INSTITUTO POLITÉCNICO DE LISBOA

ESCOLA SUPERIOR DE SAÚDE DE LISBOA

UPCOMING mTOR SIGNALING INHIBITORS IN GASTRIC CANCER TREATMENT – A SYSTEMATIC REVIEW OF PRECLINICAL STUDIES

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Master's Degree in Clinical Laboratory Technologies

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Resumo

Contexto: De acordo com a Agência Internacional para a Investigação do Cancro, o CG é o quinto tipo de cancro mais prevalente na Europa e com a sexta taxa de mortalidade mais elevada, sendo que em Portugal é o quarto tipo de cancro mais prevalente com a terceira taxa de mortalidade mais alta. mTOR é uma proteína que, em condições normais, regula atividades fundamentais mas sua sobre-expressão está associada a várias patologias, incluindo cancro. Deste modo, o estudo dos inibidores de mTOR tornou-se uma alternativa terapêutica promissora, em muitos estudos in vivo e in vitro que avaliaram a eficácia e segurança destes no tratamento do cancro gástrico, como é o caso do Everolimus, OSI-027, 2,6-DMBQ, Temsirolimus e ácido corosólico. Perante estes factos, o principal objetivo desta revisão é analisar e sistematizar a informação atualmente disponível relativa a dados de estudos pré-clínicos in vitro e in vivo que consideram os inibidores da via de sinalização mTOR para o tratamento do cancro gástrico. **Métodos:** Após a aplicação da expressão de pesquisa, apenas estudos não clínicos *in vitro* e *in vivo* publicados entre janeiro de 2013 e maio de 2025 foram considerados. **Resultados:** 14 artigos foram incluídos segundo os critérios estabelecidos Entre os inibidores analisados, o Everolimus foi o mais estudado e o que apresentou melhores resultados em modelos pré-clínicos de cancro gástrico. Demonstrou eficácia consistente in vitro (10–100 nM, 24–72 horas) e in vivo (5–10 mg/kg/dia, 2–4 semanas, via oral), com redução de marcadores como p-mTOR, p70S6K e p-AKT. A combinação com outros fármacos (como cisplatina e cloroquina) potenciou o efeito terapêutico. Outros inibidores, como Temsirolimus, OSI-027, 2,6-DMBQ e Rapamicina, também mostraram potencial, mas com dados mais limitados e menor frequência de estudo. **Conclusões:** Entre os inibidores analisados, o Everolimus destacou-se como o mais eficaz, mostrando efeitos antiproliferativos consistentes *in vitro* e *in vivo*, especialmente em combinação com outros fármacos. Estes dados apoiam o potencial do Everolimus como terapia promissora para o tratamento do CG.

Palavras-chave

Cancro Gástrico, Via de sinalização, mTOR, Inibidores da mTOR, Estudos pré-clínicos

Abstract:

Context: According to the International Agency for Research on Cancer, gastric cancer (GC) is the fifth most prevalent type of cancer in Europe and has the sixth highest mortality rate. In Portugal, it ranks as the fourth most common cancer and the third leading cause of cancer-related death. mTOR is a protein that, under normal conditions, regulates fundamental cellular activities; however, its overexpression is associated with various pathologies, including cancer. As such, the study of mTOR inhibitors has become a promising therapeutic alternative, supported by numerous in vivo and in vitro studies evaluating their efficacy and safety in the treatment of gastric cancer—examples include Everolimus, OSI-027, 2,6-DMBQ, Temsirolimus, and corosolic acid. Given these findings, the main aim of this review is to analyse and synthesise the currently available information from preclinical in vitro and in vivo studies investigating mTOR pathway inhibitors for the treatment of gastric cancer. **Methods:** Following the application of the search expression, only non-clinical in vitro and in vivo studies published between January 2013 and May 2025 were considered. **Results:** Fourteen articles were included based on the established criteria. Among the inhibitors analysed, Everolimus was the most extensively studied and showed the most promising results in preclinical models of gastric cancer. It demonstrated consistent efficacy in vitro (10–100 nM, 24–72 hours) and in vivo (5–10 mg/kg/day, 2–4 weeks, orally), with reductions observed in markers such as p-mTOR, p70S6K, and p-AKT. Combination with other drugs (such as cisplatin and chloroquine) enhanced its therapeutic effect. Other inhibitors, including Temsirolimus, OSI-027, 2,6-DMBQ, and Rapamycin, also showed potential but were less frequently studied and supported by more limited data. **Conclusion:** Among the inhibitors assessed, Everolimus emerged as the most effective, demonstrating consistent antiproliferative effects in both in vitro and in vivo settings, particularly when used in combination with other agents. These findings support the potential of Everolimus as a promising therapeutic option for the treatment of gastric cancer.

Keywords

GC, signaling pathway, mTOR, mTOR inhibitors, preclinical studies

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List of Abbreviations

Abbreviations	Name
mTORi	mTOR Inhibitors
IP	Intraperitoneal
O	Oral
SC	Subcutaneous
IV	Intravenous
GC	Gastric cancer
WB	Western Blot
FC	Flow Citometry
H	Histology
IF	Immunofluorescence
IHC	Immunohistochemistry
Edu	5-ethynyl-2'-deoxyuridine
H&E	Hematoxylin and Eosin
CCK-8	Cell Counting Kit-8
TEM	Transmission electron microscopy
MTT	3-(4,5-dimethyl thiazol-2-yl) -2,5- diphenyl tetrazoliumbromide
RT-PCR	Reverse-transcription-polymerase Chain Reaction
qRT PCR	Real-time quantitative PCR
PI	Propidium iodide
TZB	trastuzumab
C mid	Intermediate concentrations
C max	High
GEP-NENs	Gastroenteropancreatic Neuroendocrine Neoplasms
GEP-NETs	Gastroenteropancreatic Neuroendocrine Tumours
pNETs	Primary cultures of human Pancreatic Neuroendocrine Tumours

Introduction

Gastric cancer

Cells collectively work to constantly maintain homeostasis, which may be affected by some diseases, that can modify their behavior, such as it occurs in cancer [1]. Cancer is an unnatural cellular growth that originates due to loss of control mechanisms leading to a mass called a tumour, which can invade and destroy healthy organs and spread to other distant parts of the body [2]. Cancer cells can develop in any part of the body and due to multiplication, cells can go through the blood vessels allowing the cancer to travel to other parts of the body far from the original site, resulting in metastasis [2]. The accumulation of mutations within a genetically unstable heterogeneous population of cells, with the eventual emergence of a malignant subclone, results in solid tumours capable of invasion, metastasis. Each sub-clonal population of cells evolves independently from the others, competing for space and resources[3]. Worldwide, cancer kills 10 million people per year, thus reflecting how common and lethal it remains [2]. The economic impact of cancer is huge and encompasses direct medical costs, lost productivity due to illness and premature death, and the emotional and psychological effects on patients and their families. The cost of cancer to the world exceeds 1 trillion dollars per year [2]. More than 100 different types of cancer and subtypes of tumours found within specific organs[4] exist.

Gastric cancer (GC) develops in stomach cells and can disseminate through the stomach wall and reach the esophagus, small intestine, lymphatic ganglion or other organs such as the liver, pancreas, colon, lungs and ovaries[5]. Despite advances in medical imaging and diagnostics, GC remains a global health challenge, characterised by diverse risk factors and causes, aggressive nature, and many cases are diagnosed at advanced stages due to nonspecific initial symptoms [5, 6]. Late-stage diagnosis presents an obstacle that significantly affects patient outcomes. The financial burden of GC treatment is significant, covering costs of surgery, chemotherapy, radiotherapy, preventive care and long-term follow-up, compounded by reduced cost productivity and the cost of caregivers. Elderly and sick patients diagnosed in final stages bear substantial financial burdens, but early and appropriate treatment choices could reduce costs. In addition to the financial impact, there is psychological suffering and a reduction in the quality of life, highlighting the need for psychosocial support from medical professionals, caregivers and support groups. To be able to overcome these challenges, cost-effective treatment methods are required, as are detection and prevention strategies, which together with comprehensive psychological and social support services will improve patient and family well-being [5].

Epidemiology

In 2022, GC was the fifth type of cancer with the most incidence worldwide at 4,8% (n=968.784) and the fourth in mortality at 6,8% (n=660.175) of fatalities worldwide (**Figure 1**)[7].

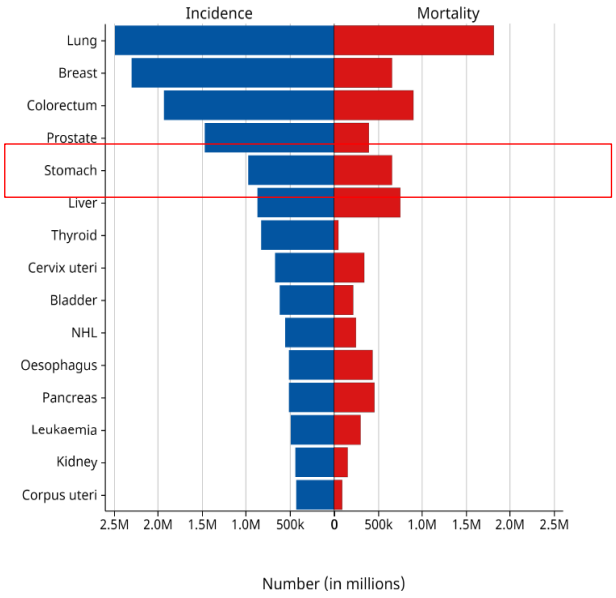

Figure 1- Incidence and mortality of different types of cancer worldwide[7].

In 2022, GC was the ninth most prevalent type of cancer in Europe with 135.610 cases and the sixth in mortality with 95.431 cases which led to death (**Figure 2**)[7].

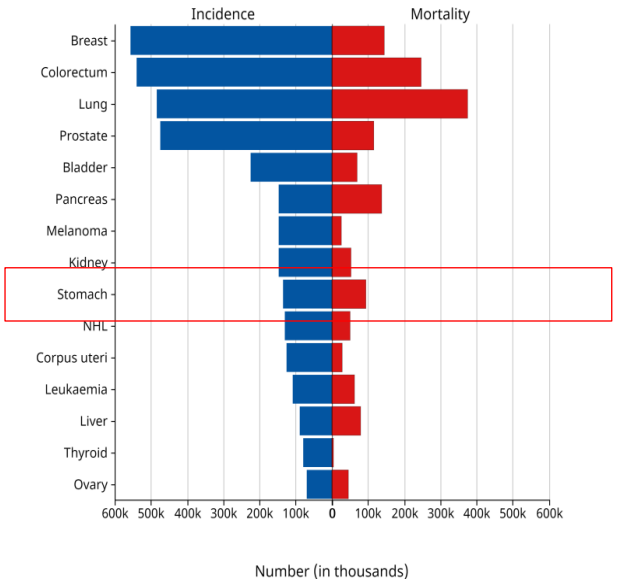

Figure 2- Incidence and mortality of the most prevalent types of cancer in Europe[7].

In Portugal in 2022, GC was the fifth cancer with the most incidents with 3.668 cases and the third in mortality with 2.578 registered cases (**Figure 3**)[7].

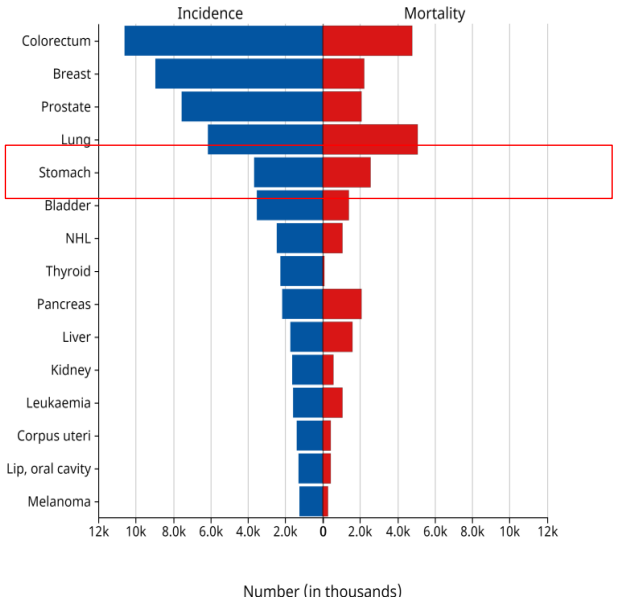

Figure 3- Incidence and mortality of the most prevalent types of cancer in Portugal[7].

The incidence varies depending on anatomical site and cancer subtype. While cases of GC associated with diet and Helicobacter pylori infection have declined over the past two decades, the incidence of proximal tumours linked to obesity and sociodemographic factors has been increasing[6].

Etiology

The etiology of GC is multifaceted, involving environmental influences, lifestyle factors, and genetic susceptibilities[5]. Studies have shown that some people are more susceptible than others to developing GC. One of the more affected groups by the disease are men older than fifty-five years old as well as African American men in Western Europe and Latin America [8]. The risk factors for GC are numerous and modifiable, which include lifestyle choices such as diet, with a high consumption of dried, salty and smoked foods, diets rich in fresh produce, smoking, and alcohol consumption, all of which can be altered to reduce risk and non-modifiable risk factors that include genetic predispositions, age and certain medical conditions that are inherent and cannot be changed (**Figure 4**) [5].

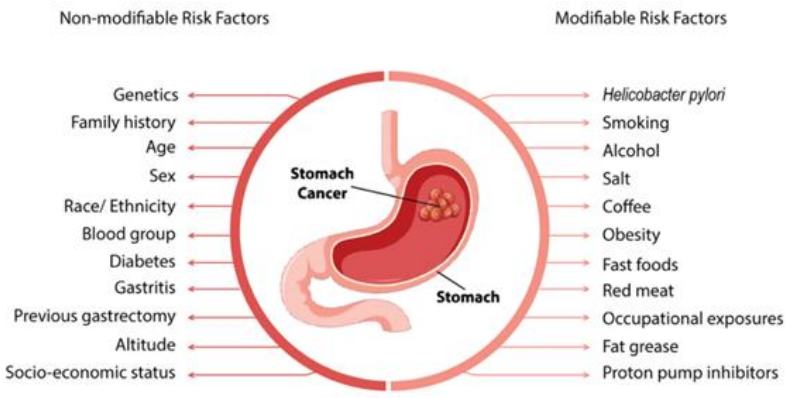

Figure 4- Modifiable and non-modifiable risk factors of GC [5].

Other reasons to increase the possibility of developing GC is inflammation and ulcers caused by the infection with *Helicobacter pylori*, which emerges as the foremost risk factor[8]. In addition to external elements, there are also intrinsic factors, such as the patient's susceptibility, which also influences GC. Whole exome sequencing analyzes show other germline mutations, such as in the tumour suppressor genes *SDHB, CTNNA1, STK11* and the *PALB2, BRCA2* and *ATM* genes involved in DNA repair. Furthermore, patients suffering from syndromes affecting DNA repair genes, like *TP53* and *APC,* as well as tumour suppressor genes (*BRCA*) are more likely to develop GC[8].

Eradication of *Helicobacter pylori* substantially reduces the incidence of GC in healthy individuals, patients with gastric atrophy and people with a family history of GC and endoscopy-based screening programs have been a success in higher detection rates of early-stage GC, with substantially reduced mortality [9].

Clinical Symptoms and Diagnosis

GC is generally asymptomatic in the early stages. At an advanced stage, the common signs and symptoms are dysphagia, poor appetite, weight loss, and abdominal pain, asthenia, indigestion, vomiting, weight loss, early satiety and/or iron deficiency anemia[9].

The gold standard for diagnosis includes endoscopic examination to locate the tumour and obtain a biopsy, followed by clinical staging to guide treatment decisions. CT scanning remains the standard method for detecting metastatic disease[6]. Endoscopic ultrasonography is a helpful tool to identify infiltrated regions of the gastric wall, and endoscopic mucosal resection and endoscopic submucosal dissection may also be used for diagnosis [9].

Endoscopic ultrasound is valuable for detecting early-stage, non-metastatic tumours that may be suitable for endoscopic resection. Peritoneal involvement is common but can be overlooked on cross-sectional imaging. Therefore, diagnostic laparoscopy is strongly advised for comprehensive perioperative staging when there is no radiological evidence of

metastasis. The presence of positive cytology in peritoneal lavage is classified as micrometastatic stage IV disease, for which routine surgical intervention is generally discouraged due to the high risk of recurrence. Nonetheless, surgery is being increasingly considered in selected patients who demonstrate negative cytology following chemotherapy[6].

There are many techniques that can be used to reach a diagnosis, although no single test can single-handedly diagnose cancer. Initial staging should include full blood count, liquid biopsy, liver and renal function assessment to identify iron deficiency and determine proper therapeutic options. Immunophenotyping and cytogenetic analysis can also help the diagnosis with analysis of antigens or markers in cell's surface of the cells or changes in chromosomes that may indicate a genetic condition for some types of cancer, respectively[9,10].

Tumour markers, proteins and other substances that are produced by cancer cells or other cells in response to cancer, can also be measured. Most tumour markers are searched in the blood, but can also be analyzed in the bone marrow, urine or in the tumour tissue itself. Some common tumour markers are **BRCA1** and **BRCA2**, **CA19.9**, **Carcinoembryonic antigen (CEA), C-kit/CD117**, **DPD** and **HER2** overexpression[10].

Imaging tests such as CT scans, MRI, ultrasound and PET scans can also provide essential information to help achieve a diagnosis[9,10]. CT and PET scans can create 3D imaging of different organs. While a CT scan uses an x-ray machine to take images from different angles of the organs, a PET scan can highlight the areas with higher glucose intake and, because cancer cells often take up more glucose than healthy cells, this technique can be a powerful diagnostic tool [9,10]. MRI uses powerful magnet and radio waves to take images of the body in slices, while ultrasound uses high-energy sound waves that echo off tissues inside the body. A computer uses these echoes to create images of areas inside the body[9,10].

Even with the variety of tests mentioned above, in most cases doctors need a biopsy to confirm a diagnosis. A biopsy consists in the removal of a tissue sample to be analyzed under the microscope[10].

GC is a biologically diverse disease. Traditionally, the Laurén classification has been used to categorise it into intestinal and diffuse subtypes. The intestinal type is associated with Helicobacter pylori infection and is characterised by well-differentiated glandular or papillary structures. In contrast, the diffuse type comprises poorly cohesive, undifferentiated tumour cells embedded in a dense stromal environment[6].

Carcinogenesis

Carcinogenesis is a complex process, divided into four phases: initiation, promotion, malignant transformation, and progression (invasion and metastasis) that change the normal function of the cell due to their abnormal growth. This process does not occur instantly and it is related to multiple factors such as the genetic predisposition or the duration of the exposition to a carcinogenic agent, resulting in an accumulation of mutations that alter the phenotype that develops characteristics (hallmarks) that facilitate tumour formation (**Figure 5)** [1,11, 12] .

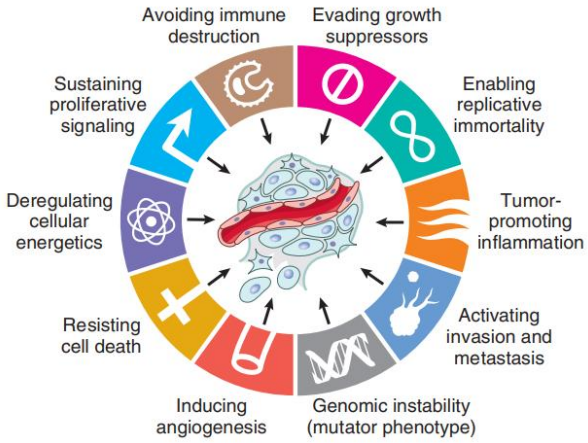

Figure 5- Hallmarks of Cancer[11].

Initiation is the transformation of genetic material of the cell due to mutations to alter a healthy cell to a cancer cell[1]. To develop a tumour, a sequence of mutations needs to occur in tumour suppressor genes and proto-oncogenes. Mutations can occur spontaneously through a random event, a mutation, or due to exposure to a carcinogenic agent [13, 14]. **Promotion** is the part responsible for cell proliferation due to promoters that can be in the environment or in drugs/medication. The promoters take the cells from the transition of the initiation phase to being carcinogenic[1]. In this phase, cells need stimulus to multiply. The stimulus can be divided into genotoxic, cytotoxic and mitogens. Genotoxic represent the stimulus that can cause DNA damage, mitogenic represent the ones that bind to cell receptors causing cell division and the cytotoxic provoke tissue damage that do not cause direct DNA damage. The accumulation of these mutations transforms a normal cell into a carcinogenic one [4]. **Progression** is the last step characterised by cellular growth independent of stimulus and the mutations that have already occurred allow for their continuous survival[4]. In this stage, a tumour can develop to enter the adjacent (invasion) or

distant tissues (metastasis) (**Figure 6**)[1]. Tumour progression can occur *via* different pathways, the hematogenous (blood vessels) and the lymphatic (lymphatic system) which are more common in sarcomas and carcinomas, respectively. There is also the transcoelomic pathway that corresponds to the metastasis through cavities and surfaces from the body, like the peritoneal, pleural, pericardial or subarachnoid cavities[1]. Tumour progression can result in mutation that can be deadly or can affect some cell functions that favor growth, proliferation, survival, invasion and metastasis [2]. Despite cancer being known to be composed of a spectrum of diseases that involves many genotypes and phenotypes, it is the accumulation of mutations in a clone of cells that is a single and unifying theme for all cancers. The minimum set of genotypes and phenotypes that a cancer cell must acquire to become malignant has been called ‘‘the hallmarks of cancer’’ [3]. As healthy cells transform into carcinogenic, it will accumulate hallmarks that are fundamental to continuous tumour growth and these hallmarks are used to classify the disease [2]. All cancer cells show ten fundamental changes [2].

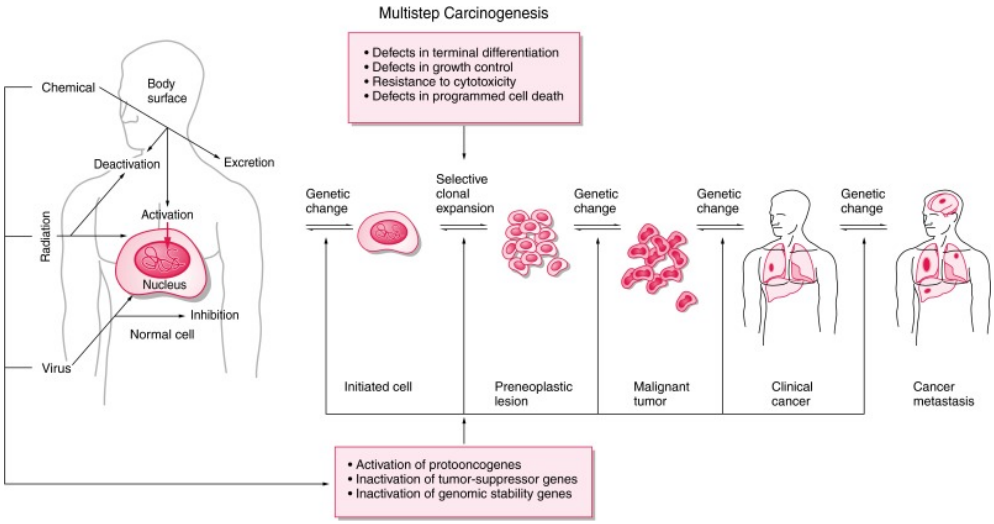

Figure 6- Multistage Carcinogenesis[15]

Cells that combined some, but not all, of these hallmarks or other differences necessary for malignancy are referred to here as ‘‘partially transformed’’. Although single random mutations are usually considered rare, the acquisition of these is a result of the accumulation of random mutations in genetically unstable cells that eventually result in a malignant cancer with the capacity to metastasize. Specific genetic mutations can contribute only partially to the acquisition of a single hallmark capability or may confer several hallmark capabilities at once [3].

Carcinogenesis in gastric cancer

It is widely accepted that a chronic inflammatory process precedes the development of GC, especially intestinal cancer. In relation to GC there are different actors of the carcinogenesis process, including ***Helicobacter pylori***, cancer stem cells, tumour microenvironment and microbiota[8,16].

In the initial process of carcinogenesis, ***Helicobacter pylori*** infection plays an important role, leading to subsequent long-lasting chronic inflammation, and has been designated as a class I carcinogen by the International Agency for Research on Cancer. Gastric microbiome is composed of numerous microbes in addition to ***Helicobacter pylori*** and recent research has shown that the composition of the gastric microbiome is different among patients with or without GC. In addition to various environmental and host factors, there is interest in the possibility that alteration of gastric flora can induce a chronic inflammatory response and precancerous changes, ultimately affecting the process of gastric carcinogenesis. However, most previous studies only revealed the differences in the microbial composition of the stomach, which are insufficient to clarify the role and importance of the microbiome in the process of gastric carcinogenesis [16]. Even so, the majority of infected individuals do not go on to develop GC [8].

Although ***Helicobacter pylori*** infects a large portion of the global population, most individuals do not develop gastric cancer (GC). This discrepancy may be partly explained by the heterogeneity of the bacterial genome and the varying virulence factors expressed by different bacterial strains. The cag pathogenicity island (cag PAI) encodes major bacterial virulence factors, including the oncoprotein CagA and proteins that make up the type IV secretion system (T4SS), which is generally associated with bacterial conjugation systems, enabling the exchange of genetic material between bacteria[8].

CagA, the first bacterial oncoprotein ever described, is translocated into the cytoplasm of gastric epithelial cells via the T4SS. Once inside the host cell, it interacts with multiple signaling pathways, destabilizes cell junctions and apico-basal polarity, and activates pro-inflammatory and oncogenic pathways. These effects impair the integrity, differentiation, and self-renewal of the gastric epithelium[8].

Studies have shown that ***Helicobacter pylori***, in a CagA-dependent manner, inhibits the tumour suppressor p14ARF, which plays a key role in the cellular response to oncogenic stress. This inhibition promotes p14ARF degradation and contributes to gastric carcinogenesis through the p53 pathway. Furthermore, the bacterium downregulates the expression of the transcription factor USF1, which stabilizes p53 under genotoxic stress. The resulting proteasomal degradation of p53 favors tumour progression. In addition, H. pylori positions itself near the host cell membrane, preventing the nuclear translocation of

the USF1/p53 complex, thereby disrupting its transcriptional function, one of the gastric epithelium's key defenses against infection[8].

Helicobacter pylori also negatively regulates the receptor for activated C kinase 1 (RACK1), a tumour suppressor involved in modulating NF-κB signaling. The downregulation of RACK1 leads to increased expression of integrin β1 and enhances oncogenic signaling via the NF-κB pathway. Studies show that RACK1 levels are significantly reduced in GC tissues compared to adjacent normal tissue and are associated with poor patient prognosis[8].

Additionally, ***Helicobacter pylori*** exploits host cell mechanisms to enhance its virulence, including the activation of the c-Abl kinase to maintain phosphorylation of the virulence factor CagA. Recent studies show that H. pylori not only activates c-Abl but also alters the intracellular localization of the activated protein. Cytoplasmic c-Abl promotes cell migration and inhibits apoptosis, thereby contributing to H. pylori-associated gastric carcinogenesis[8].

Changes in cell adhesion molecules induced by ***Helicobacter pylori*** also promote epithelial-to-mesenchymal transition (EMT), a process in which epithelial cells acquire mesenchymal features, becoming migratory and invasive key steps in tumour progression. Loss of IQGAP, a protein involved in stabilizing adherens junctions and sequestering β-catenin, enhances ***Helicobacter pylori*** induced EMT in gastric epithelial cell lines and promotes preneoplastic lesions in vivo. Furthermore, ***Helicobacter pylori*** negatively regulates other adhesion-related proteins such as Afadin, which stabilizes tight junctions, thereby facilitating EMT in gastric cancer cell lines[8].

Tumour cells are highly heterogeneous, differing in their mutations, treatment sensitivity, phenotype, and tumourigenic potential. Since the identification of cancer stem cells (CSCs), a subpopulation of cancer cells with stem-like properties multiple studies have demonstrated their presence in solid tumours such as breast, brain, colon, and gastric cancers. CSCs have the capacity for self-renewal and asymmetric division, enabling them to regenerate and give rise to differentiated tumour cells, thereby expanding the tumour mass. These highly tumourigenic cells are also known to resist chemotherapy and radiotherapy and to migrate and form metastases[8].

The tumour microenvironment (TME) has gained increasing attention, as tumours are now understood as dynamic entities engaged in constant interaction with their surrounding environment. This environment provides factors that sustain and promote cancer cell phenotypes and heterogeneity. Tumour-associated neutrophils (TANs) are among the most important stromal components contributing to carcinogenesis. TANs enhance the migration

and invasiveness of gastric cancer cells. Increased peripheral neutrophil counts, elevated neutrophil-to-lymphocyte ratios, and neutrophil infiltration in the TME have all been linked to poor prognosis. TANs produce IL-17a, which promotes EMT and GC progression via activation of the JAK2/STAT3 pathway. Neutralizing antibodies against IL-17a have been shown to reverse the effects of TANs on GC progression[8].

Gastric cancer-associated fibroblasts (GCAFs), when activated, also enhance the migratory and invasive capabilities of tumour cells. Activated GCAFs are associated with reduced patient survival. Some studies suggest that GCAFs play a role in CSC formation, tumour transformation, and therapy resistance. In addition, GCAFs produce high levels of IL-6, which contributes to JAK/STAT signaling pathway activation[8].

Tumour-associated macrophages (TAMs) may present in two phenotypes, M1 and M2. M2 TAMs are activated by cancer cells and play a crucial role in tumour growth and progression. Gastric cancer tissues are predominantly composed of M2 TAMs, which promote GC cell migration and invasion when isolated. A high abundance of TAMs in the gastric TME is associated with decreased numbers and dysfunction of Natural Killer (NK) cells, an effect that can be reversed by blocking TGF-β1. Thus, TAMs actively contribute to the immunosuppressive environment in GC. Furthermore, TAMs upregulate the expression of programmed cell death protein 1 (PD-1) as GC progresses. Increased PD-1 expression on TAMs is associated with impaired phagocytosis of tumour cells expressing PD-L1, facilitating immune evasion[8].

Recent studies show that the stomach harbors a large and diverse microbial community. Increasing evidence suggests that the complex microbiome colonizing the gastric epithelium, in combination with ***Helicobacter pylori***, can influence gastric homeostasis and disease. One critical step in the histological progression to intestinal-type GC is the development of atrophic gastritis, a condition characterized by the loss of parietal cells and elevated gastric pH. This hypochlorhydria favors the overgrowth of non-Helicobacter bacteria and promotes cancer progression, supporting the hypothesis that the microbiota plays a key role in gastric carcinogenesis[8].

The importance of the **microenvironment** in tumourigenesis is being studied further. Evidence has shown that the complex **microbiome colonizing** the gastric epithelium in combination with H. pylori might influence gastric homeostasis and disease[8].

Gastric cancer Treatment

Standard therapy

Despite advances in diagnosis and treatment, the global evolution of the prognosis of GC remains bleak, mainly due to late-stage detection and its aggressive nature[5]. Most patients are diagnosed in advanced stages, which ends up limiting treatment options and requiring palliative care. GC affects populations much more aggressively in developing nations, and although improved hygiene and the eradication of H. Pylori have contributed significantly to the improvement of GC numbers in developing countries, there is still a long way forward in terms of improving the survival statistics of advanced and metastatic bowel cancer patients. Although there are very few therapies that provide substantial benefits for survival, many different emerging therapies and targets are being evaluated [5, 17].

To date there is no gold standard therapy used in gastroesophageal junction or bowel cancer. Treatment options are selected depending on the stage of the disease, presence of biomarkers and regimen preferred by physicians, and some people will have only one treatment, while others have a combination of treatments, such as surgery with chemotherapy and radiation therapy [17, 18]. Monitoring must be adapted to each patient and to the stage of the disease, given the aggressive nature of GC and historically poor outcomes, even in the setting of disease recurrence. This means that the concept of survival is only now showing better results. Long-term implications, late effects of therapy and the psychosocial impact of treatment have been little studied to date[9].

For early-stage disease, there is greater emphasis on **tumour resection** rather than systemic chemotherapy. Patients with early-stage disease undergo resection procedures, however, most patients relapse following resection; therefore, combined modality therapies are standard [9, 17]. Surgery depends on tumour location and depth, invasion, TNM category and histological subtype and may also vary between institutions, but in general, it includes removing the entire tumour that is contained in one area, debulking a tumour, where part of a cancer tumour is removed. This technique is used when removing an entire tumour might damage an organ or the body. Removing part of a tumour can help other treatments work better and ease cancer symptoms and it is performed to remove tumours that are causing pain or pressure [18]. In GC, the techniques used are endoscopic mucosal resection, distal, subtotal or total esophagectomy gastrectomy. It is essential that physicians and surgeons optimize surgical intervention to extend the patient's quality of life, considering the comorbidities they may present [9, 17]. Although surgery is the only curative approach in the treatment of GC, the addition of pre (neoadjuvant), post (adjuvant) or perinatal **chemotherapy** operationally ensures an added survival benefit [17]. The goal of treatment

for locally advanced GC is curative. The selection of adjunctive therapies in combination with surgery for resectable cases differs across geographical regions[6]. Adjuvant Chemotherapy showed a greater benefit that has been noted in Asian studies, and the uptake of adjuvant chemotherapy in Europe for patients with resected GC remains limited due to a perceived lack of benefit and a routine use of peri operative. Being less well tolerated than neoadjuvant chemotherapy and neoadjuvant therapy leading to tumour downsizing, and allowing for more curative resections, a peri-operative approach is therefore preferred, if possible, so that more patients can benefit from a systemic treatment even if the post-operative component of treatment is unable to be delivered[9]. As demonstrated in peri-operative chemotherapy, an improvement in the 5-year survival from 23% to 36% occurs in patients with resectable stage II and III GC treated with six cycles of pre- and post-operative of epirubicinecisplatine5-FU compared with surgery alone[9]. For locally advanced disease, clinically T2–4 or positive lymph node, the National Comprehensive Cancer Network (NCCN) guidelines recommend giving preoperative chemoradiotherapy or perioperative chemotherapy[17]. In the metastatic setting, first line chemotherapy consists of a platinum-based agent, usually Oxaliplatin and a cytotoxic compound such as 5FU, mainly the FOLFOX or CAPOX regimens with or without Trastuzumab if HER2 is overexpressed [17]. Advancements in chemotherapeutic regimens have steadily improved. Although there are a variety of treatment options available for GC patients, the majority of patients succumb to their disease quickly due to the high inter and intra tumour heterogeneity and the majority of diagnoses occurring during late-stage disease. It is clear that there is a lot of work that needs to be done in order to increase survival rates for this deadly disease [17]. Locally advanced unresectable or metastatic GC has a poor prognosis. Chemoradiotherapy improves survival in comparison to best supportive care, and combination chemoradiotherapy improves survival compared with a single treatment with 5-FU.The use of nivolumab with chemoradiotherapy has recently improved survival for patients with advanced/metastatic disease and trastuzumab chemoradiotherapy has improved survival in patients with HER2-positive advanced/metastatic disease [9]. Chemotherapy can be given in many ways, such as oral, intravenous (IV), intrathecal, intraperitoneal (IP), intra-arterial (IA), topical. All the methods mentioned above, chemotherapy is most often given with an IV [18].

Other therapies available are **hormone therapy**, which slows or stops the growth of cancer, using hormones to grow; **hyperthermia**, which is a type of treatment in which body tissue is heated to 113°F which causes damage and leads to killing cancer cells with little or no damage to normal tissue; **immunotherapy** is a type of cancer treatment that helps the immune system fight cancer, being made up of white blood cells and organs and tissues of the lymphatic system. **Photodynamic therapy** is a technique that uses a light-activated

medicine called a photosensitizer or photosensitizing agent to kill cancer cells. **Radiation therapy** is a cancer treatment that uses high doses of radiation to kill cancer cells and shrink tumours[18].

Stem cell transplants are procedures that restore blood stem cells in patients who have had their cells destroyed due to high doses of chemotherapy or radiation therapy used to treat certain types of cancer, blood disorders, and autoimmune diseases. Stem cells are vital because they transform into different types of blood cells, such as white blood cells, red blood cells, and platelets[18].

Treatment for GC typically consists of a combination of surgery, chemotherapy, radiotherapy, targeted therapy, and immunotherapy. Surgical resection continues to be the main treatment option for localized disease, while chemotherapy and radiotherapy is typically used as adjuvant or neoadjuvant therapy to improve results[5].

Although mTOR inhibitors perform a potent action in cancer treatment, their clinical application was reduced by underlying mechanisms of resistance.

In addition to the molecular diversity of the disease and the frequent absence of oncogenic driver mutations that lead to suboptimal treatment responses, the complexity of cancer biology, tumour heterogeneity, and resistance mechanisms result in difficulties in developing effective therapies. This is hampered by a lack of validated biomarkers and limited driver understanding[5]. Despite advances in systemic therapies, many patients continue to face disease progression and treatment failure, often due to intrinsic problems or developed resistance mechanisms such as changes in drug target and immune evasion pathways. The molecular heterogeneity of GC further complicates the identification of optimal treatment strategies, since not all patients have actionable ocular changes such as HER2 amplification or microsatellite instability, limiting the effectiveness of targeted therapies. Resistance to standard treatments represents a significant challenge and ultimately results in treatment failure and consequently disease progression. Emerging strategies that combine immune checkpoint inhibitors with targeted agents or chemotherapy show potential by pursuing multiple pathways and promoting the development of new therapies based on understanding tumour biology and genomic alterations. The selective efficacy of current treatments highlights the need for innovative therapies in order to improve patient outcomes; this requires continued research into the molecular mechanisms of GC and the exploration of new therapeutic strategies, which includes the development of combination therapies that affect multiple pathways, immune checkpoint inhibitors and adoptive cellular therapies and identification of predictive biomarkers that allow us to personalize treatments and improve patient management [5].

mTOR pathway

mTOR corresponds to a serine/threonine kinase belonging to the family of kinases related to phosphoinositide 3-kinase (PI3K) (PIKK) [10]. In normal conditions, mTOR is important to regulate fundamental activities including cell cycle, proliferation, growth, and survival, as well as the protein synthesis, glucose metabolism and cellular division, autophagy, and homeostasis also involved in other physiological process related to health and aging but also in carcinogenic cells. Regulators of the mTOR pathway have been the object of several studies that ended up evolving towards understanding how mTOR expression itself is regulated. Furthermore, investment in development of biomarkers has been exponential and, in this context, several authors are addressing the expression of mTOR in different diseases [19].

The mTOR has two structures, **mTORC1** and **mTORC2**, that influence the production of proteins and lipids, autophagy and proteasome and ubiquitin systems. This protein regulates cell growth and survival, and controls the formation of actin cytoskeleton[10,20].Although mTOR is responsible for controlling the metabolic pathways at the cellular level, various studies defend the relation between its overexpression and the development of diseases like cancer, obesity, type II diabetes, muscle diseases and neurodegenerative diseases [19,20]. Because of those, mTOR began to be studied as a target for the treatment of those diseases[19,20]. The complex **mTORC1** is involved in cellular growth and cellular metabolism and is composed by mTOR and Raptor, mLST8, substrate of AKT and DEP domain that is composed of proteins that bond with mTOR (DEPTOR). The Raptor controls the assembly, the location and the binding of mTORC1 to the substrates, while PRAS40 and the DEPTOR negative regulate the mTORC1 kinase, working as inhibitors[19]. These complexes work according to different signals from growth factors, hormones, cytokines, nutrients and energy supply that will activate, directly or indirectly, through PI3K and AKT[19,20]. After being activated, PI3K will form phosphatidylinositol (4,5)-bisphosphate (PIP2) to create phosphatidylinositol (3,4,5)-triphosphate (PIP3) that binds and activates AKT, which is going to be responsible for activated mTORC1, inhibiting the interaction between TSC-1 and TSC-2. Stimuli like oxygen reduction and energy production and damage to DNA can inhibit the mTORC1 complex, activating REDD1 or Adenosine monophosphate (AMP) leading to the activation of protein kinase (AMPK) and inhibiting Rheb[19,20]. AMPK is responsible for, directly or indirectly, inhibiting the activity of the complex in the periods of energy shortage due to the sensibility at the intracellular AMP levels, which means that when the cell is in an energy shortage, the level of AMP is elevated activating AMPK and as a consequence, inhibiting the mTORC1 complex, phosphorylating it or the activity of the TSC-2. Therefore, it will cause the formation of the TSC-1-TSC-2 complex,

inhibit the mTORC1 complex. In moments of hypoxis, the pathway is related to damage response. This pathway will develop the transition dependent of p53, which will boost the activity of TSC-2 so as to activate REDD1 or AMPK, and consequently the mTORC1 will be inhibited [19]. The mTORC2 complex mainly controls cell proliferation and survival and is composed by mTOR, RICTOR (that is the compost insensible to Rampamycin), mLST8, DEPTOR, mammalian stress-activated map kinase-interacting protein 1(mSIN1) and the protein observed with RICTOR Protor. RICTOR is responsible for controlling the assembly of the complex and the interactions with the substrate, as well as regulating the interactions of the substrate with glucocorticoid-inducing protein kinase 1 (SGK1). The Protor facilitates the activation of SGK1by mTORC2 while DEPTOR works as an inhibitor of the complex. The presence of mLST8 is important for the maintenance of the activity of mTORC2, (**Figure 7**) [19].

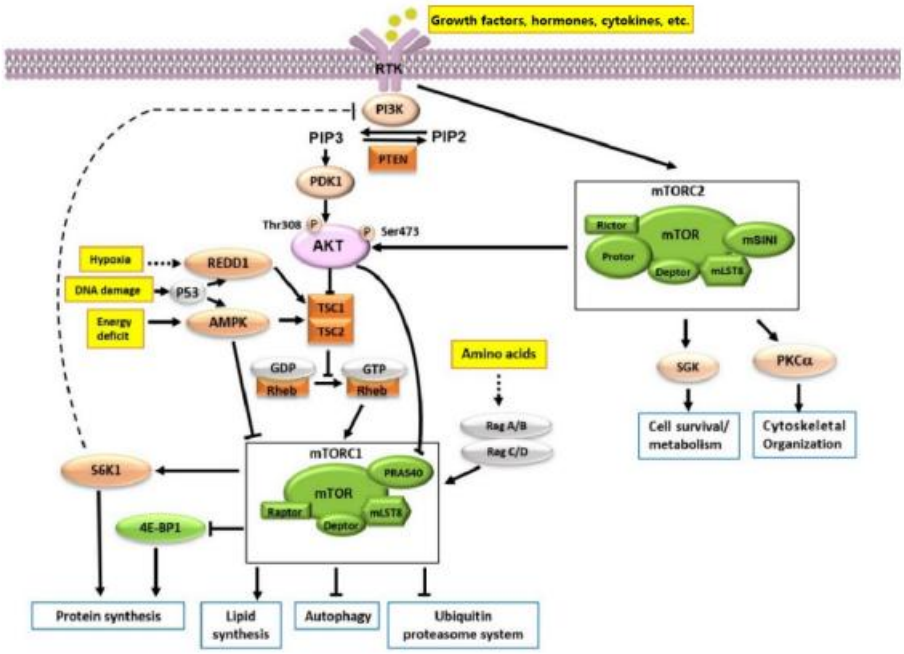

Figure 7- mTORC1[21]

Activation of mTOR signaling in cancer principally depends on three different mechanisms, mutations in the mTOR gene lead to a hyperactive mTOR signaling cascade, mutations in the components of mTORC1 and mTORC2, that result in activation of mTOR signaling and the most important one, aberrant mTOR signaling can also result from mutations in upstream genes, namely, loss-of- function mutations in suppressor genes and gain-of-function mutations in oncogenes. Examples include components of the PI3K signaling pathway which is upstream of both mTOR complexes and is commonly mutated in cancer[22]. The PTEN/AKT/TSC pathway is the principal activator of mTORC1 and genetic mutations on this pathway can lead to cancer development. In addition to this, the

expression of PTEN is frequently through epigenetic and genetic alterations and post-transcriptional mechanisms to positively regulate the PI3K/AKT/mTOR pathway in most malignant tumours [20].

In relation to tumour metabolism, mTOR is activated when there are sufficient nutrients, which favor anabolism, storage, and use of energy. When nutrients are scarce, the body inhibits the activity of mTOR[20]. Tumour cells require large amounts of nutrients to meet their needs for growth and multiplication. Therefore, abnormal mTOR pathway activity frequently occurs in tumours because mTOR plays an essential role in regulating metabolism [20]. On the other hand, tumours can also develop immune tolerance by suppressing the ability of the immune system to recognize and kill tumour cells. Studies report a variety of evidence demonstrating that the mTOR pathway, which in many cases is abnormally activated in tumours, can regulate the differentiation and function of immune cells[20].

mTOR pathway in Gastric cancer

There is no doubt that mTOR is closely associated with cancer, as its abnormal activation due to genetic alterations, such as PIK3CA mutations or amplifications, as well as PTEN loss results in constitutive activation of Akt, mTORC1, and mTORC2, there by promoting cell growth, survival, and resistance to apoptosis. Once aberrantly activated, this signalling cascade not only stimulates uncontrolled cell proliferation but also enables invasion into adjacent tissues and metastasis[11,21,22,23].

Given that the mTOR pathway regulates numerous essential biological and physiological processes, it is unsurprising that its components are frequently mutated in cancers. Dysregulation of mTOR signalling has been implicated in many human diseases, particularly in a wide range of human cancers. Indeed, mTOR pathway alterations have been detected in over 70% of human malignancies [22].

In gastric cancer, aberrant mTOR activation clearly contributes to tumour proliferation, resistance to chemotherapy (such as 5-FU) via epithelial–mesenchymal transition (EMT), inhibition of autophagy, and promotion of angiogenesis through increased expression of HIF-1α and VEGF [24]. Loss of PTEN and epigenetic modifications have been identified as key mechanisms leading to elevated levels of p-Akt, p-mTOR, p-S6K1, and p-4E-BP1, correlating with deeper tumour invasion, lymph node metastasis, and poorer overall survival in patients with gastric carcinoma [23,22,25].

With respect to targeted therapies, rapalogs such as rapamycin, temsirolimus, and everolimus preferentially inhibit mTORC1, reducing phosphorylation of S6K1 and 4E-BP1, and inducing G1 cell cycle arrest. However, clinical outcomes with rapalogs as

monotherapies have been modest [22,25]. In the phase III GRANITE-1 trial for metastatic gastric cancer, everolimus did not significantly improve overall survival, although it did reduce the risk of disease progression by approximately 34% (progression-free survival of 1.7 vs 1.4 months) [26].

Second-generation inhibitors ATP-competitive compounds that target both mTORC1 and mTORC2 have demonstrated sup cerior antiproliferative and antiangiogenic effects in preclinical models of solid tumours, including gastric cancer [26,27]. These agents avoid the compensatory feedback activation of Akt and may overcome resistance mechanisms typically associated with the exclusive use of rapalogs [26,27].

mTOR inhibitors

mTOR overexpression seems to play a role in the pathogenesis of some conditions, either cancerous and non-cancerous, and represents a potential value as a biomarker for diagnosis, disease progression and phenotype, prognosis, invasive behavior, disease recurrence and response to therapy[19].

In relation to human cancers, it is evident that the mTOR pathway is altered in a variety of tumour species and this can change throughout the course of the disease. Alteration of mTOR signaling contributes to the development of cancer through various mechanisms, such as inhibition of apoptosis, induction of chemo-resistance phenotype, metastasis, epithelial to mesenchymal transition and angiogenesis. Furthermore, some studies also demonstrated that elevated expression of mTOR is indicative of the disease, as can be seen in GC samples in relation to normal tissue samples [19]. Contributing to the pathogenesis of the gastric, prostate, esophagus, liver and ovarian tumours, which may be useful as a diagnostic biomarker. In addition to being associated with disease onset, it appears that high expression of mTOR assists disease progression[19]. In GC elevated mTOR expression seems to be associated with tumour differentiation, lymph node metastasis and staging, tumour progression and poor survival[19].

In the past decade, many mTOR inhibitors have been developed and are currently in clinical trials for cancer treatment. [28] As an objective to use the PI3K/AKT/mTOR pathway as a target for GC treatment, Rapamycin, the first generation of mTOR inhibitors, was developed into an immunosuppressive drug blocking T-cell activation.[29, 28]. Rapamycin was initially used as an antifungal agent for *Candida albicans* infections, although an *in vitro* and *in vivo* organ transplantation study showed cell cycle blockage in G1/S transition caused by Rapamycin [29,30]. Rapamycin action mechanism starts with the attachment to the FK-binding

protein 12 that binds with mTORC1, which will reduce cell growth and metabolism[29]. Due to the Rapamycin success, other derivatives with very similar mechanisms of action are in development. The Rapalogs were created by replacing the hydrogen at C-40-O position with different moieties. The first approved by the FDA was Temsirolimus.[22,28].

Genetic variations of the factors associated with the mTOR signaling pathway in cancer cells contribute to resistance. Paring the Rapalogs with cytotoxic agents and developing inhibitors that target both PI3K and mTOR, or selectively were also a strategy to circumvent the resistance. [28]

In addition to genetic variations, intratumoural heterogeneity of mTORC1 activity has been implicated as a major contributor to therapeutic resistance, with different subclonal populations within the same tumour exhibiting divergent levels of mTOR signalling and thus variable sensitivity to rapalogs [31,32,33]. Resistance may also occur via compensatory activation of parallel pathways, notably the MAPK/ERK cascade or feedback-driven re-activation of PI3K/Akt when mTORC1 is inhibited. Tumour cells frequently up-regulate autophagy as an adaptive survival mechanism, especially given that mTORC1 hyperactivation suppresses autophagic flux, thereby creating metabolic vulnerabilities that allow only resistant clones to thrive [31].

Together, these findings support the rationale for rational combination therapies such as dual PI3K/mTOR inhibitors or regimens combining mTOR inhibitors with chemotherapy, autophagy modulators or efflux pump inhibitors to circumvent both intrinsic and acquired resistance and improve therapeutic durability.

However, while simultaneously targeting PI3K and mTOR circumvents the limitation of Rapalogs in blocking PI3K/AKT signaling, the potential toxicity associated with this type of inhibitors presents a big concern. It is believed that inhibitors that are more selective for mTOR may be better tolerated than the dual inhibitors. Rapalogs, that are PI3K/mTOR and mTOR selective inhibitors are more potent in blocking cell proliferation and induction of apoptosis in many cultured cancer cells and in tumour xenograft models, including some of Rapamycin resistant tumours[28].

As with many other drugs that target intracellular signaling pathways, the challenges for new mTOR inhibitors continue to be identifying cancers that are sensitive to these inhibitors and optimizing the treatment strategy to obtain maximum benefits[28].

In relation to GC, studies have shown that PIK3CA, PIK3CB, AKT1 and mTOR are overexpressed in GC cell lines, and the mTOR pathway is active in about 60% of GC patients[22]. PTEN, a key inhibitor of the PI3K pathway, is an important tumour suppressor gene, and tends to mutate more frequently in advanced stages or in less differentiated GC.

Although the exact genomic changes that occur in mTOR signaling downstream of PI3K/Akt are not well detailed, it is reported that overexpression of phosphorylated mTOR is related to some clinicopathological features and poor prognosis in GC patients alone or combined with downregulation of TSC1[22].

Gastroenteropancreatic neuroendocrine tumours have elevated levels of mTOR, 4EBP1, p-4EBP1, p-S6K and p-eIF4E. Both mTOR expression and activity were higher in foregut tumours than in midgut tumours. In foregut tumours, mTOR expression it was greater when there were distant metastases. Immunohistochemistry of sections from GC samples demonstrated that mTOR expression was present in 51.5% of samples, compared to low/absent expression in normal tissues. A positive correlation was observed between mTOR expression and tumour differentiation, lymph node metastasis and staging. No correlation was observed with gender, age and invasive depth. Greater expression of mTOR and p-mTOR in the tumour center compared to the invasive front[19].

The mTOR inhibitors can be divided in three types:

- Allosteric mTOR inhibitors that bind to the FRB domain

- ATP-competitive mTOR inhibitors that bind to the KIN domain

- Double binding site inhibitors.

Previous studies connected mTOR to various diseases including the tumour formation and development that excessively activates the pathway AKT/mTOR. Due to these facts, the inhibitors of the mTOR pathway are considered as a possible targeted therapy for both tumours and organ transplants, rheumatoid arthritis and other diseases[11]. Numerous types of mTOR inhibitors such as Rapamycin, its Rapalogs and mTORC1/2 kinase inhibitors have been examined in various cancer models[22]. Rapamycin began to be developed as an immunosuppressive drug due to its ability to block T cell activation. It was approved by the FDA in 1997 for use in transplants to prevent allograft rejection and, in 2003, for use in coronary artery stents to prevent restenosis. Although the anticancer activity of Rapamycin was documented in the early 1980s, its application in cancer therapy only began to be explored at the end of the 1990s[28]. All Rapalogs are created by replacing the hydrogen in the C-40-O position with different portions. For Temsirolimus (CCI-779), it is an ester of dihydroxylamethyl propionic acid, Everolimus (RAD001), a hydroxylathyl group, and Ridaforolimus (AP23573), a dimethyl phosphate group[28]. However, the clinical application of Rapalogs in cancer treatment has so far met with limited success, they are effective in treating a few cancers, including renal cell carcinoma and mantle cell lymphoma, but not in the majority of solid tumours[28].

mTOR inhibitors have been used in preclinical studies and GC clinical trials. Everolimus and sirolimus showed good results of G1 cell cycle arrest and suppressed proliferation in GC *in vitro* studies. Rapamycin responded well in cancer cells containing PIK3CA and/or PTEN mutations in a preclinical study and inhibited tumour volume and microvasculature development in *in vivo* studies. Temsirolimus demonstrated a favorable toxicity profile, pharmacokinetic characteristics, and cancer-resistant efficacy in a phase I trial in advanced cancer, including GC [22]. The efficiency of this type of drug has already been proven with Temsirolimus and Everolimus in patients with renal cell carcinomas and in those with cell lymphoma[29]. This type of drug help treats GC in early stages by itself or in combination of other types of treatment.

Some of the mTORi showed in the present literature and analyzed in this study are Everolimus, OSI-027, 2,6-DMBQ and Temsirolimus.

Everolimus, is now commonly used to treat GC because PI3K/Akt/mTOR pathway plays an important role in the functions of cancer cells, and the activation of the PI3K/Akt/mTOR pathway is closely related to the progression of GC. However, dysregulation of the PI3K/Akt/mTOR pathway is related to resistance to chemoresistance in patients with GC, and Everolimus resistance in GC will not effectively increase overall survival of patients with GC[34]. Everolimus is a mammalian target of Rapamycin (mTOR) inhibitor which inhibits the mTOR complex 1 (mTORC1) and the phosphorylation of its downstream effectors: p70S6kinase (p70S6K) and 4E-BP1[35].

OSI-027 is an orally bioavailable RAPA analog that is currently in Phase II clinical trials as an antitumour drug. Unlike RAPA, OSI-027 is a specific and potent ATP-competitive inhibitor of both mTORC1 and mTORC2, which are aberrantly activated in most GC cases. Furthermore, OSI-027 has shown promise for the treatment of several malignancies, including head and neck, bladder, and breast cancer[36].

2,6-DMBQ is a benzoquinone compound that is isolated from sourdough fermentation of wheat germ. 2,6-DMBQ has been reported to possess cancer prevention properties against TPA-induced skin carcinogenesis. However, the molecular targets of 2,6-DMBQ and its potential therapeutic effect have not been investigated in cancer[37].

The mTOR inhibitors Temsirolimus and Everolimus (RAD001) have been approved as treatment for patients with metastatic renal cell carcinoma. However, monotherapy with mTOR inhibitors often yields only modest therapeutic activity in advanced GC, and the emergence of drug resistance may ultimately limit the utility of mTOR inhibitors[38].

PKI-587 is a highly potent, novel dual inhibitor of PI3K and mTORC1/C2 [39].

Preclinical studies for the development of new pharmacological approaches

Cell lines have transformed scientific research, being used in vaccine production, testing drug metabolism and cytotoxicity, antibody production, study of gene function, generation of artificial tissues and synthesis of biological compounds[40,41] .

Despite developments in diagnosis and treatment, GC remains one of the most common causes of cancer-related death. The establishment of relevant animal GC models is critical for future research. In addition to implanting patient's tissues or primary cells in immunodeficient mouse hosts for culture, different approaches have been developed for alternative hosts such as humanized mouse hosts, zebrafish hosts, and *in vitro* culture modalities also facilitated development in GC research. Current status, characteristics, interfering factors and applications emerged as the most valuable preclinical tools for studying the progression and metastasis of GC[42]. Due to the lack of specific symptoms, defined biomarkers and diagnostic methods in the early clinical stage, GC is detected mainly in intermediate to late stages. With the aim of improving treatment efficacy, the subtype and biological heterogeneity of GC needs to be defined in advance through histopathology, thus affecting the disease response to treatment. One of the factors that can lead to failure in clinical trials is the inadequate biology of the preclinical models in which drugs are developed or tested, resulting in the inability to predict an effective therapy in humans[42].

In vivo studies are often a critical component of experimental design, such as drug discovery and development process, but they end up presenting limitations that also end up being the subject of criticism. Inevitably, animal models are imperfect representations of human diseases and disturbances[43, 44].

Objectives and Research Question

The evaluation of articles that address mTOR inhibitors in the context of GC is extremely important for different reasons. First, mTOR inhibitors represent an emerging class of targeted therapies that have demonstrated potential in the treatment of diverse malignancies, such as GC. By investigating the efficacy and safety of mTOR inhibitors, we can gain crucial insights into their impact on disease progression, treatment response, and patients' quality of life. Furthermore, an analysis of articles on this topic allows us to better understand the molecular mechanisms involved in gastric carcinogenesis. Another relevant aspect is the personalization of therapies. GC presents significant heterogeneity, the current therapeutic strategies are aggressive and the evaluation of mTOR inhibitors can contribute to the development of more precise approaches that take into account the individual characteristics of tumours. This is essential to maximize therapeutic benefits and minimize side effects.

This review has as **main objective** to clarify the efficacy and safety of upcoming mTOR inhibitors in ***in vitro*** and ***in vivo*** preclinical studies data.

The **specific aims** of this project are to:

- Critically synthetize the currently available information on preclinical studies data considering the upcoming mTORi for GC treatment.
- Identify and critically assess the data regarding cell lines and animal models used in the preclinical studies of upcoming mTORi
- Evaluate and compare the outcomes regarding the efficacy and safety of mTORi
- Identify and critically assess the data regarding the treatment parameters.

To define a structured research question, the PICO+S strategy was used (**Table 1**).

Table 1: PICO + S strategy

Population	Cell lines and animal models of GC
Intervention	mTOR inhibitor, alone or in combination treatment
Comparison	No treatment with mTOR inhibitors (no drug or standard therapy only)
Outcomes	Efficacy and safety outcomes of mTOR inhibitors
Study Design	Experimental preclinical studies

This study, therefore, proposed to answer the following research question: “Do the upcoming mTOR signaling inhibitors demonstrate efficacy and safety in preclinical models of GC?”

Methods

Overview

This work followed the recommendations of the Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) guidelines[45]. This project was initiated in September of 2023 with the completion date of July 2025.

Eligibility Criteria

Studies were included for analysis whether they met the previously defined PICO+S (population, intervention, comparator, outcomes, study design):

- Population: Studies that use rodent, as *in vivo* models, and cell lines as *in vitro* models of GC. There were no limitations in terms of species, strain, age, gender, or body weight and type of cells since the purpose of this study is to analyze and synthesize all preclinical data available.
- Intervention: Studies that administer mTORi (alone or in combination) in any dosage, frequency, or treatment duration and with any route of administration in cell lines and/ or animal models of GC.
- Comparator: Studies in which a control group was without treatment with mTOR inhibitors (no drug or standard therapy only)
- Outcomes: Biochemical and histological markers related to GC, with and without mTORi treatment, were analyzed.
- Study design: Only original, analytical, experimental, *in vivo* and *in vitro* preclinical studies.

Regarding the exclusion criteria, the following were considered: studies including other comorbidities; studies that did not refer mTORi; clinical studies; review articles, expert opinions, book chapters; and studies written in languages other than English and Portuguese.

Information Sources and Search Strategy

The biomedical electronic databases used for the highly sensitive search strategy to identify and select relevant and eligible studies were MEDLINE (PubMed), Web of

Science and Scopus. The search was limited in terms of publication date between January of 2013 and may of 2025. A comprehensive search strategy was developed until May 5th of 2025 using the combination of keywords: “mtor inhibitors” and “gastric cancer” as well as their synonyms combined with the Boolean operators “AND”, and “OR” and MeSH terms to identify and select the eligible studies (**Table 2**).

Table 2: Search expressions developed for each database.

Database	Search Expression	Date	Number of Results
MEDLINE (PubMed)	("stomach neoplasms"[MeSH Terms] OR “stomach Neoplasm*”[tiab] OR “stomach Neoplasia*”[tiab] OR “Stomach Cancer*”[tiab] OR “Stomach Tumour*”[tiab] OR “Cancer of the Stomach”[tiab] OR “stomach carcinoma*”[tiab] OR “Gastric Neoplasm*”[tiab] OR “Gastric Neoplasia*”[tiab] OR “GC*”[tiab] OR “Gastric Tumour*”[tiab] OR “Gastric Carcinoma*”[tiab] OR “gastric tumour cell line*”[tiab] OR “GC cell line”[tiab] OR “stomach cancer cell line” [tiab]) AND (“mtor inhibitors”[MeSH Terms] OR “mtor inhibit*” OR rapalogs)	Consulted between January 22th, 2024 and May 5th, 2025	306
Web of Science	(TS=("stomach Neoplasm*") OR TS=("stomach Neoplasia*") OR TS=("stomach Cancer*") OR TS=("Stomach Tumour*") OR TS=("Cancer of the Stomach") OR TS=("stomach carcinoma*") OR TS=("Gastric Neoplasm*") OR TS=("Gastric Neoplasia*") OR TS=("GC*") OR TS=("Gastric Tumour*") OR TS=("Gastric Carcinoma*") OR TS=("gastric tumour cell line*") OR TS=("GC cell line") OR TS=("stomach cancer cell line")) AND (TS=("mtor inhibit*") OR TS=(rapalogs))		147
SCOPUS	(TITLE-ABS("stomach Neoplasm*") OR TITLE-ABS("stomach Neoplasia*") OR TITLE-ABS("stomach Cancer*") OR TITLE-ABS("Stomach Tumour*") OR TITLE-ABS("Cancer of the Stomach") OR TITLE-ABS("stomach carcinoma*") OR TITLE-ABS("Gastric Neoplasm*") OR TITLE-ABS("Gastric Neoplasia*") OR TITLE-ABS("GC*") OR TITLE-ABS("Gastric Tumour*") OR TITLE-ABS("Gastric Carcinoma*") OR TITLE-ABS("gastric tumour cell line*") OR TITLE-ABS("GC cell line") OR TITLE-ABS("stomach cancer cell line")) AND (TITLE-ABS("mtor inhibit*") OR TITLE-ABS(rapalogs))		100

Study Selection Process

After using the application of the search strategy in each database, the retrieved articles were exported from the PubMed, Web of Science and SCOPUS databases to a Systematic Reviews Web Application Rayyan (Rayyan QCRI; Rayyan), which was used throughout this systematic review for study screening and overall management. The first step was to detect and exclude duplicates and afterwards, the titles and abstracts were analyzed to select relevant and potentially eligible studies, according to the inclusion and exclusion criteria. After this process is completed, the full texts of each study were assessed, deciding whether the article is eligible or not and considering the eligibility criteria. The process of selection was summarized using a PRISMA flow diagram, including the reasons for excluding studies [45]. (**Figure 8**)

Data Collection Process

Upon selection of eligible studies, data was extracted from the text, graphs, and tables from each included article to a standardized extraction document in Excel (Microsoft) that was developed to extract the data of interest, including article identification (authors' names and year of publication), disease, cell and animal-related parameters, study design, drug treatment conditions, and outcomes of interest. The data of interest were extracted.

Data Items

Population: the data of interest was related to animal species, strain, gender and age and type of cell lines, to better identify the preclinical models of GC.

Intervention: the parameters of interest were mTORi's name, dose, frequency of administration, and duration of treatment; for *in vivo* studies, the route of administration was also considered.

Comparator: data from the control group(s).

Outcomes: data concerning the outcomes related to tumours growth and biochemical markers (continuous quantitative measures), macroscopic evaluation (dichotomous measures) and microscopic evaluation (dichotomous measures).

Study Design: Throughout the analyses of the preclinical studies, *in vivo* and *in vitro*, the data of interest to be extracted was related to the efficacy and safety of the compounds.

Risk of Bias Assessment

To evaluate the potential risk of bias, the internal validity of each study and the methodological quality were identified and analyzed using the risk of bias assessment tool of the Systematic Review Centre for Laboratory Animal Experimentation (SYRCLE)[46]. For each study, the classification of all components of the SYRCLE tool was done by answering with “yes”, “no”, or “unclear”, depending on the presence of this information in the article. The answer “yes” indicates low risk of bias; “no” indicates high risk of bias; and “unclear” indicates an unclear risk of bias. If one of the relevant signaling questions was answered with “no”, this indicates high risk of bias for that specific domain. For *in vivo* studies, all domains of the SYRCLE tool were considered. Since there is a lack of consensus on the risk of bias assessment tools to address *in vitro* studies, an adaptation of the SYRCLE tool was made[47] .

Registration and Reporting

The protocol for this systematic review was submitted and registered in the International Prospective Register of Systematic Reviews – PROSPERO with the code: CRD420251072047.

Results

Study Selection

The application of the search strategies to biomedical electronic databases, Pubmed, Scopus and Web Science retrieved in total 557 articles, from which 186 were identified as duplicate (**Figure 8**) [45]. Of the remaining 371 articles, 112 were considered ineligible by automation tools and 4 articles were excluded for not being related to the subject. The analysis of the titles and abstracts from the remaining articles resulted in the removal of 239 reports as this lacked relevance for the question. Next, the full texts of the 16 potentially eligible studies were critically assessed taking into consideration the inclusion and the exclusion criteria. After this process, 2 articles were excluded as one of the exclusion criteria was met. The 14 studies included for qualitative synthesis met all the eligibility criteria, on the basis of which the potential upcoming mTOR signaling inhibitors in GC treatment in *in vitro* and in *in vivo* preclinical studies were adequately evaluated.

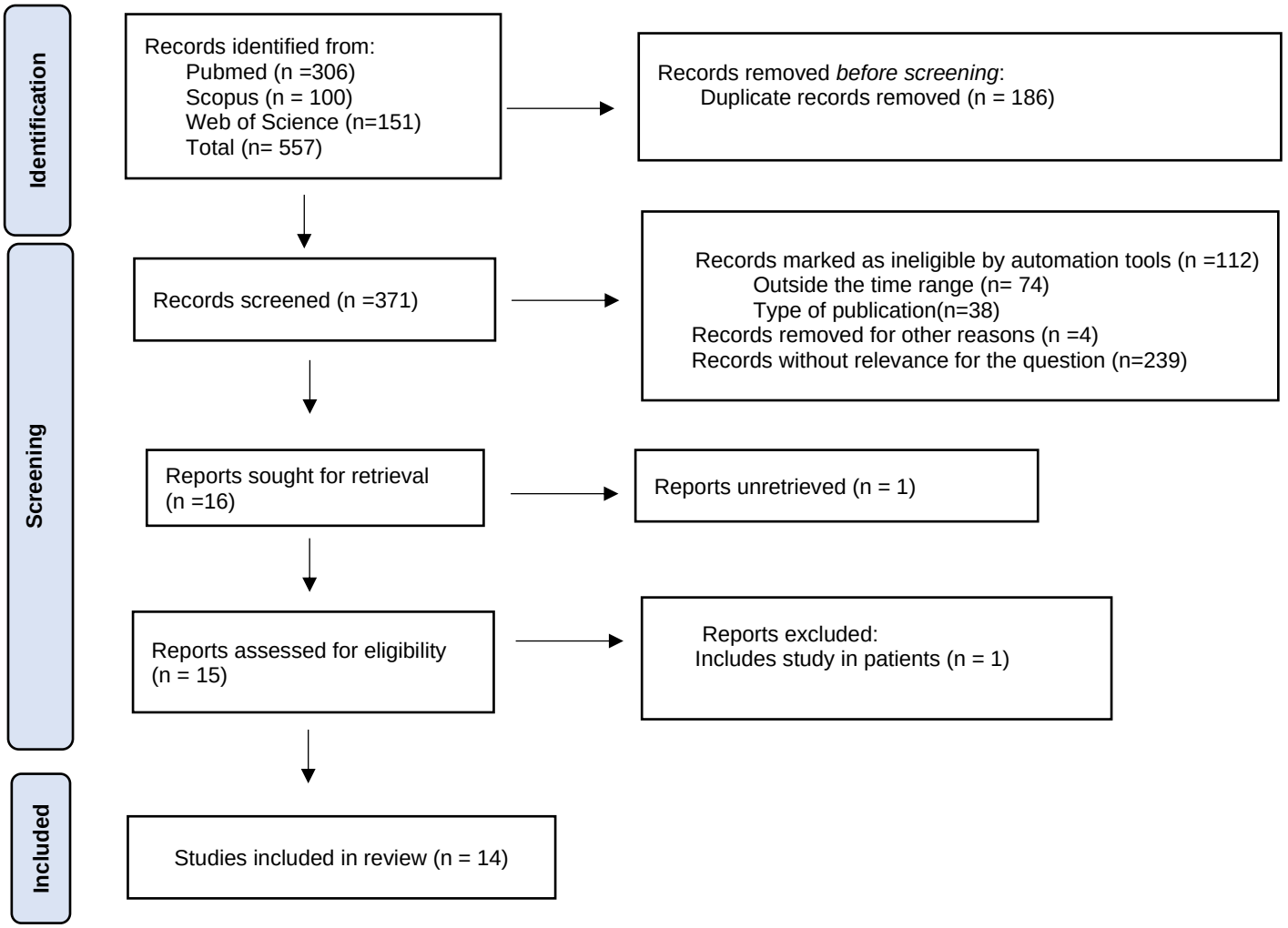

Figure 8- PRISMA 2020 flow diagram representing the selection process[45]

Study Characteristics

After reading the 14 articles in full, an extensive analysis was carried out with the aim of extracting relevant data, namely parameters related to treatment, population (cells or animals) together with the biomarkers and the used technique, for the respective studies. The collected data from *in vitro* and *in vivo* studies can be seen in Table 3 and Table 4, respectively. In addition, the data from *in vitro* and *in vivo* studies about toxicity and safety can be seen in Table 5 and Table 6, respectively.

The data extracted were used to analyze the results in the different preclinical studies that evaluated mTORi. Toxicity and safety were found in 7 different preclinical studies in 14 articles. The most prevalent compound in all 14 articles were Everolimus (experimental name RAD01) in 9 articles, while the other 4 mTORi (Temsirolimus, OSI-27, Rapamycin, 2,6-DMBQ) were present in only one article.

The studied articles included a small variety of **cell lines** (12 articles) to evaluate drug metabolism and cytotoxicity in ***in vitro*** studies was verified. Some of these cell lines are SGC7901 (4 articles), BGC823 (1 article), MKN28 (1 article), MGC803 (n=3), HGC27 (2 articles), AGS (3 articles), NCI-N87 (2 articles), MKN-45 (2 articles), SNU-1 (1 article), JB6 (1 articles), and, BON (1 article), QGP-1 (1 article), KRJI (1 article), LCC-18 (1 articles), STC-1 (1 articles), GluTag cell (1 article), MKN74 (1 article), NUGC-4 (1 article), HGC-3, -18 and -20 (1 article) and SNU-620 (1 article).

All *in vivo* studies used mice as an **animal** model (6 articles): BALB B/c nude (2 articles), CB-17SCID (1 article), Swiss nu/nu and nude (1 article each); in addition, one study did not mention the strain chosen. The studies showed a clear preference for using females (5 articles) whereas one study did not mention the selected gender. In relation to animals age, it was possible to determine that mice were 4 weeks old in 1 article, 5 weeks old in 3 articles and 6 to 9 weeks old in 1 article. One study did not mention the selected age.

Regarding efficacy parameters in ***in vitro*** studies, it was shown that the main parameter analyzed was biomarkers (11 articles), cell proliferation (5 articles), apoptosis (5 articles), cell viability (3 articles) and cell number (3 articles). For ***in vivo*** studies, tumour growth (5 articles) and biomarkers (5 articles) were the main parameters analyzed.

Considering the analyzed studies, a total of 68 different types of **biomarkers** were shown. The most prevalent were phosphorylated mTOR (p-mTOR) (7 articles), followed by phosphorylated 70 kDa ribosomal S6 kinase (p70S6K) (5 articles), phosphorylated AKT (p-AKT) (4 articles), KI-67 (3 articles), LC3A/B-Ⅱ (3 articles), 4E-BP1 (2 articles), CDK4 (2

articles), Cycledin D1(2 articles) and phosphorylated ERK1/2 (p- ERK1/2) (2 articles). Other biomarkers were shown only one time in all the analyzed studies.

A total of 26 different **techniques** were shown, being the most prevalent were Western Blot (WB) (13 articles) followed by Flow Cytometry (FC) (8 articles), 3-(4,5-dimethyl thiazol-2-yl)-2,5- diphenyl tetrazolium bromide (MTT) assay (6 articles), Immunohistochemical (IHC) (6 articles) Cell Counting Kit (CCK-8) (4 articles), Polymerase Chain Reaction (PCR) (4 articles), Immunofluorescence (3 articles), Histological analysis (3 articles), Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) and assay (2 articles). Other techniques were shown only one time in all the analyzed studies. In total, 10 different types of **staining** were shown, the most prevalent being Annexin V and propidium iodide (7 articles), EdU staining (3 articles) and Crystal violet (2 articles). Other techniques were shown only one time in all the analyzed studies.

Regarding the **frequency of administration** for ***in vitro* studies**, 10 studies did not mention the frequency chosen, leading to believe that the studies preferred a single-dose treatment. One single administration, daily administrations and administrations every three days were observed in 1 study each. The **duration of administration** for *in vitro* studies was follows 24h (n=2) 48h (n=3), 72h(n=5), 96h (n=1), 5 days (n=1), 10 days (n=1) and 14 days (n=1) (**Table 3**).

In terms of the **frequency of administration** for ***in vivo studies***, 2 studies preferred daily administrations whereas administrations every two days, 5 times a week and twice a week was observed in 1 study each; in addition, one study did not mention the frequency chosen. Among the different **routes of administration** used in the different studies analyzed, the only routes used were IP (n = 3) and oral (n = 3) (**Table 4**).

Table 3: Qualitative synthesis of the included ***in vitro*** studies.

Author, year [Ref.]	Treatment-Related Parameters				Cell-Related Parameters			Assessment Techniques	Biomarkers	Efficacy parameters
	Treatment	Dose	Frequency	Duration	Disease of origin	Type of Cell line	ID			
Huangfu L, et al. 2023[48]	Piceatannol	5 μmol L^{-1}	1 x 48h	48h	GC	Human GC	SGC7901 BGC823 MKN28 (sensitive) MGC803 HGC27 AGS (less sensitive)	IncuCyte (proliferation). TUNEL Colony forming RNAseq (gene networks). Western blot (LC3-I/II, p62, Beclin-1 phosphorylation). TEM (autophagosomes). MST/SPR (Beclin-1 binding). Synergy Analysis (Chou-Talalay CI + MuSyC dose-matrix) EdU, Anexina-V/PI	**Autophagy**: ↑LC3-II, ↓p62, ↑Beclin-1-Thr119-P, ↓Beclin-1-Ser295-P. ↓mTOR-Ser2448-P **Apoptosis**: ↑Cleaved PARP, ↑Bax/Bcl-2 ratio. **Induction of autophagic cell death**	**- Cell death:** Induced **- Proliferation:** ↓ SGC7901, BGC823, MKN28 Minimal inhibitory effect MGC803, HGC27, AGS, (but still dose-dependent). - Cell viability ↓ - DNA replication ↓ - DNA breakage ↑ - LC3-I and LC3-II: Conversion - Autophagy: ↑LC3-II, ↓p62, Beclin-1-Ser295-P↓, Beclin-1-Thr119-P↑. Disrupts Beclin-1/Bcl-2, enhances Beclin-1/UVRAG binding.
	Piceatannol + **Everolimus (RAD001)**	2,5μmol L^{-1} + 1 μmol L^{-1}								- **Proliferation**: **Stronger** ↓ across all cell lines, - **Cell Death**: Synergistically induced via **amplified autophagy-dependent mechanisms,** surpassing monotherapy effects. - **Enhanced autophagic flux** (↑↑LC3-II, ↓↓p62) due to: Piceatannol-Maintains **Beclin-1-Ser295-P**↓ and **Thr119-P**↑,Everolimus: Adds **mTOR inhibition**, blocking autophagy repression. **- DNA replication** ↓↓ **- DNA breakage** ↑↑ - **Colony formation**: Near-complete abolition of colony formation at combined doses.
Gao F, et al. 2022[34]	Rhein	80 μM	–				MGC-803	FC TUNEL WB MTT Transwell assay EdU	CDK4 Cyclin D1 - Apoptosis: ↑p53, ↓Bcl-2/Bax - EMT: ↑E-cadherin, ↓N-cadherin/Vimentin - Pathway: ↓p-PI3K/p-AKT/p-mTOR (mild)	**- Number of invasive cells:** Repressed **- Cell viability:** repressed in a dose dependent manner **- Cell invasion:** Inhibited (significant reduction at 80 μM) **- Proliferation:** ↓ (47% reduction at 80 μM) **- Apoptosis:** ↑ **- EMT Modulation:** Epithelial phenotype ↑ (functional restoration of cell adhesion); Mesenchymal phenotype ↓ (reduced metastatic potential)
	Rhein + **Everolimus (RAD001)**	80 μM + 40 nM							Apoptosis: ↑↑p53, ↓↓Bcl-2/Bax EMT: ↑E-cadherin, ↓N-cadherin/Vimentin Pathway: ↓↓p-PI3K/p-AKT/p-mTOR	**- Survival:** ↓ **- Cell apoptosis:** ↑ 4.5-fold **- Cells:** ↓ **- Number of invasive cells:** ↓ 75% **- p53, CDK4 and Cyclin D1:** Up-regulated **- p-PI3K, p-AKT, and p-mTOR:** Down-regulated **- E-cadherin:** Up-regulated **- N-cadherin and Vimentin:** Down-regulated **-Proliferation:** ↓ 72% **-EMT Modulation: Epithelial restoration:** ↑ E-cadherin, Mesenchymal suppression: ↓ N-cadherin (70%), ↓ Vimentin (68%)
Ye H, et al. 2018 [49]	TZB	1-100 μg/ml	Resistance induction: 2×/week Assays: 72h exposure.	Resistance: 10 months (50→250 μg/mL). Experiments: 72h (viability), 14 days (clonogenic).			Parental: NCI-N87 (TZB-sensitive). Resistant: POOL1, POOL2 (TZB-refractory).	WB TEM (Glutaraldehyde/uranyl acetate) MTT CCK-8 Clonogenic Crystal violet	**Autophagy:** - ↑LC3-II, ↑SQSTM1. - ↓Autolysosome formation. **Signaling:** - ↑p-Akt (Ser473), ↑p-mTOR (Ser2448) in resistant cells.	**- p-AKT and p-mTOR:** Little effect
	Everolimus (RAD001)	0.1-10 μM	Single exposure (72h for assays)						mTOR Pathway: - ↓p-mTOR (Ser2448), ↓p-Akt (Ser473). Autophagy: - ↑LC3-II (if flux restored).	**- Cell death:** Induced in parental and Tzb-refractory cells in a dose- and time-dependent manner
	TZB + **Everolimus (RAD001)**	1-100 μg/ml + 0.1-10 μM	72h exposure						Autophagy/Signaling: - ↓p-Akt, ↓p-mTOR (vs. TZB alone). - Enhanced LC3-II turnover (if flux restored).	**- Activation of Akt and mTOR:** ↓

Table 3: Qualitative synthesis of the included ***in vitro*** studies. (**Cont.**)

Author, year [Ref.]	Treatment-Related Parameters				Cell-Related Parameters			Assessment Techniques	Biomarkers	Efficacy parameters
	Treatment	Dose	Frequency	Duration	Disease of origin	Type of Cell line	ID			
Hou G, et al. 2020[50]	DDP	15 μM	Immunofluorescence assay: Culture medium changed every 3 days	Western Blot, Cell Viability assay (CCK-8): 4 hours Immunofluorescencia assay: cells were incubated with 2 separete overnight periods. Colony Formation Assay: cells were seeded overnight, followed by treatment for 6 hours Cell Apoptosis Assay: 20 minutes.	GC	Human GC	Gastric cell line: SGC-7901 Cisplatin-resistant cell line: SGC-7901/DDP	Western blot (↓LC3A/B-Ⅱ, ↓Atg5, ↑p62 in SGC-7901/DDP) Immunofluorescence (↑punctate LC3 in SGC-7901) Colony formation (↓↓SGC-7901, ↓SGC-7901/DDP) CCK-8 and flow cytometry (↓cell lines with Beclin-1 shRNA) Crystal violet (colony formation assay) Annexin V-FITC (cell apoptosis assay)	Autophagy: ↓LC3A/B- ↓Atg5 ↑p62, ↓p-mTOR ↓Raptor ↓p-p70S6K Cell viability: ↑Beclin-1 shRNA	**- Clone formation:** Inhibited **- Clone number:** ↓ 30.60% (SGC-7901) 26.68% (SGC-7901/DDP)
	DDP + CQ	15 μM + 10 μM								The strongest inhibiting effects **- Clone number:** ↓ 67.29% (SGC-7901) 71.39% (SGC-7901/DDP) **- Inhibition effect of DDP:** Enhanced by CQ on Bcl-2 in both cells, promoting the apoptotic-inducing effects of DDP **- Proliferation ability of DDP**: CQ enhanced the inhibiting, especially to the drug-resistant cells **- Inhibiting autophagy by CQ:** Strengthened the inhibiting effects of DDP on cloning formation of parental and DDP-resistant **- CQ:** Enhanced the inducing apoptotic effects of DDP, the inhibition effect of DDP on Bcl-2 in cells, promoted the apoptotic-inducing effects of DDP.
	Everolimus (RAD001) OR CQ	4 μM OR 10 μM								**- LC3A/B-II, Beclin-1 and Atg5:** Increased (Stronger in CQ). - CQ improved the chemoresistance to DDP
	DDP + **Everolimus (RAD001)**	15 μM + 4 μM								- C**loning formation:** No difference in the two cell lines, RAD001 had no effect on the ability of DDP. -**Everolimus**: No influence inhibiting effects of DDP on cell viability
Cao Y, et al. 2015 [38]	**Rapamycin**	100 nM	MTT assay (cell viability): continued incubation (up to 72hours)	Clonogenic: 15 days MTT assay: Overnight incubation followed by 48 and 72 hours treatment, plus additional 4 hours incubation. Western blot: 2 hour and a half plus overnight incubation. RT-PCR: 42 min RNA interference stable infection: 2 days			MGC803 SGC7901	MTT assay (cell proliferation) Western Blot (Cell lysate proteins) Cologenic assay (cell culture) RT-PCR Wright Giemsa	↓p-mTOR, ↓p-p70S6K p-Akt increase with rampamycin and decrease after LY294002 or Celecoxib exposure) ↑Cbl-b (with Celecoxib)	**- After 8 hours of exposure: ↓ p-mTOR, ↓p-p70S6K and ↑ p-AKT (**To investigate the mechanism of resistance) -After treatment with LY294002: ↓ p-AKT **-Rapamycin alone: ↓ cell growth.** -LY294022 + Rapamycin: ↓↓ cell growth
	Celecoxib	40 μM								**- cell survival:** Inhibited on a dose-dependent manner. - After 24 hours of exposure: ↓ p-AKT **- Increase of Cbl-b after 8 hours exposure**
	Rapamycin+ Celecoxib	100 μM + 40 μM								- Celecoxib and LY294002 showed to enhance GC cells to Rapamycin. **-Anti-proliferative response:** Enhanced to Rapamycin Inhibited growth of GC cells **- Increase of Cbl-b after 8 hours exposure**

Table 3: Qualitative synthesis of the included ***in vitro*** studies. (**Cont.**)

Author, year [Ref.]	Treatment-Related Parameters				Cell-Related Parameters			Assessment Techniques	Biomarkers	Efficacy parameters
	Treatment	Dose	Frequency	Duration	Disease of origin	Type of Cell line	ID			
Ying L, et al. 2014 [51]	DDP **OR Everolimus (RAD001)**	DDP: 2.5 mg/L RAD001: 2.5, 5, 10 mol/L	MTT assay (cell viability and survival): continuous incubation for 72 hours.	MTT assay: After 24 hours in RPMI1640 medium followed by 24,48 and 72 hours of treatment with different concentrations , followed by 4 hours incubation. (the experiment was repeted twice) Flow cytometry: 48 hours (The experiment was repeated three times) Immunohistochemical analysis: cells were treated with different concentrations for 48 hours, followed by overnight and 30 minutes incubation. Western Blot: 17 hours	GC	Human GC	Human cell lines SGC7901 and SGC7901/DDP	Immunohistochemical analysis (P-gp and MRP1) Western blot (P-gp, MRP1 and viability) MTT assay (cell survival and viiability) Annexin V-FITC/ PI and Flow Cytometry (detection of apoptosis)	P-gp MRP1 survivin	- **P-gp and MRP1**: not change in DDP alone -**Apoptosis**: Did not induce on SGC7901/DDP cells alone - No significant difference between DDP and control
	Everolimus (RAD001) (Pretreatment) + DDP	2,5 nmol/L (RAD001) + 2,5 mg/L (DDP)								- **Apoptosis:** ↑ VS control, DDP or RAD001 alone - **Proliferation:** Inhibited VS control, DDP and RAD001 - **Inhibition rate of RAD001:** Higher VS control Difference between combination group and RAD001 or DDP group alone - **P-gp, MRP1 and surivivn:** ↓ - When the cells were pretreated with RAD001 2.5,5nmol/L, the proliferation of SGC7901/DDP cells was inhibited by DDP 2.5mg/L significantly, compared to negative control group. RAD001 in combination with DDP can overcome chemoresistance RAD001 0.625 nmol/L as modulator on the reversal of resis tance to DDP
Xu E, et al. 2021 [36]	**OSI-027**	5 μM		CCK-8 (cell viability assay): After 24 hours incubation for cell attachment, the culture medium was replaced three times (24, 48 and 75 hours) Edu staining: 6 to 8 hours incubation, followed by 60 minutes wash, 20 minutes permeabilization and 20 minutes incubation. Flow cytometry: 1 hour Cell cicle analysis and siRNA transfection assay: 48 hours Western blot assay: Overnight plus 2 hours incubation			MKN-45 AGS	Flow cytometry and Annexin V–FITC/PI (cell apoptosis assay) Western blot (mTOR pathway proteins) CCK-8 (cell viability assay) EdU staining siRNA	p-mTOR mTOR Akt p-4E-BP-1 p-p70S6K Cycledin D1 CDK4	- **Proliferation:** Inhibited in a dose-dependent manner (most evident after 48 hours treatment). - **Expression of p-mTOR, mTOR, Akt, and downstream effectors of the mTOR pathway, including p-4E-BP-1 and p-p70S6K** **-Significantly reduced Cycledin D1 and CDK4:** Inhibited. -OSI-027 single treatment did not induce cell apoptosis **-Cell cycle arrest** was induced in the G0/G1 phase
	OSI-027 + Oxaliplatin	0-25 μM + 0-20μM								- **Apoptosis rate with combined treatment was increased** - **Proliferation rate:** ↓ Had a synergistic killing effect
Zu X, et al. 2020 [37]	**2,6-DMBQ**	5, 10, 20μM	–	Cell proliferation assay: Cells were seeded with 24 hours incubation followed by treatment with 2- and 48-hours incubation. Anchorage-independent cell growth: 2 weeks Western blot: overnight plus 3 hours incubation In vitro ATP assay for mTOR kinase activity: 45 minutes Cell cycle analysis and apoptosis assay: 72 hours MTT assay: 48 hours or 2 weeks			GC cells: AGS HGC27 NCI-N87 SNU-1 Mouse epithelial cells: JB6	Western blot Flow cytometry: ↓S phase fraction induced G1 phase cells. MTT assay (cell proliferation) Anchorage-independent cell growth Immunohistochemistry Annexin V/PI H&E	p21, cyclin D1, cyclin D3 (cell cycle marker proteins) PARP (apoptotic signaling pathway) ABL, AMPKα1, AURKA, BRAF, CDK2/CCNE, CHEK1, EGFR, FAK, FGFR1, FYN, GSK3β, MAPK1, MEK1, MET, PDK1, PKBα, PKCα, p70S6K, RSK2, SAPK2α, SRC, TAK1, or TBK1 (Cancer related kinases)	- 50% reduction of HGC27 and AGS with 10.1μM and 18.7μM, respectively - Increase of suspended cells (dead) and decrease in adherent cells (alive) after 48 hours treatment in AGS or HGC27 cells - **Increase in early apoptosis compared with control group.** - **S phase fraction:** ↓ - **G1 phase cell cycle arrest:** ↑ in dose-dependent manner - **Growth of GC cells:** ↓ in dose-dependent manner - **p-AKT, p-mTOR and p-p70S6K was reduced.** - 2,6-DMBQ did not affect 23 different cancer related kinases - anchorage-dependent and-independent growth GC cells were reduced by knock-down of mTOR.

Table 3: Qualitative synthesis of the included ***in vitro*** studies. (**Cont.**)

Author, year [Ref.]	Treatment-Related Parameters				Cell-Related Parameters			Assessment Techniques	Biomarkers	Efficacy parameters
	Treatment	Dose	Frequency	Duration	Disease of origin	Type of Cell line	ID			
Mohamed A, et al. 2017[52]	**Everolimus (RAD001)**	1nM and 10nM	–	Cga secretion analysis (Elisa kit): 72 hours treatment Cell viability assay: cells were seeded for 24 hours, followed by 72 hours treatment. Caspase activity determination: cells were seeded by 24 hours, followed by 24 hours treatment Protein extraction and western blot: After 24 hours in culture, cells were treated or not with the different pharmacological agents.	GEP-NETs	pNETs	–	Immunohistochemistry (PTEN, DAXX and ATRX) qRT-PCR (SST2 receptor) Cell viability Caspase activity Western blot (protein extraction) ELISA (Cga secretion) Genomic analysis (MEN1)	PTEN, DAXX ATRX, chromogranin A SST2 p-p70S6K p-Akt p- ERK1/2	**-** **Viable cell number:** ↓ from 14 % to 77% in 20 primary cultures with a median value of 59.3% - Cell viability reduced ate 1 and 10nM. Repressed from 40 to 58% in 66.6% of tested tumours. - No correlation was found in cell viability and the presence of MEN1 tumour mutations or PTEN, DAXX/ATRX expressions levels. - There was no correlation between everolimus inhibitions on cell viability and Cga secretion
	Octreotide **OR** Pasireotide	1nM								**- Cell viability:** ↓, no significant difference between analogs
	Everolimus (RAD001) + Octreotide **OR** Pasireotide	1nM + 1nM								**-** Everolimus in combination with one of the SSAs had an increase in the inhibition of ell viability in only one primary culture. **- Inhibition of cell viability:** Partially or fully reversed induced by each single treatment **-** Caspase activities were not altered by combined treatment **-** Combined treatments during 24h significantly inhibited p70S6K and increased p-Akt. Although, it was similar with everolimus treatment alone.
Freitag H, et al. 2017[39]	PKI-587	0.01 nM to 5 μ M	–	Cell viability assay: overnight followed by incubation for 24, 48 and 96 hours. Replicated 3 times. Multiplex viability, cytotoxicity apoptosis assay: overnight followed by 12- and 36-hours incubation. Replicated 3 times. Cell cycle analysis and determination of mitotic index: overnight followed by incubation for 48 hours (control) and 96 hours (cell cycle for analysis) Assessment of mitochondrial membrane potential: overnight followed by 16 hours incubation. Western blot: 25 hours. Analysis of gene expression: 60 hours.	GEP-NENs	GEP-NEN cell line models representing different tumour subtypes pancreatic BON QGP-1 cells ileal KRJ-I cells colonic LCC-18 cells	BON QGP-1 KRJI LCC-18	Cell viability assay Flow cytometry (apoptosis assay) Western blot (AKT activation) Gene Expression (treatment-induced alterations in gene regulation)	Mitochondrial membrane potential (apoptosis analysis) mTORC1 TSC2 GSK-3 FOXO1 GSK-3α GSK-3β and pan-AKT (KT activation) ERK 1/2	**-** Reduced the amount of living cells by 61-94% **- Proliferation:** Alterations dose- and time-dependent **- Cell viability:** ↓ **- Apoptotic cells** (with high concentrations)**:** ↑ in BON, KRJ-I, and LCC-18 cells **- G 0 /G 1 arrest**, ↓ amounts of cells in the **S, G2, and M phases** **-Changes in the mitochondrial membrane potential** emerged through at C max in BON and LCC-18 cells - Was more distinct than that of RAD001 as an mTORC1-selective inhibitor **Caspase 3/7 activity:** ↑ **- Living cells:** ↓ - Caused more gene alterations than everolimus
	Everolimus (RAD001)	0.01 nM to 5 μM								**-** Reduced the amount of living cells by 47-52% **- Cell viability:** Reduced. **- Caspase 3/7 activity**: Only increased in KRJ-I (in comparison with PKI-587) **- Weaker compared with PKI-587**, revealed G 0/G 1 arrest, with decreasing amounts of cells in the S, G 2, and M phases **- Phosphorylation of GSK-3α and GSK-3β in KRJ-I and QGP-1 cell lines.** slight ↑ - Increase of pan-AKT in pancreatic cell lines (BON and QGP-1)
Bollard J, et al. 2013[35]	**Everolimus (RAD001)**	11 nM	–	Maintained 72 hours under normal culture conditions. The medium was then replaced by 0.05% FCS-containing DMEM me dium for 24 h. The growth factor IGF-1 and the mTOR signaling inhibitor Everolimus were added for 24 h.	Derived from endocrine intestinal tumours developed in transgenic mice.	Cells from endocrine intestinal tumours developed in transgenic mice	STC-1 GluTagl	H WB (p70S6K and 4E-BP1) IHC Mitosis-specific Phospho (Ser10) Propidium iodide	phospho-p-70S6K phospho-4E-BP1	- can inhibit the mTOR signaling pathway in STC-1 and GluTag p-p70S6K p-4E-BP1 cells**.** - cells were treated with 3 nM exogenous IGF-1 to mimic the microenvironment conditions of the tumours. - IGF-1-induced phosphorylation of mTOR: reduced **- p-mTOR**: ↓ **- p70S6K and 4E-BP1 phosphorylati**on: Induced abrogation **- Effectors of mTOR**: Inhibited the action of the cells

Table 3: Qualitative synthesis of the included ***in vitro*** studies. (**Cont.**)

Author, year [Ref.]	Treatment-Related Parameters				Cell-Related Parameters			Assessment Techniques	Biomarkers	Efficacy parameters
	Treatment	Dose	Frequency	Duration	Disease of origin	Type of Cell line	ID			
Fukamachi H, et al. 2019 [53]	**Temsirolimus OR Everolimus**	3-100 nM	–	Effects of anti-tumour drugs on PDX: 2 weeks Effect of molecularly targeted drugs and signalling inhibitors: 5 days	Diffuse-type GCs	Human GC	MKN45, MKN74, and NUGC-4 human GC cell lines HGC-3, −18 and−20 PDXs	IHC WB Methylation-specific PCR MSI and CIN Tumourigenicity H MTT RNA extraction and sequencing mutation Hematoxylin and Alcian Blue (pH2,5)-PAS-hematoxylin	BAT25 BAT26 BAT40 BM11 CD44 EpCAM SOX9 CD49f p-mTOR EGF Noggin R-spondin1 Wnt3a	- (Diffuse type GC cells) Everolimus: gnifi cantly suppressed the growth of HGC-3 and-20 cells **- (Diffuse type GC cells) Temsirolimus:** Suppressed the growth of HGC-20 and HGC-3 cells in culture. Almost completely suppressed the growth of HGC-20 cells - Temsirolimus had a weaker effect due to the fact that is not toxic to the cells. - (Primary Culture GC cells) Temsirolimus: HGC-20 cells were the most sensitive among the cells examined to the growth-suppressive action.
	PP242									- **Weak** effect of suppression on HGC-3 and HGC-20 diffuse GC cells

Legend: ↓- reduced, **↑**-increased, **CCK-8-** Cell Counting Kit-8, **CQ-** Chloroquine, **CRA-** Corosolic Acid, **D-** Days, **Edu-** EdU staining, **FC-** Flow Citometry, **GC-** Gastric cancer, **GEP-NENs-** Gastroenteropancreatic neuroendocrine neoplasms, **GEP-NETs-** Gastroenteropancreatic neuroendocrine tumours, **H-** Histology, **H&E-** Hematoxylin and eosin, **IF-** Immunofluorescence, **IHC-** Immunohistochemistry, **MTT-** 3-(4,5-dimethyl thiazol-2-yl)-2,5- diphenyl tetrazoliumbromide, **PCR-** Polymerase chain reaction, **PI-** Propidium iodide, **p-mTOR** - phosphorylated mTOR, **pNETs-** Primary cultures of human Pancreatic neuroendocrine tumours, **qRT PCR-** Real-time quantitative-polymerase chain reaction, **rpS6** - S6 ribosomal protein, **RT-PCR-** Reverse-transcription-polymerase chain reaction, **TEM-** Transmission electron microscopy, **TZB-** Trastuzumab, **WB-** western blot.

Table 4: Qualitative synthesis of the included ***in vivo*** studies.

Author, year [Ref.]	Treatment-Related Parameters					Animal-Related Parameters					Assessment Techniques	Biomarkers	Efficacy parameters
	Treatment	Dose	Frequency	Route of Administration	Duration	Animal Model of GC	Specie	Strain	Gender	Age (weeks)			
Huangfu L et al. 2023 [48]	Piceatannol **OR** **Everolimus (RAD001)**	5 mg kg^{-1} **OR** 10 mg	2/2 D	IP		SGC7901 cells SC engrafted into each flank of the mice	Mice	BALB/c nude	Female	5	WB InCute live cell IHC (Cell proliferation, cell death and autophagy)	Ki67 PARP p-Beclin-1 LC3B-I/II Bax/Bcl-2	**- Tumour growth:** Minimal effects
	Piceatannol **+** **Everolimus (RAD001)**	5 mg kg^{-1} + 10 mg kg^{-1}			1 week								**- Tumour growth:** Inhibited **- Enhence autopahic flux though mTOR and Beclin-1 pathway** (down- regulation of phosphorylation levels of Beclin-1 at Ser-295 and mTOR at Ser-2448 and up- regulation of Beclin-1 phosphorylation at Thr 119) - increase of LC3B-I/II and Bax/Bcl-2 ratios, as well as the degradation of SQSTM1/p62. **- Strong anti-tumour effect in vivo due to autophagic cell death**
Gao F et al. 2022[34]	Rhein + **Everolimus (RAD001)**	60 mg/kg + 5 mg/kg	–	Oral	28 D	MGC-803 cells SC inoculated into the mice		CB-17SCID			IHC (cell proliferation)	p-PI3K p-AKT p-mTOR ki-67	**- Cell proliferation:** ↓ **- Tumour:** Repressed weight and volume **- p-PI3K, p-AKT, p-mTOR expression was d**own-regulated, indicating inhibition of pathway PI3K/AKT/mTOR. **- Ki-67**: ↓
Onoyama M, et al. 2013[54]	Imatinib **OR** Nolotinib	50 mg/kg	Daily		35 D	Cells injected into the gastric wall		BALB/c nude			H IF IHC qRT-PCR Ki-67	α-SMA type I collagen Ki-67 Lyve-1 phosphorylated rpS6 VEGF-A PDGF-B PDGF-Rbeta p-S6RP	**- Tumour**: Better effect **- Lymphatic vessel area:** ↓ especially in the TMK-1 tumours **-Tumour growth and metastasis:** Not inhibited in mice treated with Nilotinib alone **- p–PDGF-Rβ:** Inhibited with Nilotinib alone
	Everolimus (RAD001)	2 mg/kg											**- Apoptotic cells:** ↑ **- Tumour growth**: Inhibited **- VEGF-A:** Suppressed **-p-s6 ribossomal**: Inhibited
	Nlotinib + **Everolimus (RAD001)**	100 mg/kg + 2 mg/kg											**-Tumour growth**: More inhibited with the combination treatment. **- p–PDGF-Rβ:** Inhibited **-p-s6 ribossomal**: Inhibited **- KI-67**: ↓
Zu X, et al. 2020 [37]	**2,6-DMBQ**	20, 50, 80 mg	5 times a week		2 weeks (toxicity) 43 days (p-mTOR)	Patient tissues implanted into the neck of the mice		–		6 a 9	FC WB (protein gastric tumour tissue analysis) MTT, Anchorage-independent cell growth ATP Annexin V/P H&E IHC (tumour proliferation)	AST or ALT LSG55 (↑ p-mTOR) LSG64 (↓ p-mTOR)	**- LSG55 gastric tumours:** Reduced the volume **- LSG64 gastric tumours**: Little effect on the growth **- Ki-67, p-mTOR, p-p70S6K:** Decreased in LSG55 - ALT and AST were not altered with treatment

Table 4: Qualitative synthesis of the included ***in vivo*** studies. (**Cont.**)

Author, year [Ref.]	Treatment-Related Parameters					Animal-Related Parameters					Assessment Techniques	Biomarkers	Efficacy parameters
	Treatment	Dose	Frequency	Route of Administration	Duration	Animal Model of GC	Specie	Strain	Gender	Age (weeks)			
Bollard J, et al. 2013 [35]	**Everolimus (RAD001)**	1.5 mg/kg/day	Daily	IP	28 D	50 µl of a solution containing tumour cells adjusted to a final concentration of 5·10 7 cells/ml were injected into the spleen	Mice	Swiss nu/nu	Female	4	H WB IHC(phospho-mTOR, phospho-p70S6K, phospho-4E-BP1, Ki67, active caspase 3) Meyer's hematoxylin. hematoxylin-phloxin-safran morphometry	phospho-p-70S6K phospho-4E-BP1	- Tumour **development:** Inhibited - tumour tissue surface: STC-1-xenografted mice: evaluated to 14.84 ± 2.06% in control animals was significantly reduced to 2.51 ± 0.6% in treated animals ($p < 0.05$); GluTag-xenografted mice:was significantly decreased from 21.76 ± 3.88% in control an animals to 6.05 ± 1.84% in treated animals ($p < 0.05$) -Ki67: STC-1- was 52.0 ± 4.2% in control animals as compared to 41.33 ± 2.56% in treated animals.; GluTag- was 30.5 ± 2.4% in control animals as compared to 20.44 ± 0.95% in treated animals. **- Tumour mass:** ↓, observed under everolimus was, at least in part (Antiproliferative effect) **% cells expressing active caspase 3**: No difference was observed between control and treated animals. - Body-weight curves: significant stabilization of the weight of treated animals, whereas control animals lost weight from day 18 in both experiments **- Levels of phosphor-4E-BP1 and phosphor-p70-S6K:** remained unchanged in treated mice, the appar ent expression levels of phospho-4E-BP1 and phos pho-p70-S6K were greatly reduced in everolimus-treated animals bearing both STC-1- and GluTag-derived tumours
Fukamachi H, et al. 2019 [53]	**Temsirolimus**	1–10 mg/kg	Twice week		The injected mice were maintained for up to 6months, and killed when tumour diameters reached 10mm.	Cells injected into each flank of the mice		Nude	–	–	IHC WB Methylation-specific PCR MSI CIN Tumourigenicity H MTT RNA extration and sequencing Mutation Hematoxylin and Alcian Blue (pH2,5)-PAS-hematoxylin	BAT25 BAT26 BAT40 BM11 CD44 EpCAM, SOX9 CD49f p-mTOR EGF noggin R-pondin1 Wnt3a	**- Growth of HGC-3 and -20:** Suppressed **- Showed stronger activity than other mTOR inhibitors** - The best among the **three mTOR inhibitors** - Control the inhibition of the size of the tumour.

Legend: ↓- reduced, **↑**-increased, **CCK-8-** Cell Counting Kit-8, **D-** Days, **Edu-** EdU staining, **FC-** Flow Citometry, **GC-** Gastric caner, **GEP-NENs-** Gastroenteropancreatic neuroendocrine neoplasms, **GEP-NETs-** Gastroenteropancreatic neuroendocrine tumours, **H-** Histology, **H&E-** Hematoxylin and eosin, **IF-** Immunofluorescence, **IHC-** Immunohistochemistry, **IP-** Intraperitoneal, **MTT-** 3-(4,5-dimethyl thiazol-2-yl)-2,5- diphenyl tetrazoliumbromide, **PCR-** Polymerase chain reaction, **PI-** Propidium iodide, **p-mTOR** - phosphorylated mTOR, **pNETs-** Primary cultures of human Pancreatic neuroendocrine tumours, **qRT PCR-** Real-time quantitative-polymerase chain reaction, **rpS6** - S6 ribosomal protein, **RT-PCR-** Reverse-transcription-polymerase chain reaction, **SC** – subcutaneously, **TEM-** Transmission electron microscopy, **TZB-** Trastuzumab, **W-** Weeks **WB-** western blot.

Table 5: Toxicity and safety in ***in vitro*** **studies**.

Author, year [Ref.]	Treatment	Toxicity and safety
Ying L, et al. 2014 [51]	DDP **OR** **Everolimus (RAD001)**	Treatment with RAD001 in vitro were considered nontoxic with concentrations below 125nmol/L
	Everolimus (RAD001) (Pretreatment) + DDP	
Gao F et al. 2022[34]	**Rhein and Everolimus (RAD001)**	With concentrations of 80 μM (Rhein) and 40nM (Everolimus) it was shown to be without toxicity
Fukamachi H, et al. 2019 [53]	**Temsirolimus**	Treatment were not cytotoxic with 3 μM (3000 nM)

Table 6: Toxicity and safety in ***in vivo*** **studies.**

Author, year [Ref.]	Treatment	Toxicity and safety
Huangfu L et al. 2023 [48]	Piceatannol **OR** **Everolimus (RAD001)**	All treatments were well tolerated, with no apparent weight loss.
	Piceatannol **+** **Everolimus (RAD001)**	
Onoyama M, et al. 2013[54] **Zu X, et al. 2020** [37]	Imatinib **OR** Nolotinib	Oral administration of Nilotinib, Everolimus, and of the two drugs in combination did not significantly affect bodyweight.
	Everolimus (RAD001)	
	Nlotinib + **Everolimus (RAD001)**	
Zu X, et al. 2020 [37]	**2,6-DMBQ**	Treatment showed no morphological and functional alterations on tissues from spleen, kidney, and liver of treated mice
Bollard J, et al. 2013 [35]	**Everolimus (RAD001)**	Significant stabilization of the bodyweight of treated animals.

In *in vitro* studies (**Table 5**), available data suggest that mTOR inhibitors demonstrate a favourable safety profile when used at appropriate concentrations. In the study conducted by **Ying L, et al** [51] , treatment with Everolimus, either alone or in combination with cisplatin

(DDP), was considered non-toxic to gastric cancer cells at concentrations below 125 nmol/L. Similarly, **Gao F, et al** [34] reported no observed toxicity with the combination of Rhein (80 μM) and Everolimus (40 nM). Furthermore, in the study by **Fukamachi H, et al** [53]**,** treatment with Temsirolimus did not induce cytotoxicity at a concentration of 3 μM (3000 nM), indicating safety in a cellular environment.

In *in vivo* studies (**Table 6**), treatments involving Everolimus and other agents also demonstrated good tolerability and an absence of significant adverse effects. **Huangfu L, et al** [48] observed that both Everolimus and Piceatannol, administered alone or in combination, were well tolerated by animals, with no apparent weight loss – a common indicator of systemic toxicity. **Onoyama M, et al** [54] and **Zu X, et al** [37] investigated the oral administration of Imatinib, Nilotinib, Everolimus, and their combinations, and reported no significant impact on body weight. Specifically, **Zu X, et al** [37] also evaluated the compound 2,6-DMBQ and found no morphological or functional alterations in the spleen, kidney, or liver tissues of treated mice, further supporting the safety profile of the treatment. Lastly, **Bollard J, et al** [35] noted a significant stabilisation of body weight in animals treated with Everolimus, reinforcing its low systemic toxicity.

Overall, the assessment of toxicity and safety across both in vitro and in vivo studies revealed a consistent pattern of low toxicity associated with mTOR inhibitors, particularly Everolimus and Temsirolimus, when used within specific concentration ranges. In in vitro experiments, no cytotoxic effects were observed at concentrations considered therapeutically relevant, suggesting a favourable safety margin at the cellular level. Likewise, in vivo studies consistently reported no significant weight loss, organ damage, or histological abnormalities, even when mTOR inhibitors were combined with other agents such as Piceatannol, Nilotinib, or Rhein.

Importantly, despite the use of different experimental models and treatment combinations, there was a clear absence of systemic toxicity, reinforcing the translational potential of these agents. However, it should be noted that while the findings suggest a general trend towards safety, the heterogeneity in dosing regimens, treatment durations, and animal models limits the ability to define a precise safety threshold or dose-response pattern.

Across the preclinical studies analysed, including both in vitro and in vivo models involving mTOR inhibitors (such as Everolimus, Temsirolimus, OSI-027, PKI-587, and 2,6-DMBQ), the reporting of adverse events and safety outcomes was consistently limited or absent. In nearly all studies involving in vitro models, cytotoxicity was either intentionally

induced as a therapeutic outcome or not assessed beyond the expected antiproliferative effects. No toxicity to normal cells or off-target adverse effects were described in these settings.

In *in vivo* experiments, primarily using xenograft or patient-derived xenograft (PDX) models in immunocompromised mice mTOR inhibitors were generally well tolerated. However, most studies did not explicitly evaluate or report systemic toxicity, such as weight loss, behavioural changes, organ damage, or biochemical markers of liver or kidney function. For instance, the combination of Everolimus and Rhein was reported to reduce tumour growth without significant toxicity, but no quantitative safety data were provided. Similarly, the novel compound 2,6-DMBQ demonstrated potent antitumour effects in mice, yet no specific assessment of adverse events was included.

The study evaluating OSI-027 (a dual mTORC1/2 inhibitor) was limited to in vitro assays and did not report adverse effects beyond its intended synergistic apoptosis-inducing activity when combined with oxaliplatin. The investigation of PKI-587 (Gedatolisib), another dual PI3K/mTOR inhibitor, also focused on in vitro neuroendocrine tumour models. Although potent pro-apoptotic and anti-proliferative effects were observed, no experiments were conducted in vivo, and therefore, no systemic safety data were available. The authors referenced clinical phase I data indicating that PKI-587 exhibited a “manageable safety risk,” yet this was external to the scope of the preclinical study.

Notably, none of the analysed studies included standard preclinical safety assessments such as histopathological evaluation of non-tumour tissues, haematological analyses, or monitoring of animal survival or distress. As such, although the therapeutic efficacy of mTOR inhibitors in gastric cancer models was broadly demonstrated, there remains a significant gap in the systematic evaluation of their safety in preclinical settings.

This lack of detailed toxicity profiling represents a major limitation in the translational relevance of current preclinical data on mTOR-targeted therapies. Future studies should incorporate comprehensive safety endpoints to better inform the risk-benefit profile of these agents before clinical application.

Risk of Bias Assessment

Tables 7 and 8 provide an overview of the risk of bias assessments for each study included in this review. The majority of studies showed an unclear risk of selection,

detection, and attrition bias, mainly due to issues like inadequate sample size calculation and lack of blinding.

Regarding the ***in vitro*** studies, all articles that presented results on cells were categorized as “Unclear risk of bias” in the “Sample size calculation”, “Blinding”, “Incomplete outcome data” and “Selective reporting” domains. Originally developed to evaluate bias in animal intervention studies, the SYRCLE’s RoB tool was adapted for use in in vitro research. Selection bias was attributed to potential differences among the chosen cell lines that could influence study outcomes. Performance bias was redefined to emphasize the variability in the intervention. Regarding detection bias, the focus was placed on ensuring transparency and objectivity in the assessment of outcomes. Lastly, the evaluation of other biases considered aspects such as data presentation, clarity in the disclosure of conflicts of interest and funding sources, and the availability of data for independent verification (**Table 7**)[55].

Table 7: Risk of Bias *in vitro* studies using Modified SYRCLE tool

***In Vitro* studies Reference**	Sample size calculation	Baseline characteristics	Detailed explanation of intervention	Detailed explanation of culture conditions	Details of comparison group	Method of measurement of outcome	Blinding	Incomplete outcome data	Selective reporting	Other sources of bias
Huangfu L, et al. 2023[48]										
Gao F, et al. 2022[34]										
Xu E, et al. 2021 [36]										
Zu X, et al. 2020 [37]										
Hou G, et al. 2020[50]										
Fukamachi H, et al. 2019 [53]										
Ye H, et al. 2018										
Mohamed A, et al. 2017 [52]										
Freitag H, et al. 2017 [39]										
Lee S, et al. 2015										
Cao Y, et al [38]										
Ying L, et al [51]										
Bollard J, et al. 2013 [35]										

Legend:

Low risk of bias
Unclear risk of bias
High risk of bias

Regarding the *in vivo* studies, all articles that presented results on animals were categorized as “Unclear risk of bias” in the “Sequence generation”, “Random housing”, “Blinding”, “Random outcome assessment” and “Blinding” domains and two of them presented results categorized as “High Risk of Bias” in the “Incomplete outcome data”. All

studies were characterised as “Low risk of bias” in the “Selective outcome reporting” domain and “High risk of bias” in the “Other sources of bias” domain (**Table 8**).

Table 8: Risk of Bias in vivo studies using SYRCLE tool.

In Vivo Studies **Reference**	Sequence generation	Baseline characteristics	Allocation concealment	Random housing	Blinding	Random outcome assessment	Blinding	Incomplete outcome data	Selective outcome reporting	Other sources of bias
Huangfu L et al 2023. [48]										
Gao F et al. 2022 [34]										
Zu X, et al. 2020 [37]										
Fukamachi H, et al. 2019 [53]										
Onoyama M, et al. 2013 [54]										
Bollard J, et al. 2013 [35]										

Legend:

Low risk of bias
Unclear risk of bias
High risk of bias

By means of this analysis it was possible to observe the absence of low- and high-quality articles related to articles that present results in animals.

Efficacy and safety

Combination therapies with Everolimus: synergistic effects

Several studies demonstrated that combining Everolimus with other agents enhances its antitumour effects.

For instance, the combination with Piceatannol resulted in greater inhibition of cellular proliferation and induction of autophagy-dependent cell death **(Huangfu L, et al** [48]). Co-administration with Rhein suppressed the PI3K/AKT/mTOR pathway, reduced tumour invasiveness, and increased apoptosis **(Gao F, et al** [34]). The combination with Trastuzumab partially restored sensitivity in HER2+ resistant cell lines (Ye H). The use of Nilotinib together with Everolimus reduced metastasis and lymphatic vessel density while enhancing apoptosis (**Onoyama M, et al** [54]) (**Table 9**).

Co-treatment without everolimus also showed good results. Oxaliplatin and OSI-027 produced synergistic pro-apoptotic and anti-proliferative effects (**Xu E, et al** [36]), whereas Rapamycin combined with Celecoxib showed that the latter can reverse Rapamycin resistance by blocking Akt phosphorylation (**Cao Y, et al** [38]).

Table 9: Combination therapies with Everolimus: synergistic effects

Combination	Observed effect
Piceatannol + Everolimus	Greater inhibition of proliferation and induction of autophagy-dependent cell death (**Huangfu L, et al**)[48].
Rhein + Everolimus	Suppression of the PI3K/AKT/mTOR pathway, reduced invasion, increased apoptosis (**Gao F, et al** [34]).
Trastuzumab + Everolimus	Partial restoration of sensitivity in HER2+ resistant cells (**Ye H**[49]).
Nilotinib + Everolimus	Decreased metastasis and lymphatic vessel density; enhanced apoptosis (**Onoyama M, et al** [54]).

These findings suggest that the association of Everolimus with other compounds (antitumour agents or inducers of apoptosis/autophagy) increases therapeutic efficacy, often through synergistic action on signalling pathways, apoptosis, or cellular self-degradation.

Effects of Everolimus as monotherapy: moderate, but lineage-dependent (Table 10)

When used as a single agent, Everolimus exhibits variable therapeutic effects. In the study by **Fukamachi H, et al** [53], both Everolimus and Temsirolimus were effective in HGC-3 and HGC-20 cell lines, although the overall effects were modest. **Mohamed A. et al**[52]. reported a significant reduction in cell viability, even if with variable responses among primary cultures. **Bollard J, et al** [35] observed strong inhibition of mTOR and its downstream effectors (p70S6K and 4E-BP1), resulting in reduced tumour growth. Contrariwise, **Fukamachi H, et al** [53] found only mild effects on p-AKT and GSK-3, with inconsistent cellular responses (**Table 10**).

Table 10: Effects of Everolimus as monotherapy

Study	Key findings
Fukamachi H, et al [53]	Everolimus and Temsirolimus effective in HGC-3 and HGC-20, but modest effects overall.

Mohamed A. et al. [52]	Significant reduction in cell viability, but varied response among primary cultures.
Bollard J, et al [35]	Strong inhibition of mTOR and its downstream effectors (p70S6K and 4E-BP1); tumour growth reduction.
Freitag H, et al [39]	Mild effect on p-AKT and GSK-3; variable cellular response.

These findings indicate that Everolimus reduces proliferation and modulates apoptosis/signalling pathways; however, its efficacy varies depending on the cell line and is generally more potent in combination therapy.

Autophagy: a relevant target for mTOR inhibitors (Table 11)

Autophagy emerges as a key mechanism of action or resistance in response to mTOR inhibitors. **Huangfu L, et al** [48] showed that combining Piceatannol with Everolimus promoted autophagy. Ye H demonstrated that Everolimus induces autophagy in Trastuzumab-resistant cell lines. In contrast, **Hou G, et al** [50] found that Everolimus alone did not enhance apoptosis in combination with cisplatin (DDP), whereas the autophagy blocker chloroquine (CQ) was effective.

Table 11: Autophagy: a relevant target for mTOR inhibitors

Study	Key findings
Huangfu L, et al [48]	Autophagy promoted by Piceatannol + Everolimus combination.
Ye H[49]	Everolimus induces autophagy in Trastuzumab-resistant cell lines.
Hou G, et al [50]	Everolimus alone does not enhance apoptosis with cisplatin (DDP), but CQ (autophagy blocker) does.

These findings highlight the importance of modulating this pathway with mTOR inhibitors may induce cell death or improve response to chemotherapy.

Overcoming chemotherapy resistance (Table 12)

Some studies have explored the potential of Everolimus to reverse resistance to chemotherapeutic agents. **Ying L, et al** [51] reported that Everolimus restored sensitivity to DDP in SGC7901/DDP cells. However, **Hou G, et al** [50] found that Everolimus was ineffective in reversing DDP resistance when used alone, while the combination of DDP and

CQ was effective. Additionally, **Cao Y, et al** [38] demonstrated that Celecoxib could overcome resistance to Rapamycin.

Table 12: Overcoming chemotherapy resistance

Study	Resistance targeted	Outcome
Ying L, et al [51]	Cisplatin (DDP)	Everolimus restores sensitivity to DDP in SGC7901/DDP cells.
Hou G, et al [50]	DDP	Everolimus ineffective; CQ combined with DDP was effective.
Cao Y, et al [38]	Rapamycin	Celecoxib reverses resistance to Rapamycin.

These results suggest that Everolimus may modulate drug resistance, including to DDP and Rapamycin, particularly through regulation of P-gp/MRP1 and Akt phosphorylation. However, it is not universally effective and may require adjuvants such as CQ or Celecoxib.

Effects on cell cycle and apoptosis (Table 13)

mTOR inhibitors are known to induce cell cycle arrest and promote apoptosis, with effects that vary according to dose and cell lineage. **Xu E, et al** [36] and **Zu X, et al** [37] reported that agents such as OSI-027 and 2,6-DMBQ induced G0/G1 arrest and apoptosis. **Freitag H, et al** [39] observed that Everolimus and PKI-587 both caused cell cycle arrest and apoptotic cell death, with PKI-587 displaying greater potency. **Bollard J, et al** [35] identified a reduction in proliferation markers including Ki-67, p70S6K, and 4E-BP1.

Table 13: Effects on cell cycle and apoptosis

Study	Key findings
Xu E, et al [36] and **Zu X, et al** [37]	mTOR inhibitors such as OSI-027 and 2,6-DMBQ induce G0/G1 arrest and apoptosis.
Freitag H, et al [39]	Everolimus and PKI-587 cause cell cycle arrest and apoptosis, with PKI-587 being more potent.

Bollard J, et al [35]	Reduction in proliferation markers (Ki-67, p70S6K, 4E-BP1).

These findings support the role of mTOR inhibitors promote G0/G1 cell cycle arrest and enhance apoptosis, with dose- and lineage-dependent effects. These changes involve mTOR, p70S6K, 4E-BP1, and AKT.

Safety and tolerability (Table 14)

Table 14: Safety and tolerability

Study	Key findings
Huangfu L, et al [48] and **Zu X, et al** [37]	No weight loss or toxicity observed in animals.
Zu X, et al [37]	No alterations in ALT, AST, or target organs.

In preclinical models, mTOR inhibitors such as Everolimus, OSI-027, and 2,6-DMBQ are well tolerated, with minimal detectable systemic toxicity.Studies by **Huangfu L, et al** [48] and **Zu X, et al** [37] reported no weight loss or signs of toxicity in animals. **Zu X, et al** [37] also found no changes in liver enzymes (ALT, AST) or in target organs. These results suggest minimal systemic toxicity and good tolerability under experimental conditions, supporting the continued investigation of mTOR inhibitors in therapeutic regimens.

In summary, Everolimus demonstrated the most comprehensive and reproducible antitumour activity, supported by both functional and mechanistic data in vitro and in vivo. Temsirolimus showed similar molecular effects in vitro but lacked in vivo validation. Rapamycin displayed some efficacy, albeit with limited data. BEZ235, though mechanistically potent, requires further preclinical validation, particularly in animal models, to confirm its functional superiority. Collectively, the findings reinforce Everolimus as the most effective and translationally viable mTOR inhibitor in the current preclinical landscape for gastric cancer.

Efficacy and Biomarker

In vitro studies employing Everolimus, INK-128, 2,6-DMBQ, and other mTOR-targeting compounds demonstrated dose-dependent inhibition of cell viability and proliferation in

gastric cancer cell lines, including AGS, MKN45, NCI-N87, and SNU-1. These antiproliferative effects were consistently associated with reduced phosphorylation of mTOR (p-mTOR), 4E-BP1 (p-4E-BP1), and p70S6K (p-p70S6K). For instance, treatment of MKN45 and NCI-N87 cells with INK-128 led to potent growth suppression accompanied by strong inhibition of mTOR pathway activation, as evidenced by decreased levels of p-4E-BP1 and p-p70S6K.

Despite these consistent trends, some in vitro studies reported incomplete biomarker suppression in the presence of significant phenotypic effects, suggesting that mTORi efficacy may not be fully dependent on canonical downstream target inhibition. Additionally, autophagy activation was noted in several models following mTOR inhibition, suggesting an alternative cytostatic mechanism of action distinct from apoptosis. For example, increased LC3-II expression and autophagosome accumulation were observed in AGS and SNU-1 cells treated with Everolimus and NVP-BEZ235, supporting the role of autophagy as a non-apoptotic contributor to growth inhibition.

In vivo studies further supported the link between pathway inhibition and therapeutic response. Tumour volume reduction, observed in xenograft and PDX models treated with Everolimus, Temsirolimus, and 2,6-DMBQ, was often accompanied by decreased expression of Ki-67, confirming suppression of tumour cell proliferation. Furthermore, treatment frequently resulted in decreased levels of p-4E-BP1, p-p70S6K, and p-mTOR in tumour tissue, reinforcing their value as pharmacodynamic biomarkers.

However, similar to in vitro findings, some in vivo models demonstrated partial or inconsistent biomarker modulation despite marked tumour regression. For instance, in models derived from STC-1 and GluTag cell lines, Everolimus induced significant tumour shrinkage and reduced Ki-67 expression, yet p-4E-BP1 and p-p70S6K were only partially suppressed. This suggests that phenotypic efficacy may not require complete downstream pathway inhibition, or that compensatory mechanisms may contribute to tumour control.

Notably, the presence of PIK3CA mutations in some PDX models (e.g., HGC-3 and HGC-20) was associated with improved response to Everolimus and Temsirolimus, supporting the role of genetic alterations as predictive markers. In contrast, models lacking these mutations or showing low baseline mTOR pathway activity were less responsive, despite receiving the same therapeutic regimen.

Across both in vitro and in vivo contexts, apoptosis markers such as active caspase-3 showed variable modulation, with some studies reporting no significant changes even in models exhibiting tumour regression. This supports the notion that mTOR inhibitors may

act primarily through antiproliferative and cytostatic mechanisms, rather than through induction of apoptosis.

Taken together, the data indicate that the efficacy of mTOR inhibitors in gastric cancer models is closely, but not exclusively, associated with suppression of key biomarkers in the PI3K/AKT/mTOR pathway. While p-mTOR, p-4E-BP1, p-p70S6K, and Ki-67 remain the most frequently modulated indicators of therapeutic response, their predictive utility appears context-dependent and influenced by tumour type, baseline molecular profile, and additional resistance or compensatory pathways such as autophagy. These findings underscore the importance of combining multiple biomarkers and tumour-specific molecular characterisation to predict and enhance therapeutic outcomes.

Discussion

Animal-Related Parameters

Species and Strain

The choice of animal model is a critical component in preclinical studies, significantly influencing the translation of findings into clinical applications. Among the studies analyzed in this review, there was a predominance of immunodeficient mice, particularly nude and SCID (Severe Combined Immunodeficiency) strains, which are frequently used to support the growth of human tumour xenografts.

Three of the included studies used female BALB/c nude mice, which are immunodeficient due to the absence of T cells, thereby facilitating the engraftment of human tumour cells. For instance, **Huangfu L, et al** [48] and **Onoyama et al.** [54] employed these strains for subcutaneous (SC) and orthotopic implantation, respectively. The orthotopic model enabled the investigation of stromal-tumour interactions within a more representative tumour microenvironment. This choice is consistent with other preclinical evidence in the literature, such as the study by **Taguchi et al.**[56], which also utilised murine models with peritoneal dissemination to demonstrate the efficacy of everolimus (RAD001), reporting a significant reduction in tumour volume and number of metastatic implants [56]. SCID mice, deficient in both T and B cells, were used by **Gao F, et al** [34] and **Zu et al.**[37] to enable the engraftment of more complex tumour models, such as patient-derived xenografts (PDX). Complementarily, the clinical study by **Doi et al.**[57] assessed the use of everolimus in previously treated patients with metastatic gastric cancer, demonstrating disease control in 56% of patients and a median progression-free survival (PFS) of 2.7 months [57]. These findings suggest that the efficacy observed in immunodeficient models may partially translate into advanced clinical settings, albeit with limited outcomes.

The use of Swiss nu/nu mice, as in the study by **Bollard et al.**[35], allowed for the assessment of hepatic dissemination of murine gastrointestinal neuroendocrine tumours via splenic vein injection. Although less common, this approach adds relevant methodological diversity. Similarly, the use of NOD-SCID mice by **Fukamachi et al.**[53], which also lack functional natural killer (NK) cells, proved suitable for investigating the tumorigenicity of human cancer stem cells in PDX models.

In general terms, most studies employed young mice (4–6 weeks old) and females, which may be attributed to reduced aggressiveness, easier handling, and hormonal stability. All experiments were conducted under specific pathogen-free (SPF) conditions, ensuring

environmental control. Tumour models included SC, orthotopic, and intrahepatic xenografts, reflecting varying degrees of complexity and fidelity to human gastric cancer.

The choice of mouse strain in preclinical studies with mTOR inhibitors has a direct impact on biological fidelity, reproducibility, and the translational relevance of the findings. Immunodeficient models such as nude, SCID, and NOD-SCID mice were predominant in the studies reviewed, allowing for the engraftment of human tumour cells and the establishment of xenografts or PDX models. This controlled immunosuppression is essential for studying targeted therapies like everolimus, as it minimises interference from the adaptive immune response and enables a more direct evaluation of antitumour activity.

Age

Therapy efficacy varies depending on the age of the animal; for example, the activity of liver enzymes decreases in older animals, making age an important factor that must be taken into consideration[58].

In relation to the animals age used in the included studies, it was possible to determine that the mice were 4 weeks old, 5 weeks old and 6 to 9 weeks old.

There exist significant differences in disease-relevant systems in young or aged animals compared with middle-aged, and these differences may affect the outcome of studies investigating basic disease biology, mechanisms of drug action and efficacy. A lack of logic in the choice of the age of rodent models has the potential to impact on data quality, potentially increasing variability and reducing relevance to the human disease being studied[59].

Regarding drug development, the adaptation of compounds from non-clinical research to phase II clinical trials has been demonstrated to be poor in many therapeutic areas, with a lack of predictive rodent models for human disease, in part responsible for attrition. Use of suboptimal and variable age of animals could be contributing to this[59].

Factors such as menopause and old age are risk factors for bone diseases in humans, but are poorly modeled in younger rodents and require careful consideration of transferable biomarkers in older rodents to ensure comparability between species, alterations in blood flow and brain biochemistry with age have a significant impact in humans, but these pathologies are not reflected when modeling the disease with young rodents, with age-related phenotypes being uncovered in commonly used inbred strains[59].

Ageing can affect the composition of the mouse microbiome that plays important roles in the preservation of appropriate immune system function and response to disease and at least, drug metabolism by the liver has a critical impact on systemically administered compounds and is, therefore, of primary importance during the development of new pharmaceuticals. The gene expression of critical liver enzymes is dramatically different between young and older animals, potentially leading to discrepancies or errors affecting drug candidate selection or development[59].

Gender

Males and females react differently in the presence of certain drugs.[58]In these selected studies, a clear preference for using females was shown; in addition, one study did not mention the gender chosen[60].

Typically, researchers prefer to use male mice, mainly due to the hormonal differences observed between men and women. In fact, biological gender normally has an important impact on many inflammatory pathways, and a great variability in relation to hormonal levels in females is observed in all experiments due to the estrous cycle[60,58,61]. The effects of hormones on inflammatory responses are complicated and inconclusive. On the other hand, males are more likely to fight among themselves, probably related to the establishment of hierarchy and defense of territory[62]. This requires that males must be monitored regularly to prevent cases of serious skin lesions, which can trigger an inflammatory response, ultimately directly influencing the well-being of the animals and may distort anti-inflammatory findings in these specific models and, additionally, may result in an increased mortality rate not consequent to the studied model[63,60,64].

The use of female mice can alter the pharmacokinetic and pharmacodynamic performance of tested drugs, resulting in greater systemic exposure, differences in response mechanisms, and increased translational relevance.

Disease model

In the articles present in the study, the in vivo models were submitted to different method to induce GC. The most common method found were the SC injection of cells and the location were the flanks.

In the literature it is possible to find different methods to induce the disease, such as direct injection to the kidney [65] SC injection in the left axila [66] or posterior leg of the mice [67].

It seems that the method involved is dependent of multiple factors, such as the type of cancer or the cell lines that is analyzed.

The majority of the studies showed a waiting period after the implementation of the cancer cell lines into the mice vary depending on the study. In the articles present in this study, two showed that the time waited were dependent of the size of the tumour. Other two showed 3 and 7 days wait, independent of the tumour size, and, finally, the last two lacked to show any information regarding of the waiting period, leading to believe that the mice started treatment right after the cancer cell implementation. In the present literature, in studies about other types of cancer, such as lung, thyroid and kidney, showed prevalence on the analysis of size of the tumour to start treatment[65, 66, 67].

The type of gastric cancer induction in mice directly affects the response to treatment with mTOR inhibitors, as it influences factors such as the tumour microenvironment, vascularisation, immune response, and translational relevance. Standardization of the model including the implantation site, cell type, and waiting period before treatment initiation is essential to produce comparable results and support future clinical applications.

Treatment-Related Parameters

mTORi, Doses, Frequency and Duration of Administrations

In the collection of articles of the present study, a wide variety of mTORi treatment protocols was described in both *in vitro* and *in vivo* studies.

The maximum and the minimum dosages of **Everolimus** found *in vitro* were 10 μM and 0,01nM, respectively and *in vivo*, were 10mg/kg and 1,5mg/kg, respectively. These doses are similar to that found in other studies in GC treatment in the context of peritoneal dissemination. The same article also analyzes Everolimus treatment *in vivo* and the doses also fit the range found in this review of 5mg/kg[56]. Dosages within this rage are also shown in studies that analyze Everolimus with breast cancer. The dosages for *in vitro* analysis were 10nM and *in vivo* were 10mg/kg [68]. Not all articles in the present collection presented results for the frequency of administration for Everolimus. Only 2 out of 9 articles presented the frequency of administration for *in vitro* studies and 3 out of 4 studies presented *in vivo* results. As well as with other articles that chose to withdraw this information, it is possible that the lack of information is indicative of a single administration. With this assumption, it is possible to say that the present collection of articles shows similar results as found in pre-existing literature. The results of administration frequency for *in vivo* varied from 2 times per week to daily, which corresponds to the results demonstrated in other articles that show

daily administration in mice for gastric and breast cancer treatment [56, 68] . The duration of Everolimus treatment found in the present collection of articles varied from 24 hours to 10 days in *in vitro* results and 28 days to 49 days for *in vivo* results. The studies addressing GC treatment , it is possible to find *in vitro* values that fit the range of different duration periods presented in the present study, namely 96 and 48 hours in gastric and breast cancer treatment respectively. For in vivo results, studies analyzing gastric and breast cancer demonstrated 15 and 14 days duration periods. [56, 68]. Due to the variety of the results of dosages, frequency and duration of administration in the present collection of articles, this study was not able to find definitive specifications that point to a better protocol for Everolimus treatment in GC *in vitro* or *in vivo*.

In the included articles, only **Fukamachi H, et al** [53] described Temsirolimus as an option to GC treatment *in vitro* and *in vivo*. The maximum and the minimum dosage used *in vitro* were 3nM and 100nM respectively, while for *in vivo* analysis 1 to 10mg/kg was used. By analyzing cell viability, the authors demonstrate a dose dependent effect of temsirolimus in GC cells which is according to other studies addressing the effect of these mTORi in hepatocellular carcinoma and lymphoma[53, 69, 70]. For *in vivo* analysis, the dosage described by **Fukamachi H, et al** [53]was inferior to that found in those studies (1-10mg/kg and 25mg/kg), nonetheless in both cases significant efficacy (tumor shrinkage and increased cell death) with no additional toxicity was observed. [69, 70]. For HCC and lymphoma treatment, the frequency of administration in *in vivo* analysis was double the frequency **Fukamachi H, et al** [53], although both articles displayed no additional toxicity [69]. Regarding treatment duration for *in vitro* results displayed by **Fukamachi H, et al** [53] used 48 hours and 72 hours compared to 5 days and 2 weeks presented by **Fukamachi H, et al** [53], although this did not appear to alter the results or to lead to any additional toxicity [69, 70]. The analysis of **in vivo** results also showed that **Fukamachi H, et al** [53] had a longer duration of administration than that found in the literature. The treatment duration previously reported was 29 days while **Fukamachi H, et al** [53] reported 6 to 7 weeks, although that did not appear to alter the results or to lead to any additional toxicity [69].

The **2,6-DMBQ** compound present in the included articles had little information in pre-existing literature. It was only possible to find one article that described this compound as treatment for lung cancer [71]. In this study, **2,6-DMBQ** was only analyzed *in vitro* and the used doses were 10, 20 and 40μM, while **Zu X, et al** [37] reported results for 5, 10 and 20μM [37]. The frequency of administration was not described in both articles, suggesting that the cells were treated once in the study. The duration of **2,6-DMBQ** treatment in **Zu X, et al** [37] was not mentioned, while in the literature was 48 hours. Both articles came to the conclusion

that the 2,6-DMBQ treatment showed promising results, showing anticancer properties such as inhibiting cell proliferation via regulation of the mTOR pathway[71].It is important to highlight that both articles that studied 2,6-DMBQ were analyzed by the same team, which may be the reason for the dosage, frequency and duration of administration being similar.

The dosage and duration of **Rapamycin** administration found in the literature for various tumours is wide. The dosage found in the literature was 5μM for leukemia treatment, while **Cao Y, et al** [38] described 100nM for Rapamycin treatment alone and 100μM for combination with Celecoxib. Although the doses presented were different, both articles indicated significant efficacy for inhibiting cellular growth[38, 72]. It is important to highlight that **Cao Y, et al** [38] described enhanced cellular inhibition with combined treatment by comparison to monotherapy [38, 72]. The frequency of administration was yet again not shared in both articles, suggesting one single treatment. The duration of the Rapamycin treatment also described in the literature was significantly smaller than the one showed by **Cao Y, et al** [38].The duration found in the literature was 24 hours compared to the 72 hours analyzed by **Cao Y, et al** [38] although displayed significant efficacy results were presented in both articles showing an increase in apoptosis and autophagy.

The dosage of administration of **OSI-027** described in the literature varies from 0,01 to 30μM while the dose showed in **Xu E, et al** [36] was 5μM. The frequency of administration was not mentioned in the articles found in the literature although **Xu E, et al** [36] indicated daily OSI-027 treatment in their experiment. The duration of the studies found in the literature varies from 24 hours to 72 hours. Although the data retrieved from the literature had slight differences, all the articles seemed to indicate efficacy for cancer treatment with OSI-027, although used for treatment with different types of cancer, such as cholangiocarcinoma, gallbladder and colon cancer[73, 74, 75, 76].

Route of administration

It is well-known that the route of administration is a critical determinant of the final pharmacokinetics, pharmacodynamics as well as toxicity of pharmacological agents. [77] IV, SC, IP and oral routes are the principal paths of drug administration in laboratory animals, with each offering advantages and disadvantages depending on the specific objectives of the study. The route used to administer the medication depends on the species, volume and material to be injected, and the location where the effect should be noticed[78,63].

Among the different routes of administration used in the different analyzed studies, three studies used the IP route, and three studies used the oral route. In fact, one of the more

commonly used routes in rodent studies is the IP route where a pharmacological agent is injected into the peritoneal cavity. This technique is easy to master, quick and minimally stressful for animals[77]. The IP route allows the administration to rodents of large volumes of solution safely, which may be advantageous for agents with poor solubility. This route is especially common in longer studies involving small species for which IV access is challenging and it can be used to administer large volumes of fluid safely[77,43]. In most cases, IP is also preferred over the oral route for biological agents to avoid the GI tract and potential degradation/modification of biopharmaceuticals. The main disadvantage of this route is that it is minimally used in clinic (mostly for treatment of peritoneal cancers), because its use in experimental studies is often questioned and discouraged[77]. Other limitation is, just like what is observed with the oral route, substances absorbed from the peritoneal cavity end up in the portal vein and pass through the liver. It is generally considered that the pharmacokinetics of small molecular drugs administered through the IP route is similar to the oral, in terms of metabolic fate and leads to lower systemic exposure of IP-administered compounds[77,43].

The oral route is economical, convenient, relatively safe, and some animals can be trained to cooperate, depending on the drugs being administered. Despite its administration as voluntary consumption being ideal, this dosing technique may not be reliable in all animals, dose groups or for long-term studies, because of individual preferences or changes in behavior over time[43]. When using oral administration, it is important to ensure that the animal swallows, to ensure receiving the full dose.

Oral administration presents some limitations such as slower onset of action compared with parenteral delivery, a potentially significant first-pass effect by the liver for those substances metabolized through this route with reduced efficacy, absorption of substances, poor compliance with voluntary consumption, degradation of substances by digestive enzymes and acid, and inability to use this route in animals that are unconscious or have clinically significant diarrhea or emesis[43].

The route of administration can significantly influence both the efficacy and safety outcomes of pharmacological agents in preclinical studies. Among the studies included in this review, two main routes were employed: intraperitoneal (IP) and oral administration, each with distinct pharmacokinetic and pharmacodynamic characteristics that may have conditioned the results observed.

The IP route was used in three studies and is commonly adopted in rodent models due to its technical simplicity, low stress for animals and capacity to safely deliver relatively large

volumes of solution. This can be especially advantageous for compounds with poor solubility. Additionally, IP administration is often preferred in long-term studies involving small animals where IV access is technically challenging. Another key advantage is the ability to bypass the gastrointestinal tract, which is particularly important for biological agents susceptible to degradation or modification in acidic or enzymatic environments. However, the IP route presents notable limitations. Although it allows systemic absorption, substances delivered intraperitoneally are absorbed via the portal circulation and undergo first-pass hepatic metabolism. As such, the pharmacokinetic behaviour of small molecules administered via the IP route tends to resemble that of orally administered compounds, potentially resulting in lower systemic bioavailability. Furthermore, this route is rarely used in clinical settings except for specific indications such as peritoneal cancers limiting its translational value. Inconsistent absorption due to injection site variation or peritoneal fluid accumulation can also introduce variability in drug exposure.

Conversely, oral administration, also used in three of the included studies, is a clinically relevant and widely preferred route due to its practicality, cost-effectiveness, and non-invasive nature. In certain experimental settings, animals can even be trained for voluntary ingestion, depending on the drug's properties. Nevertheless, oral administration poses several limitations. Voluntary consumption is not always consistent across individual animals or over extended study periods, especially in cases of behavioural changes or illness. Consequently, oral gavage is often used to ensure precise dosing, although this method requires that the animal swallows the full dose. Additional challenges associated with oral administration include slower onset of action, significant first-pass metabolism by the liver (which may reduce drug efficacy), variable absorption rates, and the risk of degradation by digestive enzymes or acidic pH. This route is also unsuitable for animals with vomiting, diarrhea, or impaired consciousness.

Given these considerations, it is likely that the choice of administration route influenced the pharmacological outcomes observed across the studies. IP administration may have led to a slight overestimation of efficacy relative to oral administration due to better systemic absorption, whereas oral administration, despite its translational relevance, may have resulted in underestimation of therapeutic potential due to metabolic degradation and variability in absorption. These differences may partially account for the heterogeneity observed among study results. Therefore, when comparing the outcomes of preclinical studies employing different administration routes, it is essential to account for their pharmacokinetic implications. Future studies should aim to standardise administration

routes according to their translational goals, incorporate pharmacokinetic assessments to support efficacy data, and consider direct comparative designs (e.g., IP vs oral vs IV) to clarify the impact of delivery method on treatment outcomes.

Efficacy and safety

Efficacy refers to the ability to produce a desired or intended result. The efficacy of a drug is tested to determine whether it effectively treats a condition under ideal conditions. The summary of the efficacy and safety are presented briefly in **Tables 3, 4, 5 and 6**.

The in vitro studies reviewed show that mTOR pathway inhibitors, especially Everolimus, OSI-027, and dual PI3K/mTOR agents such as PKI-587, consistently reduce proliferation, induce apoptosis, and modulate key molecular pathways in gastric cancer models. Despite these promising preclinical results, evidence reveals a striking inconsistency when translating into clinical efficacy.

For instance **Huangfu L, et al** [48] showed that Everolimus combined with Piceatannol produced a synergistic effect, enhanced autophagy, suppressed colony formation, and increased DNA fragmentation. This synergetic effect may be due to Piceatannol's apparent role as a metabolic modulator, enhancing the effect of Everolimus through suppression of mTORC1 signalling and controlled stimulation of autophagy. Unlike ATP-competitive inhibitors, it does not directly inhibit the catalytic site of mTOR, but rather modulates upstream regulators that amplify downstream blockade of the PI3K/AKT/mTOR pathway, efficacy seems to derive from its ability to potentiate rapalogs, reducing compensatory resistance mechanisms and promoting a more robust apoptotic and autophagic response, with good systemic tolerability observed in animal models.

Hou et al.[50] found that Everolimus did not sensitize cisplatin-resistant gastric cancer cells (SGC-7901/DDP) to treatment, while chloroquine, an autophagy inhibitor, restored cisplatin efficacy, indicating the dual role of autophagy in cancer therapy [50].

This functional ambivalence is well established, as evidenced by the studies conducted by White et al. al [79] and Galluzzi et al.[80] emphasize that autophagy can either promote survival or cause cell death depending on context, supporting the idea that combined autophagy modulation is complex. In addition, **Gao F, et al** [34] observed that combining Rhein with Everolimus significantly curbed proliferation, invasion, and reversed the mesenchymal phenotype; however, this contrasts sharply with results from the phase III GRANITE-1 trial, in which Everolimus failed to improve overall survival in patients with advanced gastric cancer[26]. Although Everolimus alone did not demonstrate clinical benefit in the GRANITE-1

trial, mechanistic evidence suggests that specific combinations may help overcome its limitations. In this context, the synergistic effect observed with Rhein may be explained by its ability to block AKT phosphorylation (Ser473) and suppress Beclin-1 expression, thereby disrupting the positive-feedback mechanisms that normally reactivate the PI3K/AKT/mTOR pathway following mTORC1 inhibition. Consequently, combining Everolimus with Rhein may reduce AKT reactivation, enhance apoptosis, and limit the cytoprotective autophagy typically induced during monotherapy.

Further contradictions surround resistance mechanisms. **Ying L, et al** [51] reported that Everolimus could reverse cisplatin resistance via modulation of resistance-associated proteins like P-gp and MRP1. Conversely, **Hou et al.**[50] did not find such effects in the same resistant model, illustrating variable outcomes under similar experimental conditions.

Targeting the ERK pathway addresses another resistance mechanism. A study by Hongfang L. [81] demonstrated that Everolimus treatment activated ERK signalling in vitro and in xenografts, but the MEK inhibitor trametinib successfully restored Everolimus sensitivity, suggesting that compensatory ERK activation is a major barrier to efficacy.

The discrepancies among studies regarding the ability of Everolimus to reverse cisplatin (DDP) resistance can be explained by several experimental and molecular factors. In certain cell lines and PDX models, activating PIK3CA mutations increase dependence on the PI3K/AKT/mTOR pathway, rendering tumour cells more sensitive to Everolimus and allowing partial restoration of DDP responsiveness. In contrast, models lacking such dependence exhibit only modest effects. The dominant resistance mechanism also plays a crucial role: when resistance is mediated by efflux proteins (P-gp/MRP1), Everolimus can recover DDP cytotoxicity, whereas in cases driven by cytoprotective autophagy, reversal occurs only when combined with chloroquine, which blocks autophagosome–lysosome fusion.

Furthermore, compensatory mTORC2/AKT feedback limits Everolimus efficacy, since selective mTORC1 inhibition permits AKT (Ser473) reactivation, supporting cell survival. Accordingly, co-treatment with Rhein or chloroquine effectively suppresses this feedback, enhancing apoptosis. Variability across studies is also reinforced by methodological differences, including variations in dose, duration, and treatment sequence, as well as by heterogeneous endpoints (biomarker suppression versus cell viability).

Finally, activation of parallel signalling pathways, such as ERK, may counteract Everolimus activity, necessitating combination with MEK inhibitors.

Overall, the efficacy of Everolimus monotherapy remains variable, but tends to be more consistent and potent when combined with agents that block compensatory survival pathways, such as autophagy or AKT reactivation.

OSI-027, a dual mTORC1/C2 inhibitor, demonstrated superior efficacy compared to Everolimus in gastric cancer models by inducing G0/G1 cell-cycle arrest, reducing phosphorylation of p70S6K and 4E-BP1, attenuating p-Akt, and enhancing apoptosis when combined with oxaliplatin, while also downregulating P-gp and overcoming multidrug resistance [36]. These results align with earlier preclinical studies confirming that OSI-027 is an ATP-competitive inhibitor that blocks both mTORC1 and mTORC2, thereby preventing AKT phosphorylation and eliminating the compensatory feedback commonly observed with rapalogs. Studies have shown a marked suppression of p70S6K and p-AKT, leading to cell-cycle arrest at the G0/G1 phase and pronounced apoptosis[75]. This profile indicates a more complete and sustained inhibition of the pathway, supporting its mechanistic superiority over first-generation inhibitors.

Moreover, **Freitag H, et al.** [39] and **Mohamed A. et al.** [52] highlighted further discrepancies: FKI-587, a dual PI3K/mTOR inhibitor, induced deeper apoptosis and mitochondrial disruption compared to Everolimus, yet Mohamad's work showed Everolimus was effective in only about two-thirds of patient-derived neuroendocrine tumour cultures with no correlation to MEN1 or PTEN, stressing challenges in clinical translation. This behavior can be explained by the fact that it blocks the compensatory feedback mechanisms that Everolimus—acting only as a selective mTORC1 inhibitor cannot suppress. In other words, while Everolimus inhibits mTORC1, it fails to prevent the cell from reactivating the PI3K/AKT/mTOR pathway through feedback loops. The additional agent disrupts this compensation, thereby enhancing the overall inhibitory effect on the pathway.Indeed, by simultaneously inhibiting mTORC2, PKI-587 prevents the reactivation of AKT (Ser473), one of the most common mechanisms of resistance observed with rapalogs.

This review underlines the complexity and contradictions in using mTOR inhibitors for gastric cancer. While robust preclinical data support their antiproliferative and pro-apoptotic potential, clinical outcomes remain inconsistent. Resistance mechanisms, including ERK/PI3K feedback, autophagy dynamics, biomarker dependency, and tumour heterogeneity, highlight the need for personalized therapeutic strategies. Combination regimens, guided by molecular profiling and targeting these resistance pathways, may deliver more dependable benefits.

In *in vivo* studies, **Bollard J, et al** [35] observed a robust antiproliferative effect of Everolimus in xenografts derived from STC-1 and GluTag cell lines, with significantly reduced tumour mass and Ki67 expression. However, this occurred without consistent suppression of canonical mTOR downstream effectors such as phospho-4E-BP1 and phospho-p70S6K, which were only partially reduced.

This dissociation between biomarker response and phenotypic outcome suggests that tumour growth inhibition may not rely exclusively on canonical mTOR signalling, or that compensatory mechanisms allow tumour suppression even with incomplete pathway inhibition. This observation is echoed by Nawroth et al.[82], who reported that phospho-4E-BP1 expression correlates with Everolimus sensitivity, but also noted variable outcomes depending on tumour subtype and microenvironment. Contrasting results are evident in the study by **Zu X, et al** [37]**,** where 2,6-DMBQ suppressed tumour growth and decreased levels of Ki-67, p-mTOR, and p-p70S6K in LSG55 xenografts, yet had limited efficacy in LSG64, despite similar treatment protocols.

This highlights how biological variability between tumour models, such as differential expression of mTOR pathway components or differences in metabolic state, can lead to striking differences in therapeutic response. The importance of these intrinsic factors is supported by previous reports that variability in p-mTOR or p-AKT expression contributes to differential response to mTOR inhibitors across gastric cancer subtypes (PI3K/AKT/mTOR inhibitors in patients with breast and gynecologic malignancies harboring PIK3CA mutations).

Fukamachi H, et al [53] demonstrated that Everolimus and Temsirolimus were effective in suppressing tumour growth primarily in HGC-3 and HGC-20 PDX models, both diffuse-type gastric cancers with PIK3CA mutations.

. These findings reinforce the predictive value of genetic biomarkers such as PIK3CA mutations and basal p-4E-BP1 levels for mTOR inhibitor responsiveness. Similar associations have been described in breast and endometrial cancer models, where PIK3CA-mutant tumours are more susceptible to mTOR blockade [83].

On the other hand, the study by **Onoyama M, et al** [54] showed that Everolimus monotherapy was effective in reducing tumour growth and inducing apoptosis, but the addition of Nilotinib only modestly improved efficacy. This suggests that synergism is not guaranteed in all mTOR-targeted combination therapies and may depend on whether the second agent disrupts parallel signalling axes or angiogenesis. Indeed, the redundancy of

pathways such as ERK or PDGFRβ in some gastric tumours may reduce the additive value of such combinations unless properly matched to tumour biology.

The superior effectiveness of combining Everolimus with Temsirolimus—compared with Everolimus and Nilotinib—likely stems from essential differences in how these combinations act at the molecular level. Everolimus and Temsirolimus target overlapping components of the mTOR pathway, resulting in a deeper and more coordinated inhibition of PI3K/AKT/mTOR signaling. In contrast, Nilotinib acts on unrelated kinase targets, leading to a weaker effect on this pathway. These distinctions ultimately produce different cellular responses, with the mTOR–mTOR combination promoting stronger antiproliferative and pro-apoptotic effects.

Both Everolimus and Temsirolimus are rapamycin analogues (rapalogs) that act allosterically on the FKBP12–mTOR complex, thereby inhibiting mTORC1. However, their complementary pharmacodynamic properties allow for a more sustained inhibition of the mTOR pathway when administered together. Temsirolimus exhibits faster onset and higher initial affinity, while Everolimus provides greater intracellular stability and longer-lasting effects. This synergy ensures a more prolonged suppression of p-mTOR, p70S6K, and 4EBP1, thereby preventing compensatory AKT (Ser473) reactivation mediated by mTORC2, one of the key mechanisms of resistance associated with rapalog monotherapy.

In contrast, Nilotinib is a tyrosine kinase inhibitor (TKI) that targets BCR-ABL, PDGFR, and c-KIT, thereby affecting upstream signalling of mTOR, but without direct inhibition of the mTORC1 and mTORC2 complexes. Consequently, although the Everolimus and Nilotinib combination may temporarily reduce AKT and PI3K phosphorylation, it fails to prevent compensatory AKT and ERK reactivation, maintaining only partial suppression of the PI3K/AKT/mTOR pathway and thus limiting its antiproliferative effect.

Notably, some biomarkers remain inconsistent predictors of treatment response. For instance, active caspase-3 levels did not significantly change in Everolimus-treated xenografts, despite tumour regression and decreased proliferation markers in the same models. This raises the possibility that apoptosis may not be the dominant mechanism of action in all settings, and that biomarkers of proliferation (Ki-67) and mTOR activity (p-4E-BP1, p-p70S6K) should be interpreted with caution and in context.

The efficacy of mTOR inhibitors in *in vivo* gastric cancer models is highly variable and context-dependent, with contradictory results often emerging across studies that use different tumour models. While some demonstrate significant antitumour effects, others show only modest or selective efficacy, even under similar treatment conditions. These

discrepancies are likely driven by heterogeneity in molecular profiles, differential expression of mTOR pathway components, and activation of compensatory survival pathways.

Having examined the efficacy outcomes associated with different administration routes, it is also important to consider how these routes may influence the safety and tolerability of mTOR inhibitors in experimental models.

In preclinical investigations, mTOR inhibitors, particularly Everolimus, demonstrate a notably benign toxicity profile. In vitro, concentrations up to 125 nM in tumour cell lines did not impair viability, as reported by Ying et al.; similarly, combinations such as Rhein (80 μM) with Everolimus (40 nM) showed no cellular toxicity, and Temsirolimus concentrations up to 3 μM were well tolerated without harming cell viability. Although these findings suggest a favourable cytotoxicity profile, most experiments involved only tumour cell lines, limiting insight into effects on normal healthy cells.

Animal studies reinforce this safety narrative. For instance, **Huangfu L, et al** [48] observed no weight loss in nude mice receiving Piceatannol and Everolimus, while **Zu X, et al** [37] found no histopathological or functional organ alterations, including liver, kidney or spleen, even after repeated exposure to 2,6-DMBQ. **Bollard J, et al** [35] described stable body weights in Everolimus-treated mice, contrasting with weight loss in controls, and detected no evidence of liver enzyme elevations or increased caspase-3 activation in non-tumour tissues. Likewise, **Onoyama M, et al** [54] reported no significant changes in animal weight after oral administration of Everolimus alone or combined with Nilotinib.

Nonetheless, caution is warranted: these studies typically span only one to six weeks, and often omitted detailed histological analyses or multi-organ biochemical assessments. Immunodeficient murine models limit evaluation of immune-mediated toxicities, and few investigations employed positive controls known to induce toxicity, complicating direct comparisons of safety.

Turning to human clinical findings, meta-analyses reveal that Everolimus is associated with increased risks of fatigue, hyperglycaemia, hyperlipidaemia and elevated alanine aminotransferase (ALT), with relative risks ranging from approximately 1.3 to over 3 depending on the event [83] . Hypertension, pneumonitis, anaemia and stomatitis are also frequently reported in patients across solid tumour indications [83]. Specifically, the risk of elevated AST and ALT is roughly doubled, while stomatitis and pneumonitis may have odds ratios as high as 5 or 6.

Rare but serious events have been documented. A case report describes rapid and severe hepatic steatosis in an elderly patient receiving Everolimus for a neuroendocrine tumour, leading to liver failure and death after only four weeks of therapy [84]. Chronic exposure in animal models, particularly in the context of high-fat diets, may precipitate impaired glucose tolerance and insulin resistance despite reduced weight gain, reduced adiposity, and smaller adipocyte size, suggesting metabolic disturbances with long-term use [85].

Although preclinical studies generally report minimal systemic toxicity at therapeutic doses, these limitations underscore the need for more rigorous safety evaluations. Longer durations, systemic histopathology across organs, functional biochemical assays, and immune-competent models are essential. At the clinical level, adverse events such as hyperglycaemia and elevated liver enzymes are well-established and dose-dependent, often correlating with higher trough concentrations of Everolimus.

In summary, the preclinical and clinical evidence converge on a consistent safety profile for Everolimus: relatively mild toxicity in controlled doses preclinically, but a clear risk of metabolic and hepatic adverse events in patients. Understanding these risks through comprehensive, multiscale toxicological assessment is critical for safely translating mTOR inhibitors into clinical practice.

The preclinical studies analysed in this thesis consistently indicate that mTOR inhibitors exhibit an overall favourable safety profile, both in in vitro and in vivo models. In animal studies, no significant changes were observed in body weight, hepatorenal histology, or biochemical function (ALT/AST), suggesting good systemic tolerability for compounds such as Everolimus, Temsirolimus, and 2,6-DMBQ. However, toxicological quantification was limited, as few studies included comprehensive biochemical analyses, multi-organ histology, or behavioural parameters, which reduces the strength of safety conclusions.

Overall, the “low toxicity” reported across studies appears to be influenced by simplified methodologies (limited to weight monitoring and short observation periods) and by the absence of repeated-dose or long-term exposure studies. Therefore, the absence of overt toxicity does not equate to the absence of risk.

Taken together, the findings indicate that rapalogs and related compounds exhibit promising systemic tolerability, although toxicological assessments remain insufficiently standardised and detailed. The current evidence supports the experimental safety of Everolimus, Temsirolimus, and 2,6-DMBQ, while also highlighting the need for detailed in

vivo studies on OSI-027 and PKI-587, in order to establish a robust therapeutic window and benefit–risk ratio for next-generation mTOR inhibitors in gastric cancer.

Assessment Techniques

In the search for new compounds to help fight GC, many factors are important in understanding their action mechanism and the effect on the cells. Therefore, in the included studies, many techniques were used to clarify the efficacy and safety parameters for the new compounds.

Taking into consideration the studies analyzed, a total of 26 different techniques were shown. The most prevalent were Western Blot (WB) followed by Flow Cytometry (FC), 3-(4,5-dimethyl thiazol-2-yl)-2,5- diphenyl tetrazolium bromide (MTT) assay, Immunohistochemical (IHC), Cell Counting Kit (CCK-8), Polymerase Chain Reaction (PCR), Immunofluorescence, Histological analysis, and Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay. Other techniques were shown only once in all the studies analyzed.

WB was initially introduced in 1979 by Towbin et al. as a technique to identify and quantify proteins in a cell [86,87,88]. To summarize, the workflow of WB assay starts with the separation of the proteins inside a cell before separating them by gel electrophoresis [86,87]. It follows with the proteins being transferred to a membrane that will be incubated with primary and secondary antibodies[86,87].

Finally, the second antibodies can be identified by colorimetric, fluorescent, or luminescent methods [86,87]. In the studies analyzed, WB was most used to identify and quantify biomarkers that could indicate efficiency of the new compounds to inhibit the mTOR pathway.

FC were developed in the late 1960´s and early 1970´s with the objective of creating a technique for analysis of individual cell physicochemical properties and the quantification of cellular processes such as proliferation, cell death, and cell differentiation [89,90,91]. This technique has the ability to characterise cells by size, granularity, and marker expression using fluorescence and light-scatter properties [89,90,91].

To summarize, the workflow of FC starts at the separation of the cells with subsequent staining with fluorescent antibodies and dyes that go through a laser that that excite and transmit the information to the software [90,91]. The software can them differentiate the cells

by size, internal complexity (granularity) and by those that can bind with different antigens [89,90,91]. In the studies that were analyzed, FC was predominantly used to analyze cell characteristics to identify cell apoptosis. This parameter could indicate the efficiency of the compound to inhibit tumour growth.

MTT assay was first described in 1983 by Mosmann to measure the metabolic activity in living cells of the cells [92,93]. To summarize, the action mechanism of the MTT assay technique is based on the reduction of the water soluble yellow colored tetrazolium salt MTT to a non-water-soluble purple formazan crystal, by introducing metabolically active cells [92]. In the studies analyzed, this technique is used to analyze cell metabolism so that can be used to quantify cell viability.

IHC is a technique used to identify specific antigens using a specific antibody in a cell or tissue [94,95]. Pre-existing literature shows that this technique is usually used to identify and analyze biomarkers in a lot of diseases [95]. The workflow can be summarized in the retrieval of the antigens, followed by the addition of a primary antibody, application of a secondary antibody that binds to the primary antibody, and the addition of a detection reagent to localize the primary antibody [95]. In the studies analyzed, IHC was primarily used to identify and quantify biomarkers for cell proliferation and cell death.

In the studies analyzed, staining methods were also used to clarify the efficiency of the new compounds.

In total, ten different types of staining were found, the Annexin V and propidium iodide being the most prevalent, the EdU staining and Crystal violet. Other techniques were shown only once in all the studies analyzed.

Annexin V and propidium iodide staining first started to be used in the 1990s for apoptosis of vascular smooth muscle cells, to elucidate novel regulators for apoptosis *in vivo*, in the quantitation of cytotoxic drug-induced apoptosis and in the analysis of the cellular effects of farnesyl transferase inhibition [96]. Annexin V has high affinity to phosphatidylserine that is exposed in the early stages of apoptosis, while propidium iodide binds to double-stranded DNA and RNA, and cannot enter live cells so will bind in late stages of apoptosis [97,98].

Biomarkers Assessment

As a simple definition, biomarkers can be defined as “a defined characteristic that is measured as an indicator of normal biological processes, pathogenic processes or responses to an exposure or intervention” [99]. In recent literature, biomarkers have become

increasingly valuable in pharmaceutical studies, identifying a drug's principle of action, investigating toxicity and efficacy markers at an early stage of the development process, and analyzing cases which are likely to respond to therapy[100,101]. Also biomarkers are used for therapy supervision, further therapy selection, or surveillance recurrent diseases[101].

There are numerous benefits and disadvantages of biomarkers that must be considered before being used. Precision of measurement, economical, less bias than questionnaires and rapid warning signals are some of the benefits of using biomarkers, meanwhile the possibility of laboratory errors, storage (longevity of sample) and normal range are difficult to settle[101].

In pharmaceutical studies, biomarkers became extremely important tools to analyze action mechanisms, toxicity, efficacy signals and identifying patients who may benefit from the therapy [101].

To analyze the efficiency and safety of mTOR inhibitors, biomarkers are usually mentioned. The variety of biomarkers that can be used to analyze mTOR inhibitors are extensive, but the main intermediates of the PI3K/AKT/mTOR pathway were frequently analyzed in the studies[101].

Considering the analyzed studies, a total of 68 different types of biomarkers were shown. The most prevalent was phosphorylated mTOR (p-mTOR), followed by 70 kDa ribosomal S6 kinase (p70S6K), phosphorylated AKT (p-AKT), KI-67, LC3A/B-Ⅱ, 4-EBP1, CDK4, Cycledin D1, and phosphorylated ERK1/2 (p- ERK1/2). Other biomarkers were shown only once in all the studies analyzed.

In the studies that were analyzed, the PI3K/AKT/mTOR pathway was directly associated with cell proliferation and cell death. To assess if the compounds were inhibiting it, the main intermediates for the pathway were analyzed, namely p-mTOR, p70S6K, p-AKT.

Considering the studies analyzed, it was found that p-mTOR was down regulated in the presence of Rhein in combination with RAD001 (*in vivo* and *in vitro*), OSI-027 (*in vitro*), 2,6-DMBQ (*in vivo* and *in vitro*), RAD001 in combination of DDP (*in vitro*), CQ in combination with DDP (*in vitro*), 5-FU (*in vitro*)[34,36, 37, 50, 102].

In another study, **F. Hiroshi et al.** did not analyze p-mTOR in the presence of a new compound, instead the biomarker was used as a prognostic factor for patients with diffuse-type GC [53]. In all the studies above, a correlation of high levels of p-mTOR with the

aggressiveness of the tumour was suggested and these indicating it as worse biomarker prognostic.

Considering the analyzed studies, p70S6K was down regulated when treated with OSI-027 (*in vitro*), RAD001 in combination of DDP (*in vitro*), CQ in combination with DDP (*in vitro*), Everolimus (RAD001) (*in vivo* and *in vitro*) and Everolimus (RAD001) in combination with Octreotide or Pasireotide (*in vitro*) [36, 50, 52, 35]. It is important to highlight that two studies showed a down regulation of p70S6K *in vitro* with Everolimus (RAD001) treatment [52, 35]. This indicates that, in these seatings, mTORC1 was inhibited, as SGK is a substrate of mTORC1.

With regards to the studies analyzed, p-AKT was down regulated when treated with Rhein in combination with RAD001 (*in vivo* and *in vitro*) and OSI-027 (*in vitro*) [34, 36]. In contrast, **Mohamed A. et al.** [52] showed that p-AKT showed an increase after a 24h treatment with Everolimus (RAD001) or the combination of Everolimus (RAD001) with Octreotide or Pasireotide [52]. These contrasting findings suggest that while some mTOR inhibitors particularly when used in combination can effectively suppress p-AKT, others may lead to compensatory feedback activation, particularly through the mTORC2 complex.

Cao Y, et al [38] used p-mTOR, p70S6K and p-AKT to asses Rapamycin resistance [38]. To analyze the efficiency, the biomarkers were exposed to Rapamycin for 24h, but only the p-AKT level was increased [38].

In summary, the evaluation of key biomarkers such as p-mTOR, p70S6K, and p-AKT revealed consistent inhibition of mTORC1 signalling in most studies, particularly with Everolimus and its combinations. However, the variable regulation of p-AKT, including cases of upregulation, suggests compensatory feedback via mTORC2, which may contribute to resistance. These findings highlight the importance of using multiple biomarkers to assess both efficacy and potential adaptive mechanisms, supporting more informed therapeutic strategies in preclinical models.

Limitations

In addition, given that animal experiments in peer-reviewed publications are poor due to the omission of experimental design details such as blinding, randomization, strain, gender, and age details in some selected studies, there are also important limitations to be considered. At least, it is difficult to compare the results obtained due to the lack of indication of treatment dosage, frequency, and duration, which does not allow more robust

conclusions to be drawn. It is only possible to say whether the drug was successful, and it is not possible to indicate the best concentration of each inhibitor.

In the present review, it was determined that there were some limitations to be considered. The lack of a second reviewer for a better extraction of the information and the quantity of variables found in the included studies due to a too wide view on the topic could jeopardize this study.

This systematic review underscores the potential of mTOR pathway inhibitors as promising therapeutic agents for GC, particularly based on preclinical models. However, their translation into clinical practice remains limited, primarily due to model heterogeneity, inconsistencies in experimental protocols, and a lack of robust human data.

Conclusion

In normal conditions, the mTOR is important to regulate fundamental activities, such as and including the cell cycle, proliferation, growth, and survival, but in carcinogenic cells, mTOR has a close association with cancer, because when it is activated in an abnormal way, it starts sending signals that stimulate cellular growth but also invade nearby tissues and metastasize.

Previous studies connected mTOR to various diseases including tumour formation and development that activate excessively the pathway AKT/mTOR. Due to these facts, the inhibitors of the mTOR pathway are considered as a possible targeted therapy for both tumour and organ transplants, rheumatoid arthritis, and other diseases.

Based on the analysis of the preclinical studies included in this systematic review, Everolimus emerged as the most consistently effective mTOR inhibitor in gastric cancer models. It demonstrated robust anti-proliferative and pro-apoptotic effects across multiple studies, both as a monotherapy and in combination with other agents such as cisplatin (DDP), chloroquine (CQ), octreotide, and pasireotide.

The most effective conditions for mTOR inhibition using Everolimus were observed under the following experimental parameters:

- In vitro models: Human gastric cancer cell lines such as MKN45, AGS, and SGC-7901 showed significant inhibition of proliferation and mTOR pathway signaling when treated with Everolimus at concentrations ranging from 10 to 100 nM, with exposure times of 24 to 72 hours.

- In vivo models: Mouse xenograft models (mostly BALB/c nude mice) bearing human gastric tumor cells showed tumor growth suppression with oral doses of 5–10 mg/kg/day, administered for 2 to 4 weeks.
- The use of Everolimus in combination therapy (notably with DDP or CQ) consistently enhanced efficacy, suggesting a potential synergistic effect and highlighting the relevance of combination regimens.

The most informative biomarkers associated with treatment efficacy included p-mTOR, p70S6K, and p-AKT, with consistent downregulation observed in successful treatments. Everolimus was also linked to modulation of autophagy markers (e.g., LC3A/B-II) and proliferation indices (e.g., Ki-67), supporting its multifaceted mechanism of action.

In conclusion, Everolimus, particularly when used in combination therapies, under defined experimental conditions (10–100 nM in vitro; 5–10 mg/kg/day in vivo for up to 4 weeks), stands out as the most effective mTOR inhibitor in preclinical models of gastric cancer. Future studies should further refine these dosing strategies and assess their translational potential in molecular subtypes of GC.

To achieve meaningful progress in this area, the following future directions are recommended:

- Standardization of preclinical models: Harmonizing the cellular and animal models used across studies will allow for better comparison of results and enhance data reproducibility.
- Investigation of predictive biomarkers: Further research should focus on identifying molecular biomarkers capable of predicting response to mTOR inhibitors, thereby enabling better patient selection.
- Studies on combination therapies: Combining mTOR inhibitors with other therapeutic approaches—such as chemotherapy, immunotherapy, or inhibitors of other signaling pathways—may enhance efficacy and help overcome resistance mechanisms.
- Evaluation of toxicity and pharmacokinetics: A deeper understanding of the safety profile, bioavailability, and pharmacodynamics of these agents is crucial for their progression to clinical trials.
- Translation to clinical trials: Based on the promising preclinical data, the development of well-designed phase I/II clinical trials is imperative to assess the efficacy and safety of mTOR inhibitors in patients with GC.

In summary, although the analyzed data indicate a relevant role for mTOR inhibitors in controlling tumour progression in experimental models, their integration into the clinical

management of GC will require a multidisciplinary effort bridging basic, translational, and clinical research.

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