celular em HepG2 na faixa avaliada, de 0,001 a 50 ng/mL, e não foi possível determinar o valor de IC50. Mater et al. [63], ao usarem a mesma metodologia, mesma linhagem celular e faixa semelhante de concentração (0,01 a 10 ng/mL) também não determinaram os valores de IC50 de CP.

Yadav et al. [100] ao avaliarem a citotoxicidade em um ensaio de viabilidade celular com uma linhagem celular de rato (Raw 264.7), encontraram IC50 de 145.000 ng/mL, valor superior ao nosso intervalo de avaliação.

Quanto ao dano genotóxico, CP causou um aumento significativo no número de pontes nucleoplasmáticas, brotamentos nucleares e MN em concentrações acima de 0,001 ng/mL. Ao compararmos com os valores detectados em efluentes hospitalares de CP (0,002 a 29,1 ng/mL) [19, 23, 25, 31, 90, 91, 95], com valores de influentes antes do tratamento em ETE (0,002 a 0,08 ng/mL) [23, 33, 46, 90, 91, 95] e para efluente após tratamento (0,00019 a 0,091 ng/mL) [14, 23, 31, 33, 46, 90, 91], observamos que o dano genotóxico ocorre dentro da faixa de detecção de drogas em efluentes, mesmo após tratamento em ETE.

Lutterbeck et al. [39] observaram alterações cromossômicas em células de *A. cepa*, incluindo a formação de células micronucleadas expostas a valores de CP acima de 20.000 ng/mL.

Fernandes et al. [36] verificaram indução de estresse oxidativo no mexilhão marinho *Mytilus galloprovincialis* sob exposição in vivo de 14 dias a 1 ng/mL de CP, juntamente com aumento na prevalência de danos ao DNA e redução da viabilidade celular, resultados não observados em células humanas (HELA e RPE). Em ensaio semelhante, Fonseca et al. [38] ao

exporem a poliqueta *Nereis diversicolor* por 14 dias a 0,5 ng/mL de CP observaram danos ao DNA e altos níveis de dano oxidativo.

Linfócitos humanos tratados com CP em concentrações acima de 160 ng/mL apresentaram alterações cromossômicas estruturais, troca de cromátides irmãs (SCE's, do inglês *sister chromatid exchanges*) e aumento da frequência de MN [61, 62]. Todos esses estudos demonstraram genotoxicidade em diferentes modelos biológicos com valores superiores à concentração que demonstrou genotoxicidade em nosso estudo (0,001 ng / mL).

CP é um agente alquilante do DNA. Estes fármacos normalmente se ligam a uma ampla gama de moléculas biológicas, alterando assim suas estruturas e funções celulares. A formação de adutos de DNA pode levar a uma série de eventos genotóxicos, tais como: 1) redução da síntese de DNA, impedindo o progresso das bifurcações replicantes; 2) formação de ligações cruzadas evitando que o DNA seja separado para síntese ou transcrição; e 3) aberrações cromossômicas e mutações genéticas nas células [101]. A forma de ação dos agentes alquilantes, agindo diretamente no DNA, pode explicar os danos citotóxicos em altas concentrações e acima das concentrações da droga em efluentes [63, 100] e danos genotóxicos ao HepG2 em concentrações baixas, acima de 0,001 ng/mL.

Avaliação das drogas como mistura e o impacto ambiental nos efluentes

Neste estudo foi possível avaliar danos causados aos tecidos branquiais e hepáticos de *zebrafish*, como necrose e alterações nas brânquias, após exposição dos animais às misturas de medicamentos em concentrações equivalentes aos níveis detectados no efluente hospitalar. Estes dados em parte corroboram os de outros estudos, que enfatizam que a mistura de antineoplásicos e metabólitos tem potencial genotóxico aumentado se comparado às drogas isoladas [37, 63-65].

Os níveis de antineoplásicos detectados em nosso estudo são mais elevados se comparados a outros estudos realizados em centros maiores de países europeus [25, 28, 33], China [34] e Canadá [97, 102]. Algumas particularidades podem explicar estes achados, tais como: o alto consumo de antineoplásicos pelo HCB, com descarga de efluentes em um sistema de um município pequeno, ou seja, a problemática de ser um grande hospital em um município com apenas 120 mil residentes permanentes; excreção de antineoplásicos e metabólitos por fezes

e urina de pacientes em diferentes níveis; diferentes regimes de administração dos compostos nos hospitais; e diferentes capacidades e formas de tratamento de efluentes em ETE's.

A descarga contínua de antineoplásicos e metabólitos, substâncias persistentes no meio ambiente, mesmo que em baixas concentrações, pode levar ao seu acúmulo em níveis considerados tóxicos [103], além de causarem danos histológicos diretos a *zebrafish* em concentrações presentes nos efluentes [90].

No Brasil, onde apenas cerca de 40% dos efluentes são tratados [54], e não há legislação sobre o lançamento de medicamentos antineoplásicos em efluentes, os níveis de toxicidade são preocupantes. Além disso, é importante ressaltar que, após a infusão quimioterápica, muitos pacientes retornam às suas cidades de origem e excretam resíduos de medicamentos em locais sem tratamento de esgoto, problema comum no Brasil. É importante ressaltar também que, mesmo após tratamento municipal de esgoto, os medicamentos persistem nos efluentes, uma vez que o tratamento padrão é apenas biológico e mecânico, e o processo não remove efetivamente compostos químicos como os quimioterápicos [52].

Ao avaliarmos a problemática local, o HCB realiza uma média de 200 infusões diárias de quimioterapia com a descarga dos efluentes em um sistema de tratamento de águas residuais de baixa capacidade, além de ser específico para tratamento de efluentes domésticos, e não químicos. Estes níveis elevados, com descarga contínua, podem causar danos ao ecossistema local. É importante destacar também que, de uma média de 40 diferentes medicamentos antineoplásicos utilizados no HCB, este estudo concentrou sua avaliação em 03 drogas e 02 metabólitos, uma pequena amostra da realidade.

É necessária a realização de um número maior de estudos, para ampliar e validar a presença de medicamentos antineoplásicos e seus metabólitos em efluentes hospitalares e municipais, assim como avaliações de amostras sólidas e dos efeitos ecotoxicológicos, citotóxicos e genotóxicos desses compostos em forma isolada e como mistura. Outra abordagem sugerida recentemente por Mastroianni et al. [104] refere-se a estudos de metabolômica ambiental, abordagem utilizada para entender o impacto de xenobióticos ambientais em diferentes organismos. Com a utilização de diferentes plataformas analíticas, é possível avaliar alterações metabólicas em diferentes organismos aquáticos da cadeia trófica após a exposição a fármacos, e até o momento não se tem conhecimento científico de avaliação metabolómica em antineoplásicos [104].

O campo para estudo é vasto, uma vez que muitos antineoplásicos e metabólitos ainda não estão caracterizados e para outros o número de estudos e experimentos é irrisório. Novas drogas estão sendo desenvolvidas a todo momento com escalonamento para uso em nível mundial, sendo de extrema relevância o olhar para os aspectos ambientais deste alto consumo. Apenas com o crescente número de pesquisas e embasamento científico poderemos proporcionar uma estrutura sólida para a formulação de novas políticas públicas, que visem o controle do descarte e suportem o tratamento adequado dos efluentes.

Limitações do estudo

Apesar dos resultados obtidos, o presente estudo contém algumas limitações que devem ser consideradas.

Ao avaliarmos a presença das drogas nos efluentes, a pré-concentração das amostras por extração em fase sólida (SPE, do inglês *solid phase extraction*) não foi realizada. Kosjek et al. [105] quantificaram IF e CP e seus metabólitos em efluentes hospitalares utilizando SPE para limpeza e pré-concentração de amostra seguida de derivatização química de compostos antes da análise por meio de cromatografia gasosa acoplada a espectometria de massas, obtendo limites menores de detecção e quantificação. Devido a não utilização de SPE em nosso estudo, desenvolvemos e utilizamos dois métodos analíticos baseados em: (1) diluição direta de água da amostra de efluente e injeção em sistema CLAE-EM/EM otimizado; e (2) derivatização química usando reagente PITC (Isotiocianato de fenil do inglês *phenyl isothiocyanate*) para os compostos 5-FU e 3-NH_2-F com grupo amino primário disponível, análise prévia ao CLAE-EM/EM [106]. Esses métodos desenvolvidos forneceram resultados confiáveis com ênfase na simplicidade e especificidade sem comprometer a sensibilidade. Por meio desse método foi possível detectar drogas mesmo com limites de detecção considerados mais elevados.

Quanto aos ensaios de citotoxicidade e genotoxicidade, é importante destacar que as análises foram realizadas *in vitro*, em condições controladas, diferente do que ocorre no meio ambiente. As análises foram realizadas em células de hepatocarcinoma humano (HepG2), um modelo conhecido por ser usado para análises de genotoxicidade [58, 107], mas os testes são restritos a uma linhagem celular, e para uma avaliação mais ampla e completa, outras linhagens celulares podem ser avaliadas, como por exemplo HPBLs e ZFL. Outro ponto, não

utilizamos outros ensaios de genotoxicidade, como por exemplo, o ensaio cometa, para avaliação de danos no DNA em fita simples.

7. CONCLUSÕES

Com o presente estudo foi possível avaliar de maneira sistêmica, desde a detecção de compostos antineoplásicos e metabólitos em efluentes hospitalares e municipais, até os danos causados em nível ecotoxicológico em organismo aquático (*Danio rerio*), a danos citotóxicos e genotóxicos em linhagem de hepatocarcinoma humano (HepG2), concluindo que:

- Em efluentes hospitalares foi possível a quantificação dos antineoplásicos CP e GEM e dos metabólitos 2-DOH-DiF e 3-NH_2-F em concentrações entre 0,16 ng/mL a 116 ng/mL;
- Em efluentes na entrada da ETE, antes da realização do tratamento, foi possível a quantificação de GEM e dos metabólitos 2-DOH-DiF e 3-NH_2-F em concentrações entre 0,11 ng/mL e 13,50 ng/mL; e a detecção de CP em concentrações entre seu limite de detecção e limite de quantificação, ou seja, entre 0,3 ng/mL e 5 ng/mL;
- Após o tratamento dos efluentes na ETE, foi possível a quantificação de 0,42 ng/mL de GEM; a detecção de CP em concentrações entre seu limite de detecção e limite de quantificação, ou seja, entre 0,3 ng/mL e 5 ng/mL; e a detecção de 2-DOH-DiF entre seu limite de detecção e limite de quantificação, ou seja, entre 1,4 ng/mL e 4,7 ng/mL;
- Este foi o primeiro estudo a detectar a presença dos metabólitos 2-DOH-DiF e 3-NH_2-F em efluentes hospitalares e municipais;
- Verificou-se que os compostos, como mistura e nas quantidades presentes nos efluentes, causam danos histológicos a *zebrafish* (*D. rerio*);
- A acroleína classificou-se como extremamente tóxica (CL<0,1) em ensaio de toxicidade aguda em *zebrafish* (*D. Rerio*), com peixes vivos apenas na concentração de 1 ng/mL.
- Foi possível verificar que três (5-FU, GEM e 2-DOH-DiF) dos cinco compostos avaliados são citotóxicos para HepG2, diminuindo sua viabilidade celular em concentrações presentes no meio ambiente;
- GEM e 5-FU, drogas antimetabólicas, apresentaram dano citotóxico com alteração no índice de divisão celular; e

— Danos genotóxicos (aumento no número de MN e brotamentos nucleares) foram observados em células HepG2 para todos os compostos nas concentrações presentes nos efluentes.

8. REFERÊNCIAS

1. Ferlay J, Bray F, Steliarova-Foucher E, Forman D. *CI5 IX: cancer incidence in five continents, volumes I to X 2014*. 2020.

2. Almeida VLd, Leitão A, Reina LdCB, Montanari CA, Donnici CL, Lopes MTP. *Câncer e agentes antineoplásicos ciclo-celular específicos e ciclo-celular não específicos que interagem com o DNA: uma introdução*. **Química nova**. 2005;28:118-29.

3. Li D, Chen H, Liu H, Schlenk D, Mu J, Lacorte S, et al. *Anticancer drugs in the aquatic ecosystem: Environmental occurrence, ecotoxicological effect and risk assessment*. **Environ Int**. 2021;153:106543.

4. INCA. *Estimativa 2020 : incidência de câncer no Brasil*. Rio de Janeiro, RJ: Instituto Nacional de Câncer José Alencar Gomes da Silva. , 2019 2019. Report No.

5. Siegel RL, Miller KD, Jemal A. *Cancer statistics, 2015*. **CA: a cancer journal for clinicians**. 2015;65(1):5-29.

6. Caley A, Jones R. *The principles of cancer treatment by chemotherapy*. **Surgery (Oxford)**. 2012;30(4):186-90.

7. INCA. *Registros hospitalares de câncer: planejamento e gestão*. 2 ed. **Rio de Janeiro: Instituto Nacional de Câncer - INCA**; 2010.

8. WHO WHO-. *Guidelines for ATC classification and DDD assignment*. In: Methodology CfDS, editor.**: World Health Organization - WHO**; 2013.

9. Mullot JU, Karolak S, Fontova A, Huart B, Levi Y. *Development and validation of a sensitive and selective method using GC/MS-MS for quantification of 5-fluorouracil in hospital wastewater*. **Anal Bioanal Chem**. 2009;394(8):2203-12.

10. Brunton LL, Chabner B, Knollmann BC. *Goodman & Gilman's the pharmacological basis of therapeutics***: McGraw-Hill Medical New York**; 2011.

11. Souza DM, Reichert JF, Martins AF. *A simultaneous determination of anti-cancer drugs in hospital effluent by DLLME HPLC-FLD, together with a risk assessment*. **Chemosphere**. 2018;201:178-88.

12. Mullot JU, Karolak S, Fontova A, Levi Y. *Modeling of hospital wastewater pollution by pharmaceuticals: first results of Mediflux study carried out in three French hospitals*. **Water Sci Technol**. 2010;62(12):2912-9.

13. Bialk-Bielinska A, Mulkiewicz E, Stokowski M, Stolte S, Stepnowski P. *Acute aquatic toxicity assessment of six anti-cancer drugs and one metabolite using biotest battery - Biological effects and stability under test conditions*. **Chemosphere**. 2017;189:689-98.

14. Llewellyn N, Lloyd P, Jurgens MD, Johnson AC. *Determination of cyclophosphamide and ifosfamide in sewage effluent by stable isotope-dilution liquid chromatography-tandem mass spectrometry*. **J Chromatogr A**. 2011;1218(47):8519-28.

15. Nussbaumer S, Bonnabry P, Veuthey JL, Fleury-Souverain S. *Analysis of anticancer drugs: a review*. **Talanta**. 2011;85(5):2265-89.

16. Aherne GW, English J, Marks V. *The role of immunoassay in the analysis of microcontaminants in water samples*. **Ecotoxicol Environ Saf**. 1985;9(1):79-83.

17. Richardson ML, Bowron JM. *The fate of pharmaceutical chemicals in the aquatic environment*. **J Pharm Pharmacol**. 1985;37(1):1-12.

18. Aherne GW, Hardcastle A, Nield AH. *Cytotoxic drugs and the aquatic environment: estimation of bleomycin in river and water samples*. **J Pharm Pharmacol**. 1990;42(10):741-2.

19. Ferrando-Climent L, Rodriguez-Mozaz S, Barcelo D. *Incidence of anticancer drugs in an aquatic urban system: from hospital effluents through urban wastewater to natural environment*. **Environ Pollut**. 2014;193:216-23.

20. Franquet-Griell H, Gomez-Canela C, Ventura F, Lacorte S. *Predicting concentrations of cytostatic drugs in sewage effluents and surface waters of Catalonia (NE Spain)*. **Environ Res**. 2015;138:161-72.

21. Kummerer K, Haiss A, Schuster A, Hein A, Ebert I. *Antineoplastic compounds in the environment-substances of special concern*. **Environ Sci Pollut Res Int**. 2014.

22. Castiglioni S, Bagnati R, Calamari D, Fanelli R, Zuccato E. *A multiresidue analytical method using solid-phase extraction and high-pressure liquid chromatography tandem mass spectrometry to measure pharmaceuticals of different therapeutic classes in urban wastewaters*. **J Chromatogr A**. 2005;1092(2):206-15.

23. Cesen M, Kosjek T, Laimou-Geraniou M, Kompare B, Sirok B, Lambropolou D, et al. *Occurrence of cyclophosphamide and ifosfamide in aqueous environment and their removal by biological and abiotic wastewater treatment processes*. **Sci Total Environ**. 2015;527-528:465-73.

24. Ferrando-Climent L, Rodriguez-Mozaz S, Barcelo D. *Development of a UPLC-MS/MS method for the determination of ten anticancer drugs in hospital and urban wastewaters, and its application for the screening of human metabolites assisted by information-dependent acquisition tool (IDA) in sewage samples*. **Anal Bioanal Chem**. 2013;405(18):5937-52.

25. Ferre-Aracil J, Valcarcel Y, Negreira N, de Alda ML, Barcelo D, Cardona SC, et al. *Ozonation of hospital raw wastewaters for cytostatic compounds removal. Kinetic modelling and economic assessment of the process*. **Sci Total Environ**. 2016;556:70-9.

26. Gomez-Canela C, Cortes-Francisco N, Oliva X, Pujol C, Ventura F, Lacorte S, et al. *Occurrence of cyclophosphamide and epirubicin in wastewaters by direct injection analysis-liquid chromatography-high-resolution mass spectrometry*. **Environ Sci Pollut Res Int**. 2012;19(8):3210-8.

27. Gomez-Canela C, Ventura F, Caixach J, Lacorte S. *Occurrence of cytostatic compounds in hospital effluents and wastewaters, determined by liquid chromatography coupled to high-resolution mass spectrometry*. **Anal Bioanal Chem**. 2014;406(16):3801-14.

28. Isidori M, Lavorgna M, Russo C, Kundi M, Zegura B, Novak M, et al. *Chemical and toxicological characterisation of anticancer drugs in hospital and municipal wastewaters from Slovenia and Spain*. **Environ Pollut**. 2016;219:275-87.

29. Kosjek T, Perko S, Zigon D, Heath E. *Fluorouracil in the environment: analysis, occurrence, degradation and transformation*. **J Chromatogr A**. 2013;1290:62-72.

30. Mahnik SN, Lenz K, Weissenbacher N, Mader RM, Fuerhacker M. *Fate of 5-fluorouracil, doxorubicin, epirubicin, and daunorubicin in hospital wastewater and their elimination by activated sludge and treatment in a membrane-bio-reactor system*. **Chemosphere**. 2007;66(1):30-7.

31. Santana-Viera S, Hernandez-Arencibia P, Sosa-Ferrera Z, Santana-Rodriguez JJ. *Simultaneous and systematic analysis of cytostatic drugs in wastewater samples by ultra-high performance liquid chromatography tandem mass spectrometry*. **J Chromatogr B Analyt Technol Biomed Life Sci**. 2019;1110-1111:124-32.

32. Santos MSF, Franquet-Griell H, Alves A, Lacorte S. *Development of an analytical methodology for the analysis of priority cytostatics in water*. **Sci Total Environ**. 2018;645:1264-72.

33. Gouveia TIA, Silva AMT, Ribeiro AR, Alves A, Santos MSF. *Liquid-liquid extraction as a simple tool to quickly quantify fourteen cytostatics in urban wastewaters and access their impact in aquatic biota*. **Sci Total Environ**. 2020;740:139995.

34. Yin J, Shao B, Zhang J, Li K. *A preliminary study on the occurrence of cytostatic drugs in hospital effluents in Beijing, China*. **Bull Environ Contam Toxicol**. 2010;84(1):39-45.

35. Azuma T, Otomo K, Kunitou M, Shimizu M, Hosomaru K, Mikata S, et al. *Environmental fate of pharmaceutical compounds and antimicrobial-resistant bacteria in hospital effluents, and contributions to pollutant loads in the surface waters in Japan*. **Sci Total Environ**. 2019;657:476-84.

36. Fernandes E, Fonseca TG, Carrico T, Mestre N, Tavares A, Bebianno MJ. *Cytotoxic responses of the anticancer drug cyclophosphamide in the mussel Mytilus galloprovincialis and comparative sensitivity with human cells lines*. **Chemosphere**. 2020;261:127678.

37. Cesen M, Elersek T, Novak M, Zegura B, Kosjek T, Filipic M, et al. *Ecotoxicity and genotoxicity of cyclophosphamide, ifosfamide, their metabolites/transformation products and their mixtures*. **Environ Pollut**. 2016;210:192-201.

38. Fonseca TG, Auguste M, Ribeiro F, Cardoso C, Mestre NC, Abessa DMS, et al. *Environmental relevant levels of the cytotoxic drug cyclophosphamide produce harmful effects in the polychaete Nereis diversicolor*. **Sci Total Environ**. 2018;636:798-809.

39. Lutterbeck CA, Kern DI, Machado EL, Kummerer K. *Evaluation of the toxic effects of four anti-cancer drugs in plant bioassays and its potency for screening in the context of waste water reuse for irrigation*. **Chemosphere**. 2015;135:403-10.

40. Misik M, Filipic M, Nersesyan A, Kundi M, Isidori M, Knasmueller S. *Environmental risk assessment of widely used anticancer drugs (5-fluorouracil, cisplatin, etoposide, imatinib mesylate)*. **Water Res**. 2019;164:114953.

41. Misik M, Filipic M, Nersesyan A, Misikova K, Knasmueller S, Kundi M. *Analyses of combined effects of cytostatic drugs on micronucleus formation in the Tradescantia*. **Environ Sci Pollut Res Int**. 2016;23(15):14762-70.

42. Parrella A, Lavorgna M, Criscuolo E, Russo C, Isidori M. *Eco-genotoxicity of six anticancer drugs using comet assay in daphnids*. **J Hazard Mater**. 2015;286:573-80.

43. Cristóvão M, Torrejais J, Janssens R, Luis P, Van der Bruggen B, Dubey K, et al. *Treatment of anticancer drugs in hospital and wastewater effluents using nanofiltration*. **Separation and Purification Technology**. 2019;224:273-80.

44. Nassour C, Barton SJ, Nabhani-Gebara S, Saab Y, Barker J. *Occurrence of anticancer drugs in the aquatic environment: a systematic review*. **Environ Sci Pollut Res Int**. 2020;27(2):1339-47.

45. Buerge IJ, Buser HR, Poiger T, Muller MD. *Occurrence and fate of the cytostatic drugs cyclophosphamide and ifosfamide in wastewater and surface waters*. **Environ Sci Technol**. 2006;40(23):7242-50.

46. Ferrando-Climent L, Cruz-Morato C, Marco-Urrea E, Vicent T, Sarra M, Rodriguez-Mozaz S, et al. *Non conventional biological treatment based on Trametes versicolor for the elimination of recalcitrant anticancer drugs in hospital wastewater*. **Chemosphere**. 2015;136:9-19.

47. Zounkova R, Kovalova L, Blaha L, Dott W. *Ecotoxicity and genotoxicity assessment of cytotoxic antineoplastic drugs and their metabolites*. **Chemosphere**. 2010;81(2):253-60.

48. Zounkova R, Odraska P, Dolezalova L, Hilscherova K, Marsalek B, Blaha L. *Ecotoxicity and genotoxicity assessment of cytostatic pharmaceuticals*. **Environ Toxicol Chem**. 2007;26(10):2208-14.

49. Seira J, Claparols C, Joannis-Cassan C, Albasi C, Montrejaud-Vignoles M, Sablayrolles C. *Optimization of pressurized liquid extraction using a multivariate chemometric approach for the determination of anticancer drugs in sludge by ultra high performance liquid chromatography-tandem mass spectrometry*. **J Chromatogr A**. 2013;1283:27-38.

50. *CONAMA. Resolução Nº 358, de 29 de abril de 2005. Dispõe sobre o tratamento e a d isposição fi nal dos resíduos dos serviços de saúde e dá outras providências.*, 358 (2005).

51. *ANVISA. Resolução da Diretoria Colegiada RDC Nº 222, de 28 de março de 2018. Regulamenta as Boas Práticas de Gerenciamento dos Resíduos de Serviços de Saúde* (2018).

52. OECD. *Pharmaceutical Residues in Freshwater : Hazards and Policy Responses*. 2019.

53. Tran NH, Reinhard M, Gin KY-H. *Occurrence and fate of emerging contaminants in municipal wastewater treatment plants from different geographical regions-a review*. **Water research**. 2018;133:182-207.

54. SNIS SNdIsS-. *Diagnóstico dos Serviços de Água e Esgotos – 2016* In: Ambiental MdC-SNdS, editor. **Brasília: Ministério das Cidades - Secretaria Nacional de Saneamento Ambiental**; 2018.

55. Russo C, Lavorgna M, Piscitelli C, Isidori M. *Toxicity of anticancer drug residues in organisms of the freshwater aquatic chain*. *Fate and effects of anticancer drugs in the environment***: Springer**; 2020. p. 379-401.

56. Besse JP, Latour JF, Garric J. *Anticancer drugs in surface waters: what can we say about the occurrence and environmental significance of cytotoxic, cytostatic and endocrine therapy drugs?* **Environ Int**. 2012;39(1):73-86.

57. Grzesiuk M, Bednarska A, Mielecki D, Garbicz D, Marcinkowski M, Pilzys T, et al. *Anticancer agents found in environment affect Daphnia at population, individual and molecular levels*. **Aquat Toxicol**. 2019;215:105288.

58. Gajski G, Geric M, Zegura B, Novak M, Nunic J, Bajrektarevic D, et al. *Genotoxic potential of selected cytostatic drugs in human and zebrafish cells*. **Environ Sci Pollut Res Int**. 2016;23(15):14739-50.

59. Kovacs R, Bakos K, Urbanyi B, Kovesi J, Gazsi G, Csepeli A, et al. *Acute and sub-chronic toxicity of four cytostatic drugs in zebrafish*. **Environ Sci Pollut Res Int**. 2016;23(15):14718-29.

60. Gacic Z, Kolarevic S, Sunjog K, Kracun-Kolarevic M, Paunovic M, Knezevic-Vukcevic J, et al. *The impact of in vivo and in vitro exposure to base analogue 5-FU on the level of DNA damage in haemocytes of freshwater mussels Unio pictorum and Unio tumidus*. **Environ Pollut**. 2014;191:145-50.

61. Kocaman AY, Istifli ES, Buyukleyla M, Rencuzogullari E, Topaktas M. *In vitro evaluation of the protective effects of 4-thujanol against mitomycin-C and cyclophosphamide-induced genotoxic damage in human peripheral lymphocytes*. **Toxicol Ind Health**. 2013;29(1):23-37.

62. Yuksel S, Tasdemir S, Korkmaz S. *Protective effect of thymoquinone against cyclophosphamide-induced genotoxic damage in human lymphocytes*. **Bratisl Lek Listy**. 2017;118(4):208-11.

63. Mater N, Geret F, Castillo L, Faucet-Marquis V, Albasi C, Pfohl-Leszkowicz A. *In vitro tests aiding ecological risk assessment of ciprofloxacin, tamoxifen and cyclophosphamide in range of concentrations released in hospital wastewater and surface water*. **Environ Int**. 2014;63:191-200.

64. Brezovsek P, Elersek T, Filipic M. *Toxicities of four anti-neoplastic drugs and their binary mixtures tested on the green alga Pseudokirchneriella subcapitata and the cyanobacterium Synechococcus leopoliensis*. **Water Res**. 2014;52:168-77.

65. da Fonseca TG, Abessa DMS, Bebianno MJ. *Effects of mixtures of anticancer drugs in the benthic polychaete Nereis diversicolor*. **Environ Pollut**. 2019;252(Pt B):1180-92.

66. Mahnik SN, Rizovski B, Fuerhacker M, Mader RM. *Determination of 5-fluorouracil in hospital effluents*. **Anal Bioanal Chem**. 2004;380(1):31-5.

67. Dorr R, Von Hoff D. *Cancer chemotherapy book*. **Appleton and Lange, Norwalk**. 1994.

68. Bila DM, Dezotti M. *Fármacos no meio ambiente*. **Química Nova**. 2003;26(4):523-30.

69. Moignet A, Hasanali Z, Zambello R, Pavan L, Bareau B, Tournilhac O, et al. *Cyclophosphamide as a first-line therapy in LGL leukemia*. **Leukemia**. 2014;28(5):1134-6.

70. Wang D, Li L, Yang H, Ferguson SS, Baer MR, Gartenhaus RB, et al. *The constitutive androstane receptor is a novel therapeutic target facilitating cyclophosphamide-based treatment of hematopoietic malignancies*. **Blood**. 2013;121(2):329-38.

71. Druckrey H, Raabe S. *[Specific chemotherapy of carcinoma of the prostate]*. **Klin Wochenschr**. 1952;30(37-38):882-4.

72. Zhang J, Tian Q, Zhou S-F. *Clinical pharmacology of cyclophosphamide and ifosfamide*. **Current Drug Therapy**. 2006;1(1):55-84.

73. Li F, Patterson AD, Hofer CC, Krausz KW, Gonzalez FJ, Idle JR. *Comparative metabolism of cyclophosphamide and ifosfamide in the mouse using UPLC-ESI-QTOFMS-based metabolomics*. **Biochem Pharmacol**. 2010;80(7):1063-74.

74. Alarcon RA, Meienhofer J. *Formation of the cytotoxic aldehyde acrolein during in vitro degradation of cyclophosphamide*. **Nat New Biol**. 1971;233(42):250-2.

75. Brock N. *The history of the oxazaphosphorine cytostatics*. **Cancer**. 1996;78(3):542-7.

76. Brock N. *Oxazaphosphorine cytostatics: past-present-future. Seventh Cain Memorial Award lecture*. **Cancer Res**. 1989;49(1):1-7.

77. IRIS. *Summary on Acrolein (107-02-8)*. [Internet]: U.S. Environmental Protection Agency's Integrated Risk Information System (IRIS). ; 2000 [cited 2016/07/11];Available from: **http://www.epa.gov/iris/**.

78. McEvoy G. *American Hospital Formulary Service/AHFS Drug Information 2000. Bethesda: The American Society of Health-System Pharmacists*. **Inc**; 2000.

79. Weigel JA. *Process for making gemcitabine hydrochloride*. **Google Patents**; 1999.

80. Micromedex T. *Drug information for the health care professional*. **Volume**. 2005;1:2569-72.

81. Kasel D, Jetter A, Harlfinger S, Gebhardt W, Fuhr U. *Quantification of cyclophosphamide and its metabolites in urine using liquid chromatography/tandem mass spectrometry*. **Rapid Commun Mass Spectrom**. 2004;18(13):1472-8.

82. Powell CH, Bingham E, Cohrssen B. *Patty's Toxicology*: **John Wiley**; 2001.

83. IBGE. *Estimativa populacional 2016.* [Internet]: Instituto Brasileiro de Geografia e Estatística (IBGE); 2016 [cited 07/16/2019];Available from: **https://cidades.ibge.gov.br/brasil/sp/barretos/panorama**.

84. Palmero EI, Galvao HC, Fernandes GC, Paula AE, Oliveira JC, Souza CP, et al. *Oncogenetics service and the Brazilian public health system: the experience of a reference Cancer Hospital*. **Genet Mol Biol**. 2016;39(2):168-77.

85. Zucker E, Johnson SL. *Hazard Evaluation Division Standard Evaluation Procedure: Acute Toxicity Test for Freshwater Invertebrates*: **US Environmental Protection Agency, Office of Pesticide Programs**; 1985.

86. McKnight JA. *Principles of chemotherapy*. **Clin Tech Small Anim Pract**. 2003;18(2):67-72.

87. Russo C, Lavorgna M, Cesen M, Kosjek T, Heath E, Isidori M. *Evaluation of acute and chronic ecotoxicity of cyclophosphamide, ifosfamide, their metabolites/transformation products and UV treated samples*. **Environ Pollut**. 2018;233:356-63.

88. Weissbrodt D, Kovalova L, Ort C, Pazhepurackel V, Moser R, Hollender J, et al. *Mass flows of X-ray contrast media and cytostatics in hospital wastewater*. **Environ Sci Technol**. 2009;43(13):4810-7.

89. (ECHA) ECA. *Notified classification and labelling from ECHA's C&L Inventory. Name: 2',2'-Difluoro-2'-deoxyuridine*. [Internet]: European Chemicals Agency (ECHA); 2018 [cited 11/08].

90. de Oliveira Klein M, Serrano SV, Santos-Neto A, da Cruz C, Brunetti IA, Lebre D, et al. *Detection of anti-cancer drugs and metabolites in the effluents from a large Brazilian cancer hospital and an evaluation of ecotoxicology*. **Environ Pollut**. 2021;268(Pt A):115857.

91. Kundi M, Parrella A, Lavorgna M, Criscuolo E, Russo C, Isidori M. *Prediction and assessment of ecogenotoxicity of antineoplastic drugs in binary mixtures*. **Environ Sci Pollut Res Int**. 2016;23(15):14771-9.

92. Serdjebi C, Milano G, Ciccolini J. *Role of cytidine deaminase in toxicity and efficacy of nucleosidic analogs*. **Expert Opin Drug Metab Toxicol**. 2015;11(5):665-72.

93. Hardman JG, Limbird LE. *Goodman & Gilman's: the pharmacological basis of therapeutics*. *Goodman & Gilman's: the pharmacological basis of therapeutics*2001.

94. Turci R, Sottani C, Spagnoli G, Minoia C. *Biological and environmental monitoring of hospital personnel exposed to antineoplastic agents: a review of analytical methods*. **J Chromatogr B Analyt Technol Biomed Life Sci**. 2003;789(2):169-209.

95. Thomas KV, Dye C, Schlabach M, Langford KH. *Source to sink tracking of selected human pharmaceuticals from two Oslo city hospitals and a wastewater treatment works*. **J Environ Monit**. 2007;9(12):1410-8.

96. Negreira N, Lopez de Alda M, Barcelo D. *On-line solid phase extraction-liquid chromatography-tandem mass spectrometry for the determination of 17 cytostatics and metabolites in waste, surface and ground water samples*. **J Chromatogr A**. 2013;1280:64-74.

97. Rabii FW, Segura PA, Fayad PB, Sauve S. *Determination of six chemotherapeutic agents in municipal wastewater using online solid-phase extraction coupled to liquid chromatography-tandem mass spectrometry*. **Sci Total Environ**. 2014;487:792-800.

98. Egerton T, itanium Compounds T. *Kirk Othmer Encyclopedia of Chemical Technology*. **John Wiley & Sons, Inc., New York**; 1997.

99. Faroon O, Roney N, Taylor J, Ashizawa A, Lumpkin MH, Plewak DJ. *Acrolein health effects*. **Toxicol Ind Health**. 2008;24(7):447-90.

100. Yadav A, Mandal MK, Dubey KK. *In Vitro Cytotoxicity Study of Cyclophosphamide, Etoposide and Paclitaxel on Monocyte Macrophage Cell Line Raw 264.7*. **Indian J Microbiol**. 2020;60(4):511-7.

101. Peterson A. *DNA synthesis, mutagenesis, DNA damage, and cytotoxicity in cultured mammalian cells treated with alkylating agents*. **Cancer research**. 1980;40(3):682-8.

102. Garcia-Ac A, Segura PA, Gagnon C, Sauve S. *Determination of bezafibrate, methotrexate, cyclophosphamide, orlistat and enalapril in waste and surface waters using on-line solid-phase extraction liquid chromatography coupled to polarity-switching electrospray tandem mass spectrometry*. **J Environ Monit**. 2009;11(4):830-8.

103. Leblanc GAJAToMT. *Acute toxicity*. 2004:213-24.

104. Mastroianni G, Scognamiglio M, Russo C, Fiorentino A, Lavorgna M. *Environmental Metabolomics: A Powerful Tool to Investigate Biochemical Responses to Drugs in Nontarget Organisms*. *Fate and Effects of Anticancer Drugs in the Environment***: Springer**; 2020. p. 441-65.

105. Cesen M, Kosjek T, Busetti F, Kompare B, Heath E. *Human metabolites and transformation products of cyclophosphamide and ifosfamide: analysis, occurrence and formation during abiotic treatments*. **Environ Sci Pollut Res Int**. 2016;23(11):11209-23.

106. Kwanyuen P, Burton JWJJotAOCS. *A modified amino acid analysis using PITC derivatization for soybeans with accurate determination of cysteine and half-cystine*. 2010;87(2):127-32.

107. Quadros APO, Almeida LM, Petreanu M, Niero R, Rosa PCP, Sawaya A, et al. *Risk assessment via genotoxicity, metabolism, apoptosis, and cell growth effects in a HepG2/C3A cell line upon treatment with Rubus rosifolius (Rosaceae) leaves extract*. **J Toxicol Environ Health A**. 2020;83(13-14):495-508.

9. ANEXOS

ANEXO A – Imagens fotográficas da condução do estudo


Figuras 1 e 2. Entrada dos efluentes municipais na Estação de Tratamento de Efluentes (ETE) IV, administrada pelo SAAE (Serviço Autônomo de Água e Esgoto de Barretos) no Município de Barretos, SP. Data: 23/08/2016.

Figuras 3 e 4. Saída dos efluentes, após tratamento, da estação de tratamento (ETE IV) para o Ribeirão Pitangueiras. Data: 23/08/2016.


Figuras 5 e 6. Coleta das amostras na saída dos efluentes do Hospital de Câncer de Barretos (HCB) para a rede pública coletora de esgoto municipal.
Data: 04/05/2017.

Figura 7. Coleta dos efluentes na entrada da ETE IV. Data: 04/05/2017.

Figura 8. Coleta dos efluentes na saída da ETE IV

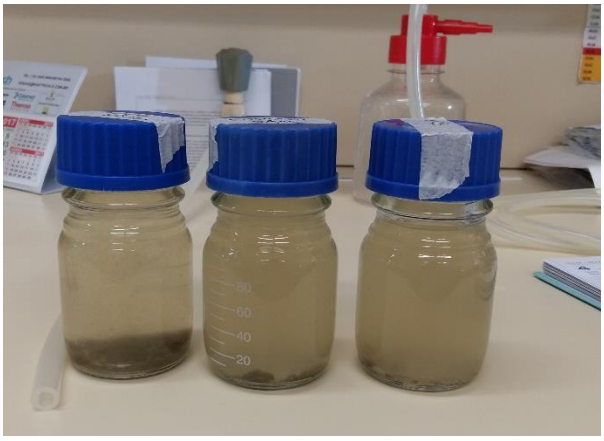
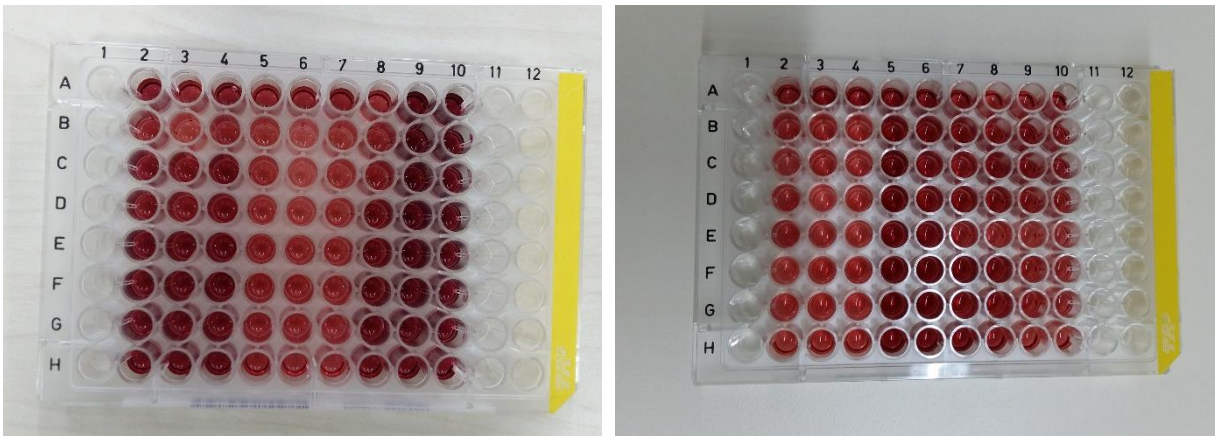


Figura 9. Amostras pré-filtragem.

Figura 10. Amostras acondicionadas para envio a CEMSA, SP.

Figuras 11 e 12. Realização dos ensaios de toxicidade aguda com *zebrafish* (*Danio rerio*). Data: 11/03/2018.



Figuras 13 e 14. Placas de realização dos ensaios de viabilidade celular (MTS) da exposição dos compostos a HepG2 após leitura de absorbância. Data: 21/06/2019.

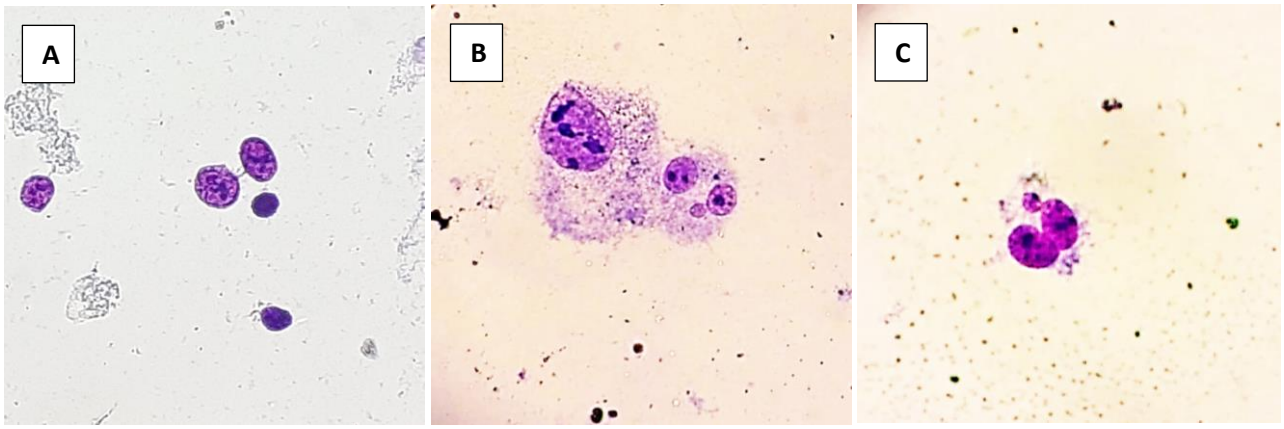


Figura 15. Exemplos de pontes nucleoplasmáticas (A), micronúcleo (B) e brotamento nuclear (C) identificados com a exposição dos compostos a HepG2 por meio de ensaio de micronúcleo. Data:

ANEXO B – Cartas de aprovação do estudo pelos Comitês de Ética em Pesquisa Animal

HOSPITAL DE CÂNCER DE BARRETOS
Fundação Pio XII
O HOSPITAL DO AMOR

Para: Henrique C. S. Silveira

De: Emilio de Almeida Belmonte

Coordenador CEUA Fundação Pio XII IRCAD Brazil

Data: 08/03/2017

Projeto de Pesquisa: **"Avaliação da presença de antioneoplásicos e seus metabólitos em efluentes no município de Barretos, SP e suas interações ecológicas e genotóxicas"**

Prezado Senhor,

Venho, por meio desta, informar que os membros do Comitê de Ética no Uso de Animais - Fundação Pio XII IRCAD Brazil, através de votação, analisaram, aprovaram a estão cientes que o projeto intitulado *"Avaliação da presença de antioneoplásicos e seus metabólitos em efluentes no município de Barretos, SP e suas interações ecológicas e genotóxicas"* fará o uso de peixes e as etapas que envolvem o uso dos animais não serão executadas nas dependências da Fundação Pio XII ou IRCAD América Latina.

Atenciosamente,

Dr. Emilio de Almeida Belmonte
Coordenador CEUA Fundação Pio XII IRCAD Brazil

FONE/FAX - (17) 3321-6600
END: RUA ANTENOR DUARTE VILELLA, 1331
CEP - 14784-400 - BARRETOS/SP - BRASIL

WWW.HCANCERBARRETOS.COM.BR

Comissão de Ética no Uso de Animais

CEUA

unifeb

COMISSÃO USO DE ANIMAIS EM EXPERIMENTAÇÃO E/OU ENSINO

Barretos, 13 de outubro de 2016.

CERTIFICADO

Certificamos para os devidos fins que o protocolo nº 160823/2016 do estudo intitulado **"Avaliação da presença de antineoplásicos e seus metabólitos em efluentes no município de Barretos, SP e suas interações ecológicas e genotóxicas"**, sob Responsabilidade do Dr. Henrique César Santejo Silveira do Hospital do Câncer de Barretos, está de acordo com os Princípios Éticos na Experimentação Animal adotado pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA), foi aprovado junto a COMISÃO DE ÉTICA NO USO DE ANIMAIS – CEUA do Centro Universitário da Fundação Educacional de Barretos, São Paulo, Brasil em reunião ordinária de 13 de outubro de 2016.

Prof. Dr. Claudinei da Cruz

-Coordenador/CEUA/UNIFEB-