INSTITUTO DE PESQUISAS ENERGÉTICAS E NUCLEARES

Autarquia Associada à Universidade de São Paulo

EVALUATION OF MCP ANTICANCER ACTIVITY USING MOLECULAR IMAGING

FÁBIO FERNANDO ALVES DA SILVA

Tese apresentada como parte dos requisitos para obtenção do Grau de Doutor em Ciências na Área de Tecnologia Nuclear – Aplicações.

Orientador: Dr. Emerson Soares Bernardes

São Paulo

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No mais, aproveitem a leitura e até mais.

RESUMO

SILVA, F. F. A. d. ***Avaliação da atividade anticâncer de MCP utilizando imagem molecular***. 2023. 157 f. Tese (Doutorado em Tecnologia Nuclear), Instituto de Pesquisas Energéticas e Nucleares, IPEN-CNEN, São Paulo.

O MCP é um polissacarídeo encontrado abundantemente na parede primária de células vegetais e apresenta atividade em diversas áreas da indústria de alimentos e na nutrição, servindo como alimento, melhorando o fluxo intestinal, reduzindo o colesterol e sendo um importante composto nutricional. MCP também pode atuar como molécula anti-tumorigênica em diversos tipos de tumores, além de evitar a quimiorresistência, modular o sistema imunológico e prevenir doenças renais causadas por radioterapia e quimioterapia em tratamentos oncológicos. Este estudo foi dividido em três partes principais. Na primeira parte, foi realizada uma revisão para estudar a atividade biológica de MCP e sua contribuição para a terapia do câncer, demonstrando doses de tratamento, tipos de MCP utilizados e desenho experimental, fornecendo uma visão crítica dos dados expostos e sua relação com a Galectina -3 e outras teorias sobre seus mecanismos. Na segunda parte, nós radiomarcamos MCP com ^{99m}Tc e verificamos sua biodistribuição e farmacocinética, na administração por via oral e intravenosa (IV). Inicialmente, a estrutura e a composição monossacarídica da MCP foram estudadas, e foi demonstrado que existe uma diversidade de monossacarídeos e pesos moleculares dentro da estrutura da MCP e que as frações MCP30 e MCP3 são ricas em galactose. Em seguida, estudamos a afinidade e a capacidade de inibição de MCP pela galectina-3 e demonstramos que a MCP se liga parcialmente à Gal-3 e que a MCP3 apresenta capacidade de inibição na concentração de 25 mg/ml. Marcamos radioativamente MCP com ^{99m}Tc e verificamos sua estabilidade em solução salina em diferentes pH, plasma e *in vivo*. O MCP-^{99m}Tc apresenta baixa absorção gastrointestinal (5,27x10-6 % da contagem total de radioatividade) e eliminação gastrointestinal quando administrado por via oral e eliminação renal e hepatobiliar quando administrado por via IV. Por fim, o ensaio de compartimentalização sanguínea mostrou que MCP-^{99m}Tc tem uma alta afinidade por proteínas plasmáticas e células sanguíneas em camundongos C57BL/6 Lgals3$^{+/+}$, e essa afinidade foi parcialmente perdida quando a galectina-3 foi inibida em animais C57BL/6 Lgals3$^{-/-}$. O estudo farmacocinético mostrou que a velocidade de

eliminação de MCP-^{99m}Tc foi maior nos camundongos C57BL/6 Lgals3$^{-/-}$ do que nos animais C57BL/6 Lgals3$^{+/+}$, indicando que a falta de galectina-3 aumenta a eliminação de MCP *in vivo*. Na terceira parte, analisamos o comportamento do MCP-^{99m}Tc em animais com tumores SKOV-3 e MKN45 usando imagem molecular. Inicialmente, demonstramos que a ligação celular e a internalização de MCP foram apenas parcialmente influenciadas pela expressão de Galectina-3 *in vitro* usando células SKOV-3 scrambled e SKOV-3 shRNAGal3 (knockdown para expressão de Gal-3). Em seguida, mostramos que o MCP (20 mg/kg) exibiu uma atividade anticancerígena em um modelo de xenoenxerto de tumor de células SKOV-3, reduzindo o crescimento do tumor em 48,5% e o peso do tumor em 50% quando administrado por via intravenosa; no entanto, a administração oral de MCP (200 mg/kg) não apresentou efeito anticancerígeno. Posteriormente, demonstramos que o MCP-^{99m}Tc atingiu o tumor e se ligou a regiões de necrose em xenoenxerto tumorais de células SKOV-3 quando administrado por via intravenosa. Por fim, demonstramos que a administração IV de MCP (10 mg/kg) em camundongos com tumores SKOV-3 (menor expressão de Gal-3) e tumores MKN45 (maior expressão de Gal-3) reduziu o crescimento tumoral em 58,7% e 35,4%, respectivamente, e reduziu o peso do tumor em 51,7% e 30,7%, respectivamente. Além disso, MCP-^{99m}Tc atingiu o tumor na mesma proporção em ambos os xenoenxertos tumorais. Esses resultados demonstram que a radiomarcação de MCP-^{99m}Tc é uma ferramenta importante para estudar a atividade anticancerígena de MCP. Também mostramos que os dados não corroboram a hipótese do efeito farmacológico direto de MCP visando exclusivamente a Gal-3, contribuindo assim para o conhecimento da atividade anticancerígena de MCP.

Palavras-chaves: Pectina cítrica modificada, imagem molecular, atividade anticancerígena e proteína galectina-3.

ABSTRACT

SILVA, F. F. A. d. ***Evaluation of MCP anticancer activity using molecular imaging***. 2023. 157 f. Tese (Doutorado em Tecnologia Nuclear), Instituto de Pesquisas Energéticas e Nucleares, IPEN-CNEN, São Paulo.

MCP is a polysaccharide found abundantly in the plant's primary wall and shows activity in several areas of the food industry and nutrition, serving in food production, improving intestinal flow, reducing cholesterol, and being an important nutritional compound. MCP can also act as an anti-tumorigenic molecule in several types of tumors, in addition to avoiding chemoresistance, modulating the immune system, and preventing renal disease caused by radiotherapy and chemotherapy in cancer treatments. This study was divided into three main parts. In the first part, a review was carried out to study the biological activity of MCP and its contribution to cancer therapy, demonstrating treatment doses, types of MCP used, and experimental design, providing a critical view of the exposed data and its relationship with galectin-3 and other theories regarding its mechanisms. In the second part, we radiolabeled MCP with ^{99m}Tc and verified the biodistribution and pharmacokinetics of MCP-^{99m}Tc orally and intravenously (IV) administrated. First, the structure and monosaccharide composition of MCP were studied, and it was demonstrated that there is a diversity of monosaccharides and molecular weights within the MCP structure and that MCP30 and MCP3 fractions are rich in galactose. Next, we studied the inhibition and binding affinity of MCP for galectin-3 and demonstrated that MCP partially binds Gal-3 and that MCP3 shows an inhibition capacity at a concentration of 25 mg/ml. We radiolabeled MCP with ^{99m}Tc and verified its stability in saline in different pH, plasma, and *in vivo*. MCP-^{99m}Tc has a low gastrointestinal absorption (5.27x10-6 % total radioactivity counts) and gastrointestinal elimination when administered via oral and renal and hepatobiliary elimination when administered via IV. Finally, the blood compartment distribution assay showed that MCP-^{99m}Tc has a high affinity for plasma proteins and blood cells in C57BL/6 Lgals3 $^{+/+}$ mice, and this affinity was partially lost when galectin-3 was deleted in C57BL/6 Lgals3 $^{-/-}$ mice. The pharmacokinetic assay showed that MCP-^{99m}Tc elimination speed was greater in the C57BL/6 Lgals3$^{-/-}$ mice than in the C57BL/6 Lgals3 $^{+/+}$, indicating that a lack of galectin-3 increases MCP elimination. In the third part, we analyzed the behavior of MCP-^{99m}Tc in

animals with SKOV-3 and MKN45 tumors using molecular imaging. First, we demonstrated that cell binding and internalization of MCP were only partially influenced by Galectin-3 expression *in vitro* using SKOV-3 scrambled and SKOV-3 shRNAGal3 cells (knockdown of Gal-3 expression). Next, we showed that MCP (20 mg/kg) exhibited anticancer activity in a SKOV-3 cell tumor xenograft model, reducing tumor growth by 48.5% and tumor weight by 50% when administered intravenously; however, oral administration of MCP (200 mg/kg) did not show an anticancer effect. Subsequently, we demonstrated that MCP-^{99m}Tc reached the tumor and bound to regions of necrosis in the SKOV-3 cell tumor xenograft model when administered intravenously. Finally, we demonstrated that IV administration of MCP (10 mg/kg) in mice with SKOV-3 tumors (lower expression of Gal-3) and MKN45 tumors (greater expression of Gal-3) reduced tumor growth by 58.7% and 35.4%, respectively, and reduced the tumor weight by 51.7% and 30.7 %, respectively. Furthermore, in both tumor xenografts, MCP-^{99m}Tc reached the tumor at the same proportion. These results demonstrate that MCP-^{99m}Tc radiolabeling is an important tool for studying the anticancer activity of MCP. We also showed that the data do not corroborate the hypothesis of the direct pharmacological effect of MCP exclusively targeting Gal-3, thus contributing to the knowledge of MCP anticancer activity.

Keywords: Modified citrus pectin, Molecular imaging, Anticancer activity e Galectin-3 protein.

Summary

CHAPTER 1

MODIFIED CITRUS PECTIN FOR CANCER TREATMENT: CURRENT STATUS AND CLINICAL POTENTIAL

MODIFIED CITRUS PECTIN FOR CANCER TREATMENT: CURRENT STATUS AND CLINICAL POTENTIAL

Highlights

- **Pectin is widely used in the biomedical fields.**
- **MCP plays a prominent role in cancer, exhibiting anti-proliferative, apoptotic, and antimetastatic activities.**
- **MCP has been shown to be important in anticancer therapies as an adjuvant in combination with chemotherapy and radiotherapy.**
- **The anticancer activity of MCP is closely linked to galectin-3 and immunomodulatory effects.**

Abstract

Cancer has taken a greater dimension in recent decades, becoming a public health problem worldwide with 10 million deaths annually. Therefore, new approaches and alternatives to treatments of cancer are important. Pectin's are complex heteropolysaccharide polymers found abundantly in plant cell primary walls and are widely used in the biomedical field to treat heart disease, nephropathy, gastric disease, and oncological diseases. Modified citrus pectin (MCP) is a subtype of pectin extracted from citrus that has been studied for several applications and has anticancer properties. The evidence demonstrating this is vast and can be verified in *in vitro*, preclinical, and clinical studies in many types of cancer. Its mechanisms of action are related to its interaction with galectin-3 (Gal-3) and immunological modulation. However, the mechanism of action of MCP is not a consensus among researchers and is the subject of study. In this review, we studied the biological activity of MCP and its contribution to cancer therapy, demonstrating treatment doses, types of MCP used, and experimental design, providing a critical view of the exposed data and its relationship with galectin-3 and other theories regarding its mechanisms. This study contributes to a better understanding of the pharmacological activity and mechanism of action of MCP.

Keywords: Modified citrus pectin; anticancer activity; clinical trials; Galectin-3

1. Introduction

The incidence of cancer worldwide has increased at a pace that accompanies the aging population owing to the increase in life expectancy. Thus, cancer has taken on a greater dimension in recent decades and has become an evident public health concern worldwide. According to the World Health Organization, cancer is the second leading cause of death globally, and is responsible for an estimated 10 million deaths by 2020 (GICR, 2020; WHO, 2020). In 2023, there will be an estimated 1.95 million new cancer cases and 609,820 cancer-related deaths in the United States. In Brazil approximately 625.000 new cases of cancer, reaching 704,000 for each year of the 2023-2025 triennium (American Cancer Society, 2023; MS/INCA, 2022; Santos et al., 2023; Siegel et al., 2023). Therefore, new approaches and alternatives for the treatment of different types of cancers are important.

Pectin's have potential preventive and adjuvant effects in several types of cancers (Emran et al., 2022). Specifically, modified citrus pectin (MCP) has been studied because of its high efficiency and action in the combat of the development and progression of cancer and metastasis (Emran et al., 2022). The relationship between MCP and Gal-3 has been supported over the years using data from *in vitro* and *in vivo* studies; however, new evidence has shown a complex interaction between the immunomodulatory and anticancer effects of these polysaccharides, contributing to the identification of the mechanisms involved in the use of this bioactive molecule (Conti et al., 2018; Gharibzahedi et al., 2022).

This article presents an extensive and in-depth review of MCP in combat of cancer progression and metastasis. Moreover, this review discusses the mechanisms of action involved in this process. These results provide a detailed understanding of the pharmacological use of MCP and its potential clinical applications.

1.1. Pectin: molecular structure and properties

Pectin is a natural anionic biopolymer composed of complex heteropolysaccharides present in the cell wall (middle lamella of plants), first identified in 1790 in apple pomace (Emran et al., 2022; Vauquelin, 1790). The french chemist and pharmacist Henri Braconnot, expert in the extraction of active components from plants, was one of the first to systematize the study a heteropolysaccharide "Pectin acid" (greek

πηκτικός, meaning coagulant) in 1825 (Leclere et al., 2013). Pectin is a thin, flexible, and hydrostatic polysaccharide that is considered to be the second most abundant and widely distributed plant polysaccharide in nature, just behind cellulose, and is responsible for morphological and mechanical resistance, providing support for growth and development (Fabi et al., 2007). Furthermore, pectin participates in specific functions, including hydraulic control, barrier protection, steer plant cell expansion, cell protection, cell-to-cell communication, cell adhesion, cell differentiation, cell proliferation, cell fate specification, morphogenesis, environmental sensing, and stress responses (Duan et al., 2020; Haas et al., 2020; Jonsson et al., 2021; Lampugnani et al., 2018; Wachsman et al., 2020). Pectin is synthesized by various interconversion enzymes, including glycosyltransferases, methyltransferases, and acetyltransferases. In this process, sugar is transferred from an activated donor to an acceptor, leading to elongation of the pectin polymer. It is excreted by the endoplasmic reticulum and Golgi complex and can undergo various modifications, such as demethylesterification and deacetylation (Atmodjo et al., 2013; Bar-peled & Neill, 2011; Cao et al., 2020; Philippe et al., 2017).

Pectin is structurally and functionally one of the most complex and highly variable polysaccharides, thus, the exact structure of pectin is does not a consensus. They are heterogeneous chain polysaccharides composed of D-galacturonic acid linked by α-1,4 glycosidic bonds, with 1–4-α-D-galactopyranuronic acid – GalpA (galacturonic acid) representing ~ 70% of the pectic structure (Antonov et al., 2021; Mohnen, 2008). The primary structure of Pectin is a family of covalently linked galacturonic acid-rich polymers with five main polysaccharides: homogalacturonan (HG), xylogalacturonan, apiogalacturonan, rhamnogalacturonan-I (RG-I), and rhamnogalacturonan-II (RG-II) (Figure 1). They are heterogeneous chain polysaccharides as well as substituted galacturonans, and their respective secondary structures vary from species to species (Erdmann et al., 2017; Gawkowska et al., 2018; Leclere et al., 2013; Wu et al., 2020a) (Figure 1). In this sense, the HG and RG-II have backbones formed by repeated motif of α(1,4)-GalA, whereas that RG-1 polymer consists of α(1,4)-GalA and α(1,2)-rhamnose. Additionally, xylogalacturonan (XGA) and apiogalacturonan (AGA) are also considered pectin's because they have the same backbone as HG. The backbones of pectin

polysaccharides receive several attachments of oligosaccharides, such as α(l,5)-arabinans, β(1,4)-galactans, complex heteropolysaccharides, and methyl and acetyl groups (Shin et al., 2021; Tan et al., 2013).

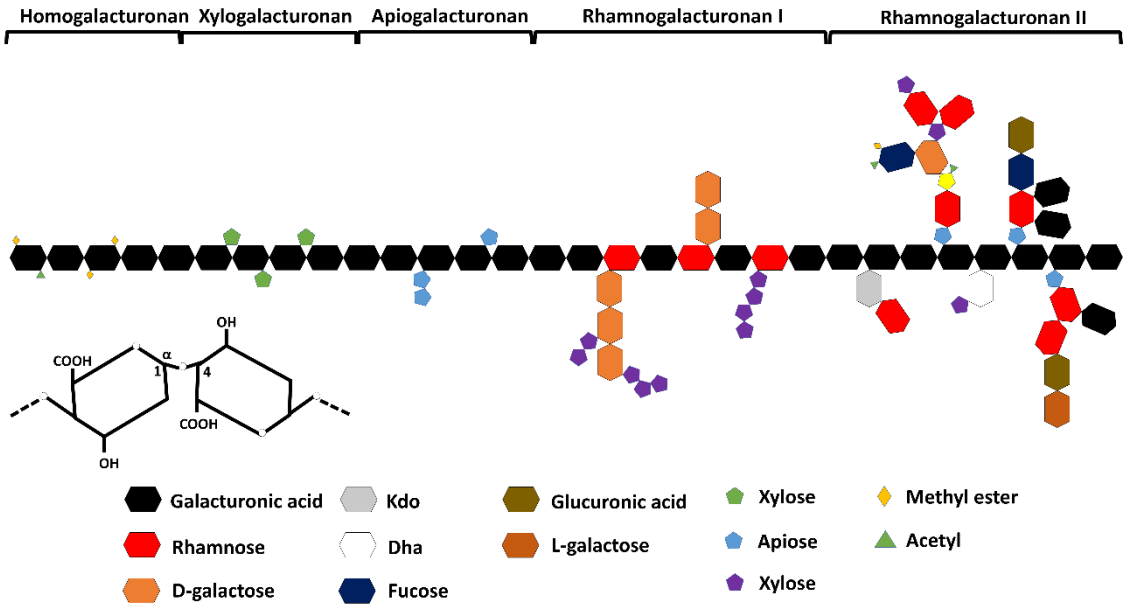

Figure 1: Structural characteristics of pectin molecules: A pectin molecule can have different structural characteristics, including homogalacturonan, xylogalacturonan, apiogalacturonan, rhamnogalacturonan I, and rhamnogalacturonan II. Kdo: 3-deoxy-D-manno-2-octulosonic acid, Dha: 3-deoxy-D-lyxo-2-heptulosaric acid.

Pectin is obtained mainly from fruits (citrus and apple) and sugar beets, but can also be extracted from different sources and plant organs, such as eggplant, pomelo, pomegranate cashew pulp, chamomile, durian, hibiscus (*sabdarffa* L.), banana, mango, jackfruit, passion, tomato, artichoke (*Cynara scolymus* L.), sisal, tobacco, and cocoa (Mellinas et al., 2020). It can be extracted using water, buffers, enzymes, alkalis and acids, chelating agents, ultrasound, microwave-assisted ionic liquids, and surfactants. Additionally, treatment with hot water, CDTA, and sodium carbonate results in pectin's with different structures, leading to the existence of a wide variety of pectin's in the market (Liu et al., 2020; Su et al., 2019; Zdunek & Pieczywek, 2021, Gharibzahedi et al., 2022). Pectin can be modified by treatment at different pH values. The most studied pectin is Modified Citrus Pectin (MCP), which was isolated from citrus and treated with pH variation. This process consists of alkaline treatment-generated ß-elimination reactions, which provoked depolymerization of the polysaccharide backbone and deesterification in the HG regions. The next step is the cleavage of the neutral sugar with

acid treatment, releasing the branched regions and arabinose residues of the pectin backbone. Thus, several arabinogalactans and galactans are produced (Leclere et al., 2013).

1.2. Galectin-3

Galectins are a family of proteins that have a carbohydrate recognition domain. Discovered first in 1976, it is distributed from lower or higher vertebrates. There are 15 different types of galectins, numbered according to the date of discovery (galectins 1 – 15) (Barondes et al., 1994; Cummings et al., 2009; Kasai & Hirabayashi, 1996;Dong et al., 2018).

The galectin-3 (Gal-3) is a multifunctional lectin protein of 27 KDa, expressed by immune, endothelial, and epithelial cells. It is located in the cytoplasm, membrane and nucleus and is secreted extracellularly. The galectin-3 through its carbohydrate recognition domain and carbohydrate-independent mechanism modulate numerous biological processes. For example, intracellular galectin-3 inhibits the intrinsic apoptotic pathway, whereas intra-nuclear galectin-3 activates cell proliferation (Barondes et al., 1994; Cummings R.D., Liu F.T. Galectins. In: Varki A., Cummings et al., 2009; Davidson et al., 2002; Hsu & Liu, 2002; Kasai & Hirabayashi, 1996; Ochieng et al., 2002; Blanchard et al., 2014).

The galectin-3 is expressed in various tissues and has several important functions in the tumor microenvironment. This protein is overexpressed in many types of tumors and is associated with tumor growth and metastasis. Several studies indicate that the expression of galectin-3 is induced in the tumor microenvironment, and that this maintains the homeostasis of tumor cells. Galectin-3 also can regulate the immune system and promotes tumor-directed immunosuppression. In addition, intracellular galectin-3 is related to glycolysis, mitochondrial metabolism, and several signaling mechanisms in tumors, contributing to tumor progression (Guo et al., 2020; Kariya et al., 2018; Rinaldi et al., 2018; Ruvolo et al., 2019; Wang et al., 2019). Thus, galectin-3 is dysregulated in cancer, has a pro-tumor effect, and is highly expressed in many types of cancer (Newlaczyl & Yu, 2011). Its relationship with MCP is also well studied, being one of the main pathways that explain the mechanism of action of MCP, as we will see below.

Reviews have been developed in medical science as a way to synthesize research results in a transparent, reproducible, and systematic way and are referred to as the gold standard among reviews (Davis et al., 2014). This type of review can be illustrated as a research method for identifying and critically analyzing research, collecting relevant data, and analyzing parameters (Liberati et al., 2009; Snyder et al., 2016; Witell et al., 2016). The principal aim of the systematic review is to identify all the evidence to answer a specific research question. The advantage of this method is that, with a systematic methodology, the trend can be minimized, generating more reliable conclusions (Liberati et al., 2009; Snyder, 2019). In this systematic review, we aimed to understand the relationship between MCP, its applications and cancer.

2. Methodology of review

In this work, we present a systematic review with the aim of contributing to the discussion on MCP and its biological effects, focusing on its application in oncology. Our research question was: “What are the biological applications of MCP and its importance for cancer treatment?”. The inclusion criteria were the presence of some type of MCP, mainly, in anti-tumorigenic application. The search for studies was performed on scientific journal search platforms: NCBI PubMed, Scienci Direct (Elsevier), OpenMD, and National Library of Medicine (ClinicalTrials.gov). The selected studies were peer-reviewed scientific articles published in reputable journals. The extracted data included the names of molecules, applications, models used, concentration of molecules, effectiveness results, and Gal-3 participation. A screening was also carried out on studies on pectin, in which its application was related to clinical studies.

3. Initial conceptions

In this review, we verified the existence of pectin’s that have been widely studied for different applications. Some of these are commercially available as MCP (modified citrus pectin, PectaSol-C, EcoNugenics, Santa Rosa, CA), CP (pectin from citrus peel (P9135, Sigma Aldrich), MCP pH modification from CP (Sigma St Louis, MO), MCP (Allergy Research Group), and GCS-100/MCP (CP modified from Sigma Chemicals). The MCP are also used with other components, being coupled to other molecules and nanoparticles, showing synergistic effects, such as PSVII@MCP-CaP (PSVII with MCP from Centrax

International Corporation, and a Cap nanoparticle), Cet-MCPCNPs (cetuximab-conjugated modified citrus pectin-chitosan nanoparticles), MCP:HNK (MCP (econugenics) and honokiol (HNK)), MCP and S-trans (transfarnesylthiosalicylic acid (FTS; Salirasib)), MCP with ginseng pectin and P-galactan, BD (BreastDefend), PC (ProstaCaid) and PTX (Paclitaxel), and some are not cited the source due to legal protection (Table 1, 2 and 3). This information makes it clear that MCP is not homogeneous and the results of biological activity presented by a molecule are individual, taking into account its extraction source, route of administration, dose and frequency used, degree of purity, molecular weight, monosaccharide composition, degree of esterification, acetylation, and methylation, among others.

Another fact that can be highlighted in this context is that modified citrus pectin (MCP) is a type of pectin. The Pectin´s are used in many applications, presenting interesting results with great therapeutic potential, and are generally extracted from alternative sources such as Papaya (*Carica papaya* L.), *Panax ginseng* flower buds, citrus, grapefruit, wheat white, cotton-boll, beet, carrot, bell pepper, *S. boulardii* pectin, among others (Cui et al., 2019a; do Prado et al., 2019; Pedrosa et al., 2020; Prado et al., 2017; L. Wang et al., 2020a; Wu et al., 2020ª, Nesterenko VB, Nesterenko AV, Babenko VI, Yerkovich TV, 2004, Cerda et al., 1988, Lutter et al., 2021; McKay et al., 2021, 2022, Challen et al., 1983, Salomov et al., 1994, Lazareva, Smirnov, et al., 2002; Lazareva, Spiridonova, et al., 2002, Lutter et al., 2021; McKay et al., 2021, 2022) (Table 1). Pectin is considered in this study because of its use in clinical studies; however, our focus will be on MCP.

Although MCP has a range of pharmacological activities, the evidence of these activities varies among studies carried out *in vitro* (Table 1), *in vivo* (pre-clinical studies) (Table 2), and in humans (clinical studies) (Table 3) in different developmental phases. Another characteristic of these molecules is that their mechanisms of action are the result of intense study, not being a consensus among researchers in the area, as we will see below.

For the purposes suggested here, we divided the evidences showed in articles into; Pectin's in clinical studies, MCP *in vitro* studies, MCP in preclinical studies and MCP in clinical studies, as well as, we extract from the articles information about name of molecules, application, concentration used, effective result (positive or negative) and

Gal-3 participation in explaining the mechanism of action (yes or no). Another part of this review deals more specifically with the mechanisms of action of MCP.

4. Pectin's in clinical studies

Our first search filter included articles that included pectin in clinical studies. In this research was seen that the pectin's used in clinical studies are natural and often used as a complement to other molecules or pharmakos, as such as; Tacrolimus 0.1 % in pectin, apple-pectin, oligofructose and pectin, grapefruit pectin (*Citrus paradisi*), wheat bran pectin, pectin and guar gum or citrus pectin, pectin with FF5005 formulation, psyllium-citrus pectin, Aronia with apple pectin, hight and low-methoxyl pectin, alginate-pectin, acacia gum and pectin, apple-pectin-guar, cotton-boll pectin, yogurt with RG (rhamnogalacturonan), pectin and beta-glucan, beet pectin, FPNS (Fentanyl Pectin Nasal Spray), cRG-1 (carrot-derived RG-I (rhamnogalacturonan-I)), bpRG-I (bell pepper (rhamnogalacturonan-I)), pectin-HPMC (pectin-hydroxypropyl methylcellulose coating), PFA (pectin-based, raft-forming, natural, anti-reflux agent), DFKP (diphenhydramine syrup plus kaolin-pectin (50:50)), SBP (sugar beet pectin) and PDX (polydextrose), *S. boulardii* pectin (lactose-free, high-mineral, low-osmolarity formula, containing rice and pectin fortified with *Saccharomyces boulardii*), scGOS (galacto-oligosaccharides), lcFOS (long-chain fructo-oligosaccharides), pAOS (ectin-derived acidic oligosaccharides), Prebiotic formula (neutral oligosaccharides and pectin-derived acidic oligosaccharides), WSDF (psyllium, pectin, guar gum, and locust bean gum), SN-263 (amylopectin sulfate),and E-440 (apple pectin "Classic AU-701") (Table 1).

The first record was made in 1966 to reduce serum cholesterol in human patients with oral administration of pectin (6–10 mg daily for 4–10 months) (Palmer & Dixon, 1966). This activity against hypercholesterolemia has been reported in several studies, all with its oral use in daily fractional doses and all with positive results (Hunninghake et al., 1994; Knopp et al., 1999; Judd & Truswell, 1982; Pirich et al., 1992; Jensen et al., 1993, 1997). Similarly, there is a relationship between lipid reduction and glycemic management, as well as the regulation of metabolism and liver parameters (Savastano et al., 2014; Remer et al., 1996; Jenkins et al., 1975, 1976, 1978; Frape & Jones, 1995; Hillman et al., 1985; Pelkman et al., 2007; Pouteau et al., 2010; Hillman et al., 1986; Schwab et al., 2006; Tadesse & Eastwood, 1978; Villaume et al., 1988; Aller et al., 2004). These studies also demonstrated the use of pectin in applications such as coronary heart

disease due to hypercholesterolemia and ischemic heart disease through its effect on platelet aggregation and blood clotting, type II diabetes, satiety, and obesity (Lorenzo et al., 1989; Savastano et al., 2014; Cerda et al., 1988; Challen et al., 1983; Asp et al., 1981; Sharafetdinov et al., 1993; Howarth et al., 2003; Liebermeister & Toluipur, 1980). However, it is important to note that some studies have shown inconclusive or even negative results (with zero effects) for these applications (Table 2).

Another important application of pectin demonstrated in clinical studies is in nutritional regulation, gastroesophageal reflux disease (negative response), nutrition, erythrocytes disorder in infants, children with acute diarrhea, fecal microbiota, desquamative gingivitis, and chronic duodenal ulcers (Farup et al., 2009; Salomov et al., 1994; Cocking, 1972; Littman, 1970, 1972; Le Luyer et al., 2010; Magne et al., 2008; Corrocher et al., 2006). Scintigraphic evaluation of the colon can be performed using pectin-HPMC and pectin with an anti-reflux formulation (FF5005), which is used as ^{99M}TC-DTPA-labelled tablet and test meal radiolabelled with indium-^{113m} and technetium-^{99m}, respectively (Hodges et al., 2009; Washington et al., 1988).

Others sporadic uses have been attributed to pectins, such as innate immune and antiviral responses (cRG-I or bpRG-I), DTaP-IPV-Hib immunization in preterm infants (negative response), neurodevelopmental outcomes measured by Bayley Scales of Infant and Toddler development in preterm infants, early atopic dermatitis infants using scGOS/lcFOS/pAOS, and inflammatory responses using yogurt with RG (Lutter et al., 2021; McKay et al., 2021, 2022; van den Berg et al., 2013; van den Berg et al., 2016; Grüber et al., 2010; Lee et al., 2016). Pectins may also reduce ^{137}Cs in contaminated patients (apple pectin), reduce the cesium (^{137}Cs) burden related to cardiovascular symptoms in children from Chernobyl (apple pectin), detoxify patients with high mercury levels (apple or beet pectin), and help in burn wounds (E-440) (Nesterenko VB, Nesterenko AV, Babenko VI, Yerkovich TV, 2004; Bandazhevskaya et al., 2004; Lazareva, Smirnov, et al., 2002; Lazareva, Spiridonova, et al., 2002; Sobolev et al., 1999).

Regarding the use of pectin in oncology, interesting applications have been reported. The first application is related to immunomodulatory activity in patients with breast cancer during postoperative radiation therapy, where 15 g of apple pectin in combination with 20 ml of aronia daily during postoperative irradiation improves immune parameters in patients (Yaneva et al., 2002). Another application is the

management of breakthrough cancer pain associated with chirurgical or radiotherapy procedures using FPNS per breakthrough pain episode or DFKP for pain caused by radiation-induced oropharyngeal mucositis (Bulloch & Hutchison, 2013; Davies et al., 2011; Fallon et al., 2011; Hui et al., 2016; Jandhyala et al., 2013; Mercadante et al., 2014, 2016; Perelman & Knight, 2013; Portenoy et al., 2010; Prieto et al., 2016; Taylor et al., 2014; Torres et al., 2014; Barker et al., 1991). FPNS is also associated with a reduction in allergic rhinitis (Fisher et al., 2010; Perelman & Knight, 2013). This result is associated with analgesic and anti-inflammatory drugs. In these cases, pectin has a secondary synergistic effect, possibly influencing the pharmacokinetics and efficiency of the drug.

These data show that pectin has a relevant clinical effect in several applications, as demonstrated in the analyzed articles. It was also verified that none of these applications was related to galectin-3, the main mechanism of action known for these molecules.

Table 1: Clinical studies using pectin.

Molecule	*Application*	*Model*	*Route of administration*	*Concentration*	*Effectiveness Result*	*Gal-3 participation*	*Reference*
FPNS	Management of Breakthrough Cancer Pain associated with chirurgic or radiotherapy procedures	Human patients	Nasal formulation	≥60 mg/day, 100 and 800 µg or 15%-25% per breakthrough pain episode	Positive	No	(Bulloch & Hutchison, 2013; Davies et al., 2011; Fallon et al., 2011; Hui et al., 2016; Jandhyala et al., 2013; Mercadante et al., 2014, 2016; Perelman & Knight, 2013; Portenoy et al., 2010; Prieto et al., 2016; Taylor et al., 2014; Torres et al., 2014)
FPNS	Allergic rhinitis	Human patients	Nasal formulation	100-800 µg	Positive	No	(Fisher et al., 2010; Perelman & Knight, 2013)
Tacrolimus 0.1 % in pectin	Desquamative gingivitis	Human patients	Topical use	0.2 mg for 4 weeks	Positive	No	(Corrocher et al., 2006)
Apple-pectin	Reduction of the 137Cs in contaminated patient	Human patients	Oral	Dried and ground apple extract containing 15-16% pectin	Positive	No	(Nesterenko VB, Nesterenko AV, Babenko VI, Yerkovich TV, 2004)
Pectin	Obesity	Human patients	Oral	15 g of pectin per day	Positive	No	(Lorenzo et al., 1989)

Oligofructose (OFS) and pectin (P)	Satiety and glycemic parameters (obesity)	Human patients	Oral	High-dose (30 g/d) and low-dose (15 g/d)	Inconclusive	No	(Savastano et al., 2014)
cRG-I or bpRG-I	Innate immune and antiviral responses	*In vitro*, *in vivo* and Human patients	Oral	0-0.3-1.5 g/day	Positive	No	(Lutter et al., 2021; McKay et al., 2021, 2022)
Grapefruit pectin (Citrus paradisi)	Coronary heart disease due to hypercholesterolemia	Human patients	Oral	27 capsules per day (15 g) based on 5 per meal for three meals	Positive	No	(Cerda et al., 1988)
Wheat bran pectin	Ischemic heart disease through an effect on platelet aggregation and blood clotting	Human patients	Oral	36 g/d pectin for 3 at 6 weeks	Positive	No	(Challen et al., 1983)
Pectin and guar gum or citrus pectin	Type II diabetes	Human patients	Oral	8.4 g for day	Positive	No	(Asp et al., 1981; Sharafetdinov et al., 1993)
Pectin	Plasma levels of dehydroepiandrosterone (DHEA) and its sulfate ester (DHEAS)	Human patients	Oral	Daily doses of 0 g, 15 g or 30 g pectin	Positive	No	(Remer et al., 1996)
Pectin	Reduction of serum cholesterol	Human patients	Oral	6 to 10 mg daily for 4 to 10 months	Positive	No	(Palmer & Dixon, 1966)
Pectin-HPMC	Scintigraphy evaluation of colon	Human patients	Oral	Dosed with one 99mTc-DTPA-labelled tablet	Positive	No	(Hodges et al., 2009)
Pectin with anti-reflux formulation (FF5005)	Scintigraphy evaluation of colon	Human patients	Oral	The formulation and test meal were radiolabeled with indium-113m and technetium-99m	Positive	No	(Washington et al., 1988)
Pectin	Post-prandial hyperglycemia	Human patients	Oral	10-36g of pectin for 2 weeks	Positive	No	(Jenkins et al., 1975, 1976, 1978)
Psyllium-citrus pectin or pectin	Postprandial responses of plasma insulin, glucose and lipids	Human patients	Oral	2.2 or 15 g daily for 3 weeks	Negative	No	(Frape & Jones, 1995; Hillman et al., 1985)
Apple pectin	Electrolytes and trace elements in patients with hyperlipoproteinemia	Human patients	Oral	15g daily for 3 months	Positive	No	(Grudeva-Popova & Sirakova, 1998)
Guar gum, pectin, soy, pea, corn bran	Hypercholesterolemia	Human patients	Oral	20 g/day for 36 weeks	Positive	No	(Hunninghake et al., 1994; Knopp et al., 1999)

Aronia with apple pectin	Immunomodulatory activity in patients with breast cancer in the course of postoperative radiation therapy	Human patients	Oral	15 g of apple pectin in combination with 20 ml of Aronia concentrate twice daily during postoperative irradiation	Positive	No	(Yaneva et al., 2002)
Hight and low-methoxyl pectin	Cholesterol-lowering	Human patients	Oral	15 g	Positive	No	(Judd & Truswell, 1982)
Alginate-pectin	Reducing food intake in overweight and obese	Human patients	Oral	1.0 g, 2.8 g twice per day (once before breakfast and once midafternoon) for 7 d	Positive	No	(Pelkman et al., 2007)
PFA	Gastroesophageal reflux disease	Human patients	Oral	Six weeks' on-demand treatment	Negative	No	(Farup et al., 2009)
Acacia gum and pectin	Lipolysis in insulin-resistant patients	Human patients	Oral	28 g for 5 weeks	Negative	No	(Pouteau et al., 2010)
Apple-pectin-guar	Hight cholesterol	Human patients	Oral	17g for day	Positive	No	(Pirich et al., 1992)
DFKP	Pain of radiation-induced oropharyngeal mucositis	Human patients	Oral	Not identified	Positive	No	(Barker et al., 1991)
Pectin, cellulose and lignin	Biliary lipid composition and bile salt metabolism	Human patients	Oral	36g daily dietary supplementation for four weeks (1:1:1).	Positive	No	(Hillman et al., 1986)
SBP or PDX	Postprandial glycemia and fasting concentrations of serum lipoprotein lipids	Human patients	Oral	Daily drinks (16g) for 12 weeks	Negative	No	(Schwab et al., 2006)
Cotton-boll pectin	Nutrition and erythrocytes disorder in infants	Human patients	Oral	1 g per 100 ml of infant milk or food 3 times a day for 45-60 days	Positive	No	(Salomov et al., 1994)
Pectin	Metabolism of dietary fiber components in man assessed by breath hydrogen and methane	Human patients	Oral	20g in 400ml of water for day	Negative	No	(Tadesse & Eastwood, 1978)
S. boulardii pectin	Children with acute diarrhea	Human patients	Oral	26mg/100ml per day	Positive	No	(Le Luyer et al., 2010)
scGOS/lcFOS/ pAOS	Response on pneumococcal vaccine in preterm infants	Human patients	Enteral supplementation	Supplementation of 1.5 g/kg/day to breast milk or preterm formula (3 and 30 days of life)	Positive	No	(van den Berg et al., 2015)

scGOS/lcFOS/ pAOS	DTaP-IPV-Hib immunization in preterm infants	Human patients	Enteral supplementation	Supplementation of 1.5 g/kg/day to breast milk or preterm formula (3 and 30 days of life)	Negative	No	(van den Berg et al., 2013)
scGOS/lcFOS/ pAOS	Neurodevelopmental outcomes measured by Bayley Scales of Infant and Toddler Development in preterm infants	Human patients	Enteral supplementation	Supplementation of 1.5 g/kg/day to breast milk or preterm formula (3 and 30 days of life)	Positive	No	(van den Berg et al., 2016)
scGOS/lcFOS/ pAOS	Early atopic dermatitis infants	Human patients	Enteral supplementation	Supplementation of 1.2 g/kg/day to breast milk or preterm formula	Positive	No	(Grüber et al., 2010)
scGOS/lcFOS/ pAOS	Faecal microbiota	Human patients	Enteral supplementation	400-gram for day, breast-fed children from 1 week to 3 months old	Positive	No	(Magne et al., 2008)
Pectin	Plasma insulin and glucagon after ingestion of meal	Human patients	Oral	5, 10 and 15 g included in a solid-liquid meal	Positive	No	(Villaume et al., 1988)
Yogurt with RG	Inflammatory response	Human patients	Oral	dairy yogurt containing 50 mg of probiotics and 100 mg of hallabong peel polysaccharide (60% RG) each day for 8 weeks	Positive	No	(Lee et al., 2016)
WSDF	Management of hypercholesterolemia	Human patients	Oral	15 g/day supplemental water-soluble dietary fiber	Positive	No	(Jensen et al., 1993, 1997)
Apple pectin	Relationship between cesium (^{137}Cs) load, cardiovascular symptoms, and source of food in 'Chernobyl' children	Human patients	Oral	5 g (taken with water or milk during meals) twice a day for 16 days.	Positive	No	(Bandazhevskaya et al., 2004)
Pectin's, gums and mucilage	Lipid and glucose levels	Human patients	Oral	10.4g per day	Positive	No	(Aller et al., 2004)
Pectin, beta-glucan	Hunger, satiety or body weight	Human patients	Oral	27 g/day	Negative	No	(Howarth et al., 2003; Liebermeister & Toluipur, 1980)
SN-263	Chronic duodenal ulcer	Human patients	Oral	Tablets of 500 mg 6 times per day for 48 weeks	Positive	No	(Cocking, 1972; Littman, 1970, 1972)
Apple or beet pectin's	Burn wounds	Human patients	Topical	Topical use whenever necessary	Positive	No	(Lazareva, Smirnov, et al., 2002; Lazareva, Spiridonova, et al., 2002)
E-440	detoxication of mercury	Human patients	Oral	Not identified	Positive	No	(Sobolev et al., 1999)

FPNS = Fentanyl Pectin Nasal Spray. cRG-1 = carrot-derived rhamnogalacturonan-I (RG-I). bpRG-I = bell pepper (rhamnogalacturonan-I). Pectin-HPMC = pectin-hydroxypropyl methylcellulose coating. PFA =

pectin-based, raft-forming, natural, antireflux agent. DFKP = diphenhydramine syrup plus kaolin pectin (50:50). SBP, sugar beet pectin; PDX, polydextrose. S. boulardii pectin = lactose-free, high-mineral, low-osmolarity formula, containing rice and pectin fortified with *Saccharomyces boulardii*. scGOS, galacto-oligosaccharides; lcFOS, long-chain fructo-oligosaccharides; pAOS, pectin-derived acidic oligosaccharides. Prebiotic formula = neutral oligosaccharides and pectin-derived acidic oligosaccharides. WSDF = psyllium, pectin, guar gum, and locust bean gum. SN-263 = Amylopectin sulfate. E-440 = apple pectin "Classic AU-701.

5. MCP *in vitro* evidences

To analyze the full spectrum of evidence supporting the clinical importance of MCP, we analyzed its prevalence in *in vitro* studies. Initially, we verified the presence of a series of MCP molecules in studies for different applications: MCP (EcoNugenics, Santa Rosa, CA), MCP* (Centrax International Corporation, San Francisco, CA, USA), MCP** (pectin from citrus peel (P9135, Sigma Aldrich)), MCP*** (unidentified), MCP# (MCP pH modification from CP Sigma St Louis, MO), and MCP## (Allergy Research Group). All of these are commercial or are produced, which facilitates the standardization, comparison, and interpretation of the articles. There are MCP coupled with other molecules, showing synergistic action, such as; PSVII@MCP-CaP (PSVII with MCP from Centrax International Corporation, and a Cap nanoparticle), Cet-MCPCNPs (cetuximab-conjugated modified citrus pectin-chitosan nanoparticles for targeted delivery of curcumin), MCP:HNK (MCP (econugenics) and honokiol (HNK)), MCP with BD (BreastDefend), PC (ProstaCaid) and PTX (Paclitaxel). GCS-100/MCP (CP purchased from Sigma Chemicals and modified) is an example of purified MCP in advanced clinical studies (Tables 2 and 4).

Regarding its applications, the MCP shows antioxidant and anti-Inflammatory effects in mouse monocytes with MCP:HNK ((9 : 1) 0–2000 μg/ml), activation of lymphocyte T-, B- and NK-cell of human blood (MCP, 10 – 800 μg/ml) and combats arthritis in synovial fibroblasts (SF) obtained from patients with rheumatoid arthritis and osteoarthritis (MCP**) (Arad et al., 2015; Ramachandran et al., 2017; Ramachandran et al., 2011). Other important activity of MCP is in calcific aortic stenosis (AS) in valvular interstitial cells (VICs) isolated from aortic valves (AVs) of patients undergoing replacement valves (MCP 10^{-6} mol/L) (Śadaba et al., 2016).

In oncology applications, MCP showed a anticancer activity in prostate cancer model LNCaP, PC3, CASP2.1 and CASP1.1 cells (MCP 1%), ovarian cancer in SKOV-3 cells

2D and 3D model (non-effective dose of PTX (0.005-100nM) and 0.1-0.025% MCP), human breast (MDA-MB-231 cells) and prostate cancer (PC-3) using MCP (0.25-1.0 mg/mL) synergistically enhanced by BD (20 µg/mL) and PC (10 µg/mL), liver (HepG2 cells) and lung cancer (A549 cells) with MCP# (3 mg/ml), colon cancer (Caco-2 and HCT-116 cells) using Cet-MCPCNPs (2.5 - 20 µg/mL) and prostate cancer (PC3 cells) with GCS-100/MCP and cisplatin (25 - 50µM of cisplatin and 0.3% of MCP) (Yan & Katz, 2010; Hossein et al., 2013; Hossein et al., 2019a; Jiang et al., 2013a; Leclere et al., 2015; Sabra et al., 2019; Y. Wang, Balan, et al., 2010). Furthermore, MCP can sensitize PC-3, DU-145, and Cl-1 cells for radiotherapy in prostate cancer (MCP 0.25 – 2 mg/ml) (Conti et al., 2018). The GCS-100 are used against human multiple myeloma, diffuse large B-cell lymphoma, B-cell chronic lymphocytic leukemia, and prostate cancer chemoresistance in myeloma cell lines, primary tumor cells (250-800 µg/mL), and PC3 cells (0.3% MCP, cisplatin (50 µM)) (Chauhan et al., 2005; Clark et al., 2012; O'Brien & Kay, 2011; Ruvolo et al., 2016; Streetly et al., 2010; Wang, Balan, et al., 2010). The GM-CT-01 also was used against cancer, alone or combined with cancer vaccination in isolated human tumor-infiltrating lymphocytes (0.6–1.8 mmol/L) with a positive effect (Demotte et al., 2014).

All these immunomodulatory, cardiovascular protective, and anticancer effects are related to the interaction of MCP with galectin-3 (Table 2).

Table 2: *In vitro* studies using MCP.

Molecule	*Application*	*Model*	*Concentration*	*Effectiveness Result*	*Gal-3 participation*	*Reference*
MCP	Aortic stenosis (AS)	Valvular interstitial cells (VICs) isolated from aortic valves (AVs) of patients	10^{-6} mol/L	Positive	Yes	(Śadaba et al., 2016)
*MCP***	Arthritis	Synovial fibroblasts (SF) obtained from rheumatoid arthritis and osteoarthritis patients	0.3%	Positive	Yes	(Arad et al., 2015)
MCP	Prostate Cancer	LNCaP, PC3, CASP2.1 and CASP1.1 cells	1%	Positive	Yes	(Yan & Katz, 2010)
MCP	Sensitizer for Radiotherapy in Prostate Cancer	PC-3, DU-145, and Cl-1 cells	0.25 – 2mg/ml	Positive	Yes	(Conti et al., 2018)

MCP and PTX	Ovarian Cancer	SKOV-3	Non-effective dose of PTX (100nM) and 0.1% MCP	Positive	Yes	(Hossein et al., 2013)
MCP and PTX	Ovarian cancer spheroids	2D and 3D SKOV-3	MCP (0.025%) and PTX (5 µM)	Positive	Yes	(Hossein et al., 2019a)
MCP, BD and PC	Human Breast and Prostate Cancer	MDA-MB-231 and PC-3	MCP (0.25-1.0 mg/mL) is synergistically enhanced by BD (20 µg/mL) and PC (10 µg/mL)	Positive	Yes	(Jiang et al., 2013a)
MCP#	Liver and Lung cancer	HepG2 and A549 cells	3mg/ml	Positive	Yes	(Leclere et al., 2015)
Cet-MCPCNPs	Colon cancer	Caco-2 and HCT-116 cells	2.5 - 20 µg/mL	Positive	Yes	(Sabra et al., 2019)
GCS-100/MCP and cisplatin	Prostate cancer	PC3 cell	25 - 50µM (cisplatin) and 0.3% of MCP	Positive	Yes	(Y. Wang, Balan, et al., 2010)
MCP:HNK	Antioxidant and anti-inflammatory effects	Mouse monocytes	MCP:HNK (9 : 1) 0–2000 µg/ml	Positive	Yes	(Ramachandran et al., 2017)
MCP	Activation of lymphocyte	T-, B- and NK-cell of human blood	10 – 800 µg /ml	Positive	Yes	(Ramachandran et al., 2011)
GCS-100,	Human multiple myeloma, diffuse large B-cell lymphoma and B-chronic lymphocytic leukemia	Myeloma cell lines, and primary tumor cells	250-800 µg/mL	Positive	Yes	(Chauhan et al., 2005; Clark et al., 2012; O'Brien & Kay, 2011; Ruvolo et al., 2016; Streetly et al., 2010)
GCS-100/ cisplatin	Prostate cancer chemoresistance	PC3 cells	0.3% MCP, cisplatin (50 µM)	Positive	Yes	(Wang, Balan, et al., 2010)
GM-CT-01	Patients with cancer, alone or combined with cancer vaccination	Isolated human tumor-infiltrating lymphocytes	0.6–1.8 mmol/L	Positive	Yes	(Demotte et al., 2014)

MCP = EcoNugenics, Santa Rosa, CA. MCP* = Centrax International Corporation, San Francisco, CA, USA. MCP** = pectin from citrus peel (P9135; Sigma-Aldrich). MCP*** = unidentified. MCP# = MCP pH modification obtained from Sigma-Aldrich CP Louis, MO, USA). MCP## = Allergy Research Group. PSVII@MCP-CaP = PSVII with MCP from Centrax International Corporation, and a Cap nanoparticle. BD = BreastDefend. PC = ProstaCaid. PTX = Paclitaxel. Cet-MCPCNPs = cetuximab-conjugated modified citrus pectin-chitosan nanoparticles for the targeted delivery of curcumin. GCS-100/MCP = CP was purchased from Sigma Chemicals and was modified according to the protocol described in the article. MCP:HNK = MCP (economics) and honokiol (HNK).

6. Preclinical studies with MCP

Concerning the use of MCP in preclinical studies, the number of studies and the variation of applications are greater. New conditions were also added, such as the animal model and the route of administration. The molecules used were practically the same as those verified in the *in vitro* studies. The animal models used were rats, and mice, which may or not be accompanied by *in vitro* experiments. The route of administration is oral (the most common), I.V. (intravenous injection), I.P. (intraperitoneally injection), I.T. (intratumorally injection), I.T.T (intrathecal injections), and I.C. (intracerebroventricularly injection). The biological effects of MCP *in vivo* can be classified into three categories: cardiovascular effects, renal injury, and anticancer activity.

The cardiovascular effect of MCP was seen in vascular fibrosis (MCP***), pulmonary hypertension (MCP), myocardial injury after ischemia/reperfusion (MCP), ischemic heart failure (MCP***), cardiac hypertrophy (MCP), atherosclerotic lesions (MCP*, MCP##), cardiac metabolic disturbance associated with obesity (MCP***), cardiac inflammation and fibrosis (MCP), dilated cardiomyopathy (MCP##), cardiomyopathy induced by β2-adrenoceptor (MCP##), high-glucose-induced cardiomyocyte (MCP***), cardiac dysfunction (MCP***), left ventricular dysfunction and fibrosis (MCP***), myocardial fibrosis and inflammation (MCP), vascular, aortic valve alterations and cardiac fibroblasts (MCP), diabetes-associated cognitive impairment (MCP*), cerebral ischemia/reperfusion injury (MCP*), subarachnoid hemorrhage (MCP), obesity (MCP***) and cardiorenal dysfunction (MCP***) (Calvier et al., 2013; Cao et al., 2021; Ibarrola et al., 2019; Le et al., 2019; Li et al., 2021; Lu et al., 2017; MacKinnon et al., 2013; Marín-Royo et al., 2018; Ernesto Martínez-Martínez, Calvier, et al., 2015; Ernesto Martínez-Martínez, López-Ándres, et al., 2015; Nguyen et al., 2019; Nguyen et al., 2018; Sun et al., 2020; Tian et al., 2020; Vergaro et al., 2016; Xu et al., 2020; Ibarrola et al., 2017, 2018; Yin et al., 2020; Cui et al., 2022; Nishikawa et al., 2018; Martínez-Martínez et al., 2016; Calvier et al., 2015).

In the immune system modulation, MCP can regulate cytokine levels in the spleen of BALB/c mice (MCP, 0–5% in drinking water for 21 days) and improve neuropathic pain

(MCP#, 100 mg/kg/day for 2 weeks, with intrathecal administration) (Ma et al., 2016; Merheb et al., 2019).

Protection against renal injury is a well-known therapeutic application of MCP. MCP is used for chronic and acute renal failure, renal damage (1% MCP-supplemented drinking water one week before inductor injection), renal injury associated with chemotherapy (MCP, 100 mg/kg/day in the drinking water, 7 days prior to cisplatin injection), and renal damage associated with obesity and aortic stenosis (MCP, 100 mg/kg/day in drinking water for 6 weeks) (Kolatsi-Joannou et al., 2011a; H. Y. Li et al., 2018; Martínez-Martínez, Ernesto; Ibarrola, Jaime; Fernández-Celis, Amaya; Calvier, Laurent; Leroy, Celine; Cachofeiro, Victoria; Rossignol, Patrick; López-Andrés, 2018; Martinez-Martinez et al., 2016).

The anticancer activity of MCP *in vivo* has been seen in metastatic breast and prostate carcinoma model (MCP#, administrated I.V., 0.25% wt/vol with metastatic model), melanoma model (MCP#, administrated I.V., 0.5% wt/vol with metastatic model), mammary adenocarcinoma metastasis model (MCP, I.T., 0.5 µL/g/day for 21 days) and colon cancer model (PSVII@MCP-CaP, I.V., 5 mg/kl every 4 days for 7 times) (Glinskii et al., 2005; Inohara & Raz, 1994ª; Wang et al., 2020ª; Bai et al., 2021). In the sarcoma model, immunomodulatory effect was see only with ginseng pectin (MCP#, ginseng pectin, and P-galactan, I.P., 10 mg/kg each day for 10 days) (Xue et al., 2019). With oral route of administration MCP is used against human breast carcinoma, melanoma, human colon carcinoma model (MCP#, 1-1.5% w/v, MCP in drink water), bladder tumor (MCP, 350-700 mg/kg MCP-fed daily for 6 weeks), colon cancer model (MCP*, 1 - 5.0% wt/vol in drink water), prostate cancer model (MCP***, 1.0% wt/vol in drinking water), breast cancer model (MCP, 350 mg/kg and 700 mg/kg every day, orally for 28 days) and thyroid carcinoma model (MCP# and S-trans, transfarnesylthiosalicylic acid FTS; Salirasib 40 mg/kg, daily and oral, and MCP 0.5% in drinking water 5 ml/day) The negative effect was see in colorectal cancer (MCP, 20% in feed mixtures for 21 days) (Dange et al., 2014; Nangia-Makker et al., 2002; Fang et al., 2018; Ferreira-lazarte et al., 2021; Liu et al., 2008; Pienta et al., 1995; Wang et al., 2022; Menachem et al., 2015).

Other important *in vivo* studies use an anti–galectin-3 antibody and GCS-100 in tumor-bearing mice (mastocytoma) vaccinated against a tumor antigen and treated with

GCS-100 (10 to 62 days of treatment, 700 µg GCS-100 three times a week:200µg were injected in the periphery of the tumor and 500µg I.P.) showed a positive effect and relationship with Gal-3 inhibition (Demotte et al., 2010).

This evidence demonstrates that the in vivo biological effects of MCP on cardiovascular, renal injury, immunomodulation, neuropathic pain/protection, and anticancer activities are related to the modulation of protein galectin-3 (Table 3), the main mechanism of action used to explain MCP activity. However, there is no consensus among researchers, as we will see below.

Table 3: Preclinical studies on MCP.

Molecule	*Application*	*Model*	*Route of administration*	*Concentration*	*Effectiveness Result*	*Gal-3 participation*	*Reference*
MCP#	Metastatic breast and prostate carcinoma model	Mice	I.V.	0.25% wt/vol final concentration with cells metastatic model	Positive	Yes	(Glinskii et al., 2005)
MCP#	Melanoma model	Mice	I.V.	0.5% wt/vol final concentration with cells metastatic model	Positive	Yes	(Inohara & Raz, 1994a)
MCP	Mammary adenocarcinoma metastasis model	Mice	I.T.	2% MCP (0.5 µL/g/day) for 21 days	Positive	Yes	(Wang et al., 2020a)
PSVII@MCP-CaP	Colon cancer model	Mice	I.V.	5mg/kl every 4 days for 7 times	Positive	Yes	(Bai et al., 2021)
MCP#	Human breast carcinoma, melanoma, and human colon carcinoma model	Mice	Oral	1-1.5% (w/v) MCP in drink water	Positive	Yes	(Dange et al., 2014; Nangia-Makker et al., 2002)
MCP	Bladder tumor	Mice	Oral	350-700 mg/kg MCP-fed daily for 6 weeks	Positive	Yes	(Fang et al., 2018)
MCP	Colorectal cancer	Rats	Oral	20% in feed mixtures for 21 days	Negative	No	(Ferreira-lazarte et al., 2021)
*MCP**	Colon cancer model	Mice	Oral	1 - 5.0% (wt/vol) in drink water	Positive	Yes	(Liu et al., 2008)

*MCP****	Prostate Cancer Model	Rats	Oral	1.0%(wt/vol) in drinking water	Positive	Yes	(Pienta et al., 1995)
MCP	Breast cancer model	Mice	Oral	350 mg/kg and 700 mg/kg every day give orally for consecutive 28 days	Positive	Yes	(Wang et al., 2022)
MCP# and S-trans, transfarnesylthiosalicylic acid (FTS; Salirasib)	Thyroid carcinoma model	Mice	Oral	FTS (40 mg/kg) was given daily by oral administration and MCP (0.5%) was given in mice drinking water (5 ml/day)	Positive	Yes	(Menachem et al., 2015)
MCP#, ginseng pectin and P-galactan	Sarcoma model and immunomodulation	Mice	I.P.	10 mg/kg body weight each day for 10 days	Negative for MCP# and P-galactan and positive for ginseng pectin	Yes	(Xue et al., 2019)
Anti–galectin-3 antibody and GCS-100	Tumor-bearing mice (mastocytoma) vaccinated against a tumor antigen treated with GCS-100	Mice and CD4/CD8 human cells	Periphery of the tumor and I.P.	10 to 62 day of treatment, 700 µg GCS-100 three times a week: 200µg were injected in the periphery of the tumor and 500µg I.P.	Positive	Yes	(Demotte et al., 2010)
*MCP****	Vascular Fibrosis	Mice	Oral	100 mg/kg per day for 3 weeks as drinking water	Positive	Yes	(Calvier et al., 2013)
MCP	Pulmonary hypertension	Mice	Oral	Gavage, 60 mg/kg, once a day, for 28th days	Positive	Yes	(Cao et al., 2021)
MCP	Myocardial injury after ischemia/reperfusion	Mice	Oral	100 mg/kg per day in the drinking water, 1 day before until 8 days after IR injury.	Positive	Yes	(Ibarrola et al., 2019)
*MCP****	Ischemic Heart Failure	Rats	Oral	2 ml/kg/d for 4 weeks	Positive	Yes	(Le et al., 2019)
MCP	Cardiac hypertrophy		Oral	100 - 200 mg/kg/day by gavage for 14 and 21 days	Positive	Yes	(Li et al., 2021)
*MCP**	Atherosclerotic lesions	Mice	Oral	1% MCP into the drinking water for 4 weeks	Positive	Yes	(Lu et al., 2017)
MCP##	Atherosclerotic	Mice	Oral	1% solution in the drinking water	Positive	Yes	(MacKinnon et al., 2013)
*MCP****	Cardiac metabolic disturbance associated with obesity	Mice	Oral	100 mg/Kg/day for 6 weeks	Positive	Yes	(Marín-Royo et al., 2018)

MCP	Cardiac Inflammation and Fibrosis	Mice	Oral	100 mg/kg per day for 3 weeks in the drinking water	Positive	Yes	(Ernesto Martínez-Martínez, Calvier, et al., 2015; Ernesto Martínez-Martínez, López-Ándres, et al., 2015)
MCP##	Dilated cardiomyopathy	Mice	Oral	200 mg·kg/day for the initial 2 month and was increased to 500 mg·kg/day for another 2 months in drink water.	Positive	Yes	(Nguyen et al., 2019)
MCP##	Cardiomyopathy induced by β2-adrenoceptor	Mice	Oral	200 - 400 mg·kg/day in drink water.	Negative	Yes	(Nguyen et al., 2018)
*MCP****	High-glucose-induced cardiomyocyte	Mice	Oral	100 mg.Kg/day	Positive	Yes	(Sun et al., 2020)
*MCP****	Cardiac dysfunction	Mice	Oral	MCP (100 mg/kg/day) in the drinking water for 6 consecutive weeks	Positive	Yes	(Tian et al., 2020)
*MCP****	Left Ventricular Dysfunction and Fibrosis	Mice	Oral	100 mg/kg per day as drinking water	Positive	Yes	(Vergaro et al., 2016)
MCP	Myocardial fibrosis and inflammation	Mice	Oral	100 mg/kg/day	Positive	Yes	(Xu et al., 2020)
MCP	Vascular, Aortic Valve Alterations and cardiac fibroblasts	Mice	Oral	100 mg/kg/day in the drinking water	Positive	Yes	(Ibarrola et al., 2017, 2018)
*MCP**	Diabetes-associated cognitive Impairment	Mice	Oral	100 mg/kg/day oral for 6 weeks	Positive	Yes	(Yin et al., 2020)
MCP	Immunomodulatory effects	Mice	Oral	0% - 5% in drinking water for 21 days	Positive	Yes	(Merheb et al., 2019)
*MCP**	Cerebral ischemia/reperfusion injury	Mice	I.P.	200 – 800 mg/Kg/day 7 days before of pathology	Positive	Yes	(Cui et al., 2022)
MCP#	Neuropathic pain	Rats	I.T.T.	100 mg/kg/day for 2 weeks	Positive	Yes	(Ma et al., 2016)
MCP	Subarachnoid hemorrhage and blood-Brain barrier disruption	Mice	I.C.	0.8 - 32μg 30min post-modeling	Positive	Yes	(Nishikawa et al., 2018)

*MCP****	Obesity	Rats	Oral	100 mg/kg per day in the drinking water	Positive	Yes	(Martínez-Martínez et al., 2016)
*MCP****	Cardiorenal dysfunction	Rats	Oral	100 mg/kg per day in the drinking water for 3 weeks	Positive	Yes	(Calvier et al., 2015)
MCP	Renal injury	Mice	Oral	1% MCP-supplemented drinking water one week before inductor injection	Positive	Yes	(Kolatsi-Joannou et al., 2011a)
MCP	Renal injury	Mice	Oral	100 mg/kg/day in the drinking water for the same period, while some were pre-treated with normal or 1% MCP-supplemented drinking water at 7 days prior to cisplatin injection	Positive	Yes	(Li et al., 2018)
MCP	Renal damage associated with obesity and aortic stenosis	Mice	Oral	100 mg/kg/day in the drinking water for 6 weeks	Positive	Yes	(Martinez-Martinez et al., 2016)
*MCP****	Renal damage	Mice	Oral	100 mg/kg/day in the drinking water	Positive	Yes	(Martínez-Martínez et al., 2018)

MCP = EcoNugenics, Santa Rosa, CA. MCP* = Centrax International Corporation, San Francisco, CA, USA. MCP** = pectin from citrus peel (P9135; Sigma-Aldrich). MCP*** = unidentified. MCP# = MCP pH modification obtained from Sigma-Aldrich CP Louis, MO, USA). MCP## = Allergy Research Group. PSVII@MCP-CaP = PSVII with MCP from Centrax International Corporation, and a Cap nanoparticle. I.V. = intravenous injection. I.P., intraperitoneal injection. I.T. = intratumoral injection. I.T.T.: intrathecal injections. I.C. = intracerebroventricular injection.

7. Clinical studies using MCP

Clinical studies provide highly reliable evidence for a few reasons: the model used is patients, its approval is conditioned to previous studies *in silico*, *in vitro*, and *in vivo* (with two animal models, depending on the biosafety agency), the toxicity, stability, prototype, and validation tests were carried out, and all these parameters were positive. However, the clinical phase of a drug involves the elimination of many molecules (Dagenais et al., 2022; Fountzilas et al., 2022; Gloy et al., 2022; He et al., 2022; Tong et al., 2022). There are great models in silico as analytical techniques based on artificial intelligence, however studies in animal and human are still essential and it is for this reason that we condense the results of our searches in *in vitro*, *in vivo*, and pre-clinical studies. In this way, we seek to support the application of MCP with reliable articles, robust results, and the necessary biopharmacological models.

In the clinical studies on MCP, there are not many published articles, some of which are still in the conclusion/validation phase and others have hidden information. However, MCP, MCP*, GR-MD-02, and MCP unidentified (MCP***) have been subjected to clinical studies for various applications. The MCP was related to prostate cancer treatment of non-metastatic biochemically relapsed prostate cancer in a prospective phase II study, with oral administration (18 capsules per day (14.4 g) taken in three divided doses 4.8 grams/day for six months) with a positive effect and is related to galectin-3 (Guess et al., 2003; Keizman et al., 2021). MCP was also related to cardiac fibrosis and hypertension after oral administration (4.8 g thrice daily for 6 months), and related to Gal-3 inhibition, but the result was inconclusive (Lau et al., 2021). MCP is related to urinary excretion of toxic elements (5 or 15 g of MCP daily), urinary/fecal excretion of uranium (3 capsules 750 mg/capsule twice daily of MCP: sodium alginate 2:1), and acute kidney injury (400 mg/kg/day for 7 days) with a positive effect. The second application is associated with galectin-3 (Eliaz et al., 2006; Eliaz et al., 2007; Sun, Peng, et al., 2021; Zhao et al., 2008; Eliaz et al., 2019). Sepsis-associated acute kidney injury (AKI) is also treated by MCP in human patients and rat models with oral administration (pretreated with 400 mg/kg/day and 1200 mg/kg/day for 7 days) with Gal-3 association (Sun, Jiang, et al., 2021).

MCP* is associated with type 2 diabetes mellitus in human patients and rat models (100 mg/kg/day for 4 weeks), showed a positive effect, and was associated with Gal-3 (Ma et al., 2020). GR-MD-02, a known pectin that is associated with moderate-to-severe plaque psoriasis following I.V. administration (8 mg/kg every week for 6 months), showed a positive effect. Hepatic fibrosis and inflammation in I.V. administration (8 mg/kg biweekly over a 16-week), however, showed a negative effect (Ritchie et al., 2017; Harrison et al., 2018). In the unidentified MCP group, was see applications in knee osteoarthritis with oral administration (4 g 5 capsules twice daily for 12 weeks) with an inconclusive effect, and abdominal aortic aneurysm (AAA) with an IV administration (10 mg/kg, every day) with a positive effect, both associated with Gal-3 (Andrews et al., 2020; Fernandez-García et al., 2017).

An interesting molecule that has undergone phase 2 clinical studies, GCS-100, a galectin-3 antagonist, reduces fibrosis. Furthermore, GCS-100 has been used to treat

patients with chronic kidney disease (CKD). This study consisted of a fixed dose of 3 mg IV used every 30 days for a total of 1 year (ClinicalTrials.gov Identifier: NCT01717248, NCT02333955). This molecule has also been associated with anti-cancer applications in prostate cancer chemoresistance, human multiple myeloma, diffuse large B-cell lymphoma, B-cell chronic lymphocytic leukemia, and mastocytoma (Demotte et al., 2010; Wang, Balan, et al., 2010; Chauhan et al., 2005; Clark et al., 2012; O'Brien & Kay, 2011; Ruvolo et al., 2016; Streetly et al., 2010).

The commercial product Glyconutrient Ambrotose™ (aloe vera extract inner leaf gel, arabinogalactin, ghatti gum, glucosamine hydrogen chloride, gum tragacanth, vitamin A, beta carotene, wakame algae extract, rice starch, RiFiber (rice bran), and Modified Citrus Pectin with Sodium Alginate) underwent initial clinical studies with application in healthy (immunity, improved cognitive performance, and enhanced antioxidant capacity) 2-4 grams daily for 8 weeks, not related to Gal-3 (ClinicalTrials.gov Identifier: NCT04386226).

Table 4: Clinical studies using MCP.

Molecule	*Application*	*Model*	*Route of administration*	*Concentration*	*Effectiveness Result*	*Gal-3 participation*	*Reference*
*MCP****	Knee osteoarthritis	Human patients	Oral	4 g (5 capsules) twice daily for 12 weeks	Inconclusive	Yes	(Andrews et al., 2020)
MCP	Prostate cancer	Human patients	Oral	18 capsules per day (14.4 g) taken in three divided doses. 4.8 grams X3/day for six months	Positive	Yes	(Guess et al., 2003; Keizman et al., 2021)
*MCP****	Abdominal aortic aneurysm (AAA)	Human patients and mice	I.V.	10 mg/kg, every other day	Positive	Yes	(Fernandez-García et al., 2017)
MCP	Cardiac fibrosis and Hypertension	Human patients	Oral	4.8 g thrice daily for 6 months in a 1:1 ratio	Inconclusive	Yes	(Lau et al., 2021)
*MCP**	Type 2 diabetes mellitus	Human patients and mice	Oral	100 mg/kg/day for 4 weeks	Positive	Yes	(Ma et al., 2020)
MCP	Urinary excretion of toxic elements	Human patients	Oral	15 g of MCP each day for 5 days and 20 g on day 6.	Positive	No	(Eliaz et al., 2006)

MCP	Urinary excretion of toxic elements	Human patients	Oral	5 or 15 g of MCP daily.	Positive	No	(Eliaz et al., 2007)
MCP	Acute kidney injury	Human patients and rats	Oral	Pretreated with MCP 400 mg/kg/day for 7 days	Positive	Yes	(Sun, Peng, et al., 2021)
MCP	Sepsis-associated acute kidney injury (AKI)	Human patients and rats	Oral	Pretreated with 400 mg/kg/day and 1200 mg/kg/day for 7 days	Positive	Yes	(Sun, Jiang, et al., 2021)
MCP	Urinary excretion of toxic elements	Human patients	Oral	3 months before received, 15 g in 3 divided doses per day	Positive	No	(Zhao et al., 2008)
MCP	Urinary/fecal excretion of uranium	Human patients	Oral	3 capsules (750 mg/capsule) twice daily of modified citrus pectin: sodium alginate (2:1)	Positive	No	(Eliaz et al., 2019)
GR-MD-02	Moderate-to-severe plaque psoriasis	Human patients	I.V.	8 mg/kg lean body mass every other week for 6 months	Positive	No	(Ritchie et al., 2017)
GR-MD-02	Hepatic fibrosis and inflammation	Human patients	I.V.	8 mg/kg biweekly over a 16-week period	Negative	No	(Harrison et al., 2018)
GCS-100							

MCP = EcoNugenics, Santa Rosa, CA. MCP* = Centrax International Corporation, San Francisco, CA, USA. MCP*** = unidentified. I.V. = intravenous injection.

The results of these tables provide an overview of the wide range of applications verified for MCP, its forms of administration, doses used, levels of studies, potential applications in patients, and mechanisms of action. In the next section, we will analyze in more detail the mechanisms of action of MCP, mainly with regard to oncology.

8. MCP, dietary fiber and cancer

Pectin can be used in the food industry to regulate several physiological functions, such as dietary fiber, which can be degraded by colonic intestinal microbial species and is not enzymatically digested in the small intestine, maintaining its gelling property, and slowing intestinal flow (Lattimer & Haub, 2010). Pectin also shows activity in several areas of nutrition, serving as a food, improving intestinal flow, reducing cholesterol, and being an important nutritional compound.

In relation to anticancer activity, pectin can prevent colon cancer by acting as a dietary fiber. For example, rats exposed to azoxymethane or methylnitrosourea, chemical inducers of colon cancer, and treated with a pectin diet, developed fewer colon tumors (Watanabe K, Reddy BS, Weisburger JH, 1979). Mice exposed to 1,2-dimethylhydrazine (another chemical inducer of colon cancer) and treated with pectin also develop fewer colon tumors (Heitman DW, Hardman WE, 1992). Rats treated with azoxymethane plus citrus and apple pectin in the diet showed decreased carcinogenesis, and treatment with apple pectin also inhibited B-glucuronidase, an enzyme produced by colon bacteria and related to cancer development (Ohkami H, Tazawa K, Yamashita I, Shimizu T, Murai K, Kobashi K, 1995). The Balb-C mice with colon tumors, having a daily intake of 0.8 and 1.6 mg/ml of MCP demonstrated a decrease of tumor size up to 70% (Hayashi et al., 2000).

Colon carcinogenesis results from an imbalance between the proliferation of colonocytes at the base of the crypt and the loss of colonocytes at the luminal surface, which occurs mainly by apoptosis. Avivi-Green et al. (2000a) showed that a pectin-rich diet activated apoptosis in colon tumors (high activity of caspase-1, pro-caspase-3, and cleaved PARP) and protected rats exposed to 1,2-dimethylhydrazine. This effect was also observed *in vitro*; colon adenocarcinoma HT29 cells treated with pectin for 3 days showed increased apoptosis and DNA fragmentation (Olano-Martin et al., 2003). A mixture of n-3 polyunsaturated fatty acid-rich fish oil and pectin has also shown chemoprevention in colon cancer models induced by azoxymethane in rats (Cho et al., 2012). Butyrate is a molecule produced by the fermentation of intestinal bacterial flora and causes the activation of colonocyte apoptosis in a p53-independent manner (Kolar et al., 2007) and mitochondrial Ca2+ overload (Kolar et al., 2011,Cho et al., 2012). Animals fed pectin showed an increase in butyrate levels, thus inhibiting carcinogenesis (Avivi-Green et al., 2000b).

On the other hand, a study make by Ferreira-lazarte et al., 2021 showed that citrus pectin and modified citrus pectin could not prevent tumorigenesis in rats treated with azoxymethane and dextran sodium sulfate. Moreover, in both cases, more than 50% of animals died, and the metabolism study concluded that this effect occurs due to severe dysbiosis in the gut and an increase of *Proteobacteria* (Enterobacteriaceae).

Furthermore, the pH in the cecum lumen decreased, as well as the levels of propionic and butyric acids. The levels of acetate and lactic acid increased, but the glucose and triacylglyceride levels decreased (Ferreira-lazarte et al., 2021). However, this study used a universal feed with 20% pectin in free alimentation, which could be characterized as an excess and could provoke the death of rats and the lack of cancer prevention. Further studies are needed to confirm these results.

Thus, evidence shows that pectin and MCP, in addition to being important dietary fibers that regulate digestion and intestinal flow, have a preventive effect against colon cancer at controlled concentrations.

9. Modified citrus pectin (MCP) and cancer

Avraham Raz et al. were the first to study MCP in cancer application. They see that injection of MCP in mice C57BL/G with xenograf B16-F1 melanoma cells decreased the total of metastases in lung. They were also the first to verify that MCP competes with ligands of "galactose-binding proteins, mainly galectin-3, prejudicing cell-cell interactions (an important function of Gal-3) (Inohara & Raz, 1994b; Platt & Raz, 1992). Pienta et al. (1995) showed that in rats with xenografts of MAT-LuLu prostate cancer cells, MCP reduced lung metastasis after oral administration (a dose-dependent way) (Pienta et al., 1995). They demonstrated that breast cancer (MDA-MB-231) and colon tumor (LSLiM6) in NRC nu/nu mice decreased after MCP treatment, as well as the number of metastases in the lung lymph nodes. In the same study, this effect was related to anti-angiogenic activity, with a reduction in the number of capillaries *in vivo* and a decrease in tubulogenesis (Nangia-Makker et al., 2002). Evidence regarding the action of pectin as an anti-angiogenic agent is known. For example, pectin-poly (vinyl pyrrolidone) (PVP)-based curcumin (PECTIN-PVP CUR) showed antiangiogenic activity and cytotoxicity in lung cancer cells A549 *in vitro* (Gaikwad et al., 2017).

Studies have shown that pectic acid, citric pectin, and apple pectin (*Malus* sp.) can inhibit cell growth, induce apoptosis, reduce Gal-3 levels, increase reactive oxygen species levels, and inhibit cyclin activity and cell division in the human breast cancer cell line MDA-MB-231 (Delphi et al., 2015; Salehi et al., 2018). Pectic acid can also induce apoptosis, inhibit cell growth, reduce cell attachment, chromatin, membrane blebbing,

fragmentation, and block the sub-G1 phase cellular division of 4T1 cells (breast cancer model) *in vitro* and *in vivo*, which could increase apoptosis and p53 protein levels and decrease tumor progression (Delphi et al., 2016). In addition, pectic acid (PA) induced apoptosis in rat pituitary tumor cells GH3/B6 treated with concentrations of 100 µg/ml to 1 mg/ml, whereas concentrations of 2.5 5 mg/ml caused necrosis (Attari et al., 2009).

An *in vitro* experiment with JCA-1 cells (a human prostate cancer cell line) showed that MCP decreased cell proliferation and mm23, a protein marker related to metastasis (Hsieh and Wu, 1995). GCS-100, a commercially available form of modified citrus pectin, has demonstrated efficacy against cell lines of multiple myelomas resistant to chemotherapy, activating cleavage of caspase-3, caspase-8, and PARP, and the inhibitor of pan-caspase Z-VAD-fmK also inhibited the activity of GCS-100, showing that the activity of this molecule regulates caspase-dependent apoptosis (Chauhan et al., 2005).

In a phase II clinical study, PectaSol ®, a commercially available form of MCP, elevated PSADT (PSA doubling time (PSADT) in 7 of 10 patients with prostate cancer (Guess et al., 2003; Keizman et al., 2021). This study showed that oral administration of PectaSol-C can doubling PSA time increase in men with biochemical relapsed prostate cancer and serial increases in PSA levels (U.S., 2020). A new clinical phase III study using PectaSol-C is ongoing to determine PSA Kinetics in Prostate Cancer in the setting of serial increases in PSA levels. PectaSol® and PectaSol-C® (MCP) are also cytotoxic to many prostate cancer cell lines *in vitro*, including BPH-1, CASP1.1, CASP2.1, PC3, and LNCaP. It can inhibit MAP kinase activation and increase caspase-3 cleavage as well as Bim expression (pro-apoptotic protein) in CASP1.1 and PC3 cells (Yan & Katz, 2010). These molecules can also inhibit invasion of prostate and breast cancer cells *in vitro* (Jiang et al., 2013b).

Isolated pectin from ginseng was tested in colon cancer HT-29 cells and showed that fractions rich in HG stopped the G2/M cell cycle phase and cell proliferation. The fractions rich in HG can increase the effects of anti-proliferative and active apoptosis (Cheng et al., 2011, 2013). The work done by Ho et al. (2006) showed that pectin, exposed to 20kGy of irradiation and dialyzed (WT<10,000), has antioxidant activity, and

can inhibit cancer cell growth of B16F10 (skin cancer), HT29 (colon cancer), and SKMEL (human melanoma) (Ho et al., 2006).

A pectin-like polysaccharide named RP02-1, purified from the roots of *Polygala tenuifolia*, might decrease tumor growth *in vitro* and *in vivo* in pancreatic ductal adenocarcinoma (PDA) through the inhibition of apoptosis and autophagy-dependent tumorigenic growth. The RP02-1 could inhibit proliferation, migration and colonic formation of AsPC-1 and BxPC-3 cells (pancreatic cancer cell). In addition, RP02-1 provokes BCl2-down regulation, Bax-up regulation and activated capase-3. Autophagy is also regulated by RP02-1, and the levels of ATG5 and LC3B (autophagy markers) are decreased (Bian et al., 2020).

These results show that pectin and modified citrus pectin (MCP) has antitumor, antiangiogenic, and antioxidant effects in several tumor models *in vitro* and *in vitro*. In the next section, we discuss more about the mechanisms by which MCP promotes such effects.

10. Modified citrus pectin (MCP), cancer and Galectin-3

As demonstrated by this review, the mechanism of action of MCP is directly related to the binding and inhibition of galectin-3, but many questions have arisen over the years (Pedrosa et al., 2022). Por example, pectin isolated from okra (tropical plant), rich in RG-I, induces apoptosis and arrests the cell cycle of B1610 (skin melanoma) cells through the interaction between MCP and galectin-3 (Vayssade et al., 2010), and the probable action of MCP on Gal-3 occurs in the RG-I regions and galactan residues (Gao et al., 2012). Gunming et al. (2009) isolated neutral galactan side chains of the RG-I regions from potato pectin and showed that galactan selectively binds to recombinant Gal-3 (Gunning et al., 2009).

PancTu1 and Panc1 cells (pancreatic ductal adenocarcinoma (PDAC)) treated with differentially processed pectin's and arabnogalactan-proteins (AGPs) did not show inhibition of migration and adhesion to the microvascular liver endothelium cell line (TMNK-1) when galectin-3 was decreased by siRNA knockdown. Thus, pectin-derived polysaccharides can inhibit PDAC cell adhesion to liver endothelial cells in a galectin-3

dependent manner (Schöll-naderer et al., 2020). In mice with colon cancer, MCP treatment decreased liver metastasis in a dose-dependent manner. These metastasis has higher expression of gal-3 and MCP act inhibiting this proteins, according to the authors (Liu et al., 2008). Sathisha et al. (2007) also demonstrated that pectin rich in arabinogalactan, arabinose, and galactose can significantly inhibited hemagglutination of red blood cells induced for Gal-3 (Sathisha et al., 2007).

The uronic fraction of pectin extracted from the pulp of papayas at fourth day ripening presented the biological effects in galectin-3 inhibition, with the minimum inhibitory concentrations (MIC) of 0.08 µg/µL in hemagglutination assay, and viability assays using HT-29 and HCT-116 (colorectal cancer cells) (0.2, 0.1, 0.05 and 0.025% – w/v) (Pedrosa et al., 2020). In contrast, papaya pectin extracted from the intermediate phases of ripening induced necrosis and decreased viability in HCT116, HT29, and PC3 cells (colon and prostate cancer models), but the hemagglutination assay not showed inhibition of the Gal-3 protein, indicating that the biological activity of this pectin is independent of Gal-3 (Prado et al., 2017). The enrichment of deesterified HG oligomers and the AGI and RG reduction in MCP3 (fraction of MCP smaller than 3 kDa) and the increase in AGI and loss of RGI in MCP30/10 (fraction of MCP between 30KDa and 10 kDa) improved the anticancer activity by inhibiting the aggregation, migration, and proliferation of HCT116, HT29, and PC3 cells. This work also showed that the MCP3 fraction in the hemagglutination assay inhibited Gal-3 at a lower concentration (300 µg/ml) than the brute fraction or higher molecular weight fractions (400-500ug/ml) (do Prado et al., 2019). Zhang et al. (2016) argued that the limited use of brute MCP due to its large size, which acts as an obstacle, demonstrated that MCP-2, 6-116KDa rich in RG-I (β1,4-galactan) is the best inhibitor of Gal-3-mediated agglutination, with an MIC of 0.06 µg/ml (ten-fold more potent than parent MCP), and the RG-I-rich pectin's with (1 → 4)-linked β-D-galactan side chains are more active than other fractions (Zhang et al., 2016).

Low-molecular-weight citrus pectin (LCP) has antitumor properties in gastrointestinal cancer cells *both in vitro* and *in vivo*. Ciclin B1 (cell cycle-related protein) in AGS and SW-480 cells was decreased by LCP treatment. *In vivo* studies shown that LCP

reduces tumor progression and decreases the expression of Gal-3, Bcl-xl and Survivin, thereby promoting apoptosis (Wang et al., 2016).

Wu et al. (2020) used a gal-3-binding affinity KD study and an IC50 study with MCF-7 cells (breast tumor model) and showed that cooperation between HG and the RG-I segment of water-soluble pectin plays an important role in the inhibition activity of galectin-3 (Wu et al., 2020b). Zeng et al. (2020) used crystallography and NMR spectroscopy to demonstrate that negatively charged homogalacturonan (HG) binds to the galectin-3 carbohydrate recognition domain (Zheng et al., 2020). Fang et al (2018) studying the effect of MCP in urinary bladder cancer (UBC) verified an inhibition of cell viability in human UBC cells (T24 and J82) *in vitro*. The MCP-treated UBC cells showed G2/M phase arrest, reduction of cyclin B1 and phosphorylated Cdc2, increased cleavage of caspase 3 and PARP, Gal-3 reduction, and Akt signaling pathway inactivation. knockdown of Galectin-3 by siRNA inhibits MCP-mediated apoptosis, cell cycle arrest, and anti-proliferative effects. Oral administration of MCP to T24 xenograft-bearing nude mice (bladder tumor model) significantly reduced tumor growth, Ki67 (a marker of proliferation), and caspase 3 (Fang et al., 2018). These results demonstrate an intrinsic relationship between MCP and Gal-3.

Due to its immunosuppressive capacity and role in promoting invasion, migration, and angiogenesis in the tumor microenvironment, galectin-3 has been identified as a potential target for cancer treatment (Guo et al., 2020). Several molecules that are capable of inhibiting Gal-3 have been studied (Blanchard et al., 2014). GM-CT-01, a galactomannan extracted from *Cyamopsi* sp., is a galectin-3 antagonist that shows increased CD8 + T cell activity against tumors (Demotte et al., 2014). The GR-MD-02 molecule, is a drug based on complex carbohydrates that interacts with the galectin-3 binding site and promotes the proliferation of antigen-specific T cells in advanced tumors (Dong et al., 2018; Traber & Zomer, 2013). In addition, GR-MD-02 in combination with anti-OXI40 promotes survival in mice with MCA-205 sarcoma, 4T1 breast cancer, and prostate adenocarcinoma (TRAMP-C1) (Bayes-Genis et al., 2014; Dong et al., 2018). These applications of galectin-3 inhibitors demonstrate that this lectin is a potential target for cancer treatment.

Following these arguments, MCP was demonstrated to act as an anti-tumorigenic molecule both *in vivo* and *in vitro* in several types of cancer, and many of these effects, if not all, occur due to the binding properties between MCP and gal3, which are highly expressed in cancer. For example, GCS-100, a of the most potent inhibitor of Gal-3, is used for maintenance therapy in B-chronic lymphocytic leukemia relapse patients (O'Brien & Kay, 2011). However, there are also works that do not corroborate the relationship between MCP and Gal-3.

The first point we can address in this discussion is whether MCP can specifically inhibit galectin-3. The work done by Stegmayr et al., 2016 using fluorescent anisotropy assay and *in vitro* studies to test the direct interaction of polysaccharides with physiological concentrations of gal-3, showed that Pectin and Galactomanna (another polysaccharide related to anti-tumor and anti-inflammatory activity) has no affinity for the galectin-3 protein. On the other hand, using a biolayer interferometry assay, Cui et al. (2019) showed that water-soluble pectin isolated from *Panax ginseng* flower buds exhibited strong bond activities with galectin-3 (Cui et al., 2019b). Jackson et al. (2007) used a distinct form of pectin in LNCaP (androgen-dependent prostate cancer) and LNCaP C4-2 (androgen-independent prostate cancer cells) which did not express galectin-3, and verified that HG, RG-I, and RG-II showed no cytotoxic effects (Jackson et al., 2007). They also demonstrated that pH-modified PectaSol ®, a citrus pectin, did not show pro-apoptotic effects in these cell lines.

In a "letter to the editor, " Hakon Leffler, criticizes the work of Nishikawa et al., 2018 (Leffler, 2019). He argued that the aforementioned article, which relates MCP and prevention of blood-brain barrier disruption in cases of subarachnoid hemorrhage in mice by inhibiting Gal-3, could not draw these conclusions. He explains that several studies, although indicating a link between MCP and gal-3, do not verify the inhibition of galectin-3, and that MCP can act by other mechanisms that are not yet known (Fang et al., 2018; Johannes et al., 2018). Another "letter to the editor", which comments on the same article cited, was made by neuroscientists Shao, Wu, and Zhang (Shao et al., 2019), they also found inconsistency in this work in relation to gal-3 inhibition by MCP. Several studies have shown that MCP binds to gal-3, but not all of them prove that this

polysaccharide causes some kind of inhibition (Glinsky & Raz, 2009b; Kolatsi-Joannou et al., 2011b; Z. Ma et al., 2016; Vergaro et al., 2016).

These results demonstrate that there are many *in vitro*, *in vivo*, and clinical studies showing that the anti-cancer activity of MCP can be related to its binding to Gal-3 and possible inhibition of this protein, but this is not a consensus (figure 2). Further studies are needed to elucidate the mechanism of action of MCP.

11. Modified citrus pectin (MCP) stimulate immune system

Some pectins can stimulate the immune system, and this immunomodulatory activity is often related to its antitumorigenic action. The angelans, pectin polysaccharides isolated from *Angelica gigas* Nakai (Chinese medical species), can increase the immune activity of macrophages, B-lymphocytes, T-helper lymphocytes, cytotoxic lymphocytes, and Natural Killer cells. It also has antimetastatic properties against B16F10 cells (skin melanoma). Apple oligogalactan (AOG) in a colon cancer model has preventive activity against toxic and carcinogenic outcomes of sodium dextran sulfate and 1,2-dimethylhydrazine, through the binding of AOG and TLR4, preventing LPS ligation and the activity of the NF-κB pathway (Liu et al., 2010). Pectin isolated from red ginseng (*Ginseng panax*) modulates NO production by macrophages and exerts antitumor effects (Choi et al., 2008). Complementarily, this immunomodulatory effect leads to improving the paclitaxel chemotherapy in mice with B16 melanoma tumors (Shin et al., 2004).

Pectin esterified up to 90% (DE90) can bind LPS (lipopolysaccharides), preventing its connection with the TL4 receptor, and inhibiting COX2 and iNOS expression, MAPK phosphorylation, as well as IKK, NF-kB, and AP-1 activation, which are important for cancer inflammation and chemoprevention (Chen et al., 2006). Ramachandran et al. (2011) showed that MCP can exert immunomodulatory effects in human blood by activating B-lymphocytes, cytotoxic T cells, and NK cells, reducing chronic myeloid leukemia K562 cells. Pectin can also stimulate the activation of T-cells and apoptosis of tumor cells, as shown by Xue et al. (2019), who demonstrated that ginseng pectin inhibits gal-3-induced T cell apoptosis and activation in the sarcoma-180 mouse model. Ginseng pectin significantly increased T-cell proliferation and IL-2 expression and

reduced tumor growth by 45%. They also showed that the PKC/ERK and ROS/ERK pathways are involved in the activation and apoptosis of T-cells, while Ras/PI3K/Akt is active in T-cell stimulation (Xue et al., 2019).

Pectin FB extracted from broccoli stalks (*Brassica oleracea*) increases the number of activated macrophages and phagocytic activity in mice, stimulates lymphocytes from the spleen, bone marrow proliferation, and interleukin IL-10 (an anti-inflammatory interleukin), but does not induce nitric oxide (NO) production by macrophages or levels of pro-inflammatory interleukins IL-1β and IL-12 (Busato et al., 2020). Another study demonstrated that two modified citrus pectin's (MCP4 and MCP10) prepared by UV/H_2O_2 can suppress the production of the pro-inflammatory cytokines TNF-α and IL-1β, in addition to decreasing NF-κB expression in THP-1 cells (human monocytic leukemia). MCP10 also inhibits the proliferation of Caco-2 cells (human colorectal adenocarcinoma) (Cao et al., 2020).

The inhibition of the immune system by MCP has been described in previous studies. A study made by Wang et al. (2020) showed that hypoxia increases the synthesis and secretion of galectin-3 by tumor-associated macrophages (TAM). TAM secretion by gal-3 induces nucleation of the transcription factor NF-Kβ, activation of reactive oxygen species (ROS), tumor growth, and metastasis. This effect is inhibited by administration of modified citric pectin (MCP), a galectin-3 inhibitor, in mammary adenocarcinoma and metastasis models, or by the macrophage-depletion agent clodronate liposomal (CP). In addition, major inhibition was observed by co-administration of CL and MCP with sorafenib and bevacizumab (anti-angiogenesis agents). These results show that hypoxia-induced galectin-3 expression is secreted by tumor-associated macrophages, and that this secretion promotes tumor growth and metastasis; however, these effects can be inhibited by MCP (Wang et al., 2020).

Thus, MCP can modulate the immune system against tumor cells and inhibit galectin-3 secretion by tumor-associated macrophages (figure 2). This is an important indirect effect of the anticancer action of MCP.

12. MCP to overcome chemoresistance

Chemoresistance is the capacity of the tumor and its metastasis to avoid the toxic effects of antitumor treatments (chemotherapy and radiotherapy). Several mechanisms determine cancer chemoresistance, among than is the overexpression of galectin-3 that in many types of cancer suppresses cell apoptosis and diminish sensitivity of cancer cells to chemotherapeutic pharmaceuticals (Glinsky & Raz, 2009a; Rebucci & Michiels, 2013). For example, MCP and lactosyl-L-leucine (LL), a specific inhibitor of gal-3, sensitize malignant endothelial cells to doxorubicin and decrease cell proliferation (Johnson et al., 2007). These molecules also sensitize metastatic breast cancer cells (MDA-MB-435) *in vitro* and in vivo (Glinsky et al., 2009). GCS-100, a commercial pH-MCP, with bortezomide and dexamethasone, sensitizes multiple myeloma cells to apoptosis, decreases their viability, and reduces gal-3 protein levels. In addition, GCS-100 sensitizes prostate cancer cells to cisplatin treatment via calpain activation (Chauhan et al., 2005; Wang et al., 2010).

The combination of MCP and two integrative polybotanical molecules, BreastDefend (BD) and ProstaCaid (PC), synergistically inhibits the adhesion and migration of breast and prostate cancer cells. This combination also decreasied urokinase plasminogen activator (uPA), a serine protease that helps in the migration of metastases (Jiang et al., 2013b). The work made by Hossein et al. (2013) sensitized human ovarian cancer cells (SKOV-3) to paclitaxel (PTX) MCP how specific gal-3 inhibitor. rGal-3 significantly increased cell proliferation and substrate-dependent adhesion and decreased caspase 3 activity. A non-effective dose of PTX and 0.1% MCP showed a synergistic cytotoxic effect, increased caspase-3 activity, and decreased substrate-dependent adhesion (Hossein et al., 2013). Another study, using ovarian cancer cell SKOV-3 in multicellular tumor spheroids (MCTS), demonstrated that the IC50 of PTX and MCP in combination was significantly smaller than that of the controls. This combination also increased apoptosis and decreased migration, invasion and gal-3 expression. These effects occur because of the inhibition of the STAT5 signaling pathway (Hossein et al., 2019b). This study demonstrated that the effect of MCP on chemotherapy resistance may be related to gal3 inhibition.

A study made by Adami et al. (2020) showed that co-administration of polysaccharides from sweet green pepper (*Capsicum annuum*) (CAP) and methotrexate

(adjuvant therapy for breast cancer) (MTX) improved antineoplastic effects in mammary tumors *in vitro* and *in vivo*. This combination reduces tumor growth, decreases VEGF gene expression and vessel area (angiogenesis markers), increases IL-6, and decreases IL-4 and IL-10, and the degree of necrosis. These effects occur due to immunomodulation and modulation of angiogenesis induced by MCP (Adami et al., 2020). Conti et al. (2018) evaluated the efficacy of combining ionizing radiation (IR) and MCP on prostate cancer cells (PCa cells). They observed that MCP significantly increased radiosensitivity and decreased cell viability, gal-3 expression, poly ADP-ribose polymerase (DNA repair protein), proliferating nuclear antigen, and increased cleavage of caspase-3 and Bax (pro-apoptotic protein). MCP also reduces the migratory and invasive potential of PCa cells (Conti et al. 2018). These results show that MCP can act as a radiosensitizer in prostate cancer models.

Thus, the combination of MCP with chemotherapy drugs and radiotherapy represents a good strategy to treat cancer by increasing the sensitivity of tumors, and this effect is linked to the interaction of MCP with Gal-3 protein (figure 2).

13. MCP, Galectin-3 and Kidneys

Galectins-3 promotes nephrogenesis during embryonic development and plays an important role in kidney function; however, it can also be related to the etiology of some pathologies, such as kidney damage (Iacobini et al., 2004; PUGLIESE et al., 2001). This way, some studies have shown that predisposition to MCP binds galectin-3 in the kidney, inhibits this protein in renal pathologies, and protects against kidney damage in certain contexts (Henderson et al., 2008; Kolatsi-Joannou et al., 2011b; O'Seaghdha et al., 2013; Okamura et al., 2011; Vansthertem et al., 2010).

Acute kidney injury (AKI) and chronic kidney disease (CKD) are major risks associated with the use of multiple chemotherapeutic drugs for cancer treatment, causing high morbidity and mortality (Siew & Davenport, 2015; Zuk & Bonventre, 2016). A study conducted by Li et al. (2018) showed that overexpression of galectin-3 in HEK 293 (primary embryonic human kidney) decreased cell viability and induced cell cycle arrest and apoptosis. The inhibition of Gal-3 by MCP antagonizes this pro-apoptotic effect. In addition, mice pretreated with MCP before cisplatin treatment showed

improvement in serum creatinine levels, decreased renal tissue damage, increased renal function, decreased apoptosis, and decreased renal fibrosis provoked by cisplatin-induced injury (Li et al., 2018). A study produced by Wang et al. (2010) also demonstrated that the inhibition of galectin-3 by siRNA or GCS-100 (an MCP antagonist of gal-3) increases cisplatin-induced apoptosis in prostate tumor cell models (PC3). This response is related to the activation of calpain (a calcium-dependent protease) which leads to androgen receptor cleavage into an androgen-independent isoform in prostate cancer cells (Wang et al., 2010).

These results show that MCP, in addition to its antitumor activity, protects against kidney damage caused by chemotherapy used in cancer treatments (figure 2).

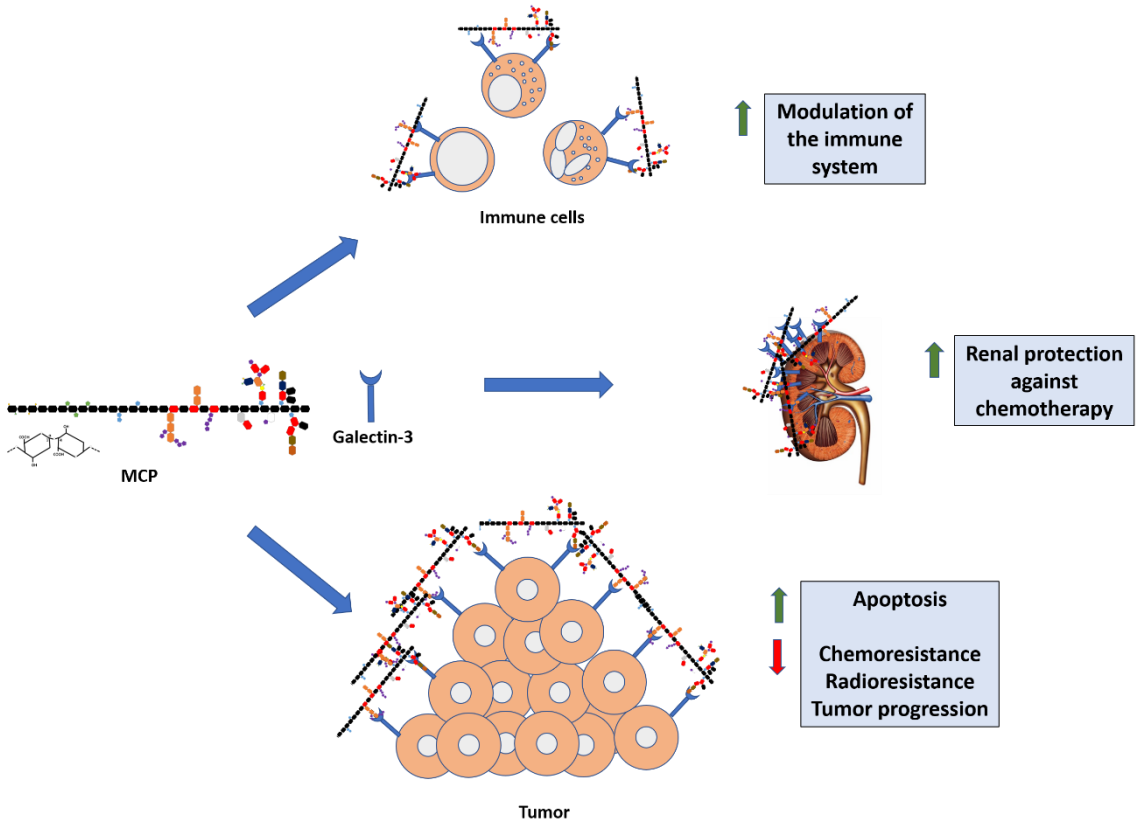

Figure 2: Pectin acts as an antitumor agent. MCP can increase apoptosis and decrease chemoresistance, radioresistance, and tumor progression. It can also modulate the immune system and protect against kidney damage caused by the chemotherapy used in cancer treatment.

14. Conclusions

In this review, we demonstrate that MCP has been widely studied and shows biological activities in many types of diseases, as evidenced by in vitro, in vivo, and clinical studies. MCP has a prominent role in cancer, presenting anti-proliferative,

apoptotic, and anti-metastatic activities. In addition, MCP is important in anticancer therapies as an adjuvant in combination with chemotherapy and radiotherapy. Furthermore, MCP can act as a renal protector against damage caused by chemotherapy in tumor treatments. These activities could be linked to its ability to bind to the galectin-3 protein, acting directly through molecular targets in the tumor and indirectly through systemic immunomodulation. Thus, MCP is an important molecule for the treatment of tumors, can protect against chemotherapy-induced kidney damage, and is a good strategy for oncological therapy. However, further research is required to understand its mechanism of action.

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CHAPTER 2

RADIOLABELING, BIODISTRIBUTION, AND PHARMACOKINETICS OF MCP-^{99m}Tc ADMINISTERED ORALLY AND INTRAVENOUSLY

RADIOLABELING, BIODISTRIBUTION, AND PHARMACOKINETICS OF MCP-^{99m}Tc ADMINISTERED ORALLY AND INTRAVENOUSLY

Abstract

MCP is a polysaccharide found abundantly in the plant's primary wall and shows activity in several areas of the food industry and nutrition, serving in food production, improving intestinal flow, reducing cholesterol, and being an important nutritional compound. Furthermore, MCP can be used as an antitumor, nephroprotective, and cardiovascular protective agent, acting *in vivo* and in clinical studies with different routes of administration, such as oral and intravenous. In this study, we radiolabeled MCP with ^{99m}Tc and verified the biodistribution and pharmacokinetics of MCP-^{99m}Tc orally and intravenously (IV) administrated. First, the structure and monosaccharide composition of MCP were studied using HPSEC-RID and HPAEC-PAD, demonstrating that there is a diversity of monosaccharides and molecular weights within the MCP structure and that MCP30 and MCP3 fractions are rich in galactose (19.11 ± 4.11 % and 16.71 ± 4.91 %, respectively). Next, we studied the inhibition and binding affinity of MCP for galectin-3 using hemagglutination and sepharose/Gal-3 assay. The hemagglutination studies demonstrated that MCP partially bind Gal-3 and that MCP3 showed an inhibition capacity in a concentration of 25 mg/ml. Sepharose/Gal-3 assay demonstrated that MCP has a partial affinity for galectin-3, showed 20.01 ± 3.8% of retention, and MCP3 had a greater affinity for galectin-3, with 37.47 ± 7.94 % of retention. We radiolabeled MCP with ^{99m}Tc and verified its stability in saline in different pH, plasma, and *in vivo*. MCP-^{99m}Tc exhibited good radiochemical purity (>95%) and stability. The kinetics and biodistribution of MCP-^{99m}Tc were analyzed orally (oral) and intravenously (IV), and their absorption and route of elimination were demonstrated. MCP-^{99m}Tc has a low gastrointestinal absorption (5.27x10-6 % total radioactivity counts) and gastrointestinal elimination when administered via oral and renal and hepatobiliary elimination when administered via IV. Finally, we studied the influence of galectin-3 on blood compartmentalization and IV pharmacokinetics of MCP-^{99m}Tc. The blood compartment distribution assay showed that MCP-^{99m}Tc has a high affinity for plasma proteins and blood cells in C57BL/6 Lgals3 $^{+/+}$ mice, and this affinity was partially lost when galectin-3 was deleted in C57BL/6 Lgals3 $^{-/-}$ mice. The pharmacokinetic assay showed that MCP-

^{99m}Tc elimination speed was greater in the C57BL/6 Lgals3$^{-/-}$ mice than in the C57BL/6 Lgals3 $^{+/+}$, indicating that a lack of galectin-3 increases MCP elimination. This knowledge is important for elucidating the mechanisms of action of MCP *in vivo*.

1. INTRODUCTION

Pectin is a polysaccharide found abundantly in plant primary wall to give mechanical strength, hydraulic control of cell and barrier from external environment. The french chemist and pharmacist Henri Braconnot, expert in the extraction of active components from plants, was the first to discover a heteropolysaccharide “Pectin acid” (greek πηκτικός, meaning coagulant) in 1825. The exact structure of pectin is not a consensus; however, it is known that pectin is a family of covalently linked galacturonic acid-rich polymers with three main polysaccharides: homogalacturonan (HG), rhamnogalacturonan-I (RG-I), and substituted galacturonans (GS), and their respective secondary structures vary from species to species (Leclere et al., 2013).

Pectin is used in the food industry to regulate several physiological functions (Lattimer & Haub, 2010). It is a dietary fiber that can be degraded by colonic intestinal microbial species and is not enzymatically digested from the small intestine, maintaining its gelling property and slowing intestinal flow. This property is beneficial in patients with Dumping syndrome, which is characterized by rapid digestion in the stomach (Lawaetz et al, 1983). Pectin can decrease blood cholesterol levels, stimulate lipid excretion, and can increase ^{137}Cs clearance (radioisotope found in Chernobyl area). MCP (modified citrus pectin) reduce the diarrhea in children and decreasing a risk of intestinal infection (Brown et al., 1999; Nesterenko et al 2004; Olano-Martin et al., 2002).

MCP is an important nutritional compound that shows activity in several areas of the food industry and nutrition, serving in food production, improving intestinal flow, reducing cholesterol, and being an important nutritional compound. However, one of the biggest contributions is as an anticancer molecule, acting in several types of tumors both *in vitro*, *in vivo,* and in clinical studies, acting alone or synergistically with other treatments. In addition, MCP regulates the immune system, acts against chemoresistance, and prevents acute and severe renal syndromes caused by

radiotherapy and chemotherapy in cancer treatment. All these effects have been reported to bind Galectin-3 protein (Fang et al., 2018).

Galectins are a family of proteins with a carbohydrate recognition domain that can bind to B-galactoside sugars by either N-linkers or C-linkers (Barondes et al., 1994; Cummings et al., 2009; Kasai & Hirabayashi, 1996). The galectin-3 (Gal-3) is a multifunctional lectin protein of 32-35 KDa, expressed by immune, endothelial, and epithelial cells, and modulates numerous biological processes through a carbohydrate-dependent and independent mechanism (Barondes et al., 1994; Cummings R.D., Liu F.T. Galectins. In: Varki A., Cummings et al., 2009; Davidson et al., 2002; Hsu & Liu, 2002; Kasai & Hirabayashi, 1996; Ochieng et al., 2002). The galectin-3 is expressed in various tissues and has several important functions in the tumor microenvironment, immune system, tumor-directed immunosuppression, glycolysis, mitochondrial metabolism, and several signaling mechanisms in tumors (Guo et al., 2020; Kariya et al., 2018; Rinaldi et al., 2018; Ruvolo et al., 2019; Wang et al., 2019). It is dysregulated in many malignancies, such as cardiomyopathies and kidney disease, has a pro-tumor effect, and is highly expressed in many types of pathological tissues, thus contributing to disease progression (Newlaczyl & Yu, 2011).

The galectin-3 is a target of MCP, and the relationship between inhibition of gal-3 provoked by MCP has been widely studied. Pectin-based inhibitors of Ga-3 are associated with the induction of cell death, apoptosis, cell cycle arrest, and inhibition of migration and adhesion in many types of tumors *in vitro* and *in vivo*. In addition, these molecules modulate the immune system and promote the recovery from cardiomyopathies, protection from renal toxicity, diabetes, atherosclerosis, obesity, and subarachnoid hemorrhage. The main mechanism of action explaining the effects of MCP is its interaction with Gal-3 (Liu et al., 2008; Sathisha et al., 2007; Schöll-naderer et al., 2020; Wang et al., 2016; Fang et al., 2018; Bayes-Genis et al., 2014; Dong et al., 2018; Xue et al., 2019; Cao et al., 2020; Li et al., 2018; Ma et al., 2020; MacKinnon et al., 2013; Martínez-Martínez et al., 2016; Nishikawa et al., 2018; Xu et al., 2020; Tian et al., 2020).

Pharmacokinetics is a science that studies the kinetics of molecules and drugs in the body, demonstrating their absorption, distribution, metabolism, excretion, and elimination. Pharmacokinetic studies involve experimental and theoretical approaches,

such as biological sampling techniques, measurement of molecules and their metabolites, and analytical methods. The most efficient route of administration is intravenous injection, in which the molecule arrives intact and enters the systemic circulation more quickly. Many other routes of administration can be used, including oral, transdermal, rectal, intramuscular, sublingual, inhalation, and subcutaneous. Absorption indicates the transfer of a drug from the site of administration to the systemic circulation. Distribution refers to the transport of molecules from the systemic circulation throughout the organs and tissues of the body. The volume of distribution (VD) is related to the concentration of molecules in the body compared with the amount in the plasma. Elimination is the speed at which drugs are removed from the body and is directly related to clearance, which is defined as the speed at which the molecule is cleared away from circulation. Elimination usually occurs in two main ways: renal elimination, in which the drug is excreted from the circulation by renal filtration, and hepatic elimination, in which molecules are metabolized in the liver and removed by the biliary tract. Therefore, pharmacokinetics is a tool that benefits the development of new formulations and new therapeutic systems, demonstrating the adequate biopharmaceutical behavior of drugs and molecules for determined therapeutic objectives (Madden & Thompson, 2022; Nwabufo & Aigbogun, 2022; Shargel & Yu, 2016).

Radiolabeling and molecular imaging allow the evaluation of biological and chemical processes at the cellular level in the organism, unlike conventional imaging techniques such as computed tomography (CT), x-rays, and ultrasound, which offer pictures of the body physical structure (Rowe & Pomper, 2022). Fundamentally, molecular imaging enables visualization and localization of biochemical processes in the body through specific targets at the anatomical level. (Böhmer et al., 2021; Du et al., 2019; Haider et al., 2020; Zhou & Lu, 2017). Molecular imaging can improve the pre-clinical and clinical steps of drug development with the identification of the appropriate target, evaluation of dose response, drug biodistribution, pharmacokinetics, and pharmacodynamics. It can also identify disease-related processes and biological effects of drugs, such as receptor availability, enzymatic activity, and gene and protein

expression in pre-clinical studies (Lindner and Link 2018; Mezzanotte et al. 2017; Murphy, et al. 2017; de Vries et al. 2019).

The ^{99m}Tc is a short-half-life radionuclide that was first used in 1961 for medical applications. This nuclide has several features that make it safer for nuclear medicine or pre-clinical study of drugs: short physical half-life (t1/2 = 6h), biological half-life (1 d), low isotope cost, gamma energy of 140 keV (the same wavelength used in conventional X-ray diagnostics), easy availability and handling in nuclear medicine laboratories, and high sensitivity and measurements in physical and medical equipment. ^{99m}Tc can be incorporated into many types of molecules that target specific receptors, including monoclonal and polyclonal antibodies, cytokines, nanoparticles, and small molecules, and can be used in biological and pharmacological studies of different types of molecules and in the diagnosis of various diseases (Alberto et al., 2020; Hanna et al., 2020; Papagiannopoulou, 2017; Richards et al., 1982; Saad et al., 2022; Tahara et al., 2022).

In this study, we radiolabeled MCP with ^{99m}Tc and verified the biodistribution and pharmacokinetics of MCP-^{99m}Tc in oral and intravenously routes of administration. First, the structure and monosaccharide composition of MCP were studied, as well as its binding and inhibition of galectin-3. Next, we radiolabeled MCP with ^{99m}Tc and verified its stability in saline in different pH, plasma, and *in vivo*. The kinetics and biodistribution of MCP-^{99m}Tc were analyzed orally (oral) and intravenously (IV), and their absorption and route of elimination were demonstrated. Finally, we studied the influence of galectin-3 on MCP blood compartmentalization and IV pharmacokinetics. This knowledge is important for unveiling the mechanisms of action of biological activities and applications of MCP.

2. MATERIAL AND METHODS

2.1. Production of MCP fractions

The MCP (PectaSol-C – Modified Citrus Pectin – ecoNugenics) fractions were produced as described by Prado et al., 2019. MCP samples (triplicate) were water-solubilized and fractionated according to different molecular sizes by sequential ultrafiltration using 30, 10, and 3 kDa MWCO Amicon Ultra-4 Centrifugal Filters (Millipore). Extracts were then lyophilized, resulting in four MCP fractions: (1) MCP

higher than 30 kDa (MCP30), (2) MCP between 30 and 10 kDa (MCP30/10), (3) MCP between 10 and 3 kDa (MCP10/3), and (4) MCP lower than 3 kDa (MCP3).

2.2. Molecular weight distribution and homogeneity analysis

Samples of each fraction (3 mg) were diluted in 1 mL of deionized water, and the molecular weight distribution was analyzed by High-performance Size-exclusion Chromatography with Refractive Index Detection (HPSEC-RID) using an Infinity 1250 system (Agilent, Santa Clara, CA, USA). The system was equipped with four PL aquagel-OH columns (60, 50, 40, and 30; 300 × 7.5 mm; Agilent) connected in tandem. The eluent was 0.2 M NaNO3/0.02% NaN3 (0.6 mL/min) and the RID temperature was set at 30 °C. Molecular sizes were estimated using Dextran T-series (25, 50, 80, 150, 410 and 750 kDa; Sigma, St. Louis, MO, USA) as molecular size curve standards (do Prado et al., 2019).

2.3. Monosaccharide hydrolysis/polysaccharide composition

Hydrolyzed monosaccharides were obtained by hydrolysis of each MCP fraction and CP (pectin from citrus peel; P9561 Sigma-Aldrich) (1 mg) using 2 M trifluoroacetic acid (TFA). After hydrolysis, the samples were dried, resuspended in water, and subjected to analysis of neutral sugars and uronic acids by High-Performance Anion-Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD) using an ICS5000+ system (Thermo-Dionex, Waltham, MA, USA). Neutral sugars (L-arabinose, D-galactose, d-glucose, D-fucose, D-mannose, L-rhamnose and D-xylose) and uronic acids (D-glucuronic and D-galacturonic acids) were used as external standards (Sigma, St. Louis, MO, USA) (do Prado et al., 2019).

2.4. Radiolabeling of MCP and Quality control

The MCP radiolabeling was performed according to Sanguri et al 2016 (Sanguri et al., 2016). MCP (2.5 mg) was dissolved in saline saline (NaCl 0.9%) and mixed with 20 µg stannous chloride ($SnCl_2$ 4 mg/ml in 0.01 N HCl solution nitrogenized for 5 min). The pH was adjusted to 7 with a 0.01 N NaOH solution. ^{99m}Tc (130 MBq) was added and the mixture was nitrogenized for 5 min and incubated for 25 min. The $SnCl_2$ concentration and pH were optimized for the maximum labeling efficiency (≥95%) and minimum colloid percentage. All labeling procedures were performed in a hot laboratory under lead shielding.

Quality control assay of the MCP-^{99m}Tc complex was carried out by ITLC-SG (Agilent Technologies, CA, USA) ascending paper with two different mobile phases: 100% acetone and ethanol: ammonia: water (1:2:5). The labeling efficiency of the complex was determined by separating the radioactivity into complex (MCP-^{99m}Tc complex), free (^{99m}TcO4 -), and reduced hydrolyzed technetium states (R/H ^{99m}Tc), which were counted using a Perkin Elmer (Waltham, MA, USA) Wizard2 2480 automatic gamma counter.

2.5. Stability of MCP-^{99m}Tc in saline and plasm

The quantification of MCP-^{99m}Tc, R/H ^{99m}Tc, and ^{99m}TcO4- was measured in saline and blood plasma to demonstrate its stability. 10ul of MCP-^{99m}Tc was added to 100 µL of saline (NaCl 0.9%) or 100 µL blood plasma of C57BL/6 mice, extracted by cardiac puncture, heparinized (60 U/ml at 10%), and centrifuged at 12.000 RPM for 5 min. The times analyzed were 0, 0.5, 1, 2, 3, 4, 5, 6, and 24 hours. Quality control was carried out by ITLC-SG (Agilent Technologies, CA, USA) ascending paper with two different mobile phases: 100% acetone and ethanol: ammonia: water (1:2:5). The labeling efficiency of the complex was determined by separating the radioactivity into complex (MCP-^{99m}Tc complex), free (^{99m}TcO4 -), and reduced hydrolyzed technetium states (R/H ^{99m}Tc), which were counted using a Perkin Elmer Wizard2 2480 automatic gamma counter (Waltham, MA, USA).

2.6. Stability of MCP-^{99m}Tc in different pH

In the pH variation stability assay, the sample with pH variation was prepared with 5 ml of PBS buffer and the pH was adjusted to 0, 1, 2, 3, 4, 5, 6, 7, and 8 with 0.1M HCl and 0.1M NaOH. The pH was measured using a Kasvi pH-meter (K39-1420A). Quality control assay of the MCP-^{99m}Tc complex in the pH variation was carried out by ITLC-SG (Agilent Technologies, CA, USA) ascending paper with two different mobile phases: 100% acetone and ethanol: ammonia: water (1:2:5). The labeling efficiency of the complex was determined by separating the radioactivity into complex (MCP-^{99m}Tc complex), free (^{99m}TcO4 -), and reduced hydrolyzed technetium states (R/H ^{99m}Tc), which were counted using a Perkin Elmer (Waltham, MA, USA) Wizard2 2480 automatic gamma counter.

2.7. Western blotting

The western blotting study was done in accordance with Santos et al. 2017. The cells were lysed in RIPA buffer and 50µg of proteins were separated by Novex NuPAGE SDS-PAGE gel system (Invitrogen) and then transferred overnight to a PVDF membrane (Invitrogen). The membrane was incubated with anti-gal-3 produced with M3/38 hybridoma (M3/38, ATCC TIB166). Anti-β-actin-peroxidase (A3854 from Sigma-Aldrich) was used as a loading control. Biotinylated secondary antibodies anti-rat (BA-4001) were from Vector Laboratories. Horseradish peroxidase (HRP)-conjugated secondary antibodies (DAKO) were detected using the enhanced chemiluminescence (ECL) reagent (GE Healthcare). Image J software was used to analyze the densitometry value of Western blots bands.

2.8. Hemagglutination assay

Recombinant galectin-3 was produced as described previously (Nangia-Makker et al. 2012). Inhibition of hemagglutination was performed as previously described (Nowak et al., 1976; Ochieng et al., 1993). Briefly, erythrocytes were isolated from rabbit blood, and a final suspension of 3% was used in the experiment. Each well of a V plate had 100 µL of galectin-3, 3% of erythrocytes, 1% bovine serum albumin (BSA), and the sample diluted in PBS; sucrose (10 - 100mM), lactose (12.5, 25, 50, and 100mM), and MCP and the MCP30, MCP30/10, MCP10/3 (5 – 25 mg/ml). The plates were incubated at room temperature for 120 min. The occurrence of hemagglutination was verified by the formation of buttons at the center of the wells.

2.9. Sepharose/Gal3 column assay

The Sepharose/Gal3 column was prepared with 1 g of powdered gel (Cyanogen bromide-activated-Sepharose CNBR C9142-5G) resuspended in 1 mM HCl. The gel was placed on a column PD-10. The column was washed for approximately 15 min with 1 mM HCl (equivalent to approximately 200 mL/g powder). The ligand (1 mg of Gal3) was dissolved in coupling buffer (0,1M $NaHCO_3$ pH=8.3 containing 0.5M NaCl), 5mL of coupling buffer for 1 gram of powder (sepharose). The coupling solution + binder was added to the column and incubated overnight at 4°C (under rotation) with upper and lower caps. The excess binder was washed five times with coupling solution (5 ml). The reactive groups were block with 0.1M Tris-HCl at pH = 8 buffer (5 ml) for 4 hours.

Subsequently, the column was wash with 3 alternating pH cycles. Each cycle consisted of washing the column with acetate buffer (pH 4) containing NaCl (0.5M), followed by washing with Tris-HCl (pH 8 containing 0.5M NaCl. Finally, the column was stored in PBS with 0.03% azide (4°C) with the upper and lower caps, and the protein concentration of the washed samples was measured using a Nanodrop.

The affinity experiment was performed using a Sepharose/Gal3 column. First, the Sepharose/Gal3 column was washed with PBS (20 ml). MCP-^{99m}Tc or MCP3-^{99m}Tc (1mg/ml with 37 MBq) was incubated in a column for 5 min with upper and lower caps. The column was washed with 1 ml of PBS 1x under continuous flow for 15 min. The column was washed 15 times with fractions of lactose 100 mM (1ml) under continuous flow. All fractions of PBS or lactose (100 mM) were stored in Eppendorf tubes (1.5 ml) and measured using a Perkin Elmer (Waltham, MA, USA) Wizard2 2480 automatic gamma counter. Radiological activity was corrected for the radionuclide half-life, and the percentage of elution by lactose was verified.

2.10. Stability of MCP-^{99mTc} *in vivo*

Stability tests of MCP-^{99m}Tc *in vivo* were performed according to Sanguri et al. (2016). 37 MBq of MCP-^{99m}Tc was administered either intravenously or orally by gavage to the C57BL/6 mice. Quantitative and qualitative assays of the MCP-^{99m}Tc complex were performed using blood plasma fractions at 0.001, 5, 15, 30, 45, and 60 min (IV), and at 5, 30, 60, 120, and 240 min (oral). Blood cells were separated by centrifugation (12.000 rpm for 5 min). Protein precipitation in the supernatant was achieved by adding methanol (100 µL) to the plasma sample and by centrifugation (12.000 rpm for 5 min). The plasm sample (5 µl) was carried out by ITLC-SG (Agilent Technologies, CA, USA) ascending paper with two different mobile phases. The labeling efficiency of the complex was determined by separating the radioactivity using a Perkin Elmer (Waltham, MA, USA) Wizard2 2480 automatic gamma counter. Mobile phase acetone: run the mobile phase; free (^{99m}TcO4^{-}), stay at the application point, complex (MCP-^{99m}Tc complex), and reduced hydrolyzed technetium states (R/H ^{99m}Tc). Mobile phase ethanol: ammonia: water (1:2:5): run the mobile phase; complex (MCP-^{99m}Tc complex) and free (^{99m}TcO4^{-}), stationary phase; reduced hydrolyzed technetium states R/H ^{99m}Tc). The percentage

proportions of the agents (MCP-^{99m}Tc, R/H ^{99m}Tc, and $^{99m}TcO4^-$) were quantified and the radiochemical purity was measured.

2.11. Biodistribution studies

The animal models used were submitted for analysis by the ethics committee and biosafety committee of the Research Institute of Nuclear Energy (IPEN) under registration No. 16/22 and 2021/05, respectively.

Normal C57BL/6 mice and C57BL/6 knockout to galectin-3 were intravenously injected with 10 MBq of MCP-^{99m}Tc (100 µl) into the tail vein or 37 MBq of MCP-^{99m}Tc (200 µl) in oral administration via gavage. One hours after injection, mice were euthanized, and organs of interest were harvested, rinsed in PBS, weighed, and counted in a Perkin Elmer (Waltham, MA, USA) Wizard2 2480 automatic gamma counter to quantify the percentage of injected dose per gram of tissue (%ID/g).

2.12. Kinetic studies

Kinetic studies of MCP-^{99m}Tc were performed in C57BL/6 mice. In the IV study, 100ul with 10-20 MBq of MCP-^{99m}Tc (approximately 0.3 mg) was injected into the tail vein of the animals. In the oral study, 37 MBq of MCP-^{99m}Tc (approximately 0.7 mg) was administered via gavage. Venous blood samples were collected at 0.001, 5, 10, 15, 30, 60, 120, 240, and 1440 min (IV), and 5, 30, 60, 120, and 240 min (oral), through the tail vein. To minimize trauma to the tail vein, a single sample puncture was performed for the first sample, which was collected by removing the blood and collecting the sample (5µL) from the bleeding site in a microtube for further counting of the radioactivity. Blood activity was measured using a Wizard 2 2480 Perkin Elmer automatic γ-counter (Waltham, MA, USA) to determine radioactivity per sample. The radiological activity was corrected for the half-life of radionuclides, and the injected activity and mass were correlated.

2.13. Pharmacokinetic studies

Pharmacokinetic studies were performed in C57BL/6 (C57 / B16 Lgals3$^{+/+}$) and knockout mice for galectin-3 (C57BL/6 Lgals3$^{-/-}$). 100ul with 10-20 MBq of MCP-^{99m}Tc was injected into the tail vein of the animals. Venous blood samples were collected at 0.001, 5, 10, 15, 30, 60, 120, 240, and 1440 min p.i. via the tail vein. To minimize trauma

to the tail vein, a single sample puncture was performed for the first sample, which was collected by removing the blood and collecting the sample (5µL) from the bleeding site in a microtube for further counting of radioactivity. Blood activity was measured using a Wizard 2 2480 Perkin Elmer automatic γ-counter (Waltham, MA, USA) to determine the radioactivity per sample. The radiological activity was corrected for the half-life of radionuclides, and the injected activity and mass were correlated.

The half-lives of the distribution and elimination were determined using a statistical equation. We used nonlinear exponential regression of the "two-phase decay" type and obtained a two-dimensional exponential plot with two half-lives (distribution and elimination).

The (CL) values were calculated using Equation 2:

$$CL = \frac{\text{Total Injected mass (mg)}}{ASC} \quad (2)$$

where ASC = Area on the curve, calculated with the statistical equation using the x/y analysis (area under curve).

The volume of distribution (Vd) was calculated using equation 3:

$$Vd = CL \times \frac{T_{1/2}}{0{,}693} \quad (3)$$

Where $T_{1/2}$ is the half-life of the drug elimination.

2.14. µSPECT/CT imaging

In the µSPECT/CT imaging MCP-^{99m}Tc (37 MBq) was injected intravenously (into the tail vein) or administrated orally (gavage) with 6 hours of food fasting in C57BL/6 mice. Imaging was conducted at 1 hour and 4 hours post injection on an Albira µPET/SPECT/CT imaging system (Bruker Biospin Corporation, Woodbridge, CT, USA). The imaging protocols used was: 3D FOV 80 45proj/s to SPECT and high 400µA 45Kev to CT. Images were processed using PMOD software (PMOD Technologies, Zurick, CH, USA).

2.15. Blood compartment distribution assay

Blood compartment distribution assay was performed as previously described by Jans et al. (2018). Normal C57BL/6 mice and C57BL/6 knockout to galectin-3 were inject

with 10–20 MBq of MCP-^{99m}Tc, intravenously. Venous blood samples were collected at 5, 15, 30, 45, and 60 min p.i. via the tail vein and further processed. To minimize trauma to the tail artery, a single sampling puncture was used for the first sample, which was obtained by carefully removing the scab and collecting the sample (10 µL) from the bleeding site into a calibrated heparinized micro-hematocrit capillary tube. Blood cells were separated by centrifugation (12,000 rpm for 5 min). Protein precipitation in the supernatant was achieved by adding methanol (100 µL) to the plasma sample and centrifugation (12,000 rpm for 5 min). Fractions of blood cells, proteins, and plasma were measured using a Perkin Elmer (Waltham, MA, USA) Wizard2 2480 automatic gamma counter to determine the radioactivity per sample. The radiological activity was correct for the radionuclide half-life.

2.16. Partition coefficient

The partition coefficient of MCP-^{99m}Tc was determined by extraction in a physiological saline sample (0.9% NaCl) system with n-octanol as the aqueous and organic phases, respectively (Wilson et al., 2001). Briefly, an Eppendorf tube containing 500 µL of each phase was added to 50 µL (<1 MBq) of the labeled compound solution. The vial was capped and shaken vigorously for 5 minutes at room temperature. After stirring, the mixture was centrifuged at 2000 × g for 2 min to separate the layers. Aliquots (100 µL) were removed from each phase and the amount of labeled compounds present in each phase was measured using a Wizard gamma counter. The experiments were performed in triplicate, and the partition coefficient (P) values were determined using the following formula:

$$P = \frac{c(fase.org)}{c(SSF)}$$

Where,

c (fase.org) corresponds to the activity of the counts in the n-octanol sample.

c (SSF): corresponds to the activity of counts in the physiological saline sample (0.9% NaCl).

The decimal logarithm of this parameter (log P) was calculated.

2.17. Statistical analysis

All data are expressed as the mean ± SD of at least three independent experiments. Statistical analysis was performed using the GraphPad Prism software (version 8.0; San Diego, CA, USA). Statistical significance was set at $p < 0.05$. Data were analyzed using an unpaired t-test (multiple t-tests). Outliers were removed before data analysis. Statistical significance was set at $P < 0.05$.

3. Results

3.1. Structure and monosaccharide composition of MCP

The study of the structure and monosaccharide composition was performed to understand the molecular characteristics of MCP. MCP sample were water-solubilized and fractionated according to different molecular sizes by sequential ultrafiltration using 30, 10, and 3 kDa Centrifugal Filters, producing four fractions: (1) MCP higher than 30 kDa (MCP30); (2) MCP between 30 and 10 kDa (MCP30/10); (3) MCP between 10 and 3 kDa (MCP10/3); and (4) MCP less than 3 kDa (MCP3). The molecular weight distribution was analyzed by high-performance size-exclusion chromatography with refractive index detection (HPSEC-RID) that determined the yield % of the MCP fractions, the peak of molecular weights as well as their maximum and minimum values of molecular weight by a fraction. The yield % of the MCP fractions were MCP30 (32.34 ± 0.26 %), MCP30/10 (7.98 ± 0.51 %), MCP10/3 (8.73 ± 0.14 %) and MCP3 (50.07 ± 0.54 %). The peak molecular weights of the MCP fractions were MCP (9.42 KDa), MCP30 (28.93 KDa), MCP30/10 (23.03 KDa), MCP10/3 (17.33 KDa), and MCP3 (8.78 KDa), along with their maximum and minimum values MCP (>750 – 1.10 KDa), MCP30 (28.93 – 3.91 KDa), MCP30/10 (247.30 – 4.12 KDa), MCP10/3 (17.33 – 4.24 KDa), and MCP3 (29.08 – 1.04 KDa) (Figure 1 A, B, C and D).

Based on the yield % and molecular weight of MCP and MCP fractions, the monosaccharide composition of MCP was verified through the hydrolyzed monosaccharides obtained by TFA, dried, resuspended in water, and analyzed by High-performance Size-exclusion Chromatography with Refractive Index Detection (HPAEC-PAD). Neutral sugars L-arabinose (Ara), D-galactose (Gal), D-glucose (Glc), D-fucose (Fuc), D-mannose (Man), L-rhamnose (Rha), D-xylose (Xyl), and uronic acids D-glucuronic acid

and D-galacturonic acid (GalA) were used as external standards. In this study, we showed that the main composition of the MCP samples was GalA in almost all fractions (> 80 of GalA), with MCP30 (51.71 ± 13.01) and MCP3 (77.63 ± 6.07) containing less than 80% GalA. Associated with this, the MCP30 is rich in Ara (6.10 ± 2.08 %), Rha (19.11 ± 4.11 %) and Gal (19.15 ± 5.26 %), and MCP3 has a significant concentration of Gal (16.71 ± 4.91 %). These results showed that there is a diversity of monosaccharides and molecular weights within the MCP structure (Figures 1 E, and Table 1). The MCP30 and MCP3 fractions have drawn attention because they are rich in Gal, a known inhibitor of galectins, a protein related to several pleiotropic functions in the body. (Dahlqvist et al., 2019).

Yield (%)	Mean	SD
MCP >30	32,34	0,26
MCP <30>10	7,98	0,51
MCP <10>3	8,37	0,14
MCP <3	50,07	0,54

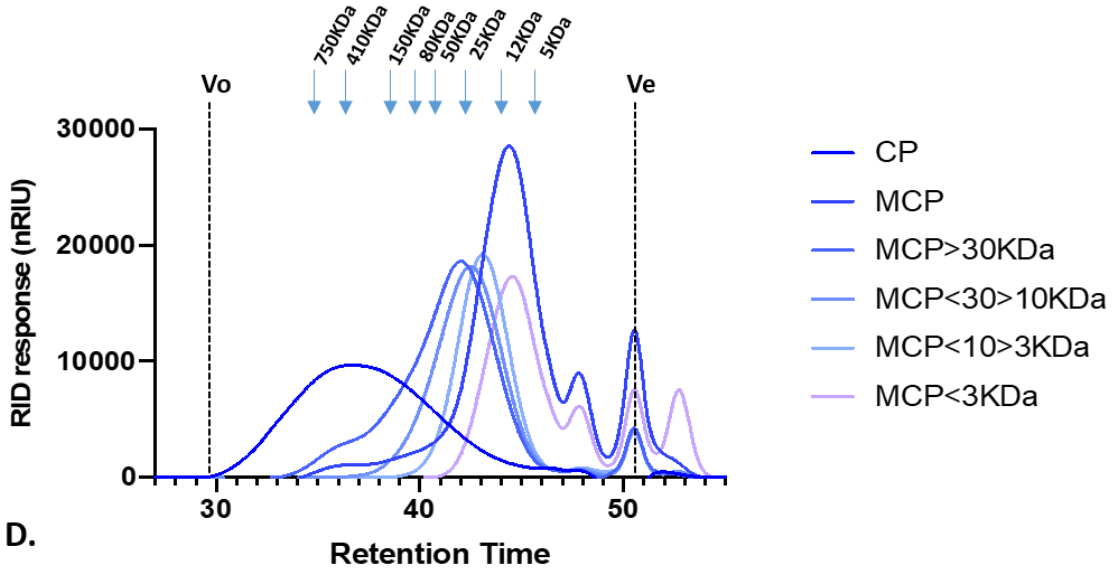
A.

Peak molecular weight (KDa)	
CP	363,89
MCP	9,42
MCP 30	28,93
MCP 30-10	23,03
MCP 10-3	17,33
MCP 3	8,74

B.

Maximum and minimum (kDa)		
	Max	Min
CP	>750KDa	3,91
MCP	>750KDa	1,10
MCP 30	28,93	3,91
MCP 30-10	247,30	4,12
MCP 10-3	17,33	4,24
MCP 3	29,08	1,04

C.



D.

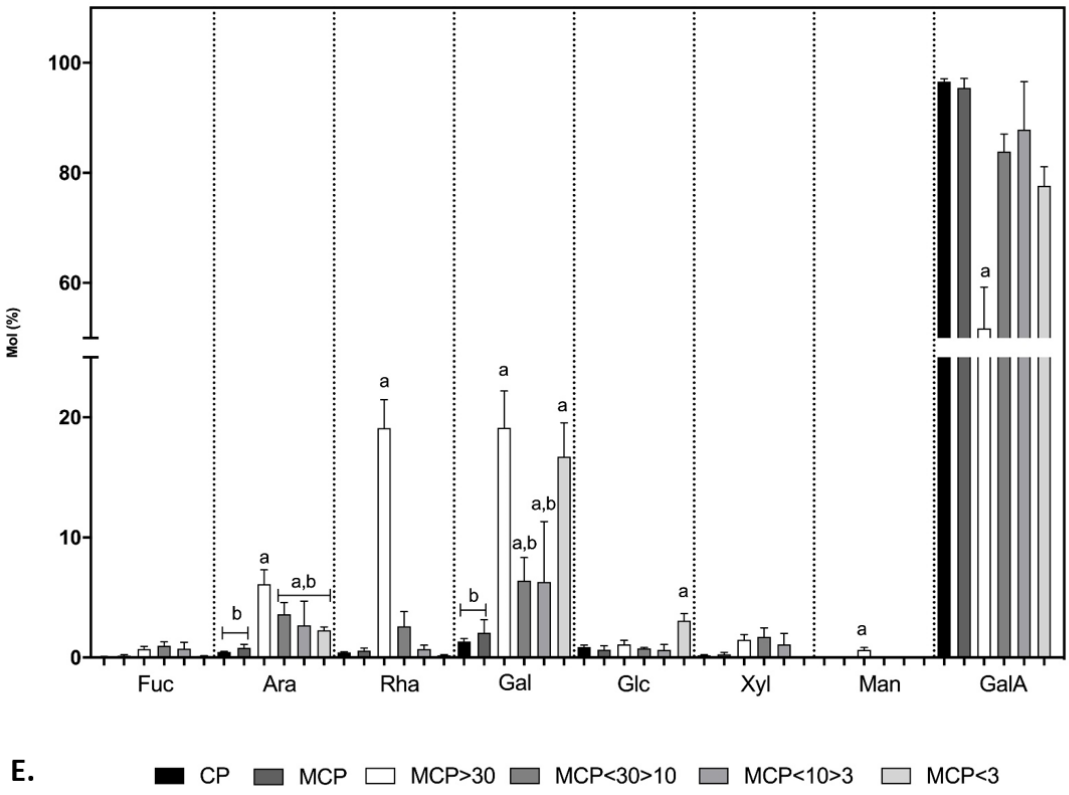


FIGURE 1: Structure and monosaccharide composition of MCP. (A) Results of Yield (%) of CP, MCP, MCP30, MCP30/10, MCP10/3, and MCP3. (B) Peak molecular weight (KDa) of CP, MCP, MCP30, MCP30/10, MCP10/3, and MCP3. (C) Maximum and minimum weight (KDa) of CP, MCP, MCP30, MCP30/10, MCP10/3, and MCP3. (D) molecular weight distribution of MCP and its fractions in the HPSEC-RID analysis. (E) Monosaccharide composition of MCP CP, MCP, MCP30, MCP30/10, MCP10/3, and MCP3. L-arabinose (Ara), D-galactose (Gal), D-glucose (Glc), D-fucose (Fuc), D-mannose (Man), L-rhamnose (Rha), D-xylose (Xyl), and uronic acids D-glucuronic acid and D-galacturonic acid (GalA). Data are representative of three independent experiments. $^{a,\ a\text{-}b,\ b}$ $p > 0.05$.

TABLE 1: Monosaccharide composition of CP, MCP, MCP30, MCP30/10, MCP10/3, and MCP3. Fucose (Fuc), arabinose (Ara), rhamnose (Rha), galactose (Gal), glucose (Glc), xylose (Xyl), mannose (Man) and galacturonic acid (GalA). Data are representative of three independent experiments.

	Ara	Rha	Gal	Glc	Xyl	GalA
CP	0.45 ± 0.10	0.44 ± 0.09	1.34 ± 0.44	0.87 ± 0.27	0.18 ± 0.07	96.61 ± 0.86
MCP	0.52 ± 0.02	0.75 ± 0.28	0.98 ± 0.08	0.35 ± 0.02	0.11 ± 0.02	97.14 ± 0.38
MCP>30	6.10 ± 2.08	19.11 ± 4.11	19.15 ± 5.26	1.11 ± 0.57	1.47 ± 0.75	51.71 ± 13.01
MCP<30>10	4.58 ± 0.04	3.83 ± 0.16	8.22 ± 1.54	0.77 ± 0.13	2.33 ± 1.07	83.92 ± 5.42
MCP<10>3	0.67 ± 0.03	3.83 ± 0.60	1.29 ± 0.22	0.19 ± 0.02	0.16 ± 0.04	96.56 ± 0.04
MCP<3	2.28 ± 0.47	0.19 ± 0.12	16.71 ± 4.91	3.07 ± 1.01	-	77.63 ± 6.07

3.2. MCP can be radiolabeled with ^{99m}Tc and is stable in saline and blood plasma

In the radiolabeling assay, we demonstrated the possibility of radiochemical labeling of MCP with ^{99m}Tc. For this, MCP (2.5 mg) was dissolved in saline and mixed with 20 µg stannous chloride ($SnCl_2$ in 0.01 N HCl solution nitrogenized for 5 min). The pH was adjusted to 7 with a 0.01 N NaOH and ^{99m}Tc (130 MBq) was added, mixed and nitrogenized for 5 min and incubated for 25 min. The quality control assay of the MCP-^{99m}Tc complex was carried out by ITLC-SG with two different mobile phases: 100% acetone and ethanol: ammonia: water (1:2:5). The labeling efficiency of the complex was determined by separating the radioactivity into complex (MCP-^{99m}Tc complex), free (^{99m}TcO4 -), and reduced hydrolyzed technetium states (R/H ^{99m}Tc). The data demonstrated that direct radiolabeling of MCP with ^{99m}Tc is efficient, showing a radiochemical purity greater than 95% (95.4 ± 1.7%), with low rates of $^{99m}TcO_4^-$ (1.3 ± 0.3%) and colloid R/H ^{99m}Tc (3.4 ± 1.8%) (Figure 2 A).

The quantification of MCP-^{99m}Tc, R/H ^{99m}Tc, and ^{99m}TcO4- was performed in saline and blood plasma to demonstrate its stability. MCP-^{99m}Tc was added to 100 µL of saline or blood plasma and the times analyzed were 0, 0.5, 1, 2, 3, 4, 5, 6, and 24 hours. Quality control was carried out by ITLC-SG with two different mobile phases: 100% acetone and ethanol: ammonia: water (1:2:5). The labeling efficiency of the complex was determined by separating the radioactivity into a complex (MCP-^{99m}Tc complex), free (^{99m}TcO4 -), and R/H ^{99m}Tc. The results showed that MCP-^{99m}Tc was stable for 5 h in saline and for 24 h in plasma with a radiochemical purity greater than 95% in the ITL-C stability assay (Figure 2 B and C). These results demonstrate that the stability of MCP-^{99m}Tc is greater in plasma and partially in saline, however, for the purpose of this study, good stability in plasma is essential for further studies.

To verify whether the orally administered MCP-^{99m}Tc remained stable after passing through the gastrointestinal tract, a stability study in saline was performed using a pH gradient to mimic the acidic environment of the stomach. For this, the sample with pH variation was prepared in PBS and the pH was adjusted to 0, 1, 2, 3, 4, 5, 6, 7, and 8 with 0.1M HCl and 0.1M NaOH. Quality control of the MCP-^{99m}Tc complex in the pH variation was carried out by ITLC-SG with two different mobile phases: 100% acetone and ethanol: ammonia: water (1:2:5). The labeling efficiency of the complex was determined by separating the radioactivity into a complex (MCP-^{99m}Tc complex), free

(^{99m}TcO4 -), and R/H ^{99m}Tc. The results showed that MCP-^{99m}Tc was stable at a pH equal to or greater than 3 at 1 h and 4 h (> 95% radiochemical purity), and partially stable at a pH of 2 or less at 1 h and 4 h (> 82% radiochemical purity) (Figure 2 D and E).

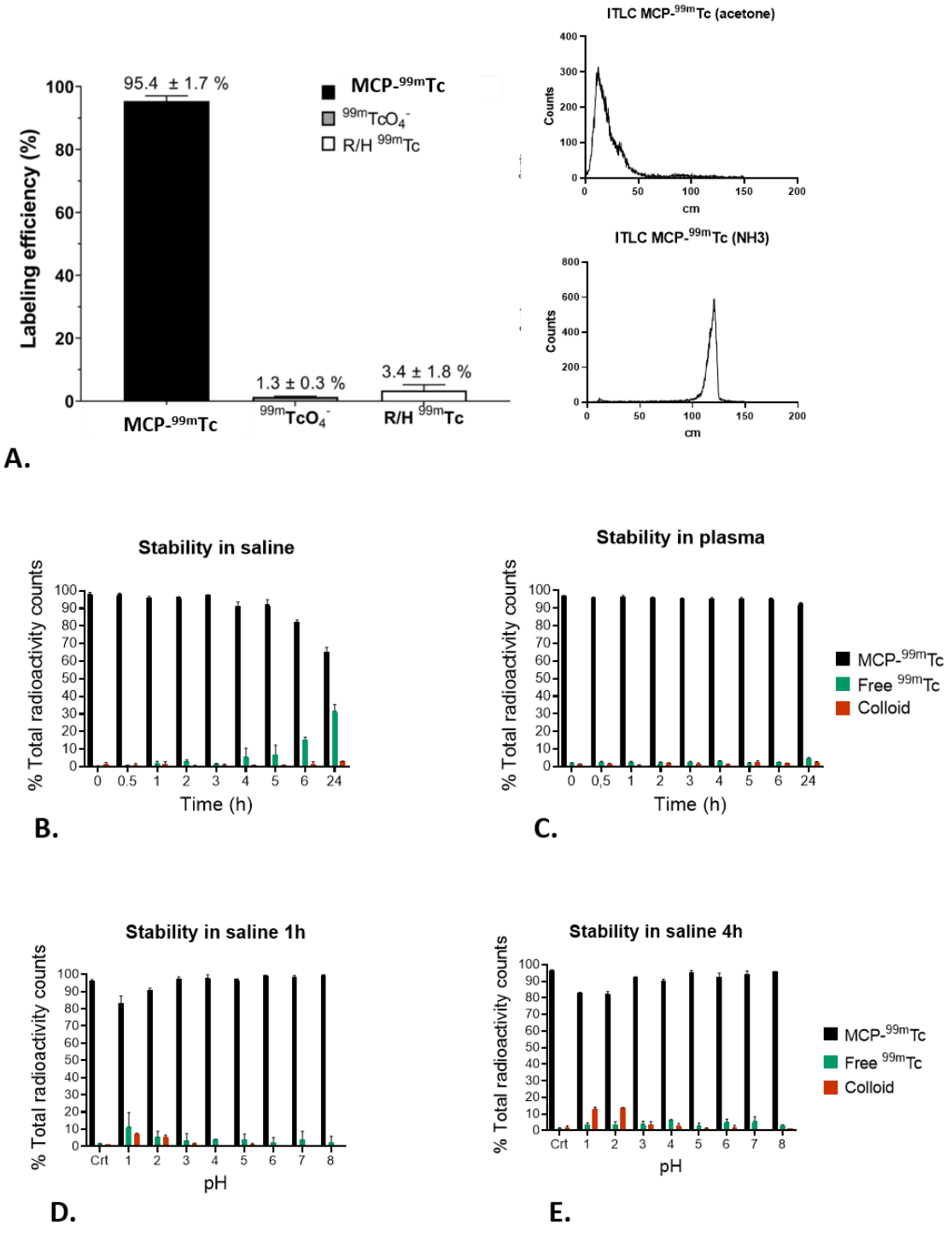


FIGURE 2: MCP can be radiolabeled with ^{99m}Tc and is stable in saline and blood plasma. (A) Labeling efficiency of MCP-^{99m}Tc with activity counts in ILTC-acetone and ILTC- NH3. (B) The % of total radioactivity

counts of MCP-^{99m}Tc in saline. (C) The % of total radioactivity counts of MCP-^{99m}Tc in blood plasma. (D) The % of total radioactivity counts of MCP-^{99m}Tc in saline at different pH values at 1 h. (E) The % of total radioactivity counts of MCP-^{99m}Tc in saline at different pH values at 4 h. Data are representative of three independent experiments.

3.3. MCP has a partial affinity for galectin-3

To verify the affinity and inhibition of MCP against galectin-3, sepharose/gal3 and hemagglutination assays were performed, respectively. The hemagglutination assay was initiated with the production of recombinant galectin-3, as described previously (Nangia-Makker et al. 2012). Gal-3 stability was analyzed by western blotting, and its integrity was confirmed (Figure 3 A). Hemagglutination assay was performed by isolating erythrocytes from rabbit blood in a final suspension of 3%. Each well of a V plate contained 100 µL of rabbit blood (3%), 1% bovine serum albumin (BSA), and PBS with the samples. The plates were then incubated at room temperature for 120 min. In the control, concentrations of 1, 5, 10, and 20 µM of Gal-3 caused the clumping of red cells and the formation of the net, preventing the production of buttons in the center of the well (hemagglutination) (Figure 3 B). In the inhibition control, the concentrations of 10 and 100 mM of sucrose (negative control) did not inhibit the action of Gal-3; however, 12.5, 25, 50, and 100 mM of lactose inhibited Gal-3, producing buttons of erythrocytes in the center of the well (Figure 3 C). The treatments were performed using MCP and the MCP30, MCP30/10, MCP10/3, and MCP3 fractions at concentrations of 5 and 25 mg/ml. The data of this study showed that MCP partially inhibits the Gal-3 in the concentration of 25 mg/ml, and the fraction that had more ability to avoid hemagglutination of erythrocytes was MCP3 at a concentration of 25 mg/ml (Figure 3 D).

The affinity assay was performed by passing MCP-^{99m}Tc and MCP3-^{99m}Tc throw a Sepharose/Gal3 column. First, the Sepharose/Gal3 column was washed with PBS, and MCP-^{99m}Tc or MCP3-^{99m}Tc (1mg/ml with 37 MBq) was incubated in the column for 5 min. Next, the column was washed with PBS under a continuous flow for 15 min. The column was washed 15 times with fractions of lactose 100 mM (1ml) under continuous flow, all fractions of PBS or lactose (100 mM) were stored in tubes, and the radiation activity was measured. Finally, radiological activity was corrected for time, and the percentage retention of MCP was determined. In this study, we verified that approximately 20% of MCP (20.01 ± 3.8%) remained in the column sepharose/gal3, binding galectins-3 and

could be removed with lactose 100mM (Figure 3 E and F). Thus, MCP-^{99m}Tc has partial affinity for galectin-3. In contrast, the sepharose/gal-3 assay using MCP3^{-99mTc} showed that MCP3 has a greater affinity for galectin-3, with 37.47 ± 7.94 % of retention (Figure G and H). These results show that MCP has partial affinity for galectin-3, and that MCP3 shows an increase in affinity, indicating that only a portion of the oligosaccharides in MCP can bind Gal-3.

These affinity and inhibition assay data show that there are fractions within MCP with a low molecular weight that can partially bind and inhibit galectin-3, an important protein for the discussion of the mechanism of action of MCP.

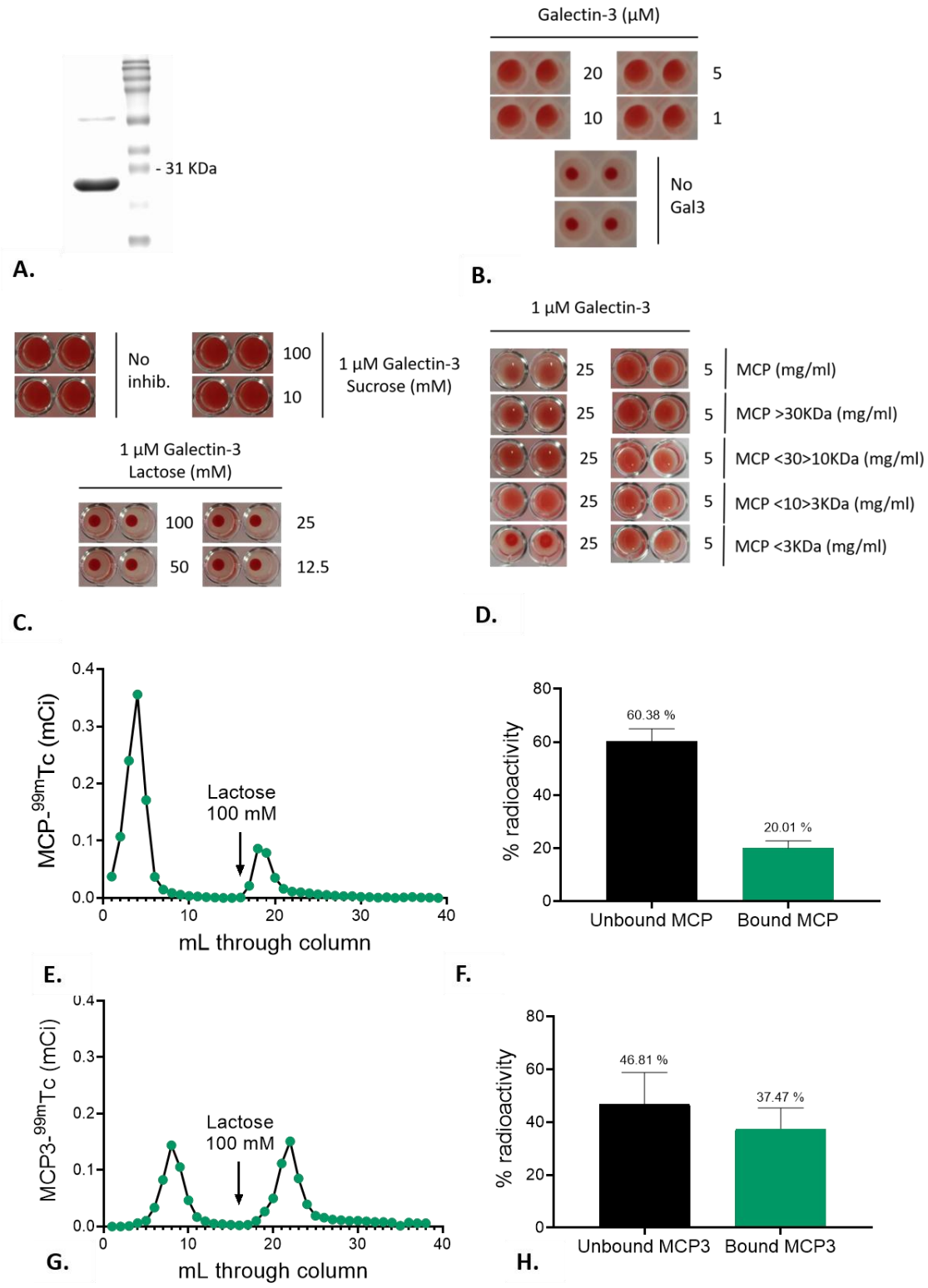


Figure 3: MCP has a partial affinity for galectin-3. A) Western blotting of galectin-3 samples. (B) Control of hemagglutination assay with and without galectin-3. (C) Control of hemagglutination assay with sucrose, lactose, and no inhibition. (D) Hemagglutination assays with MCP, MCP30, MCP30/10, MCP10/3, and MCP3. (E) Affinity assay using sepharose/gal3 column with MCP-^{99m}Tc. (F) The % radioactivity of unbound and bound MCP-^{99m}Tc. (G) Affinity assay column using sepharose/gal3 with MCP3-^{99m}Tc. (H) The % radioactivity of unbound and bound MCP3-^{99m}Tc. Data are representative of three independent experiments.

3.4. MCP-^{99m}Tc exhibits renal/hepatobiliary elimination when administered intravenously and low abortion and gastrointestinal elimination when administered orally

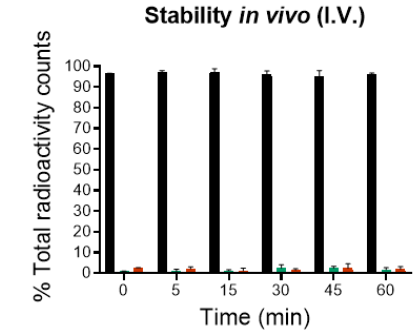
To verify the radiochemical purity of MCP^{-99m}Tc *in vivo*, a stability study was performed using oral and intravenous (IV) routes of administration. 37 MBq of MCP-^{99m}Tc was administered intravenously or orally to the C57BL/6 mice. Quantitative and qualitative assays of the MCP-^{99m}Tc complex were performed using blood plasma fractions at 0.001, 5, 15, 30, 45, and 60 min (IV), and at 5, 30, 60, 120, and 240 min (oral). Blood cells were separated by centrifugation (12.000 rpm for 5 min). Protein precipitation was achieved by adding methanol (100 µL) to the plasma sample and centrifugation (12.000 rpm for 5 min). The plasm sample (5 µl) was carried out by ITLC-SG ascending paper with two different mobile phases, acetone and ethanol: ammonia: water (1:2:5). The percentage proportions of the agents (MCP-^{99m}Tc, R/H ^{99m}Tc, and ^{99m}TcO4^{-}) were quantified and the radiochemical purity was measured. The results show that MCP^{-99m}Tc injected intravenously was stable for 1 h with a radiochemical purity greater than 95%. In contrast, MCP^{-99m}Tc injected orally, was partially stable for 4 h with a radiochemical purity greater than 87% (Figure 4 A and B).

Kinetic studies were performed in C57BL/6 mice to verify the *in vivo* absorption of MCP-^{99m}Tc. In the IV study, 100ul with 10-20 MBq of MCP-^{99m}Tc (approximately 0.3 mg) was injected into the tail vein of the animals. In the oral study, 37 MBq of MCP-^{99m}Tc (approximately 0.7 mg) was administered via oral gavage. Venous blood samples were collected at 0.001, 5, 10, 15, 30, 60, 120, 240, and 1440 min (IV), and 5, 30, 60, 120, and 240 min (oral), through the tail vein. A single sample puncture was performed for the first sample, which was collected by removing the blood and collecting the sample (5µL) from the bleeding site in a microtube for further counting of radioactivity. Blood activity was measured to determine the radioactivity per sample. The radiological activity was corrected for the half-life of radionuclides, and the injected activity and mass were correlated. The intravenous kinetics study showed that MCP-^{99m}Tc has a progressive elimination, with the highest 1.3 ± 0.24 % total radioactivity counts in the first minute, a %D.I. equivalent to approximately 3.9 mg/5µl, and an elimination half-life of 502.89 ± 35.37 min (Figure 4 C and Table 3). The oral kinetics study, however, showed that MCP-

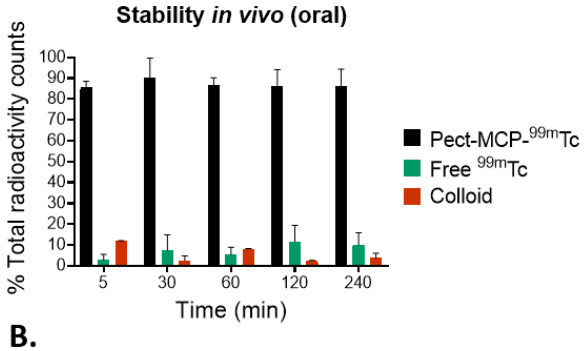
^{99m}Tc could be absorbed by the gastrointestinal tract, but at low concentrations, with the best absorption time of 1 hour with $5.27x10^{-6} \pm 7.5\times10^{-7}$ % total radioactivity counts (%D.I.), a %D.I. equivalent to an effector dose of approximately 37,6 pg/5µl that reaches the circulation system (Figure 4 D).

A biodistribution study using MCP-^{99m}Tc was conducted to verify the organs in which MCP-^{99m}Tc arrived when administered orally and intravenously and its elimination route. Normal C57BL/6 mice were intravenously injected with 10 MBq of MCP-^{99m}Tc (100 µl) or 37 MBq of MCP-^{99m}Tc (200 µl) in oral injection. One hours after injection, mice were euthanized, and organs of interest were harvested, rinsed in PBS, weighed, and counted in a automatic gamma counter to quantify the percentage of injected dose per gram of tissue (%ID/g). The results of intravenous injection showed that the main route of elimination of MCP-^{99m}Tc is a renal and hepatobiliary elimination pathway, verified by the activities taken up in the kidneys (12.25 ± 2.67 %D.I/g), bladder (35.57 ± 5.93 %D.I/g), and liver (5.71 ± 1.59 %D.I/g) in 1 hour (Figure 4 E). The oral biodistribution study showed that MCP-^{99m}Tc presents a renal elimination route for the absorbed molecules, with activity detected in the kidney (0.06 ± 0.007 %D.I/g), bladder (0.295 ± 0.1 %D.I/g) and pancreas (0.13 ± 0.08 %D.I/g), however, the main route of elimination becomes gastrointestinal, with high activities in the stomach (56.95 ± 14.18 %D.I/g), large intestine (29.6 ± 11.8 %D.I/g) and small intestine (33.7 ± 18.7 %D.I/g) in 1 hour (Figure 4 E).

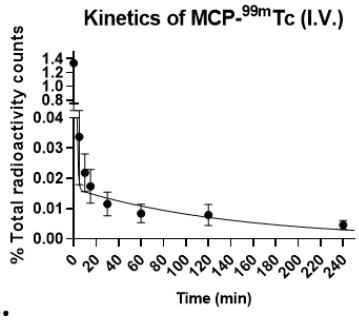
An imaging study using µSPECT/CT was performed to demonstrate the distribution of MCP-^{99m}Tc in the body at predetermined times. MCP-^{99m}Tc (37 MBq) was injected intravenously or orally after 6 h of fasting in C57BL/6 mice. Imaging was conducted at 1 and 4 h post-injection on an Albira µPET/SPECT/CT imaging system, and processed using PMOD software. The imaging study with MCP-^{99m}Tc administrated IV showed high activity in the bladder, confirming renal elimination after 1 h (Figure 4 F). However, the oral imaging study with MCP-^{99m}Tc showed high activity in the small intestine and larger intestine at 1 h time and in the small intestine, larger intestine, and rectum at 4 h, confirming progressive gastrointestinal elimination (Figure 4 G and H).


Stability *in vivo* (I.V.)
% Total radioactivity counts
Time (min)

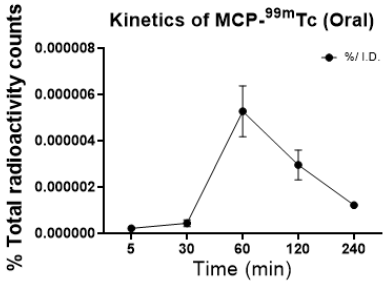
A.


Stability *in vivo* (oral)
% Total radioactivity counts
Time (min)
Pect-MCP-99mTc
Free 99mTc
Colloid

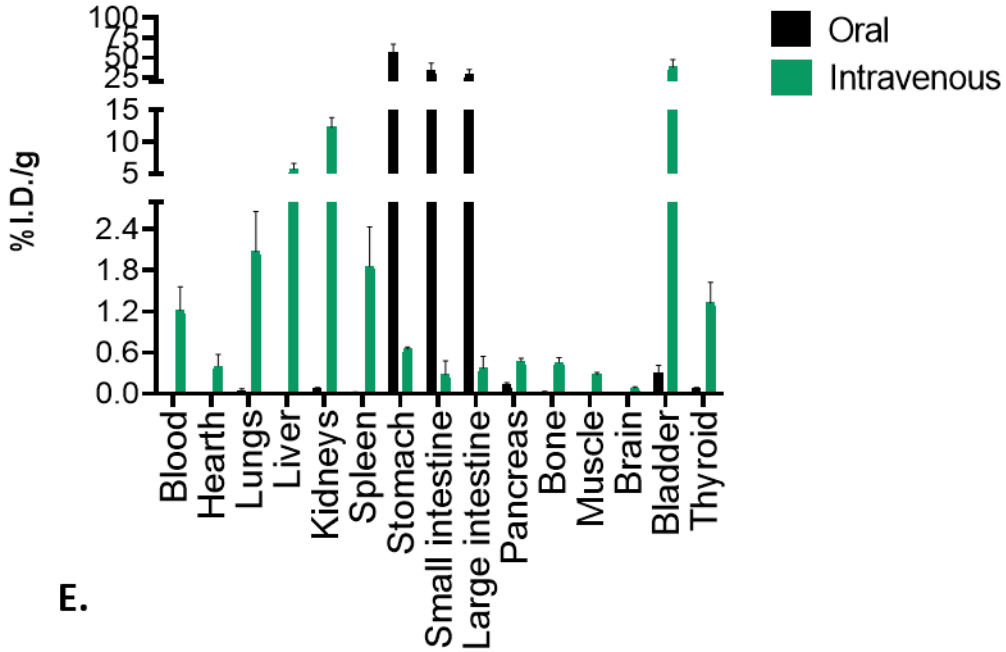
B.


Kinetics of MCP-99mTc (I.V.)
% Total radioactivity counts
Time (min)

C.

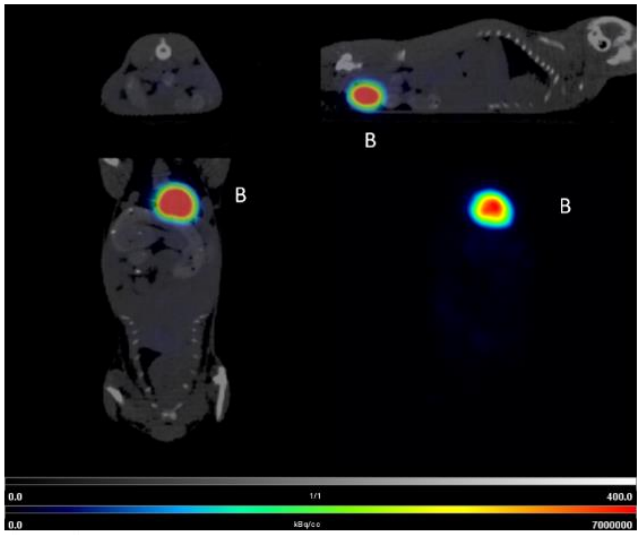

Kinetics of MCP-99mTc (Oral)
% Total radioactivity counts
%/ I.D.
Time (min)

D.


Oral
Intravenous
%I.D./g
Blood
Hearth
Lungs
Liver
Kidneys
Spleen
Stomach
Small intestine
Large intestine
Pancreas
Bone
Muscle
Brain
Bladder
Thyroid

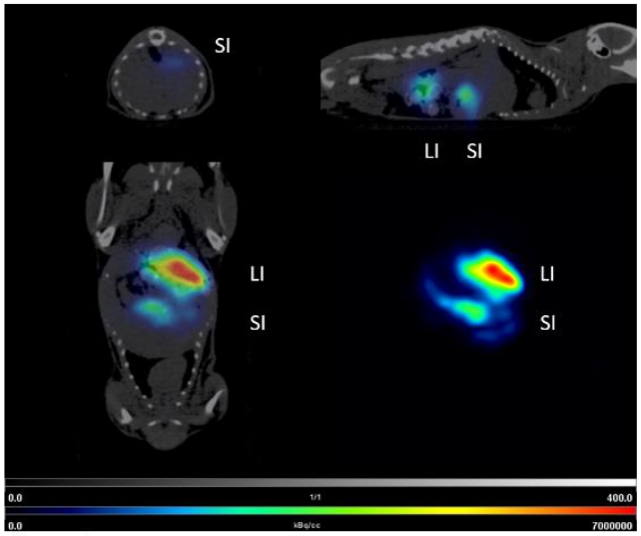
E.

MCP-^{99m}Tc I.V. (1h)



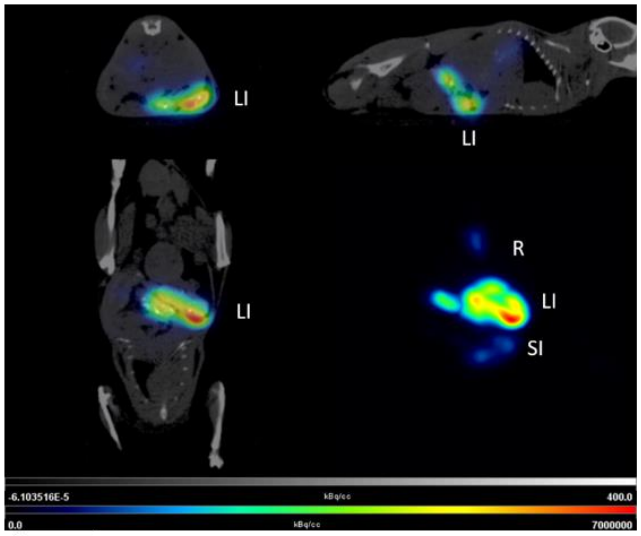
F.

MCP-^{99m}Tc oral (1h)



G.

MCP-^{99m}Tc oral (4h)



H.

FIGURE 4: MCP-^{99m}Tc exhibits renal/hepatobiliary elimination when administered intravenously and low abortion and gastrointestinal elimination when administered orally. (A) Stability *in vivo* assay of MCP-^{99m}Tc administered I.V. (B) Stability *in vivo* assay of MCP-^{99m}Tc administered orally. (C) Kinetics of MCP-^{99m}Tc administered intravenously. (D) Kinetics of orally administered MCP-^{99m}Tc. %D.I.: percentage of injected dose. (E) Biodistribution study of MCP-^{99m}Tc administered orally and intravenously at 1 h. (F) µSPECT/CT image study of MCP-^{99m}Tc administered intravenously at 1 h (B: bladder). (G) µSPECT/CT image study of MCP-^{99m}Tc administered orally at 1 h (SI: small intestine, LI: larger intestine) (H) µSPECT/CT image study of MCP-^{99m}Tc administered orally at 4 h (SI: small intestine, LI: larger intestine C: rectum). Data are representative of three independent experiments.

3.5. Galectin-3 influences MCP-^{99m}Tc blood compartmentalization, biodistribution, and IV pharmacokinetics *in vivo*

The blood compartment distribution assay was performed to verify the behavior of MCP in the blood, whether it is maintained in plasma proteins, cells, or soluble fraction, in addition to analyzing whether galactin-3 influences this distribution. A blood compartment distribution assay was performed using C57BL/6 Lgals3 $^{+/+}$ mice (normal galectin-3 expression) and C57BL/6 Lgals3 $^{-/-}$ mice (galectin-3 knockout). 10–20 MBq of MCP-^{99m}Tc was injected intravenously. Venous blood samples were collected at 5, 15, 30, 45, and 60 min p.i. via the tail vein and processed further. Blood cells were separated by centrifugation (12,000 rpm for 5 min). Protein precipitation in the supernatant was achieved by adding methanol (100 µL) to the plasma sample and centrifuging (12,000 rpm for 5 min). The fractions of blood cells, proteins, and plasma were measured using an automatic gamma counter to determine the radioactivity per sample. The results showed that C57BL/6 Lgals3 $^{+/+}$ have more MCP-^{99m}Tc in plasma proteins, with a % total radioactivity counts that varies from 56.01 ± 3.14% in 5 min to 32.24 ± 2.19% in 60 min, followed by a regressive reduction of soluble fraction concentration of 26.47 ± 2.79% in 5 min and 7.12 ± 1.89% in 60 min. It was also verified that the concentration of MCP-^{99m}Tc in blood cells for up to 1 h showed little variation, remaining at 20% (Figure 5 A). In C57BL/6 Lgals3 $^{-/-}$ mice, there was more MCP-^{99m}Tc in the blood plasma in 5 min (56.09 ± 1.38 %), followed by regression of concentration in longer times up to 60 min (7.64 ± 0.43 %). In protein plasma, the concentration of MCP-^{99m}Tc showed little variation, remaining at 22.7%, and in the cell blood, the greater concentration was 22.12 ± 1.59% in 5 min, followed by a reduction until 1 h with 8.7% of MCP-^{99m}Tc (Figure 5 B). In short, the blood compartment distribution assay showed that MCP-^{99m}Tc has a high affinity for plasma proteins and cells in C57BL/6 Lgals3 $^{+/+}$ mice, and this affinity was partially lost when galectin-3 was deleted in C57BL/6 Lgals3 $^{-/-}$ mice.

A biodistribution study using MCP-^{99m}Tc in mice knockout for galectin-3 was made with the objective to verify whether galectin-3 influences the distribution of MCP in the body. C57BL/6 Lgals3 $^{+/+}$ and C57BL/6 Lgals3 $^{-/-}$ mice were intravenously injected with 10 MBq of MCP-^{99m}Tc. One hour after injection, the mice were euthanized, and the organs of interest were harvested, rinsed in PBS, weighed, and counted in an automatic gamma counter to quantify the percentage of injected dose per gram of tissue (%ID/g). The results showed that the main route of elimination of MCP-^{99m}Tc in both groups was renal, with high activity in the bladder (average of 45%ID/g) and kidney, followed by high activity in the liver (average of 2.7%ID/g) and intestine (average of 1.9%ID/g), indicating hepatobiliary elimination. However, a significant difference was observed in the kidneys of C57BL/6 Lgals3 $^{+/+}$ (5.92 ± 2.65%ID/g) and C57BL/6 Lgals3 $^{-/-}$ (11.1 ± 1.63%ID/g), as well as in the thyroid with 2.22 ± 0.05%ID/g in C57BL/6 Lgals3 $^{+/+}$ and 1.24 ± 0,07%ID/g in C57BL/6 Lgals3 $^{-/-}$mice, possibly due to filtration rates in the kidneys (clearance) of MCP-^{99m}Tc, as shown in the pharmacokinetics study (Figure 5 C and Table 3).

Log-P measurements are fundamental physicochemical parameter relationships in medicinal chemistry. In this parameter, the smaller the log P in relation to O, the more hydrophilic the molecules, and the larger the log P in relation to 0, the more lipophilic they are (Wilson et al., 2001). The partition coefficient of MCP-^{99m}Tc was determined by extraction in a physiological saline sample (0.9% NaCl) system with n-octanol as the aqueous and organic phase. In a tube containing 500 µL of each phase, 50 µL (< 1 MBq) of labeled compound solution was added. The vial was capped and shaken vigorously for 5 minutes at room temperature. After stirring, the mixture was centrifuged at 2000 × g for 2 min to separate the layers. Aliquots (100 µL) were removed from each phase, the amount of radioactivity present in each phase was measured using a gamma counter, and the partition coefficient (P) values were determined. In this study, we verified that the log P value of MCP was 1.78 (Table 2). Thus, MCP was shown to be highly lipophilic, which is a possible explanation for its affinity for plasma proteins such as serum albumin but does not explain the loss of affinity in C57BL/6 Lgals3 $^{-/-}$ mice or the difference observed in blood cells.

A pharmacokinetic study was performed to evaluate the influence of gelatin-3 on the IV kinetic behavior of MCP. Pharmacokinetic studies were performed on C57BL/6

Lgals3 $^{+/+}$ and C57BL/6 Lgals3 $^{-/-}$ mice. 100ul–10-20 MBq MCP-^{99m}Tc was injected into the tail vein of the animals. Venous blood samples were collected at 0.001, 5, 10, 15, 30, 60, 120, 240, and 1440 min p.i. via the tail vein. Blood sample was collected (5µL) from the bleeding site in a microtube. Blood activity was measured to determine the radioactivity per sample. The radiological activity was corrected for the half-life of radionuclides, and the injected activity and mass were correlated. The half-lives of distribution and elimination were determined using a statistical equation. We used a nonlinear exponential regression of the "two-phase decay" type and obtained a two-dimensional exponential plot with two half-lives (distribution and elimination). The clearance (CL) values were calculated using the area on the curve, calculated using the statistical equation of the x/y analysis. The volume of distribution (Vd) was calculated using the CL and $T_{1/2}$. This study showed a significant difference between the pharmacokinetic of MCP-^{99m}Tc in C57BL/6 Lgals3 $^{+/+}$ and C57BL/6 Lgals3 $^{-/-}$ mice in some parameters (*$p < 0.05$) (Table 3). There is no a significant difference of the distribution half-life ($T_{1/2\ \alpha}$) between C57BL/6 Lgals3 $^{+/+}$ (0.77 ± 0.06 min) and C57BL/6 Lgals3 $^{-/-}$ (2.40 ± 1.06 min). This result demonstrates that MCP reaches its targets at the same speed in the presence or absence of galectin-3. The elimination half-life ($T_{1/2\ \beta}$) of the C57BL/6 Lgals3 $^{+/+}$ (502.89 ± 35.37 min) was significantly higher than the C57BL/6 Lgals3 $^{-/-}$ (303.53 ± 30.63 min), demonstrating that the elimination of MCP in mice without galectin-3 expression is faster. These results are corroborated by the values of Clearance (CL) that which showed a significant difference between C57BL/6 Lgals3 $^{+/+}$ (1693.64 ± 368.39 µl/min) and C57BL/6 Lgals3 $^{-/-}$ (2863.14 ± 129.40 µl/min). However, the volume of distribution (Vd) did not show a significant difference between C57BL/6 Lgals3 $^{+/+}$ (1007865 ± 496145µl) and C57BL/6 Lgals3 $^{-/-}$ (1023093 ± 465654µl), proving that the tissue/blood ratio does not change with galectin-3 deficiency (Table 3). These results showed that the MCP elimination speed was greater in the C57BL/6 Lgals3$^{-/-}$ group than in the C57BL/6 Lgals3 $^{+/+}$ group, indicating that the lack of galectin-3 increases MCP elimination, possibly due to a lack of active sites for MCP in the blood and other tissues.

These results show that the blood compartment distribution, biodistribution, and pharmacokinetic of MCP-^{99m}Tc are influenced by galectin-3 expression in vivo, especially with regard to its distribution in the blood and its elimination speed.

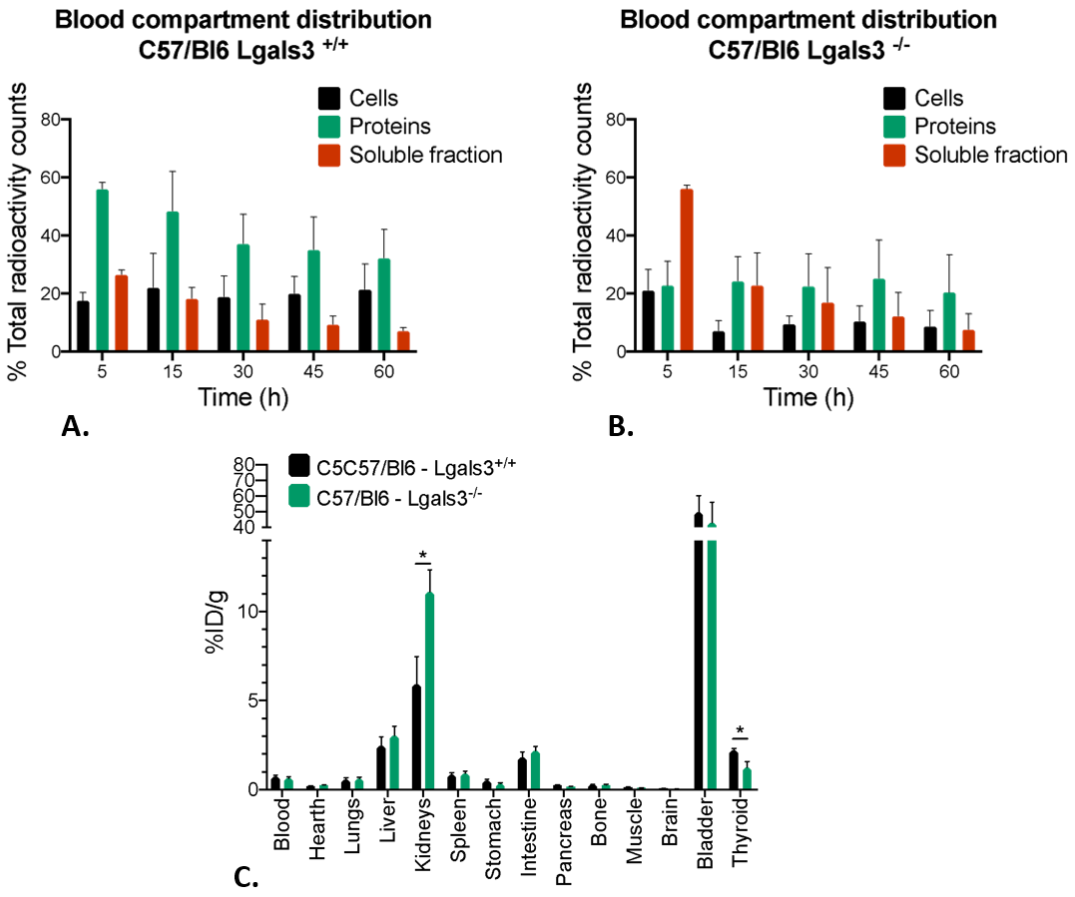


Figure 5: Galectin-3 influences MCP-^{99m}Tc blood compartmentalization and biodistribution *in vivo*. (A) Percentage of total radioactivity counts of MCP-^{99m}Tc in the blood compartment distribution in C57BL/6 Lgals3 $^{+/+}$ mice. (B) Percentage of total radioactivity counts of MCP-^{99m}Tc in the blood compartment distribution in C57BL/6 Lgals3 $^{-/-}$mice. (C) The biodistribution of MCP-^{99m}Tc administrated I.V. at 1 hour in C57BL/6 Lgals3 $^{+/+}$ and C57BL/6 Lgals3 $^{-/-}$. Data are representative of three independent experiments. *p>0.05.

Table 2: Partition coefficient (log P) of MCP-^{99m}Tc. Data are representative of three independent experiments.

Molecule	Partition coefficient (*P*)	Partition coefficient (*log P*)
MCP-^{99m}Tc	**60.57 ± 3.57**	**1.78**

Table 3: Galectin-3 influences MCP-^{99m}Tc pharmacokinetics. Parameters presented: distribution half-life ($T_{1/2\ \alpha}$), elimination half-life ($T_{1/2\ \beta}$), clearance (CL), and volume of distribution (Vd) of MCP-^{99m}Tc in wild type (WT) and Knockout gal3 mice (KO). Data are representative of three independent experiments.

PK parameter	Control WT	Knockout KO	P value
Distribution Half-life (min)	0.77 ± 0.06	2.40 ± 1.06	0.1653
Elimination Half-life (min)	502.89 ± 35.37	303.53 ± 30.63	0.0027
Clearance (µl/min)	1693.64 ± 368.39	2863.14 ± 129.40	0.0172
Volume of distribution (µl)	1007865 ± 496145	1023093 ± 465654	0.9827

4. Discussion

In this study, we radiolabeled MCP with ^{99m}Tc and verified the biodistribution and pharmacokinetics of MCP-^{99m}Tc in oral and intravenously routes of administration. First, the structure and monosaccharide composition of MCP were investigated. Pectin is a complex polysaccharide with many sources and a variable number of substructures, including homogalacturonans (HG), rhamnogalacturonans (RG-I and II), and xylogalacturonan (Maxwell et al., 2012). The monomeric structure of polysaccharides is also extremely variable and depends on the food source, extraction method, and modification strategy used (Pedrosa et al., 2022). Analyses of MCP used in this work were carried out through their separation into four fractions by HPSEC-RID: (1) MCP > 30 kDa (MCP30); (2) MCP between 30 and 10 kDa (MCP30/10); (3) MCP between 10 and 3 kDa (MCP10/3); and (4) MCP lower than 3 kDa (MCP3). The yield %, peak molecular weight, and maximum and minimum values of the MCP fractions were quantified (Figure 1 A, B, C, and D). The composition of monosaccharides in MCP samples was considered, including fucose (Fuc), arabinose (Ara), rhamnose (Rha), galactose (Gal), glucose (Glc), xylose (Xyl), mannose (Man), and uronic acids (D-glucuronic and D-galacturonic acids) (GalA). The main component of the MCP samples was GalA, while MCP30 is rich in Ara (6.10 ± 2.08 %), Rha (19.11 ± 4.11 %) and Gal (19.15 ± 5.26 %), and MCP3 has a significant concentration of Gal (16.71 ± 4.91 %) (Figure 1 E and table 1). These results demonstrate the diversity of molecular weights and monosaccharides within MCP and are fundamental for predicting its impacts on human health, molecular modulation, and mechanism of action.

We radiolabeled MCP with ^{99m}Tc to study its behavior in an organism. For this, was use a direct ^{99m}Tc radiolabeling protocol for MCP-^{99m}Tc production, and for the first time, we demonstrated the radiolabeling of MCP with a radiochemical purity bigger than > 95%. All controls of saline, saline at different pH (to mimic stomach pH), and blood *in vivo* were prepared and showed good radiochemical purity (Figure 2 and 4 A and B). This result demonstrates that the MCP^{99m}Tc radiolabeling protocol is efficient and that the molecule is stable. The protocol for direct labeling of polysaccharides with technetium was developed by Sanguri et al. (2016), where a similar radiochemical efficiency was verified for the production of Mannan-^{99m}Tc, a complex carbohydrate molecule TLR2

agonist used in many pharmacological applications. Colon scintigraphy evaluation studies performed with technetium-labeled pectin have also shown efficient radiochemical purity and have interesting application in intestinal flow studies (Hodges et al., 2009; Majumdar et al., 2011).

One of the most explored biological activities of MCP is the possibility of targeting galectin-3 protein regulation, a protein related to several pleiotropic functions in the body. Interestingly, in this work was show that MCP30 and MCP3 are rich in Gal, a known inhibitor of galectins (Dahlqvist et al., 2019). Therefore, we studied the inhibition and binding affinity of MCP for galectin-3 using hemagglutination and Sepharose/Gal-3 assays. The hemagglutination studies demonstrated that MCP partially inhibited Gal-3 and that MCP3 showed an inhibition capacity in a concentration of 25 mg/ml (Figure 3 A – D). Sepharose/Gal-3 assay demonstrated that MCP has a partial affinity for galectin-3, with 20.01 ± 3.8% of retention, and MCP3 had a higher affinity for galectin-3, with 37.47 ± 7.94 % of retention (Figure 3 E - H). These inhibition and affinity data show that there are fractions within MCP that can partially bind and inhibit galectin-3 protein, which is important for the discussion of the mechanism of action of MCP. Important studies related to molecular modeling of Gal-3 inhibition have demonstrated that low molecular weight sugar molecules (<1Kda) have a high potential for protein-inhibition ligands (Blanchard et al., 2016; Laaf et al., 2017; Stegmayr et al., 2016). The monosaccharide galactan and galactosyl residues contribute to the pharmacological effects observed between MCP and galectin-3, because of its connection site β-D-galactopyranoside (Gao et al., 2013; Gunning et al., 2009). However, other monomeric compounds such as monosaccharides Gal*p*A and Ara*f*, in addition to galactosyl, are important targets of interest, demonstrating high inhibition of galectin-3 through techniques such as hemagglutination assays (G3H), surface plasmon resonance assays (SPR), and binding through biolayer interferometry assays (BLI) (Cui et al., 2019; Shi et al., 2017; Wu et al., 2020; L. Zhou et al., 2020). Thus, structural studies of MCP are essential to unravel the pharmacological mechanisms of action of these molecules, and more studies are needed for further details.

With regard to hemagglutination assays involving Gal-3, studies have shown both positive and negative interactions between pectin and Gal-3. The uronic fraction of

pectin extracted from the pulp of papayas extracted from intermediated phases of ripening presented the biological effects and galectin-3 inhibition, with MIC (minimum inhibitory concentrations) of 0.08 µg/µL in hemagglutination assay, and positive viability assays using HT-29 and HCT-116 (colorectal cancer cells) (0.2, 0.1, 0.05 and 0.025% – w/v) (Pedrosa et al., 2020). Another study demonstrated that papaya pectin induced necrosis and decreased viability in HCT116, HT29, and PC3 cells (colon and prostate cancer models); however, the hemagglutination assay did not show inhibition of recombinant human Gal-3 protein, indicating that it activity is independent of Gal-3 (Prado et al., 2017). A distinct study showed that MCP3 (fraction of MCP smaller than 3 kDa) and MCP30/10 (fraction of MCP between 30KDa and 10 kDa) of CP (treated citrus pectin) improved anticancer activity by inhibiting the aggregation, migration, and proliferation of HCT116, HT29, and PC3 cells in a cell line-dependent manner. This work also showed that the MCP3 fraction in the hemagglutination assay inhibited Gal-3 at a lower concentration (300 µg/ml) than the higher molecular weight fractions or brute fraction (400-500ug/ml) (do Prado et al., 2019). Zhang et al. (2016) debated the limited use of brute MCP due to its large size, which acts as an obstacle, and showed that MCP-2, 6-116KDa rich in RG-I (β1,4-galactan) is the best inhibitor of Gal-3-mediated agglutination, with an MIC of 0.06 µg/ml (ten-fold more potent than parent MCP), and the RG-I-rich pectins with (1 → 4)-linked β-D-galactan side chains are more active than other fractions (Zhang et al., 2016). Despite all the data presented and the diverse binding sites and different chemical conformations that explain the interaction between these polysaccharides and Gal-3, the mechanism of action is not fully understood. Furthermore, the range of mechanisms of action unrelated to Gal-3 specific inhibition cannot be ignored, such as systemic immunological modulation (Pedrosa et al., 2022).

In the animal studs, the stability in plasma assay was perform *in vivo* administrated orally or intravenously. We verified that MCP^{-99m}Tc injected intravenously was stable for 1 h with a radiochemical purity greater than 95% and MCP^{-99m}Tc injected orally has partially stable for 4 h with a radiochemical purity greater than 87% (Figure 4 A and B). These results corroborate the stability data obtained with blood plasma and saline at different pH values, showing that MCP^{-99m}Tc administered I.V. is stable in the blood and suffers the effects of pH variation when passing through the stomach during

oral administration. The intravenous kinetics study showed that MCP-^{99m}Tc has a progressive elimination, with the highest 1.3 ± 0.24 % total radioactivity counts in the first minute, a %D.I. equivalent to approximately 3.9 mg/5µl, and an elimination half-life of 502.89 ± 35.37 min (Figure 4 C and Table 3). The oral kinetics study, however, showed that MCP-^{99m}Tc could be absorbed by the gastrointestinal tract, but at low concentrations, with the best absorption time of 1 hour with $5.27x10^{-6} \pm 7.5\times10^{-7}$ % total radioactivity counts (%D.I.), a %D.I. equivalent to an effector dose of approximately 37,6 pg/5µl that reaches the circulation system (Figure 4 D). These data are important to show that MCP administered orally, compared to IV administration, does not have a significant absorption. In addition, the vast majority of MCP studies in animal models and patients (clinical studies) have used oral administration (usually in drinking water or capsules). Other less frequent routes of administration are intravenous, intraperitoneal, intratumoral, intrathecal, and intracerebroventricular injection (Andrews et al., 2020; Guess et al., 2003; Keizman et al., 2021; Fernandez-García et al., 2017; Lau et al., 2021; Ma et al., 2020; Eliaz et al., 2006; Eliaz et al., 2007; Sun et al., 2021; Zhao et al., 2008; Eliaz et al., 2019; Nishikawa et al., 2018; Ma et al., 2016; Cui et al., 2022; Xue et al., 2019; Wang et al., 2020).

The biodistribution and image µSPECT/CT studies using MCP-^{99m}Tc were performed orally and intravenously to verify the route of elimination and the organs in which MCP was found *in vivo*. The IV biodistribution showed that the main route of elimination of MCP-^{99m}Tc is a renal and hepatobiliary elimination pathway, verified by the activities taken up in the kidneys, bladder, and liver in 1 hour (Figure 4 E). The oral biodistribution study showed that MCP-^{99m}Tc presents a renal elimination route for the absorbed molecules, with activity detected in the kidney, bladder and pancreas, however, the main route of elimination becomes gastrointestinal, with high activities in the stomach, large intestine and small intestine in 1 hour (Figure 4 E). The imaging study with MCP-^{99m}Tc administrated IV showed high activity in the bladder, confirming renal elimination after 1 h (Figure 4 F). However, the oral imaging study with MCP-^{99m}Tc showed high activity in the small intestine and larger intestine at 1 h time and in the small intestine, larger intestine, and rectum at 4 h, confirming progressive gastrointestinal elimination (Figure 4 G and H). These results can be compared with work

made by Sanguri et al. 2016 which Mannan-^{99m}Tc was administered intravenously, intraperitoneally, and orally. Mannan-^{99m}Tc has renal and hepatobiliary elimination in IV and intraperitoneally administration, and no absorption when administered orally. Mannan is a complex carbohydrate that remains indigested in the gut owing to its bulky structure, which restricts enzyme access (Cuskin et al., 2015). However, in the case of pectin, evidence demonstrates that MCP can be fermented by groups of enzymes produced by the bacterial flora of the intestines, depending on the degree of esterification and methylation of the sample (Dongowski et al., 2000, 2002). Evidence also shows that there is a group of bacteria in the microbiota, unique amongst Firmicutes known to be present in the human gut, called *Monoglobus pectinilyticus*, which has a highly specialized glycobiome for MCP transformation. Its genome encodes a simple set of metabolic pathways relevant to pectin sugar degradation, with an unusual distribution of carbohydrate-active enzymes (CAZymes) with numerous extracellular methyl/acetyl esterases and pectate lyases, demonstrating that MCP can be metabolized in the human intestine (Kim et al., 2019).

The discussion about the mechanisms of action of orally administered MCP considers some hypotheses, such as pectin structures influencing the gastrointestinal immune barrier, impacting the intestinal microbiota, or directly interacting with immune cells (Beukema et al., 2020). Studies have demonstrated that pectin is degraded by colonic enzymes but is unresponsive to upper gastrointestinal enzymes, and is a promising biopolymer for the construction of microbial-triggered colon-specific carriers (Das, 2021). Other studies have shown that oral administration of MCP from broccoli (*Brassica oleracea*) in mice increased the number of activated macrophages and lymphocytes (Busato et al., 2020). Despite this, MCP can be an indigestible polysaccharide by human enzymes, but can be easily degraded by gut bacteria through the production of short-chain fatty acids. The interaction between pectin and gut microbiota affects the gut microbiome, and fermentation products produced in response to gut bacteria can affect the immune system (Elshahed et al., 2021). However, others studies have shown a systematic and direct effect of oral administration of MCP. A study showed that antibody recognition of RG-I from *Bupleurum falcatum* is present and reactive in the bloodstream of mice after oral treatment with MCP, demonstrating partial

absorption of MCP (Maxwell et al., 2012; Sakurai et al., 1996). A hypothesis of MCP absorption is related to the activity of galactoside-terminal glycoprotein transporters, such as asialoglycoprotein receptors, that could play a role in absorbing MCP throughout the intestine (Majee et al., 2018; W. Zhang et al., 2015). These data with our results indicate that the biological effect of oral administration of MCP may be indirect and not dependent on its gastrointestinal absorption. However, further studies are required.

The blood compartment distribution assay was performed to verify the behavior of MCP in the blood, whether it is maintained in plasma proteins, cells, or plasm, in addition to analyzing whether galactin-3 influences this distribution. We evaluated MCP-^{99m}Tc blood compartment distribution in both C57BL/6 wild-type (Lgals3$^{+/+}$) and galectin-3 knockout (Lgals3$^{-/-}$) mice after intravenous injection. This analysis showed that MCP-^{99m}Tc mainly bound to blood proteins and blood cells in Lgals3$^{+/+}$ mice, and to a lesser extent, in the soluble fraction (Figure 5 A). In lgals3$^{-/-}$ mice, on the other hand, 5 min post-injection, MCP-^{99m}Tc was found mostly in the soluble fraction of blood and, in a lower amount, in blood cells and proteins, being gradually excreted from the body (Figure 5B). These data indicate that MCP-^{99m}Tc has a higher affinity for blood proteins and cells in Lgals3$^{+/+}$ mice than in lgals3 $^{-/-}$ mice. A higher binding to blood proteins and cells may translate into an increased blood circulation time of MCP-^{99m}Tc in Lgals3$^{+/+}$ mice in comparison to lgals3$^{-/-}$ mice, as observed in the pharmacokinetic study (Table 3).

The *Log P* measurements are fundamental physicochemical parameter relationships in medicinal chemistry and are important in areas of cell membrane penetration (including skin and blood barrier penetration), multiple drug resistance, enzyme inhibition, receptor affinity, and blood protein binding (Nario et al., 2022; Ortega Pijeira et al., 2020; Wilson et al., 2001). To verify the lipophilicity of MCP-^{99m}Tc, a partition coefficient study was carried out, and we verified that the *log P* of MCP was 1.78 (Table 2). Thus, MCP was shown to be highly lipophilic, which is a possible explanation for its affinity for plasma proteins such as serum albumin, but does not explain the loss of affinity in C57BL/6 Lgals3 $^{-/-}$ mice. Evidence showing the presence of galectin-3 in fluids, such as blood plasma and urine, is well known and is used as a prognostic and biomarker for the diagnosis of several diseases, such as heart disease, kidney disease, schizophrenia, sarcoidosis, diabetes mellitus, and cancer. The Laboratory

reference ranges (Laboratory Corporation of America, Burlington, N.C., USA) of Gal-3 concentration in human blood is about 17.8 – 22.2 ng/ml (Andrejic et al., 2019; Dong et al., 2018; Eliaz, 2013; Kara et al., 2022; Kılıç et al., 2020; S. Liu et al., 2022). In blood, galectin-3 is expressed on endothelial cells, red blood cells, platelets, microparticles, and leukocytes and possesses prothrombotic properties in the context of venous thrombosis (DeRoo et al., 2015). Our hypothesis is that the differences in blood compartmentation of MCP between Lgals3$^{+/+}$ and Lgals3$^{-/-}$ mice may be due to the binding of MCP to galectin-3 receptors in blood plasma and blood cell membranes.

A biodistribution study using MCP-^{99m}Tc in mice knockout for galectin-3 was made with the objective to verify whether galectin-3 influences the distribution of MCP in the body. One-hour post-injection we could observe that MCP-^{99m}Tc was mainly found in the bladder, kidneys and liver of both Lgals3$^{+/+}$ and Lgals3$^{-/-}$ mice, suggesting that MCP presents a renal/hepatobiliary excretion *in vivo* independent of galectin-3 (Figure 5 C). However, in the kidneys %I.D./g, we observed a significant increase in MCP-^{99m}Tc activity in Lgals3$^{-/-}$ mice (11.1 ± 1.63%ID/g), compared to that in Lgals3$^{+/+}$ mice (5.92 ± 2.65%ID/g), which could suggest a faster elimination of MCP-^{99m}Tc through the kidneys in Lgals3$^{-/-}$ mice, as shown in the pharmacokinetics study (Table 3). In pharmacokinetics study, was shown a significant difference between the kinetic behavior of MCP-^{99m}Tc in C57BL/6 Lgals3 $^{+/+}$ and C57BL/6 Lgals3 $^{-/-}$ mice in some parameters (Table 3). There is no a significant difference of the distribution half-life ($T_{1/2\,\alpha}$) between C57BL/6 Lgals3 $^{+/+}$ and C57BL/6 Lgals3 $^{-/-}$. This result demonstrates that MCP reaches its targets at the same speed in the presence or absence of galectin-3. The elimination half-life ($T_{1/2\,\beta}$) of the C57BL/6 Lgals3 $^{+/+}$ (502.89 ± 35.37 min) was significantly higher than the C57BL/6 Lgals3 $^{-/-}$ (303.53 ± 30.63 min), demonstrating that the elimination of MCP in mice without galectin-3 expression is faster. These results are corroborated by the values of Clearance (CL) that which showed a significant difference between C57BL/6 Lgals3 $^{+/+}$ (1693.64 ± 368.39 µl/min) and C57BL/6 Lgals3 $^{-/-}$ (2863.14 ± 129.40 µl/min). However, the volume of distribution (Vd) did not show a significant difference between C57BL/6 Lgals3 $^{+/+}$ and C57BL/6 Lgals3 $^{-/-}$, proving that the tissue/blood ratio does not change with galectin-3 deficiency (Table 3). These results showed that the MCP elimination speed was greater in the C57BL/6 Lgals3$^{-/-}$ group than in the C57BL/6 Lgals3 $^{+/+}$ group, indicating that the

lack of galectin-3 increases MCP elimination, possibly due to a lack of active sites for MCP in the blood and other tissues. Thus, these results show for the first time that galectin-3 influences the blood compartmentalization, biodistribution, and pharmacokinetics of MCP, conditioning its elimination, mainly through its binding to plasma proteins and cells in the blood. This result describes the behavior of MCP *in vivo* and may contribute to our understanding of the pharmacological mechanisms of action involved in its various applications.

5. Conclusion

Altogether, our data demonstrate in an unprecedented way that MCP can be radiolabeled with ^{99m}Tc with good radioactive purity, and that this technology can be used as a tool to study the biological activity of MCP *in vivo*. We determined the structure and monosaccharide composition of MCP and its partial affinity and inhibitory capacity against galectin-3. We demonstrated that IV administration of MCP-^{99m}Tc resulted in renal/hepatobiliary elimination and that oral administration of MCP resulted in low gastrointestinal absorption and intestinal elimination. Moreover, we verified the influence of Gal-3 on biodistribution, blood compartmentalization, and IV pharmacokinetics of MCP-^{99m}Tc *in vivo*. These results describe the pharmacological behavior of MCP *in vivo* and may contribute to our understanding of the mechanisms of action involved in its various applications.

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CHAPTER 3

STUDY OF ANTICANCER ACTIVITY OF MCP USING MOLECULAR IMAGING

STUDY OF ANTICANCER ACTIVITY OF MCP USING MOLECULAR IMAGING

Abstract

Cancer is one of the main public health problems worldwide and is the second leading cause of death. Modified citrus pectin (MCP) is a polysaccharide found abundantly in the primary wall of plants and shows activity in several areas of the food industry and nutrition. MCP can also act as an anti-tumorigenic molecule in several types of tumors, in addition to avoiding chemoresistance, modulating the immune system, and preventing acute and chronic renal disease caused by radiotherapy and chemotherapy in cancer treatments. In this study, we investigated the *in vivo* anticancer activity of MCP using molecular imaging. To this end, we radiolabeled MCP with ^{99m}Tc and analyzed the behavior of MCP-^{99m}Tc in animals with SKOV-3 and MKN45 tumors. First, we demonstrated that cell binding and internalization of MCP were only partially influenced by Galectin-3 expression *in vitro* using SKOV-3 scrambled and SKOV-3 shRNAGal3 cells (knockdown of Gal-3 expression). Next, we showed that MCP (20 mg/kg) exhibited anticancer activity in a SKOV-3 cell tumor xenograft model, reducing tumor growth by 48.5% and tumor weight by 50% when administered intravenously; however, oral administration of MCP (200 mg/kg) did not show an anticancer effect. Subsequently, using biodistribution, autoradiography, and μSPECT/CT imaging studies, we demonstrated that MCP-^{99m}Tc reached the tumor and bound to regions of necrosis in the SKOV-3 cell tumor xenograft model when administered intravenously. Finally, we demonstrated that IV administration of MCP (10 mg/kg) in mice with SKOV-3 tumors (lower expression of Gal-3) and MKN45 tumors (greater expression of Gal-3) reduced tumor growth by 58.7% and 35.4%, respectively, and reduced the tumor weight by 51.7% and 30.7 %, respectively. Furthermore, in both tumor xenografts, MCP-^{99m}Tc reached the tumor at the same proportion. Thus, these results do not corroborate the hypothesis of the direct pharmacological effect of MCP exclusively targeting Gal-3 and contribute to the knowledge of MCP anticancer activity.

1. Introduction

Cancer is one of the main public health problems worldwide and the second leading cause of death globally, responsible for 10 million deaths in 2020 (GICR, 2020;

WHO, 2020). The estimates indicate 1.9 million of the new cancer cases diagnosed and 609,360 cancer deaths in the United States in 2022 and, 625.000 new cases of cancer in Brazil, reaching up to 704,000 by 2025 (American Cancer Society 2022; MS/INCA 2022; Siegel et al. 2022). Thus, new alternatives to cancer treatment are important.

Pectin is a polysaccharide first identified in 1790 in apple pomace as a component of plant cells (Vauquelin, 1790; Leclere et al., 2013). Pectin is a flexible, thin, and hydrostatic sugar that is abundant in plant cell primary walls and serves as a barrier, hydraulic control, steering plant cell expansion, cell protection, cell-to-cell communication, cell differentiation, cell proliferation, cell adhesion, cell fate specification, morphogenesis, stress response, and environmental sensing (Duan et al., 2020; Haas et al., 2020; Jonsson et al., 2021; Lampugnani et al., 2018; Wachsman et al., 2020). This macromolecule can be obtained from different sources, such as from fruit (citrus and apple), sugar beet, eggplant, pomelo, chamomile, durian, pomegranate cashew pulp, hibiscus (*sabdarffa* L.), passion, tomato, banana, mango, jackfruit, artichoke (*Cynara scolymus* L.), sisal, tobacco and cocoa (Mellinas et al. 2020). These polysaccharides can be extracted using enzymes, water, buffers, ionic liquids, different pH, chelating agents, alkalis and acids, ultrasound, microwave-assisted surfactants, hot water, CDTA, and sodium carbonate, resulting in pectins with different structures and molecular weights (Liu et al., 2020; Su et al., 2019; Zdunek & Pieczywek, 2021, Gharibzahedi et al., 2022).

Pectin is a complex heteropolysaccharide polymers. The your primary structure are a family of covalently linked galacturonic acid-rich polymers with five main polysaccharides identified; homogalacturonan (HG), apiogalacturonan, rhamnogalacturonan-I (RG-I), xylogalacturonan, and rhamnogalacturonan-II (RG-II) with substituted galacturonans and secondary structures (Erdmann, Iacomini, and Cordeiro 2017; Gawkowska, Cybulska, and Zdunek 2018; Leclere, Cutsem, and Michiels 2013; Wu et al. 2020a). The pectin studied in this work was modified citrus pectin (MCP), which was isolated from citrus and extracted at different pH with alkaline treatment, which generated ß-elimination reactions, depolymerization of the polysaccharide backbone, and deesterification in HG regions. The next step was the cleavage of the neutral sugar

with acid treatment, dismissing the pectin backbone, arabinose residues, and branched regions. Thus, several arabinogalactans and galactans are produced (Leclere et al., 2013).

Pectin can be used as a dietary fiber being degraded by colon intestine microbial species, and not enzymatically digested from small intestine, maintaining its gelling property and slowing intestinal (Lattimer and Haub 2010). This polysaccharide shows activity in several areas of the food industry and nutrition, such as food production, cholesterol reduction, and intestinal flow improvement. However, one of its most significant contributions is as an anticancer molecule, acting in several types of tumors *in vitro*, *in vivo* and in clinical studies, against cancers of prostate, ovarian, breast, liver, lung and colon (Yan & Katz, 2010; Hossein et al., 2013; Hossein et al., 2019a; Jiang et al., 2013a; Leclere et al., 2015; Sabra et al., 2019; Y. Wang, Balan, et al., 2010; Chauhan et al., 2005; Clark et al., 2012; O'Brien & Kay, 2011; Ruvolo et al., 2016; Streetly et al., 2010; Wang, Balan, et al., 2010; Demotte et al., 2014). The anticancer activity of MCP is related to the pharmacological direct effect, commonly related to galectin-3 protein, and indirect effect, related to the bacterial microbiota metabolism and immune system modulation (Aureli et al. 2023; Donadio et al. 2022). MCP can also avoid chemoresistance, modulate the immune system, and prevent acute and chronic renal diseases caused by radiotherapy and chemotherapy in cancer treatment (Silva et al., 2023, in production).

The galectin-3 (Gal-3) is a multi-functional lectin with 27 KDa, expressed by different cells, such as immune, endothelial, and epithelial cells. This protein is located in the membrane, cytoplasm, and nucleus, and can be secreted extracellularly, presenting many functions. The Gal-3 modulate numerous biological processes through its carbohydrate recognition domain and carbohydrate-independent mechanism, such as inhibition of apoptotic pathway, activation of cell proliferation, cell-cell junction, tissue remodeling and cell protection (Barondes et al., 1994; Cummings R.D., Liu F.T. Galectins. In: Varki A., Cummings et al., 2009; Davidson et al., 2002; Hsu & Liu, 2002; Kasai & Hirabayashi, 1996; Ochieng et al., 2002; Blanchard et al., 2014). It is overexpressed in altered tissues, has several important functions in the tumor microenvironment in many types of tumors, and is associated with tumor growth, metastasis, and homeostasis of tumor cells. Galectin-3 also regulates immune cells and promotes tumor-directed

immunosuppression. In addition, Gal-3 is involved in mitochondrial metabolism, glycolysis, and several signaling mechanisms in tumors, thus contributing to tumor progression (Guo et al., 2020; Kariya et al., 2018; Rinaldi et al., 2018; Ruvolo et al., 2019; Wang et al., 2019; Newlaczyl & Yu, 2011). The relationship between MCP and Gal-3 has been studied extensively, and its inhibition is considered one of the main pharmacological pathways that explains the mechanism of action of MCP (Silva et al., 2023, in production).

Molecular imaging is a term coined in the late 1990s and can be defined as "a type of medical imaging that provides detailed pictures of what is happening inside the body at the molecular and cellular level" according to the Society of Nuclear Medicine and Molecular Imaging (SOCIETY OF NUCLEAR MEDICINE & MOLECULAR IMAGING 2022). Unlike conventional imaging techniques, such as radiography, computed tomography (CT), and ultrasound, which offer pictures of the body's physical structure, molecular imaging allows the measurement of the biological and chemical processes of cellular component and study the functioning of the organism (Rowe and Pomper 2022). Fundamentally, molecular imaging allows the visualization and localization of biochemical processes in healthy or pathological tissues through specific targets that are invisible at the anatomical imaging level.

Molecular imaging is based on an imaging agent labeled with probes, such as fluorescent molecules, bioluminescence molecules, or radionuclides, usually administered intravenously or orally, which interact with the respective target. This target is a biomarker that is generally overexpressed in the studied tissues and can serve as a marker for radiotracers in nuclear medicine. Trackers can be small molecules, analogs of molecules, enzyme substrates or products, antibodies, nanobodies, peptides, sugars, aptamers, polyglycosaccharides, proteins, and nanoparticles that participate in metabolic pathways and bind to specific targets, such as enzymes, receptors, antigens, proteins, glycopolysaccharides, and glycoproteins. The other important component of molecular imaging is an applicable hardware, scanner, or sensor that detects the tracer in the body and translates this energy into spatial information using appropriate software (Böhmer et al., 2021; Du et al., 2019; Haider et al., 2020; Zhou & Lu, 2017).

Molecular imaging can improve and accelerate the preclinical steps of drug development, including the identification of appropriate targets, assessment of on-target and off-target effects, evaluation of dose response, drug biodistribution, blood compartmentalization, pharmacodynamics, and pharmacokinetics. This technology can assess disease-related processes and the biological effects of drugs, such as gene expression, metabolic activity, enzymatic activity, receptor availability, protein synthesis and trafficking, cell migration, and cell proliferation, *in vivo* and *in vivo*. Molecular imaging and pharmaceutical research have always been related, including tumor evaluation in oncology trials, joint space in osteoarthritis, and brain measurement in neurology (Lindner and Link 2018; Mezzanotte et al. 2017; Murphy, Patel, and McCarthy 2017; de Vries et al. 2019).

The ^{99m} technetium (^{99m}Tc) is a radionuclide that has several features that make it safer for pre-clinical study of drug development and nuclear medicine, such as biological half-life (1 day), short physical half-life (t1/2 = 6h), gamma energy of 140 keV (same wavelength used by conventional X-ray diagnostics), low isotope cost, easy handling and availability in pharmacological and nuclear medicine laboratories, and high sensitivity and measurements in physical equipment. ^{99m}Tc can be integrated into molecules that can bind to specific receptors, such as antigens, enzymes, proteins, lipids, glycoproteins, and sugars, and can be used in pharmacological and biological studies for various applications (Alberto, Braband, and Nadeem 2020; Hanna et al. 2020; Papagiannopoulou 2017; Richards, Tucker, and Srivastava 1982; Saad et al. 2022; Tahara et al. 2022).

In this study, we investigated the pharmacological activity of modified citrus pectin (MCP) in an *in vivo* ovarian and gastric cancer model, with oral and intravenous routes of administration, using molecular imaging to understand its anticancer effect and its mechanisms of action. These results are important for the study of the pleiotropic functions of MCP in cancer.

2. Materials and Methods

2.1. Radiolabeling of MCP and quality control

The MCP radiolabeling was performed according to Sanguri et al 2016 (Sanguri et al., 2016). MCP (PectaSol-C – Modified Citrus Pectin – ecoNugenics) (2.5 mg) was dissolved in normal saline and mixed with 20 µg stannous chloride ($SnCl_2$ 4 mg/ml in 0.01 N HCl solution nitrogenized for 5 min). The pH was adjusted to 7 with a 0.01 N NaOH solution. ^{99m}Tc (130 MBq) was added and the mixture was nitrogenized for 5 min and incubated for 25 min. The $SnCl_2$ concentration and pH were optimized for the maximum labeling efficiency (≥95%) and minimum colloid percentage. All labeling procedures were performed in a hot laboratory under lead shielding. Quality control assay of the MCP-^{99m}Tc complex was carried out by ITLC-SG (Agilent Technologies, CA, USA) ascending paper with two different mobile phases: 100% acetone and ethanol: ammonia: water (1:2:5). The labeling efficiency of the complex was determined by separating the radioactivity into complex (MCP-^{99m}Tc complex), free (^{99m}TcO4 -), and reduced hydrolyzed technetium states (R/H ^{99m}Tc), which were counted using a Perkin Elmer (Waltham, MA, USA) Wizard2 2480 automatic gamma counter.

2.2. Cell culture

The SKOV-3 (human ovary cancer) tumor cells were cultured in Dulbecco modified Eagle medium (DMEM, Gibco, Life technologies, MD, USA) supplemented with 10% of fetal bovine serum (FBS, Gibco, Life technologies, MD, USA), 1% sodium pyruvate and penicillin (100 U/mL) and streptomycin (100 g/mL) (Sigma-Aldrich, St. Louis, MO, USA), and kept at 37° C and 5% CO_2. The MKN45-mock (human gastric cancer) tumor cells ware cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco, Life technologies, MD, USA), supplemented with growth factors and 5% of fetal bovine serum (Gibco, Life technologies, MD, USA), penicillin (100 U/mL) and streptomycin (100 g/mL) (Sigma-Aldrich, St. Louis, MO, USA), and kept at 37° C and 5% CO_2. The cells were used at passages 2 and 6. For virus production, HEK293t cells (ATCC CRL-3216) were cultured in DMEM supplemented with 10% FBS, antibiotics, antimycotics, penicillin (100 U/mL), and streptomycin (100 g/mL (Sigma-Aldrich, St. Louis, MO, USA). Louis, MO, USA), and kept at 37° C and 5% CO_2. Mycoplasma contamination in cultured cells was examined using a Lonza Mycoplasma Detection Kit.

2.3. Western blotting

Western blotting was performed as described by Santos et al. 2017. The cells were lysed in RIPA buffer and 50µg of proteins were running using a Novex NuPAGE SDS-PAGE gel system (Invitrogen) and then transferred overnight to a PVDF membrane (Invitrogen). The membrane was incubated with anti-gal-3 produced by M3/38 hybridoma (M3/38, ATCC TIB166). Anti-β-actin peroxidase (A3854, Sigma-Aldrich) was used as the control. Biotinylated secondary antibodies anti-rat (BA-4001), were obtained from Vector Laboratories. Horseradish peroxidase (HRP)-conjugated secondary antibodies (DAKO) were detected using an enhanced chemiluminescence (ECL) reagent (GE Healthcare). ImageJ software was used to analyze the densitometry values of the western blot bands.

2.4. Generation of SKOV-3 galectin-3 knockdown cells

SKOV-3 galectin-3 knockdown was performed as described by Santos et al. 2017. SKOV-3 cell line were transduced with stable shRNA targeting galectin-3 (TRCN0000029305, Sigma) and negative control (SHC016, Sigma), generated after co-transfection of 30 µg of shRNA-containing plasmids with 15µg pPAX2 and 5 µg of pMDG.2 (Addgene) into the HEK293t packaging cell line using the CaCl2 method for viral production. The viral supernatant was recovered, and transduced cells were generated by infection with two multiplicity of infectious units (MOI) of shRNA lentiviral particles. The next day, cells were replaced with fresh medium, and selected with 2 µg/mL puromycin for 1 week. Galectin-3 knockdown (shRNA-gal-3) and negative control (scramble) cells were generated and subjected to western blotting to investigate galectin-3 expression.

2.5. Cell binding and internalization assay

Cell binding and internalization assays were performed based on previous reports (Oroujeni et al. 2019), with modifications. SKOV-3 scrambled and SKOV-3 shRNAGal3 (4×10^5 cells) were plated in a 6-well plate overnight and then incubated for one hour at 37°C in internalization study, or 4°C in cell binding study with 10 µCi (370 kBq) of MCP-^{99m}Tc per well, in the presence or absence of MCP (200 ×), sucrose 100 mM (negative control), and lactose 100 mM (positive control). For the inhibition study, the cells were pretreated for 30 min before adding the radiolabeled compound. At the end of the

incubation period, the supernatant was aspirated and cells were washed six times with ice-cold PBS. The cells were removed with a cell scraper, and transferred to a gamma counter tube. Cellular activity was measured using a Perkin Elmer Wizard2 2480 automatic gamma counter (Waltham, MA, USA). The quantification of protein was determined with Pierce™ BCA Protein Assay Kit, and percentage of injected dose by mass was determined (%D.I./µg).

2.6. Subcutaneous inoculation of tumor cells in mice

The animal models used were submitted for analysis by the ethics committee and biosafety committee of the Research Institute of Nuclear Energy (IPEN) under registration No. 16/22 and 2021/05, respectively. Subcutaneous inoculation of tumor cells was made in Balb/c nude mice. When the animals reached between 6 and 8 weeks of age, 1×10^6 SKOV-3 and MKN45 cells were inoculated subcutaneously. Each group consisted of five animals. When the tumors reached approximately 0.5 cm^3, biodistribution, µSPECT/CT imaging, and *in vivo* tumor assays were performed.

2.7. *In vivo* tumor assay

In the *in vivo* tumor assay, Balb/C nude mice were subcutaneously injected with xenografts of SKOV-3 cells (10^6 cells/animal) and treated with oral and intravenous (IV) administration of MCP. When the tumors reached approximately 50 mm3, the animals were divided into 4 groups and daily treated orally with Vehicle (saline 100µl); MCP (200 mg/Kg in 100µl); or daily I.V. with; vehicle (saline 100µl); MCP (20mg/kg in 100µl). In another study, Balb/c nude mice were subcutaneously inoculated with 1×10^6 SKOV-3 cells or MKN45 cells. When the tumors reached 50 mm^3, the animals were separated into three groups and treated daily with intravenous administration of MCP: vehicle (saline 100ul), MCP 5 mg/kg, and MCP 10 mg/kg for 21 days. Each group consisted of five animals. Tumor volume was measured each day using an automatic caliper and quantified using the formula 0.5 × larger diameter × smaller $diameter^2$. When the tumors reached approximately 1.6 cm^3 or after 21 days of the experiment, the animals were euthanized, and the tumor, organs, and blood samples were collected for later histological and biochemical analyses. The weight of the tumor, animals and their feed intake were monitored every seven days using a precision scale.

2.8. Histological analysis

In the histological analysis, the kidneys of mice groups were collected, cleaved, and immediately fixed in 4% buffered paraformaldehyde pH 7.4 for 24 h at 4 °C, after 21 days of treatment. The samples were dehydrated in increasing concentrations of ethanol (70–100%) for 30 min each and washed three times in xylene for 30 min. They were then individually embedded in paraffin, sectioned into 4 µm thick slices and stained with hematoxylin and eosin (H&E). Masson's trichrome (MT) staining was used to identify extracellular matrix deposits. Bright-field images were acquired using LAS X Core Leica Microsystems Software, Germany.

2.9. Biochemical analysis

For biochemical analysis, blood samples from healthy or animals treated with MCP or vehicle (saline) were collected and centrifuged in a microcentrifuge (Hettich MIKRO 185) at 10.000 rpm for 5 min for blood plasma separation. Blood plasma samples were analyzed with reagent kits; Transaminase AST (TGO) (aspartate aminotransferase/ oxalacetic transaminase) kinetic test (Bioclin KO48-9), Creatine Automated kinetic test (Bioclin), Alkaline phosphatase (FAL) IFCC kinetic test (Bioclin), Transaminase ALT (TGP) (alanine aminotransferase) kinetic test (Bioclin KO49-6), Urea UV (Bioclin K056- 1) and Glucose monoreagent enzymatic reagent (Bioclin KO82). Analyses were carried out using an automatic biochemical analyzer ChemWell-T (Labtest).

2.10. Autoradiography

The autoradiography assay was performed as described by Pijeira et al. 2020. The tumors harvested from Balb/C nude mice (after the biodistribution studies) were rinsed in PBS and frozen in an isopentane/dry-ice bath. The slides of the tumor were obtained using a cryostat (Leica, Wetzlar, Germany), and thaw mounted onto Superfrost Plus microscope slides (Fischerbrand from Thermo Fisher Scientific, Toronto, ON, Canada) (Zhang et al. 2017). Next, tumor slides were exposed to a flexible phosphor screen (Carestream Kodak 10x12" Poc 140 260 360 Vita Cr Flex), and incubated overnight in Cassette (Carestream 10x12" 25x30cm + Screen Poc 140 260 360 Vita Flex Cr). Images were obtained using a Typhoon FLA 9500 scanner (GE Healthcare, Chicago, IL, USA). The

area of necrotic regions was counted and calculated in square millimeters using the ImageJ software. Three or more fields per tumor were analyzed and averaged.

2.11. Immunostaining assay

Immunostaining was performed as described by Santos et al. 2017. The tumors harvested from Balb/C nude mice (after the biodistribution studies) were rinsed in PBS and frozen in an isopentane/dry-ice bath. The slides of the tumor were obtained using a cryostat (Leica, Wetzlar, Germany) and thaw mounted onto Superfrost Plus microscope slides (Fischerbrand from Thermo Fisher Scientific, Toronto, ON, Canada) (Zhang et al. 2017). Tissue sections were stained with anti-GAL3 antibody (M3/38, ATCC TIB166). Secondary biotinylated antibodies of anti-rat (BA-4001) was obtained from Vector Laboratories. Next, streptavidin peroxidase (Sigma) was added, and color development was performed using DAB (DAKO). Nuclei were counterstained with hematoxylin, and the cytoplasm was stained with eosin. Tissue areas were traced using TissueFAXS plus microscopy (Tissue Gnostics) and measured with TissueFAXS viewer NIH ImageJ software. The area of necrotic regions was counted and calculated in square millimeters using the ImageJ software. Three or more fields per tumor were analyzed and averaged.

2.12. Biodistribution studies

In the biodistribution study the Balb/C nude mice with xenografts of SKOV-3 and MKN45 cells (10^6 cells/animal) were intravenously injected with 10 MBq of MCP-^{99m}Tc. One hour after injection, the mice were euthanized, and the organs of interest were harvested, rinsed in PBS, weighed, and counted using a Perkin Elmer (Waltham, MA, USA) Wizard2 2480 automatic gamma counter to quantify the percentage of injected dose per gram of tissue (%ID/g).

2.13. µSPECT/CT imaging

In the µSPECT/CT imaging study MCP-^{99m}Tc (37 MBq) was intravenously injected into Balb/c nude mice containing xenografts of SKOV-3 cells (10^6 cells per animal). Imaging was conducted 1 hour post-injection using an Albira µPET/SPECT/CT imaging system (Bruker Biospin Corporation, Woodbridge, CT, USA). The imaging protocols used was: 3D FOV 80 45proj/s to SPECT and high 400µA 45Kev to CT. Images were processed using PMOD software (PMOD Technologies, Zurick, CH, USA).

2.14. Statistical analysis

All data are expressed as the mean ± SD of at least three independent experiments. Statistical analysis was performed using the GraphPad Prism software (version 8.0; San Diego, CA, USA). Data were analyzed using an unpaired t-test (multiple t-tests). Outliers were removed before data analysis. Statistical significance was set at $P < 0.05$.

3. Results

3.1. Cell binding and internalization of MCP is only partially influenced by Galectin-3 expression *in vitro*

In the cell binding and internalization study, SKOV-3 scrambled cells (ovary tumor model) and SKOV-3 shRNAGal3 cells (knockdown of Galectin-3 expression) were used to understand the role of Gal-3 in MCP binding and internalization. Western blotting analysis showed that SKOV-3 scrambled cells had 1000 times more Gal-3 expression (1.05 ± 0.1 Gal-3/β-actin expression level) than SKOV-3 shRNAGal3 cells (0.001 ± 0.0001 Gal-3/β-actin expression level) (Figure 1 A).

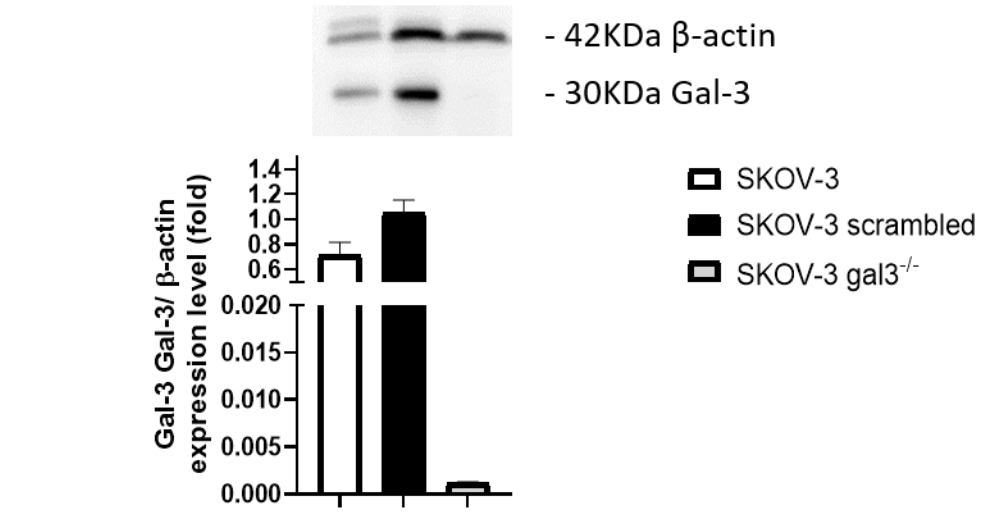
Cell binding and internalization assays were performed with SKOV-3 scrambled and SKOV-3 shRNAGal3 cells (4×10^5 cells) plated in a 6-well plate overnight and then incubated for one hour at 37°C in internalization study, or 4°C in cell binding study with 370 kBq of MCP-^{99m}Tc per well. For the inhibition study, the cells were pretreated for 30 min before adding the radiolabeled compound with MCP (200 ×), sucrose 100 mM (negative control), and lactose 100 mM (positive control). At the end of the incubation period, the supernatant was aspirated and cells were washed with ice-cold PBS. The cells were removed, and transferred to a gamma counter tube, and percentage of injected dose by mass was determined (%D.I./μg).

An affinity study was performed at 4 °C to observe the cell membrane binding of MCP. The results showed that MCP-^{99m}Tc binds significantly more in SKOV-3 scrambled cells (0.522 ± 0.194 %I.D./μg) than SKOV-3 shRNAGal3 (0.066 ± 0.003 %I.D./μg) cells in PBS, an 87.35 % of binding reduction. The negative control, performed with pretreatment of sucrose (100mM), a sugar that does not inhibit Gal-3, demonstrated that MCP-^{99m}Tc binds more in SKOV-3 scrambled cells (0.006 ± 0.001 %I.D./μg) than

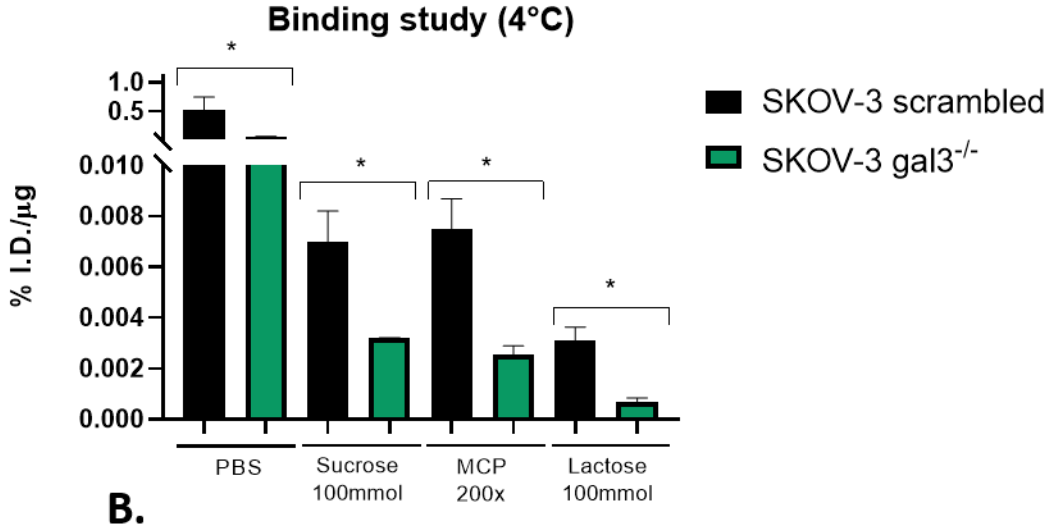
SKOV-3 shRNAGal3 (0.003 ± 0.001 %I.D./μg), 50 % of binding reduction. However, this result was less than that in the PBS group, which demonstrated the non-specific binding of MCP. Pre-treatment with MCP 200x showed that MCP-^{99m}Tc binding was significantly higher in SKOV-3 scrambled cells (0.007 ± 0.001 %I.D./μg) than in SKOV-3 shRNAGal3 cells (0.002 ± 0.001 %I.D./μg), approximately 71.42 % of binding reduction. These data demonstrated that MCP (200 ×) had less binding than PBS, but not sucrose (100 mM). This result is evidence of the non-specific binding of MCP to membrane cells. The positive control was lactose (100 mM), which specifically inhibited Gal-3, and showed a significant difference in binding of MCP-^{99m}Tc in SKOV-3 scrambled cells (0.003 ± 0.001 %I.D./μg) and SKOV-3 shRNAGal3 cells (0.0006 ± 0.0001 %I.D./μg), demonstrating the lowest binding among all previous groups (Figure 1 B). These results demonstrate that the cell membrane binding of MCP in SKOV-3 cells is only partially influenced by the expression of Gal-3, and others unspecific or unknown targets also regulate its connection.

An affinity study was performed at 37 °C to determine the cellular binding and internalization of MCP. The results showed that MCP-^{99m}Tc binds significantly more in SKOV-3 scrambled cells (0.160 ± 0.009 %I.D./μg) than SKOV-3 shRNAGal3 (0.079 ± 0.004% I. D./μg) cells in PBS, a 50.6 % of binding reduction. The negative control, performed with pretreatment of sucrose (100mM), a sugar that does not inhibit Gal-3, demonstrated that MCP-^{99m}Tc binds significantly more in SKOV-3 scrambled cells (0.023 ± 0.006 %I.D./μg) than in SKOV-3 shRNAGal3 (0.019 ± 0.001% I. D./μg), 17.19 % of binding reduction. However, this result was smaller than PBS group, which demonstrated non-specific binding of MCP. The pre-treatment with MCP 200x showed that MCP-^{99m}Tc binding significantly more in SKOV-3 scrambled (0.013 ± 0.001 %I.D./μg) than SKOV-3 shRNAGal3 cells (0.007 ± 0.001 %I.D./μg), approximately 46% of binding reduction. It was also demonstrated that the group treated with MCP (200 ×) had less binding of MCP than the PBS or sucrose (100 mM) group. The positive control was lactose (100 mM), a specific inhibitor of Gal-3, and showed that the binding of MCP-^{99m}Tc in SKOV-3 scrambled cells was 0.003 ± 0.0003 %I.D./μg and 0.001 ± 0.0002 %I.D./μg in SKOV-3 shRNAGal3 cells, demonstrating the lowest binding among all groups, as expected (Figure 1 C).

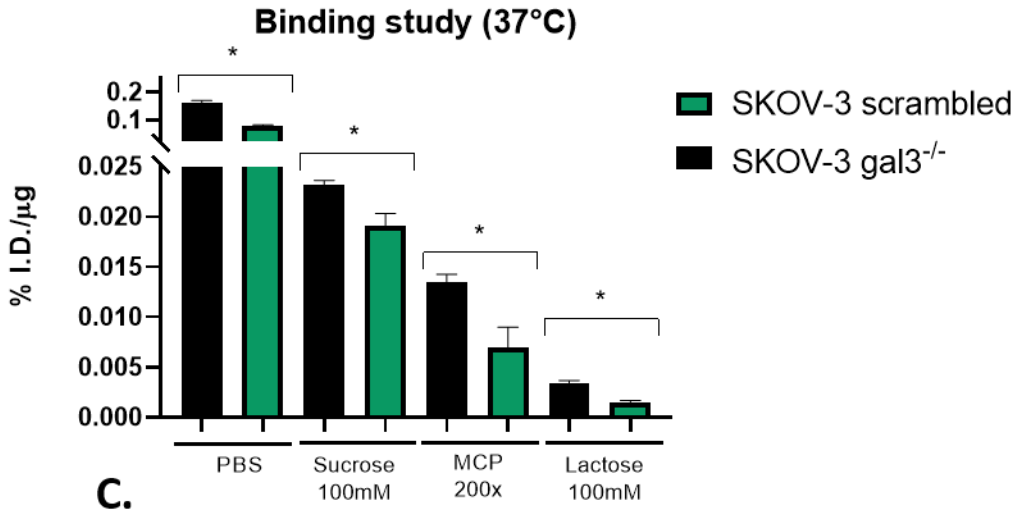
These results demonstrate that cellular binding and internalization of MCP in SKOV-3 cells are not only influenced by the expression of Gal-3, but also by non-specific or unknown targets that may influence its biological processes. Further studies are needed to identify this non-specific target.


- 42KDa β-actin
- 30KDa Gal-3
Gal-3 Gal-3/ β-actin expression level (fold)
1.4
1.2
1.0
0.8
0.6
0.020
0.015
0.010
0.005
0.000
SKOV-3
SKOV-3 scrambled
SKOV-3 gal3-/-

A.


Binding study (4°C)
% I.D./µg
1.0
0.5
0.010
0.008
0.006
0.004
0.002
0.000
*
PBS
Sucrose 100mmol
MCP 200x
Lactose 100mmol
SKOV-3 scrambled
SKOV-3 gal3-/-

B.


Binding study (37°C)
% I.D./µg
0.2
0.1
0.025
0.020
0.015
0.010
0.005
0.000
*
PBS
Sucrose 100mM
MCP 200x
Lactose 100mM
SKOV-3 scrambled
SKOV-3 gal3-/-

C.

Figure 1: Cell binding and internalization of MCP is only partially influenced by Galectin-3 *in vitro*. (A) Western blotting and quantification of Gal-3. (B) Cell binding study of MCP in SKOV-3 scrambled and SKOV-3 $gal^{-/-}$ cells. (C) Internalization study of MCP in SKOV-3 scrambled and SKOV-3 $gal^{-/-}$ cells. Data are representative of three independent experiments. *, #, ## $p > 0.05$.

3.2. MCP exhibits anticancer activity in a tumor xenograft model when administered intravenously but not via oral

To verify the antitumor effect of MCP administered intravenously and orally, an anticancer activity assay was performed using a SKOV-3 tumor xenograft model. In the *in vivo* tumor assay, Balb/C nude mice were subcutaneously injected with xenografts of SKOV-3 cells (10^6 cells/animal) and treated with oral and intravenous (IV) administration of MCP. When the tumors reached approximately 50 mm^3, the animals were divided into four groups and daily treated orally with vehicle (saline), MCP (200 mg/kg), or daily IV with vehicle (saline) and MCP (20 mg/kg). Tumor volume was measured each day using an automatic caliper and quantified using the formula 0.5 × larger diameter × smaller $diameter^2$. When the tumors reached approximately 1.6 cm^3 or after 21 days of the experiment, the animals were euthanized, and the tumor, organs, and blood samples were collected for later histological and biochemical analyses. The weight of the tumor, animals, and their feed intake were monitored every seven days using a precision scale. The results showed that 20 mg/kg of MCP daily I.V. administered for 21 days significantly reduced tumor growth by 48.5%, with a control group with 1610.8 ± 624 mm^3 (Crt I.V.), and the treated group with 829.3 ± 219 mm^3 (MCP I.V.) (Figure 2 A). On the other hand, MCP treatment at 200 mg/kg, orally administered daily, did not show a significant difference between the control and treated groups (both with an average of 1650 mm^3) (Figure 2 B). The tumor weight of Crt I.V. was 1.2 ± 0.24 g and of the MCP IV was 0.59 ± 0.20 g, a reduction of 50%, while the tumor weight of Crt oral and MCP oral has an average of 1.3 g (Figure 2 C and D). Thus, these results demonstrated that IV administration of MCP (20 mg/kg) reduced tumor growth in SKOV-3 cells tumor xenografts; however, oral administration of MCP did not show an anti-tumorigenic effect.

To verify the toxicity of MCP treatment, body weight was measured, and histochemical and biochemical analyses were performed. The presence of tumoral xenografts of SKOV-3 cells reduced the body weight of the animals after 21 days of treatment, independent of MCP utilization, with a body weight reduction of 14.4 % in

Crt IV, 15.45 % in IV treatment, 9.25 % in Crt oral and 8.5 % in oral treatment (Figure 2 E and F).

For histological analysis, the kidneys of mice were collected, cleaved, and immediately fixed in paraformaldehyde after 21 days of treatment. The samples were dehydrated in increasing concentrations of ethanol (70–100%) for 30 min each and washed three times in xylene for 30 min. They were then individually embedded in paraffin, sectioned into 4 μm thick slices, and stained with hematoxylin and eosin (H&E). Masson's trichrome (MT) staining was used to identify extracellular matrix deposits. Histochemical analysis showed that oral and IV administration of MCP did not cause kidney damage, as shown by the normal structure of renal glomeruli, proximal convoluted tubules, distal convoluted tubules, and extracellular matrix (ECM) deposition (Figure 2 G and H).

For biochemical analysis, blood samples from healthy animals or animals treated with vehicle (saline) or MCP were collected and centrifuged in a microcentrifuge for blood plasma separation. Blood plasma samples were analyzed using reagent kits and an automatic biochemical analyzer (ChemWell-T). Biochemical analysis showed that the levels of TGP (ALP), TGO (AST), FAL, urea, glucose, and creatine remained unchanged compared with those in healthy animals after IV administration of MCP. However, the levels of TGO (AST) and creatine were outside the normal range of healthy animals in the group treated with oral MCP. These results demonstrate that intravenous treatment with MCP at a concentration of 20 mg/Kg did not cause toxicity; however, oral treatment with MCP (200 mg/kg) presented evidence of hepatic damage (Table 1).

These studies demonstrated that treatment with MCP (20 mg/kg) exhibits anticancer activity in a tumor xenograft model when administered intravenously, without evidence of kidney damage or hepatic toxicity. However, treatment with oral MCP (200 mg/kg) did not exhibit anticancer activity and showed an indication of hepatic toxicity.

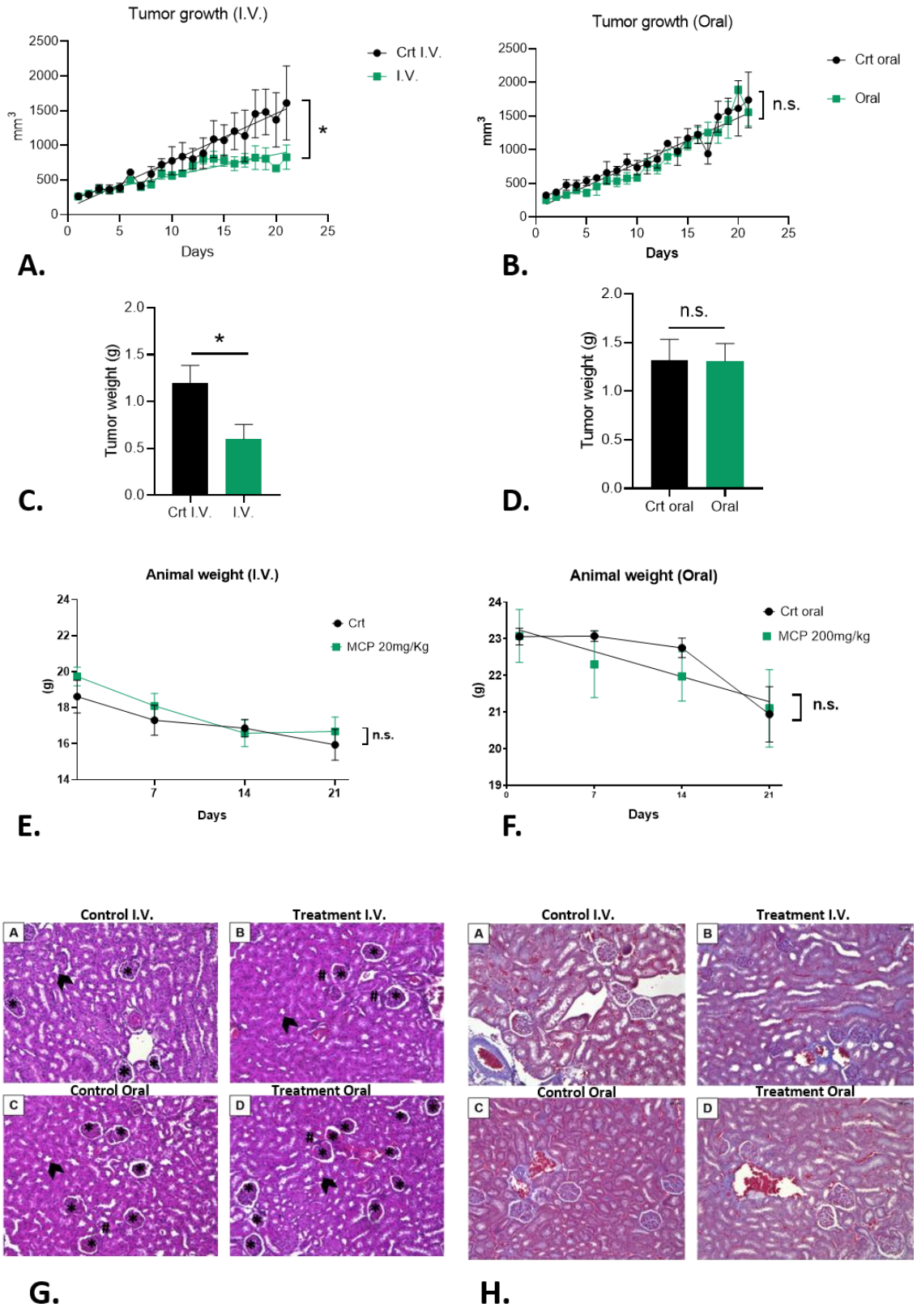


Figure 2: MCP exhibits anticancer activity in a tumor xenograft model when administered intravenously but not via oral. (A) Tumor growth study with IV administration of MCP (20 mg/kg). (B) Tumor growth study with oral administration of MCP (200 mg/kg). (C) Tumor weight after intravenous treatment of MCP (20 mg/kg). (D) Tumor weight after oral treatment of MCP (200 mg/kg). (E) Animal weight study of MCP treatments with IV administration. (F) Animal weight study of MCP treatments with oral administration.

(G) Photomicrographs of Kidneys after 21 days of treatment with MCP. Representative images of cortical renal parenchyma obtained from the control (A – I.V. and C - oral) and from treatment with MCP (B – I.V. and D - oral). A, B, C, and D show the histological structure using hematoxylin and eosin staining, showing the renal glomeruli (*), proximal convoluted tubules (arrowhead), and distal convoluted tubules (#). Data are representative of three independent experiments. Magnification: A-D (100x). (H) Extracellular matrix (ECM) of kidneys after 21 days of treatment with MCP. Representative images of cortical renal parenchyma obtained from the control (A – I.V. and C - oral) and from treatment with MCP (B – I.V. and D - oral). A, B, C, and D show extracellular matrix (ECM) deposition evidenced in blue beams by Masson's trichrome stain. Data are representative of three independent experiments. Magnification: A-D (100x). * $p > 0.05$.

Table 1: Biochemical analysis of animals treated with MCP, both orally and intravenously.

	HEALTHY	CRT ORAL	ORAL	CRT I.V.	I.V.
TGO (AST)	40.53 ± 18.22 U/L	55.7 ± 1.1 U/L	84.45 ± 4.55 U/L *	34.32 ± 11.8 U/L	28.54 ± 11.8 U/L
TGP (ALT)	54.03 ± 20.6 U/L	56.47 ± 5.45 U/L	42.11 ± 7.25 U/L	30.08 ± 10.12 U/L	42.27 ± 8.67 U/L
FAL	78.57 ± 31.2 U/L	58.53 ± 16.84 U/L	86.37 ± 9.88 U/L	80.21 ± 48.29 U/L	98.09 ± 0.84 U/L
Urea	43.08 ± 22.6 mg/dL	54.22 ± 18.4 mg/dL	49.3 ± 5 mg/dL	46.39 ± 2.89 mg/dL	52.7 ± 8.5 mg/dL
Glucose	95.1 ± 20.9 mg/dL	113.6 ± 47.1 mg/dL	134.1 ± 35.5 mg/dL	122.6 ± 27.4 mg/dL	86.9 ± 21.7 mg/dL
Creatine	1.065 ± 0.2 mg/dL	0.852 ± 0.07 mg/dL	0.589 ± 0.05 mg/dL *	1.070 ± 0.3 mg/dL	1.01 ± 0.6 mg/dL

TGO (AST): aspartate aminotransferase/ oxalacetic transaminase. FAL: alkaline phosphatase. TGP (ALT): alanine aminotransferase. Data are representative of three independent experiments. * $p > 0.05$.

3.3. MCP binds to the tumor in the SKOV-3 cell xenograft model when intravenously injected

To verify whether MCP can reach the tumor tissue in animal models with tumor xenografts of SKOV-3 cells, biodistribution and µSPECT imaging experiments were performed. In the biodistribution study the Balb/C nude mice with xenografts of SKOV-3 (10^6 cells/animal) were intravenously injected with 10 MBq of MCP-^{99m}Tc. One hour after injection, the mice were euthanized, and the organs of interest were harvested, rinsed in PBS, weighed, and counted with an automatic gamma counter to quantify the percentage of injected dose per gram of tissue (%ID/g). The biodistribution study demonstrated that MCP-^{99m}Tc can reach that tumor tissue after 1 h of delivery (0.78 ± 0.045 %I.D./g), in intravenous application. However, MCP-^{99m}Tc was not detected in the tumor when administered orally (0.015 ± 0.005 %I.D./g) (Figure 3 A).

In the µSPECT/CT imaging study MCP-^{99m}Tc (37 MBq) was intravenously injected into Balb/c nude mice containing xenografts of SKOV-3 cells (10^6 cells/animal). Imaging was conducted 1 h post-injection using Albira µPET/SPECT/CT, and processed using the PMOD software. µSPECT/CT imaging studies demonstrated that tumor activity in MCP-^{99m}Tc I.V. administered was greater than that in MCP-^{99m}Tc injected orally, as shown in

Figure 3 B. These results demonstrate that MCP can reach the tumor tissue when injected intravenously, but not when administered orally.

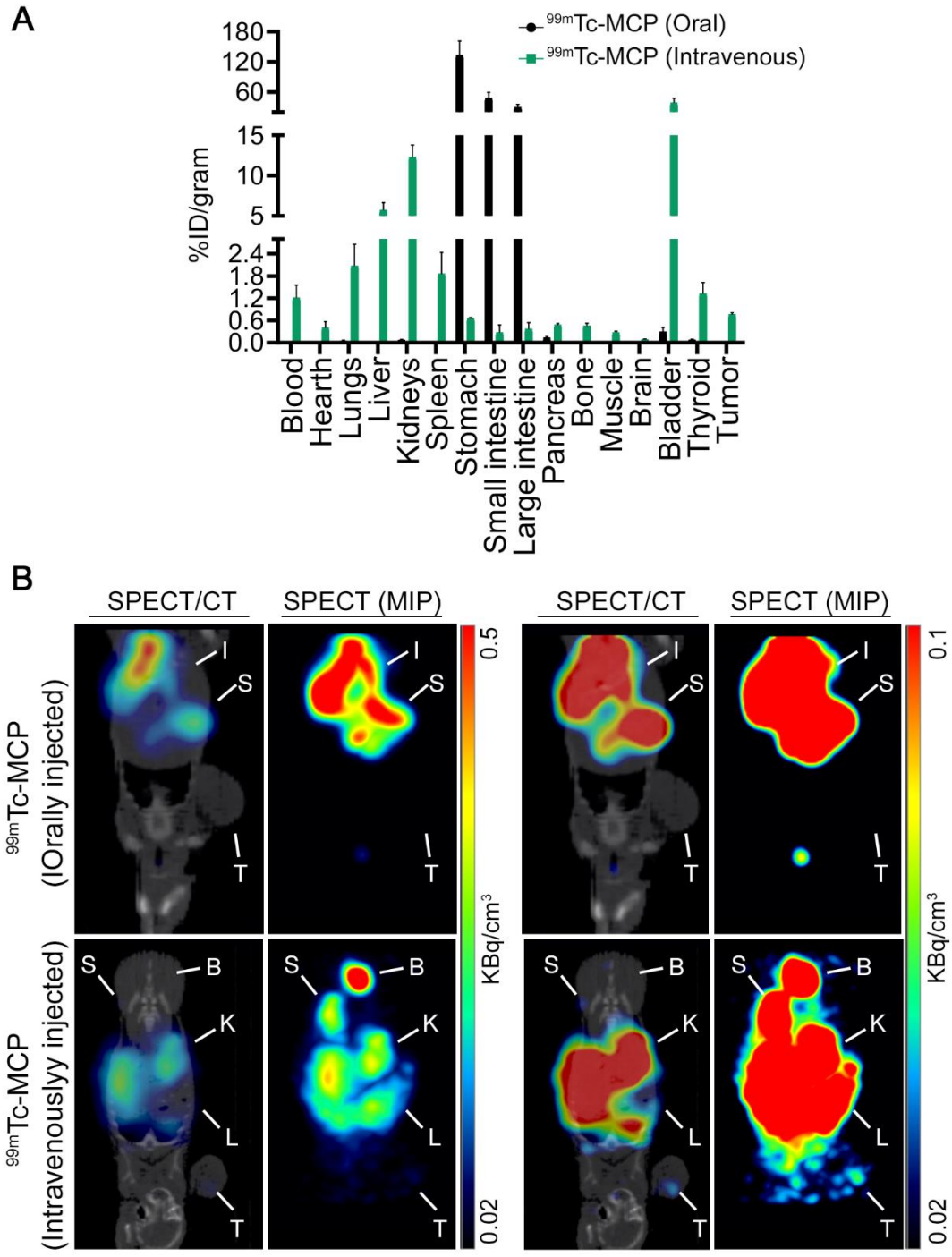
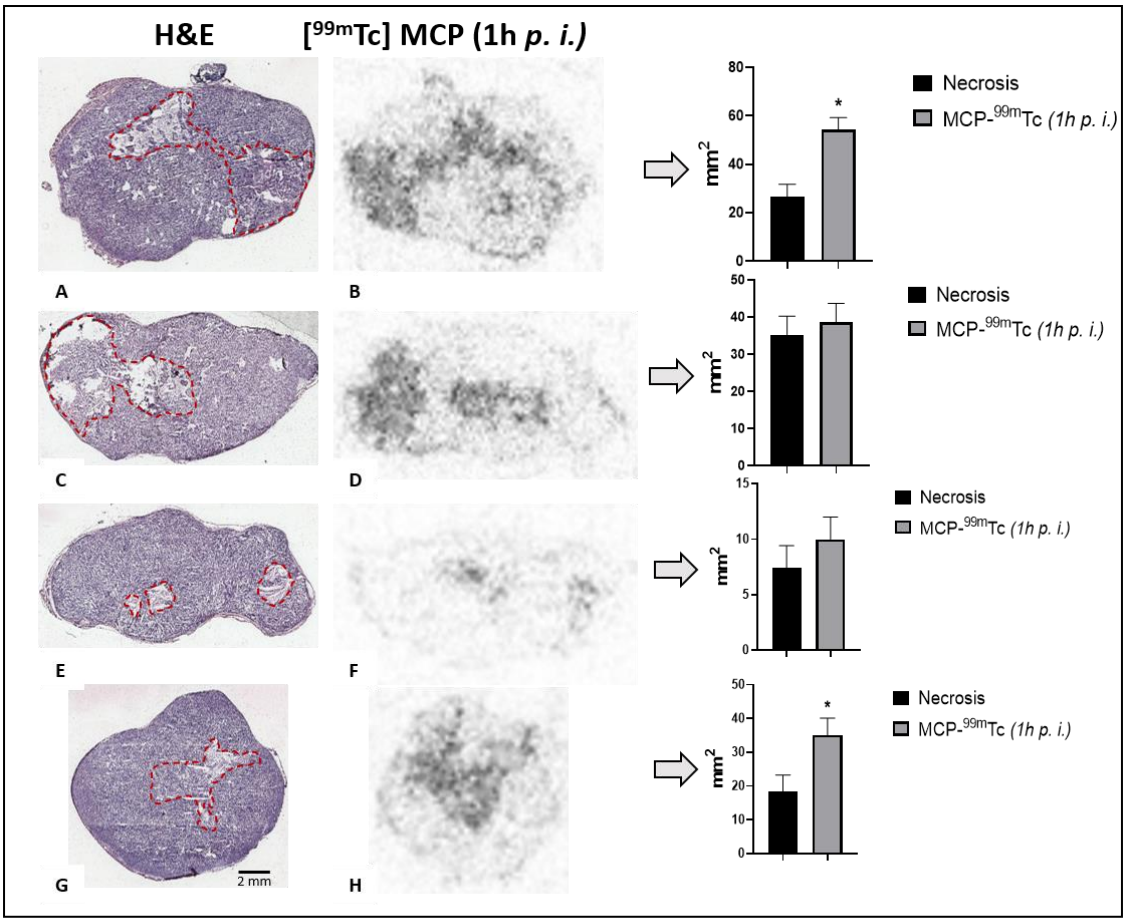


Figure 3: MCP binds to the tumor in the SKOV-3 cell xenograft model when intravenously injected. (A) Biodistribution study of MCP-^{99m}Tc administered orally and intravenously at 1 h, with measurement of %I.D./g of indicated organs. (B) µSPECT/CT image study of MCP-^{99m}Tc administered intravenously and orally at 1 h; T = tumor; L = liver; K = kidney; B = bladder; S = spleen; I = intestine; MIP = maximum intensity projection. Data are representative of three independent experiments.

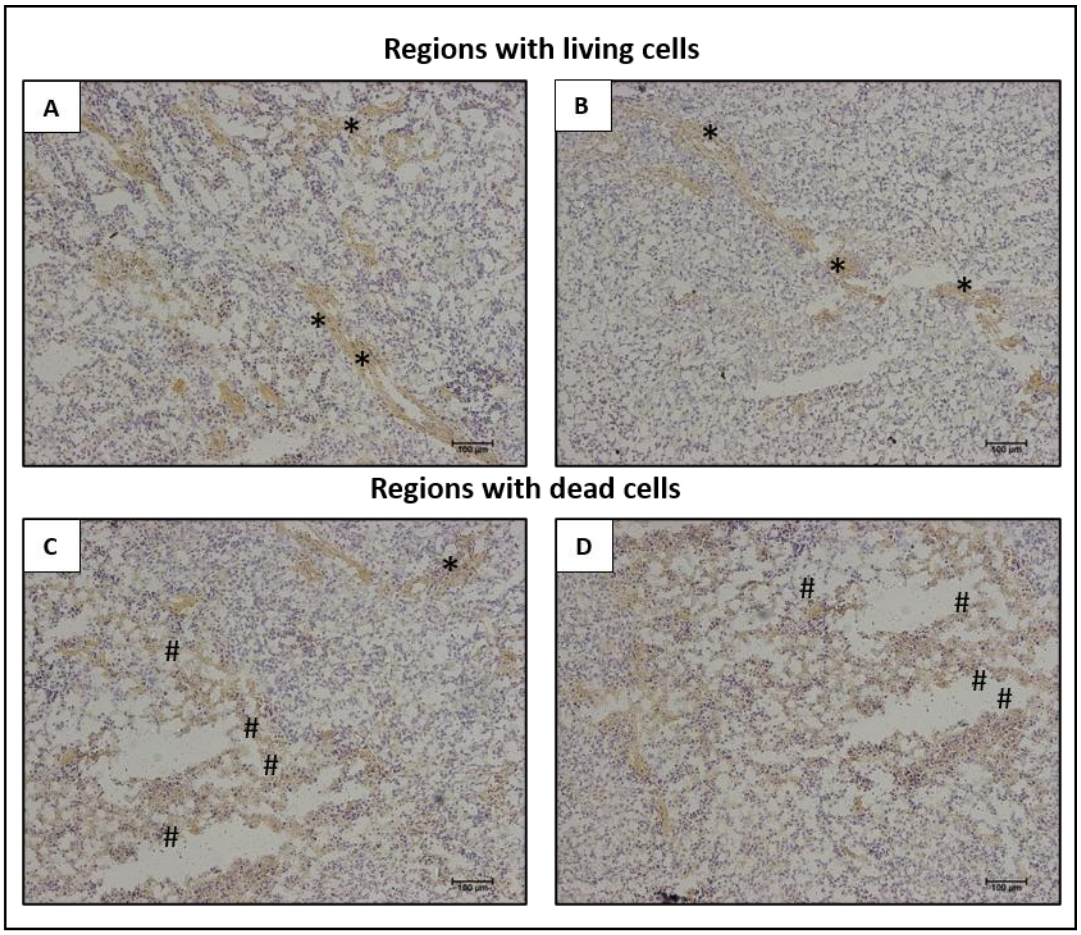
3.4. MCP binds to regions of tumor necrosis in the SKOV-3 cells xenograft model when intravenously injected

To analyze where MCP binding when reaching the tumor tissue in animal models of SKOV-3 tumors, an *in vivo* autoradiography study was performed. In the autoradiography assay, tumors were harvested from Balb/C nude mice (after the biodistribution studies), rinsed in PBS, and frozen in an isopentane/dry-ice bath. Tumor slides were obtained using a cryostat. Next, the tumor slides were exposed to a flexible phosphor screen and incubated overnight in a cassette. Images were obtained using an autoradiography scanner. The areas of necrotic regions were counted and calculated in square millimeters using the ImageJ software. This study showed that MCP-^{99m}Tc intravenously injected binds to the internal regions of the tumor tissue, with evidence of cell death and necrosis. Quantification analysis demonstrated that these regions of necrosis in the center of the tumor were correlated to the regions with MCP-^{99m}Tc activity, but not restricted to such regions. (Figure 4 A). It was also verified that the expression of Gal-3 in tumors of SKOV-3 cells was reduced in living cells and restricted to regions of the fibers, extracellular matrix, and necrosis sites (Figure 4 B).

These results demonstrate that MCP reaches tumor xenograft models in IV administration by binding to regions of necrosis rich in Ga-3 expression. However, these data do not necessarily indicate a direct relationship between Gal-3 expression and MCP binding in tumors.



A.



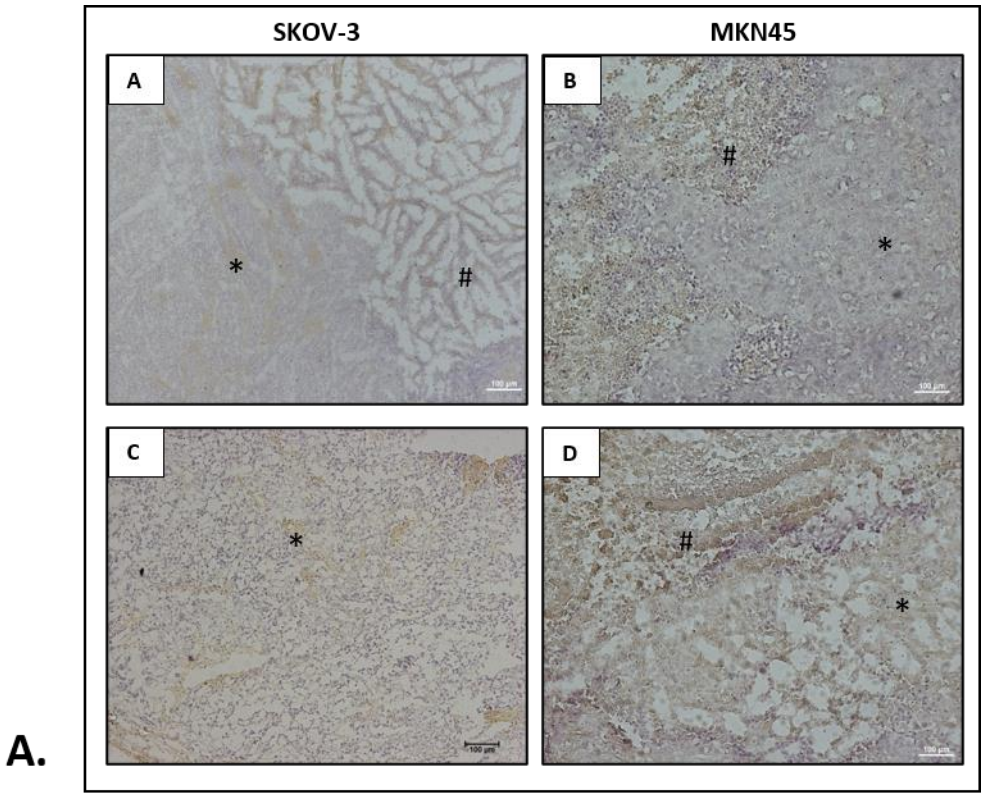
B.

Figure 4: MCP binds to regions of tumor necrosis in the SKOV-3 cells xenograft model when intravenously injected. (A) Autoradiography of SKOV-3 xenograft tumor cells. Left images = H&E staining, right images = autoradiography slices. The dotted lines indicate the regions of necrosis quantified and indicated on the graphs on the sides. Magnification: A (2x), scale bar: 2 mm. (B) Immunostaining assay of xenograft SKOV-3 cells for Gal-3 labeling. (*) regions of fibers and extracellular matrix. (#) cell death and necrosis. Magnification: B (10x), scale bar: 100 µm. Data are representative of three independent experiments. * $p > 0.05$.

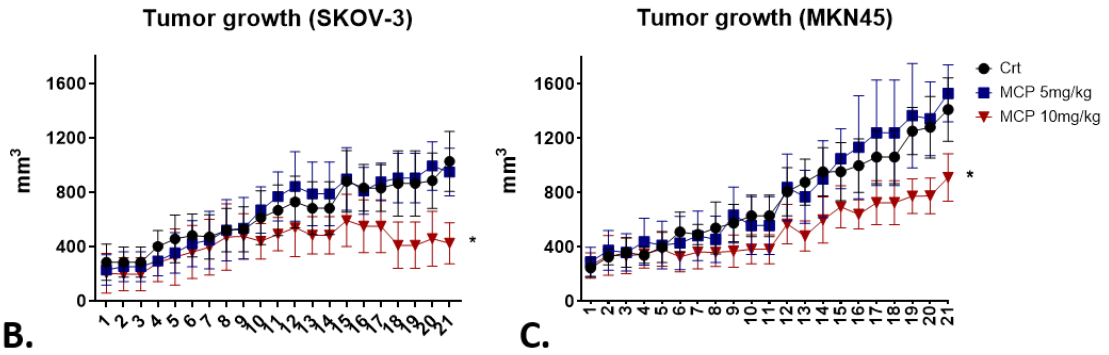
3.5. Gal-3 expression did not influence MCP antitumor activity and MCP binding in tumors *in vivo*

To unravel the correlation between MCP and Gal-3 in anticancer response, a new study of antitumor activity using tumor xenografts of SKOV-3 and MKN45 cells (gastric cancer model) was carried out. Balb/c nude mice were subcutaneously inoculated with 1×10^6 SKOV-3 or MKN45 cells. When the tumors reached 50 mm^3, the animals were separated into three groups and treated daily with intravenous administration of MCP: vehicle (saline 100ul), MCP 5 mg/kg, and MCP 10 mg/kg for 21 days. Tumor volume was measured daily using an automatic caliper. When the tumors reached approximately 1.6

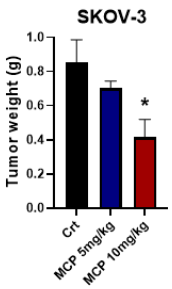
cm^3 or after 21 days of the experiment, the animals were euthanized and the tumor, organs, and blood samples were collected for analysis. The weight of the tumor was measured, and feed intake was monitored every seven days. The immunostaining assay showed that MKN45 cell xenografts showed a greater expression of Gal-3 than SKOV-3 cell xenografts (Figure 5 A). To verify whether doses lower than 20 mg/kg have antitumor activity, concentrations of 10 mg/kg and 5 mg/kg IV were used daily for 21 days. The results showed that in both tumor xenograft strains, a concentration of 10 mg/kg showed antitumor activity, with a significant reduction in tumor growth and weight, but 5 mg/kg did not. In the SKOV-3 cells, the control of tumor growth was 1026.86 ± 220.36 mm^3 (Crt SKOV-3) and the treatment was 423.78 ± 151.60 mm^3 (MCP SKOV-3 10 mg/kg), concurrently, the tumor weight control was 0.85 ± 0.28 g (Crt SKOV-3) and the treatment 0.41 ± 0.10 g (MCP SK0V-3 10 mg/kg), an average of 58.7% and 51.7% of reduction, respectively. In the MKN45 cells, the tumor growth control was 1410.06 ± 233.84 mm^3 (Crt MKN45) and the treatment 909.85 ± 174.40 mm^3 (MCP MKN45 10 mg/kg), while the tumor weight control was 0.823 ± 0.07 g (Crt MKN45) and the treatment 0.57 ± 0.14 g (MCP MKN45 10 mg/kg), a more modest reduction, with 35.4 % and 30.7 %, respectively (Figure 5 B -G). These results demonstrated that the expression of Gal-3 in the tumor xenografts of SKOV-3 and MKN45 cells did not influence the antitumor effect of MCP *in vivo*.


SKOV-3
MKN45
A
B
C
D
*
#

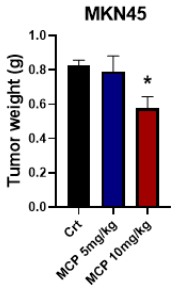
A.


Tumor growth (SKOV-3)
Tumor growth (MKN45)
mm3
1600
1200
800
400
0
Crt
MCP 5mg/kg
MCP 10mg/kg
*

B. C.


SKOV-3
Tumor weight (g)
1.0
0.8
0.6
0.4
0.2
0.0
*
Crt
MCP 5mg/kg
MCP 10mg/kg

D.


MKN45
Tumor weight (g)
1.0
0.8
0.6
0.4
0.2
0.0
*
Crt
MCP 5mg/kg
MCP 10mg/kg

E.

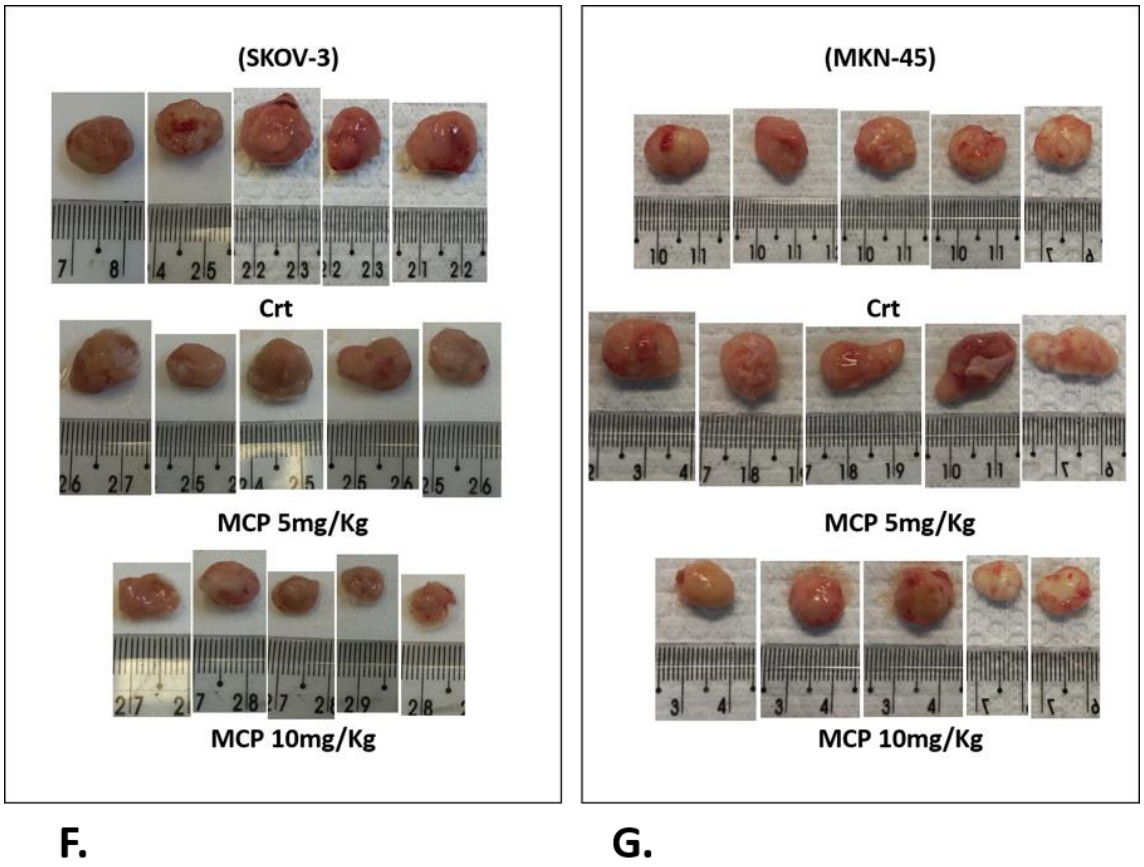


Figure 5: Gal-3 expression did not influence MCP antitumor activity *in vivo*. (A) Immunostaining assay of xenografts of SKOV-3 and MKN45 cells for Gal-3 labeling. (*) regions of fibers and extracellular matrix. (#) cell death and necrosis. Magnification: B (10x), scale bar: 100 µm. (B) Tumor growth study of SKOV-3 cells with intravenous administration of MCP (5 and 10 mg/kg). (C) Tumor growth study of MKN45 cells treated with IV administration of MCP (5 and 10 mg/kg). (D) Tumor weight of SKOV-3 cells after treatment with I.V. administration of MCP. (E) Tumor weight of MKN45 cells after treatment with I.V. administration of MCP. (F) Imaging of SKOV-3 tumors after treatment with I.V. administration of MCP. (G) Imaging of MKN45 tumors after treatment with I.V. administration of MCP. Data are representative of three independent experiments. * $p > 0.05$.

We also verified the proportion of MCP in the tumors *in vivo* using a biodistribution study in tumor xenograft models, SKOV-3 cells, and MKN45 cells. The results showed that MCP binds in the same proportion in both tumor xenografts, 0.55 ± 0.20 %I.D./g for SKOV-3, and 0.50 ± 0.18 %I.D./g to MKN45 (Figure 6 A). The two tumor xenograft strains reduced the weight of the animals during 21 days of treatment, independent of MCP treatment (Figure 6 B and C). There were also small, but significant differences, in food consumption between the groups, Crt (2.28 ± 0.1 g/day/animal), MCP 5 mg/kg (2.69 ± 0.12 g/day/animal), and MCP 10 mg/kg (3.19 ± 0.11 g/day/animal), with increased food consumption as the concentration of treatment increases (Figure 6 D). However, none of the MCP treatment concentrations changed glycemia in the mice

(Figure 6 E). These results demonstrate that the expression of Gal-3 in the tumor xenografts of SKOV-3 and MKN45 cells did not influence the tumor binding of MCP and that its treatments did not show *in vivo* toxicity.

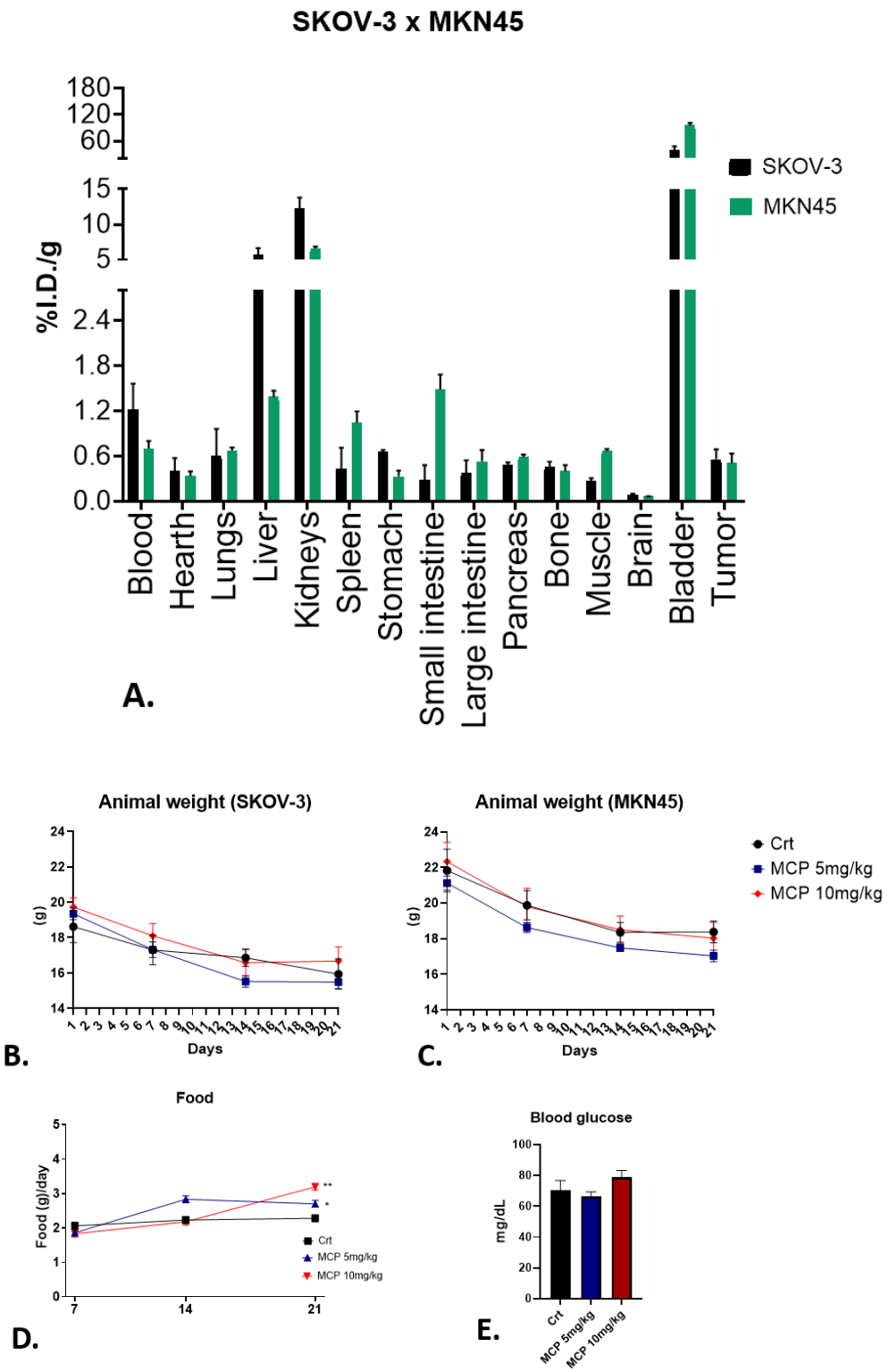


Figure 6: Gal-3 expression did not influence MCP binding in tumors *in vivo*. (A) Biodistribution study of MCP-^{99m}Tc administrated I.V. in SKOV-3 or MKN45 xenograft tumors at 1 h, with measurement of %I.D./g

of indicated organs. (B) Animal weight study of tumor xenografts of SKOV-3 cells with IV administration of MCP (5 and 10 mg/kg). (F) Animal weight study of tumor xenografts of MKN45 cells with IV administration of MCP (5 and 10 mg/kg). (C) Food intake of animals subjected to MCP treatment (5 and 10 mg/kg) monitored for 21 days (g/day). (D) Blood glucose levels of animals subjected to MCP treatment (5 and 10 mg/L) (mg/dL). Data are representative of three independent experiments. * $p > 0.05$.

4. Discussion

In this study, we investigated the anticancer activity of modified citrus pectin (MCP) *in vivo* using molecular imaging with oral and intravenous administration. MCP was radiolabeled with ^{99m}Tc in a previous study carried out by our research group (Silva et al., 2023, in production). In this study, we verified the biodistribution and pharmacokinetics of MCP-^{99m}Tc orally and intravenously (IV) administrated, as well as the structure and monosaccharide composition of MCP for HPSEC-RID and HPAEC-PAD. It has been demonstrated that there is a diversity of monosaccharides and oligosaccharides with different molecular weights within the MCP structure and that MCP30 and MCP3 fractions are rich in galactose. Next, the inhibition and binding affinity of MCP for galectin-3 were demonstrated using hemagglutination and Sepharose/Gal-3 assays, which showed that MCP partially binds to Gal-3, and that MCP3 has a greater affinity and inhibits galectin-3. MCP-^{99m}Tc exhibited a good radiochemical purity (>95%) and stability. MCP-^{99m}Tc has low gastrointestinal absorption and gastrointestinal elimination when administered via oral, and, renal and hepatobiliary elimination when administered via IV. Finally, the blood compartment distribution assay showed that MCP-^{99m}Tc had a high affinity for plasma proteins and blood cells in C57BL/6 Lgals3 $^{+/+}$ mice, and this affinity was partially lost when galectin-3 was deleted in C57BL/6 Lgals3 $^{-/-}$ mice. The pharmacokinetic assay showed that MCP-^{99m}Tc elimination speed was greater in C57BL/6 Lgals3$^{-/-}$ than in C57BL/6 Lgals3 $^{+/+}$ mice, indicating that a lack of galectin-3 increases MCP elimination *in vivo* (Silva et al., 2023, in production).

In this study, cell binding and internalization studies were performed in SKOV-3 scrambled cells (ovarian tumor model) and SKOV-3 shRNAGal3 cells (knockdown of Galectin-3 expression) to understand the role of Gal-3 in MCP binding and its cell internalization (Figure 1). An affinity study was performed at 4 °C to observe the cell membrane binding of MCP, and the results showed that MCP-^{99m}Tc binds significantly more in SKOV-3 scrambled cells than in SKOV-3 shRNAGal3 cells in PBS, with 87.35 % of binding reduction. The negative control, which was pretreated with sucrose (100mM),

demonstrated that MCP-^{99m}Tc binds more in SKOV-3 scrambled cells than SKOV-3 shRNAGal3, with a 50 % of binding reduction; however, it had a smaller proportion than the PBS group, which demonstrated the non-specific binding of MCP. Pre-treatment with MCP 200x showed that MCP-^{99m}Tc binding was higher in SKOV-3 scrambled cells than in SKOV-3 shRNAGal3 cells, with a binding reduction of approximately 71.42 %, with the same proportion than sucrose (100 mM). This result demonstrates the non-specific binding of MCP to membrane cells. The positive control with lactose (100 mM) showed a significant difference in the binding of MCP-^{99m}Tc in SKOV-3 scrambled cells and SKOV-3 shRNAGal3 cells, demonstrating the lowest binding among all previous groups. An affinity study was performed at 37 °C to determine cellular binding and internalization of MCP. The results showed that MCP-^{99m}Tc bound significantly more in SKOV-3 scrambled cells than in SKOV-3 shRNAGal3 cells in PBS, with a 50.6 % of binding reduction. The negative control, demonstrated that MCP-^{99m}Tc binds significantly more in SKOV-3 scrambled cells than in SKOV-3 shRNAGal3cells, with a 17.19 % of binding reduction, and the proportion of this group was smaller than the PBS group, which demonstrated the non-specific binding of MCP. Pre-treatment with MCP 200x showed that MCP-^{99m}Tc binding was significantly higher in SKOV-3 scrambled cells than in SKOV-3 shRNAGal3 cells, with approximately 46% of binding reduction, and that this group had a lower binding proportion than the PBS or sucrose (100 mM) group. The positive control was performed with lactose (100 mM) and showed that the binding of MCP-^{99m}Tc in SKOV-3 scrambled cells was greater than in SKOV-3 shRNAGal3 cells, demonstrating the lowest binding among all groups. These results demonstrated that cellular binding and internalization of MCP, in SKOV-3 cells, is not only influenced by the expression of Gal-3, but for other unspecific or unknown targets, and more studies need to be carried out to discover this non-specific target.

These results corroborate the data obtained by Silva et al. 2023 (in production) regarding the structure and monosaccharide composition of MCP obtained by HPSEC-RID and HPAEC-PAD analysis. These results demonstrate that there is a diversity of monosaccharides and oligosaccharides with different molecular weights within the MCP structure, with fractions of 30 KDa (MCP30) and 3 KDa MCP3, which are rich in galactose monomers. The hemagglutination and Sepharose/Gal-3 assays also indicated that

inhibition of Gal-3 by MCP occurs mainly in low molecular weight fractions (MCP3) that have a greater affinity for galectin-3. The importance of Gal-3 in explaining the mechanism of action of MCP has been exhaustively described in studies involving some applications of MCP. There are a series of MCP molecules in studies for different applications, and there are also MCP molecules coupled with other molecules that show synergistic actions (Silva et al. 2023, in production). This variation in molecules classified as pectin or MCP must be particularly analyzed, since they all have their respective monomeric properties (monosaccharide composition), molecular weight, number of branches, esterification, extraction source, and extraction method, among other characteristics (Silva et al., 2023, in production). There are also several references demonstrating that MCP binds to Gal-3, but not all of them prove its inhibition (Glinsky and Raz 2009; Kolatsi-Joannou et al. 2011; Ma et al. 2016; Vergaro et al. 2016). Therefore, the mechanism of action of MCP is not a consensus and should not be generalized.

Inside of oncology application, MCP showed an anticancer activity *in vitro* to prostate cancer (LNCaP, PC3, CASP2.1, CASP1.1 cells), ovarian cancer in SKOV-3 cells 2D and 3D model, human breast (MDA-MB-231 cells), liver cancer (HepG2 cells), lung (A549 cells) and colon cancer (Caco-2 and HCT-116 cells), alone or synergistically enhanced by BD (BreastDefend), PC (ProstaCaid), Cet-MCPCNPs (cetuximab-conjugated modified citrus pectin-chitosan nanoparticles for targeted delivery of curcumin) or cisplatin (Yan & Katz, 2010; Hossein et al., 2013; Hossein et al., 2019a; Jiang et al., 2013a; Leclere et al., 2015; Sabra et al., 2019; Y. Wang, Balan, et al., 2010). Moreover, MCP can sensitize cancer for radiotherapy to prostate cancer cells (PC-3, DU-145, and Cl-1 cells) (Conti et al. 2018). A great example of this application is the GCS-100 and GM-CT-01 molecules used against myeloma, lymphoma, leukemia, and prostate cancer chemoresistance, alone or in combination with cisplatin (Chauhan et al., 2005; Clark et al., 2012; O'Brien & Kay, 2011; Ruvolo et al., 2016; Streetly et al., 2010; Wang, Balan, et al., 2010; Demotte et al., 2014). All of these molecules are related to a mechanism of action that involves the interaction between MCP and galectin-3.

To verify the antitumor effect of MCP, an anticancer activity assay was performed using a tumor xenograft model administered intravenously and orally (Figure 2). The

animals were divided into four groups and treated orally with vehicle (saline), MCP (200 mg/kg), or IV administration of vehicle (saline) and MCP (20 mg/kg). Tumor volume was measured daily, and tumor growth and tumor weight were quantified. The results showed that 20 mg/kg of MCP daily I.V. administered for 21 days significantly reduced tumor growth by 48.5 %, and tumor weight by 50%. However, MCP treatment (200 mg/kg orally administered daily) did not reduce tumor growth or tumor weight in this study. To verify the toxicity of MCP treatment, body weight was measured, and histochemical and biochemical analyses were performed. These results demonstrate that the presence of tumoral xenografts of SKOV-3 cells reduced the body weight of the animals after 21 days of treatment, independent of MCP utilization (Figure 2 E and F). Histochemical analysis showed that oral and IV administration of MCP did not cause kidney damage, as shown by structure of renal glomeruli, proximal convoluted tubules, distal convoluted tubules, and extracellular matrix (ECM) deposition (Figure 2 G and H). Biochemical analysis showed that the levels of TGP (ALP), TGO (AST), FAL, urea, glucose, and creatine remained unaltered after IV administration of MCP. However, the levels of TGO (AST) and creatine changed in the group treated with oral MCP. These studies demonstrated that treatment with MCP (20 mg/kg) exhibits anticancer activity in an SKOV-3 cells tumor xenograft model when administered intravenously, without evidence of kidney damage or hepatic toxicity. However, treatment with oral MCP (200 mg/kg) did not exhibit anticancer activity and showed hepatic toxicity.

Despite this, the use of MCP in preclinical and clinical studies has been realized principally through oral administration. Other routes of administration that are less often used are intravenous, intraperitoneal (I.P.), intratumoral, intrathecal, and intracerebroventricular injection. In contrast to our results, oral administration of MCP showed positive effects against human breast carcinoma, melanoma, and human colon carcinoma (1-1.5% w/v, in drinking water), bladder tumor (350-700 mg/kg fed daily for 6 weeks), colon cancer (1–5.0% wt/vol in drinking water), prostate cancer (1.0% wt/vol in drinking water), breast cancer (350 mg/kg and 700 mg/kg every day orally for consecutive 28 days), and thyroid carcinoma (MCP and S-trans, transfarnesylthiosalicylic acid FTS; Salirasib 40 mg/kg, daily by oral administration and MCP 0.5% in mice drinking water 5 ml/day). However, in colorectal cancer, 20% MCP in feed mixtures for 21 days

has a negative effect. (Dange et al., 2014; Nangia-Makker et al., 2002; Fang et al., 2018; Ferreira-lazarte et al., 2021; Liu et al., 2008; Pienta et al., 1995; Wang et al., 2022; Menachem et al., 2015). Corroborating our results, the anticancer activity of MCP *in vivo* with alternative routes of administration has been observed in metastatic breast and prostate carcinoma models (I.V., 0.25% wt/vol), melanoma models (I.V., 0.5% wt/vol), mammary adenocarcinoma metastasis models (I.T., 0.5 µL/g/day for 21 days), and colon cancer models (PSVII@MCP-CaP, I.V., 5 mg/kl every 4 days for 7 times) (Glinskii et al., 2005; Inohara & Raz, 1994; Wang et al., 2020ª; Bai et al., 2021). MCP also had an immunomodulatory effect in a sarcoma model (MCP of ginseng pectin, I.P., 10 mg/kg each day for 10 days) (Xue et al. 2019).

It is important to analyze that our models were tumor xenografts subcutaneously injected and that for a pharmacological treatment of this tumor, the molecule needs to reach the tumor through blood circulation, this way, treatments of MCP administrated orally against chemically induced tumors, like a colon cancer model, can demonstrate better results. For example, the rats exposed to azoxymethane or methylnitrosourea, chemical inducers of colorectal cancer, treated with a pectin diet reduced the incidence of cancer development (Watanabe K, Reddy BS, Weisburger JH 1979). Other studies have shown that mice exposed to 1, 2-dimethylhydrazine (a chemical inducer of colon cancer) and treated with pectin show reduced colon tumors (Heitman et al. 1992). Rats treated with azoxymethane plus citrus and apple pectin in the diet decreased carcinogenesis and inhibited the B-glucoronidase, an enzyme produced by colon bacteria related with cancer development (Ohkami H, Tazawa K, Yamashita I, Shimizu T, Murai K, Kobashi K 1995). Finally, mice with colon tumors treated with daily intake of 0.8 and 1,6 mg/ml of MCP showed a reduction in tumor size by almost 70% (Hayashi, Gillen, and Lott 2000). However, a study make by Ferreira-lazarte et al. (2021) showed that citrus pectin and modified citrus pectin did not prevent tumors in rats treated with azoxymethane and dextran sodium sulfate. In this study, with both inductors of tumorigenesis, more than 50% of the animals died, and metabolic studies demonstrated that this effect occurs due to severe dysbiosis in the gut and an increase of *Proteobacteria* (Enterobacteriaceae). Furthermore, the pH in the cecum lumen and the levels of propionic and butyric acids decreased. The levels of acetate and lactic acid increased, and the levels of glucose and

triacylglycerides decreased (Ferreira-lazarte et al., 2021). However, the authors argued that in this study, they used a feed with 20% pectin in free alimentation, an excess that could provoke the death of rats and the lack of cancer prevention. In our study, we did not verify an antitumor effect at doses of 200 mg/kg of MCP administered orally in tumor xenografts of SKOV-3 cells, possibly because of the low intestinal absorption of MCP *in vivo*, as demonstrated in a previous study (Silva et al. 2023, in production). More studies are needed to complement these results, and our data corroborate the current literature in that it is important to be careful with the doses used in studies of oral administration of MCP to avoid toxicity.

To demonstrate where MCP binds when reaching the tumor tissue in animal models of SKOV-3 tumors, an *in vivo* autoradiography study was performed. This study demonstrated that MCP-^{99m}Tc intravenously injected binds to the internal regions of the tumor tissue, with cell death and necrosis sites. Quantification analysis demonstrated that the regions of necrosis were correlated to the regions with MCP-^{99m}Tc activity, but were not restricted to it (Figure 4 A). We also verified that the expression of Gal-3 in tumors of SKOV-3 cells was higher in the regions of fibers, extracellular matrix, and necrosis sites (Figure 4 B). These results demonstrate that MCP reaches SKOV-3 cell tumors after IV administration by binding to regions of necrosis rich in Ga-3 expression and other sites. However, these data do not necessarily indicate a direct relationship between Gal-3 expression and MCP binding in tumors.

To determine the correlation between MCP and Gal-3 expression in their anticancer response, anticancer activity was studied using tumor xenografts of SKOV-3 and MKN45 cells. First, the immunostaining assay showed that MKN45 cell xenografts showed greater expression of Gal-3 than SKOV-3 cell xenografts (Figure 5 A). The anticancer activity results showed that in both tumor xenograft models, a concentration of 10 mg/kg of MCP showed antitumor activity, whereas 5 mg/kg did not. In the tumor of SKOV-3 cells, MCP treatment (10 mg/kg) reduced tumor growth by 58.7% and tumor weight by 51.7%. In the tumor of MKN45 cells, MCP (10 mg/kg) reduced tumor growth and tumor weight by 35.4% and 30.7 % (Figure 5 B -G). We also verified the proportion of MCP in tumors using a biodistribution study. The results showed that MCP binds in the same proportion in both SKOV-3 and MKN45 tumor xenografts (Figure 6 A). Both

tumor xenografts reduced the weight of the animals during the 21 days of treatment, independently of MCP treatment (Figure 6 B and C), and food consumption increased as the concentration of treatment increased (Figure 6 D). None of the MCP treatment concentrations changed glycemia in the mice (Figure 6 E). These results demonstrate that the expression of Gal-3 in the tumor xenografts of SKOV-3 and MKN45 cells did not influence the antitumor effect or tumor binding of MCP *in vivo*, as well as showed that treatment of MCP did not show toxicity. These results demonstrate evidence that corroborate the null hypothesis of the direct pharmacological effect of MCP exclusively against Gal-3 and show that other targets that are not yet known may contribute to the antitumor effect of MCP.

The mechanism of action of MCP has been related to the direct effect of MCP binding and inhibition of Gal-3 proteins, as well as indirect effects such as systemic immune modulation (Pedrosa et al., 2022, Silva et al. 2023 in production). For example, pectin isolated from okra (a tropical plant) rich in RG-I induces apoptosis and arrests the cell cycle of B1610 cells (skin melanoma) through the interaction between MCP and galectin-3 (Vayssade et al. 2010). This interaction occurs between the RG-I regions and galactan residues (Gao et al., 2012; Gunning et al., 2009). Pancreatic ductal adenocarcinoma (PDAC) treated with pectins and arabnogalactan-proteins (AGPs) did not show inhibition of adhesion and migration when galectin-3 was deleted by siRNA knockdown (Schöll-naderer et al. 2020). In mice with colon cancer, MCP treatment reduces metastasis, resulting in less expression of Gal-3 (Liu et al., 2008). Sathisha et al. (2007) also demonstrated that pectin rich in arabinogalactan, arabinose, and galactose could inhibit hemagglutination induced by Gal-3 (Sathisha et al. 2007). Low-molecular-weight citrus pectin (LCP) has anti-tumor properties in gastrointestinal cancer *in vitro* and *in vivo*, with reduction of ciclin B1 (cell cycle-related protein) Gal-3, Bcl-xl and Survivin, promoting apoptosis, and reducing tumor growth (Wang et al., 2016).

A study carried out by Wu et al. (2020) using gal-3-binding affinity KD and IC50 studies in MCF-7 cells showed the cooperation between HG and RG-I in the inhibition of galectin-3 (Wu et al. 2020b). Zheng et al., 2020 used crystallography and NMR spectroscopy to demonstrated that homogalacturonan (HG) bind to the galectin-3 carbohydrate recognition domain. Fang et al (2018) also studied the effect of MCP in

urinary bladder cancer (UBC) and showed an inhibition of cell viability of UBC cells, apoptosis, cell cycle arrest and anti-proliferation marker, Gal-3 reduction and Akt signaling pathway, and that Gal-3 deletion by siRNA inhibited the MCP-mediated apoptosis, cell cycle arrest and anti-proliferation effect. In the same study, oral administration of MCP in T24 xenograft-bearing nude mice reduced tumor growth (Fang et al. 2018). The GR-MD-02 molecule, a complex carbohydrate that interacts with Gal-3, promotes the proliferation of antigen-specific T cells in advanced tumors (Dong et al. 2018; Traber and Zomer 2013). In addition, GR-MD-02 with anti-OXI40 promoted survival of animals in MCA-205 sarcoma model, 4T1 breast cancer model, and transgenic rat prostate adenocarcinoma (TRAMP-C1) (Bayes-Genis et al. 2014; Dong et al. 2018). Cui et al. (2019) showed that pectin from *Panax ginseng* flower buds exhibited an affinity for Gal-3 in a biolayer interferometry assay (Cui et al., 2019b).These applications of galectin-3 inhibitors demonstrate that Gal-3 is a potential target in cancer treatment. However, there is evidence that not show an anticancer activity of MCP or its interaction with Gal-3 to explain its mechanism of action. Stegmayr et al. (2016) used a fluorescent anisotropy assay and *in vitro* studies to show that pectin and galactomannan (polysaccharide related to anti-tumor and anti-inflammatory activity) did not have affinity for Gal-3. Jackson et al., 2007 used distinct pectin in LNCaP (androgen-dependent prostate cancer) and LNCaP C4-2 cells (androgen-independent prostate cancer that does not express galectin-3) and verified that HG, RG-I, and RG-II did not demonstrate any cytotoxic effect. Many other studies have corroborated this hypothesis. (Nishikawa et al., 2018; Leffler, 2019; Fang et al., 2018; Johannes et al., 2018; Shao et al., 2019).

MCP can also stimulate the immune system, as shown by Xue et al. (2019), who demonstrated that ginseng pectin inhibits gal-3-induced T-cell apoptosis and allows its activation in the sarcoma-180 mouse model. This molecule increased T-cell proliferation and IL-2 expression and reduced tumor growth by 45% through of the PKC/ERK and ROS/ERK pathways, involved in activation and apoptosis of T-cells, and Ras/PI3K/Akt in activation of T-cell stimulation (Xue et al., 2019). A study made by Wang et al. (2020) showed that regions of hypoxia in the tumor increase the synthesis and secretion of galectin-3 by tumor-associated macrophages (TAM). Gal-3 secreted by TAM has a pro-tumorigenic effect and provokes the nucleation of NF-κβ, activation of ROS, tumor

growth, and metastasis. In turn, this action is inhibited by administration of MCP or by a macrophage-depletion agent (clodronate liposomal [CP]) in mammary adenocarcinoma and metastasis models. Greater inhibition of tumor growth was seen by co-administration of CL and MCP with sorafenib and bevacizumab (anti-angiogenesis agents). These results show that hypoxia-induced galectin-3 expression can be secreted by tumor-associated macrophages, and that this secretion promotes tumor growth and metastasis, with these effects being inhibited by MCP via Gal-3 inhibition (Wang et al., 2020).

Thus, the literature and our results show the complexity of the mechanisms involved in the anticancer activity of MCP. It is possible that this activity is not related to a target or a single mechanism of action but to several targets, and several mechanisms still unknown. The data acquired in this study provide evidence that corroborates the null hypothesis of the direct pharmacological effect of MCP exclusively against Gal-3, widely disseminated in studies with pectin, and shows us that more studies need to be carried out to discover possible molecular targets that are not yet known.

5. Conclusion

In this study, we investigated the *in vivo* anticancer activity of MCP using molecular imaging. To this end, we radiolabeled MCP with ^{99m}Tc and analyzed the behavior of MCP-^{99m}Tc in animals with SKOV-3 and MKN45 tumors. First, we demonstrated that cell binding and internalization of MCP were only partially influenced by Galectin-3 expression in SKOV-3 scrambled and SKOV-3 gal$^{-/-}$ cells. Next, we showed that MCP exhibits anticancer activity in an SKOV-3 cell tumor xenograft model when administered intravenously, but not orally. Subsequently, we demonstrated that MCP reaches the tumor and binds to regions of necrosis in SKOV-3 tumors *in vivo*. Finally, we demonstrated that the tumor expression of Gal-3 *in vivo* did not influence the anticancer activity of MCP and MCP tumor binding in SKOV-3 and MKN45 tumors. Thus, these results do not corroborate the hypothesis of the direct pharmacological effect of MCP exclusively targeting Gal-3 and contribute to the understanding of MCP anticancer activity.

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Conclusions

In the first part of this work, we demonstrate with a review that MCP has been widely studied and shows biological activities in many types of diseases, as evidenced by *in vitro*, *in vivo*, and clinical studies. MCP has a prominent role in cancer, presenting anti-proliferative, apoptotic, and anti-metastatic activities. In addition, MCP is important in anticancer therapies as an adjuvant in combination with chemotherapy and radiotherapy. Furthermore, MCP can act as a renal protector against damage caused by chemotherapy in tumor treatments. These activities could be linked to its ability to bind to the galectin-3 protein, acting directly through molecular targets in the tumor and indirectly through systemic immunomodulation. In the second part, we demonstrated in an unprecedented way that MCP can be radiolabeled with ^{99m}Tc with good radioactive purity, and that this technology can be used as a tool to study the biological activity of MCP *in vivo*. We determined the structure and monosaccharide composition of MCP and its partial affinity and inhibitory capacity against galectin-3. We demonstrated that IV administration of MCP-^{99m}Tc resulted in renal/hepatobiliary elimination and that oral administration of MCP resulted in low gastrointestinal absorption and intestinal elimination. Moreover, we verified the influence of Gal-3 on biodistribution, blood compartmentalization, and IV pharmacokinetics of MCP-^{99m}Tc *in vivo*. In the final part of this study, we investigated the *in vivo* anticancer activity of MCP using molecular imaging. For this, we radiolabeled MCP with ^{99m}Tc and analyzed the behavior of MCP-^{99m}Tc in animals with SKOV-3 and MKN45 tumors. First, we demonstrated that cell binding and internalization of MCP were only partially influenced by Galectin-3 expression in SKOV-3 scrambled and SKOV-3 gal$^{-/-}$ cells. Next, we showed that MCP exhibits anticancer activity in an SKOV-3 cell tumor xenograft model when administered intravenously, but not orally. Subsequently, we demonstrated that MCP reaches the tumor and binds to regions of necrosis in SKOV-3 tumors *in vivo*. Finally, we demonstrated that the tumor expression of Gal-3 *in vivo* did not influence the anticancer activity of MCP and tumor binding in SKOV-3 and MKN45 tumors. Thus, we conclude that radiolabeling MCP with ^{99m}Tc is an important tool for studying the anticancer activity of MCP. We also conclude that the data obtained in this work do not corroborate the hypothesis of the direct pharmacological effect of MCP exclusively targeting Gal-3, which has been extensively reported in the literature. Thus, our results contribute to the

understanding of MCP anticancer activity. However, more studies need to be carried out to analyze the mechanisms of action of MCP.

Final considerations

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Supplementary material

TABLE 1: Monosaccharide composition of CP, MCP, MCP30, MCP30/10, MCP10/3, and MCP3. Fucose (Fuc), arabinose (Ara), rhamnose (Rha), galactose (Gal), glucose (Glc), xylose (Xyl), mannose (Man) and galacturonic acid (GalA). Data are representative of three independent experiments.

	Ara	Rha	Gal	Glc	Xyl	GalA
CP	0.45±0.10	0.44±0.09	1.34±0.44	0.87±0.27	0.18±0.07	96.61±0.86
MCP	0.52±0.02	0.75±0.28	0.98±0.08	0.35±0.02	0.11±0.02	97.14±0.38
MCP>30	6.10±2.08	19.11±4.11	19.15±5.26	1.11±0.57	1.47±0.75	51.71±13.01
MCP<30>10	4.58±0.04	3.83±0.16	8.22±1.54	0.77±0.13	2.33±1.07	83.92±5.42
MCP<10>3	0.67±0.03	3.83±0.60	1.29±0.22	0.19±0.02	0.16±0.04	96.56±0.04
MCP<3	2.28±0.47	0.19±0.12	16.71±4.91	3.07±1.01	-	77.63±6.07

Table 2: Doses used per experiment in MBq

Experiment	**Dose (MBq)**
Radiolabeling of MCP	130
Quality control	20
Stability of MCP-^{99m}Tc in saline, plasm and different pH	20
Sepharose/Gal3 column assay	37
Stability of MCP-^{99mTc} *in vivo*	37
Biodistribution studies (IV)	10
Biodistribution studies (oral)	37
Kinetic studies (IV)	10-20
Kinetic studies (oral)	37
Pharmacokinetic studies	10-20
µSPECT/CT imaging	37
Blood compartment distribution assay	10-20
Partition coefficient	1

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