Taste signalling in glioblastoma

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“All our dreams can come true, if we have the courage to pursue them. If you can dream it, you can do it.”

Walt Disney

Dedicatory

À minha família e amigos, a família que escolhi, por sempre me terem apoiado nos melhores e nos mais difíceis momentos.

Em memória da "tia" Anabela, o maior exemplo de coragem, luta, resiliência e esperança desde o primeiro dia do diagnóstico de um tumor cerebral.
Nunca iremos esquecer o seu sorriso, simplicidade, vivacidade e bondade.

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Resumo Alargado

Nos mamíferos, o paladar é um sentido químico e consiste na perceção dos sabores amargo, doce, azedo, salgado ou *umami*, tendo início na cavidade oral, quando os alimentos ou outras substâncias contactam as papilas gustativas, originando respostas aversivas ou atrativas. Os recetores do sabor pertencem à superfamília dos recetores acoplados à proteína G correspondem a proteínas transmembranares que podem ser divididas nos tipos 1 e 2. Existem três subunidades de recetores do tipo 1 (TAS1Rs) que medeiam os sabores doce e *umami* através da formação dos heterodímeros TAS1R2+TAS1R3 e TAS1R1+TAS1R3, respetivamente. Por outro lado, o grupo de recetores do tipo 2 (TAS2Rs) é constituído por 25 membros que medeiam o sabor amargo e podem ser ativados por um único ou vários tipos de compostos. Apesar de inicialmente identificados nas papilas gustativas da cavidade oral, a expressão dos recetores do sabor tem vindo a ser reportada em diversos outros órgãos extraorais e também em vários tipos de cancro, onde a sua função tem sido associada a importantes funções biológicas, como a proliferação celular, apoptose, regulação do metabolismo da glicose, entre outros.

O cancro permanece como um grande problema de saúde em todo o mundo. De acordo com a Organização Mundial de Saúde (OMS), estima-se que em 2020 tenham surgido 19.3 milhões de novos casos e 9.96 milhões de mortes causadas pelo cancro. Em 2040, estima-se que estes números aumentem mais de 40%, realçando a urgência na identificação de novos alvos terapêuticos para favorecer o prognóstico. Apesar dos grandes avanços na terapia do cancro, quase todos os fármacos utilizados na quimioterapia despoletam quimiorresistência, especialmente devido à sobre-expressão de transportadores de efluxo responsáveis pela extrusão dos compostos terapêuticos. Curiosamente, um dos potenciais mecanismos de regulação dos transportadores de efluxo envolve os TAS2Rs, pois muitos dos fármacos anticancerígenos ou coadjuvantes são compostos de sabor amargo, ligandos dos TAS2Rs. Por outro lado, também a reprogramação metabólica das células cancerosas, conhecida como efeito Warburg, está associada à quimiorresistência e a piores prognósticos. Esta alteração metabólica permite a proliferação e sobrevivência das células tumorais devido aos reduzidos níveis de oxigénio e elevada disponibilidade de glicose no microambiente tumoral, largamente assegurada pelo aumento de absorção de glicose e de glutamina pelas células. Curiosamente, o recetor do paladar do sabor doce (STR) já foi descrito como um importante sensor da disponibilidade de glicose e regulador do metabolismo glicolítico em diversas células e órgãos, nomeadamente em células de cancro colorretal. Neste

contexto, o bloqueio do recetor do sabor doce poderá ter um importante papel na prevenção de absorção de glicose pelas células cancerosas e, como consequência, impedi-las de reprogramar o metabolismo para o efeito Warburg.

O glioblastoma é um tumor cerebral de grau 4, segundo a classificação da OMS, devido à sua agressividade, invasividade e pouca diferenciação. É o glioma mais prevalente e compreende cerca de 16% dos tumores do sistema nervoso central, sendo por isso o tumor cerebral primário mais comum. O tratamento-padrão do glioblastoma consiste na remoção cirúrgica, seguida de rádio- e quimioterapia com temozolomida, e ainda medicação para o alívio dos sintomas neurológicos. No entanto, a recorrência deste tipo de tumores é ainda bastante elevada, devido não só à elevada proliferação, vascularização e infiltração das células cancerosas, mas também à existência da barreira hematoencefálica e do microambiente hipóxico, e à elevada resistência à rádio- e quimioterapia. As condições do microambiente tumoral também poderão determinar a preferência das células tumorais, incluindo as células de glioblastoma, pela glicólise ou fosforilação oxidativa, o que lhes permite uma rápida proliferação e sobrevivência. No geral, todos estes fatores contribuem para o mau prognóstico dos doentes com glioblastoma.

Uma vez que os TAS2Rs são ativados por compostos amargos, incluindo muitos dos fármacos anticancerígenos ou coadjuvantes, surge a hipótese destes recetores estarem envolvidos no mecanismo de ação destas drogas, podendo assim ser utilizados como novos alvos terapêuticos para o glioblastoma. Para além disso, surge também a hipótese de que o bloqueio do STR desencadeie a reversão do efeito Warburg nas células de glioblastoma, uma vez que poderá iludir as células de que não há glicose disponível no meio, levando à sua morte por privação de glicose. Assim, o trabalho desenvolvido nesta tese de doutoramento teve como principal objetivo a análise da expressão dos recetores do paladar e vias de sinalização, particularmente dos recetores do sabor amargo e doce, em três linhas celulares de glioblastoma. Foi também analisado o potencial terapêutico destes dois tipos de recetor do paladar no glioblastoma.

No primeiro trabalho de investigação, pretendeu-se caraterizar a relevância funcional dos recetores do sabor amargo no sentido de serem utilizados como novos alvos terapêuticos para o glioblastoma. Inicialmente foi possível identificar a presença de 19 transcritos de TAS2Rs nas linhas celulares de glioblastoma e ainda numa linha celular humana de astrócitos. As diferenças de expressão génica e proteica de 4 TAS2Rs em astrócitos e células de glioblastoma foram também analisadas. Adicionalmente, foi também validada a expressão dos TAS2Rs em cortes histológicos provenientes de biópsias humanas de glioblastoma. De forma a estudar a funcionalidade da via de

sinalização do sabor amargo, nomeadamente a capacidade de as células de glioblastoma responderem ao quimioterápico temozolomida, realizaram-se estudos de Ca^{2+} *imaging*. O efeito da ativação dos TAS2Rs na resposta das células de glioblastoma à temozolomida, na presença ou ausência do conhecido antagonista probenecida, foi também avaliado. Adicionalmente, investigou-se o efeito do silenciamento de TAS2Rs específicos na resposta das células de glioblastoma à temozolomida, tendo sido demonstrado que os efeitos anticancerígenos da temozolomida nestas células é parcialmente mediado pela ativação dos TAS2Rs, particularmente pelo TAS2R43.

Na segunda parte do trabalho de investigação apresentado nesta tese avaliou-se a relevância funcional do recetor do sabor doce no metabolismo da glicose das células de glioblastoma. À semelhança dos resultados anteriores, procedeu-se à identificação e análise dos níveis de expressão das subunidades do STR, TAS1R2 e TAS1R3, em linhas celulares e amostras histológicas humanas de glioblastoma. De seguida, analisou-se o efeito da inibição do STR na proliferação e apoptose das células de glioblastoma, tanto em normoxia como em condições de privação de glicose/oxigénio. Por fim, foi avaliado o efeito do STR na reprogramação metabólicas das células de glioblastoma através da medição da absorção da glicose e dos níveis de L-lactato extracelulares. Observou-se que a inibição do STR com lactisole, um inibidor específico da subunidade TAS1R3, reduziu a viabilidade e migração das células de glioblastoma, particularmente sob privação de glicose e oxigénio, limitando a absorção de glicose pelas células de glioblastoma.

No conjunto, os resultados apresentados nesta tese de doutoramento permitiram evidenciar a potencialidade dos recetores dos sabores amargo e doce como possíveis alvos no tratamento do glioblastoma, como forma de contornar a quimiorresistência ou impedir a absorção de glicose necessária para a sobrevivência celular, no sentido de conseguir melhorar o prognóstico desta doença devastadora.

Palavras-chave

Via de sinalização do paladar; recetores de sabor amargo; recetor de sabor doce; glioblastoma; quimiorresistência; temozolomida; TAS2R43; metabolismo da glicose; efeito Warburg; TAS1R3

Abstract

In mammals, taste perception is a primordial function triggered by food ingestion which is transduced as bitter, sweet, sour, salty or umami flavours to the central nervous system. Taste receptors belong to the G protein coupled receptors superfamily and correspond to transmembrane proteins that can be divided into type 1 and type 2. There are 3 type 1 receptor subunits (TAS1Rs) that mediate sweet and umami tastes through the heterodimers TAS1R2+TAS1R3 and TAS1R1+TAS1R3, respectively. On the other hand, the group of type 2 receptors (TAS2Rs) consists of 25 members that mediate bitter taste. Although initially found in the taste buds of the oral cavity, the expression of taste receptors has been reported in several other extraoral organs and also in several types of cancer, where their function has been associated with important biological functions, such as cellular proliferation, apoptosis, glucose metabolism regulation, among others.

Glioblastoma is a grade 4 brain tumour, according to the World Health Organization classification, due to its aggressiveness, invasiveness, and poor differentiation. The standard treatment for glioblastoma is surgical resection, followed by radio- and chemotherapy with temozolomide. However, the recurrence of this type of tumour is still very high, not only due to the high proliferation and infiltration of cancer cells, but also to the existence of the blood-brain barrier and hypoxic microenvironment, and to the high radio- and chemotherapy resistance. Overall, all these factors contribute to the poor prognosis of patients with glioblastoma. One of the chemoresistant mechanisms is associated with the overexpression of efflux transporters, that can be regulated by TAS2Rs. On the other hand, the metabolic reprogramming of cancer cells, known as Warburg effect, is also associated with chemoresistance and worse prognosis. Interestingly, the sweet taste receptor (STR) is an important sensor of the glucose availability and glucose metabolism in several cells and organs.

Since TAS2Rs are activated by bitter tasting compounds, including several anticancer drugs, these receptors could be used as new therapeutic targets for glioblastoma. In addition, the STR inhibition could trigger the Warburg effect reversal in glioblastoma cells, as it may deceive the cells that there is no glucose available, leading to their death by starvation. Thus, the work developed during this doctoral thesis aimed at analysing the expression and function of taste receptors, particularly bitter and sweet taste receptors, in three glioblastoma cell lines. The therapeutic potential of these 2 types of taste receptor in glioblastoma was also analysed.

In the first research work, we aimed to characterize the functional relevance of bitter taste receptors to be used as new therapeutic targets for glioblastoma. We firstly identified the presence of 19 TAS2Rs transcripts in glioblastoma cell lines and in a human astrocytes cell line. Differences in gene and protein expression of 4 TAS2Rs were also addressed. Moreover, the TAS2Rs expression in human tumour samples of glioblastoma was also validated. Most importantly, we also showed that the anticancer effects of temozolomide in glioblastoma cells is partially mediated by TAS2Rs activation, particularly by TAS2R43.

In the second part of the research work presented in this doctoral thesis, the functional relevance of the sweet taste receptor in the glucose metabolism of glioblastoma cells was investigated. We proceeded to the identification and analysis of the 2 STR subunits expression in glioblastoma cell lines and human samples. Then, it was observed that the STR inhibition with lactisole, a TAS1R3 subunit inhibitor, reduced the viability and migration of glioblastoma cells, particularly under glucose/oxygen deprivation conditions, by limiting the glucose uptake by glioblastoma cells.

Altogether, the results presented in this doctoral thesis provided evidence for the potential of bitter and sweet taste receptors as possible targets for glioblastoma treatment, to improve the prognosis of this devastating disease.

Keywords

Taste signalling pathway; bitter taste receptors; sweet taste receptor; glioblastoma; chemoresistance; temozolomide; TAS2R43; glucose metabolism; Warburg effect; TAS1R3

Thesis Overview

This Doctoral thesis is divided in 6 chapters.

The first and second chapters enclose the introduction and contextualize the putative role of the bitter and sweet taste signalling pathways in glioblastoma. In the first chapter, the taste signalling pathway is introduced, particularly bitter and sweet taste receptors, and their expression and function in cancer. Moreover, the anticancer effects of bitter tasting compounds are explored, as well as their function as chemosensitizers of therapeutics by regulating the efflux transporters. Finally, the role of the sweet taste receptor role in glucose metabolism, and its functioning as a glucosensor in cancer cells is discussed. In the second chapter, the prevalence, incidence, and sex-bias of glioblastoma are reviewed, as well as the role of chemoresistance and glucose metabolism in the search for novel therapeutic strategies to improve the outcome of glioblastoma patients.

The general and specific aims established for the work plan of this Doctoral thesis are presented in the third chapter.

The fourth and fifth chapters present the results of the research works entitled "The anti-cancer effects of temozolomide are partially mediated by activation of the bitter taste receptor TAS2R43 in glioblastoma cells" and "The sweet taste receptor controls cell proliferation, migration, and glucose uptake in glioblastoma cells".

Finally, the sixth chapter contains the concluding remarks of this research work and discuss the future perspectives of the bitter and sweet taste signalling in glioblastoma treatment.

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Abbreviations and Acronyms

ABC	ATP-binding cassette
ABCB1	ABC subfamily B member 1 *or* P-glycoprotein (P-gP)
ABCC2	ABC subfamily C member 2 *or* multidrug resistance-associated protein 2 (MRP2)
ABCG2	ATP-binding cassette subfamily G member 2 or breast cancer resistance protein (BCRP)
Akt	Protein kinase B
ANOVA	Analysis of variance
AR	Androgen receptor
ATP	Adenosine triphosphate
BBB	Blood-brain barrier
BSA	Bovine serum albumin
BTB	Blood-tumour barrier
Ca^{2+}	Calcium ion
CaCl2	Calcium chloride
cDNA	Complementary deoxyribonucleic acid
CNS	Central nervous system
CO2	Carbon dioxide
COX-2	Cyclooxygenase 2
CREB	cAMP response element-binding protein
DAB	Diaminobenzidine
DMEM	Dulbecco’s modified eagle medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DUSP	Dual specificity phosphatase
E2	Estradiol *or* 17β-estradiol
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EMT	Epithelial-mesenchymal transition
ER	Estrogen receptor
ERK	Extracellular signal-regulated kinase
FBS	Foetal bovine serum
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GLUT	Glucose transporter
GNAT3	G protein subunit alpha transducin 3 *or* α-gustducin

GPCR	G protein couple receptor
H2O2	Hydrogen peroxide
HIF	Hypoxia-inducible factor
HRP	Horseradish peroxidase
IC50	Half-maximal inhibitory concentration
IDH	Isocitrate dehydrogenase
IP3	Inositol 1,4,5-trisphosphate
KCl	Potassium chloride
MAPK	Mitogen-activated protein kinase
MgCl2	Magnesium chloride
MGMT	Methylguanine DNA methyltransferase *or* O6-alkylguanine DNA alkyltransferase
mRNA	Messenger ribonucleic acid
mTOR	Mammalian target of rapamycin
mTORC	Mammalian target of rapamycin complex
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-dophenyltetrazolium bromide
NaCl	Sodium chloride
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced)
NaHCO3	Sodium bicarbonate
NF- κB	Nuclear factor kappa B
P4	Progesterone
PAGE	Polyacrylamide gel electrophoresis
PARP	Poly (ADP-ribose) polymerase
PBS	Phosphate-buffered saline
PFA	Paraformaldehyde
PI3K	Phosphoinositide 3-kinase
PLCβ2	Phospholipase beta 2
PMSF	Phenylmethylsulfonyl fluoride
PR	Progesterone receptor
PVDF	Polyvinylidene difluoride
RIPA	Radio-immunoprecipitation assay buffer
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT	Room temperature
RT-PCR	Reverse-transcribed polymerase chain reaction
RT-qPCR	Real-time quantitative polymerase chain reaction
SDS	Sodium dodecyl sulfate
SEM	Standard error of mean

siRNA	Small interfering ribonucleic acid
STR	Sweet taste receptor
TAS1R	Taste receptor, type 1
TAS2R	Taste receptor, type 2 *or* bitter taste receptor
TBS	Tris-buffered saline
TBS-T	Tris-buffered saline containing Tween-20
TGF	Transforming growth factor
TMZ	Temozolomide
TRPM5	Transient receptor potential cation channel, subfamily M, member 5
TTF	Tumour treating fields
WHO	World Health Organization

Chapter 1

Introduction, Part I

Taste Signalling Pathways

Part of this chapter is in the original publications which I co-authored:

1) Ana C. Duarte*, **Ana R. Costa***, Isabel Gonçalves, Telma Quintela, Robert Preissner, Cecília R.A. Santos. (2022). The druggability of bitter taste receptors for the treatment of neurodegenerative disorders. *Biochemical pharmacology*, *197*: 114915. https://doi.org/10.1016/j.bcp.2022.114915

2) **Ana R. Costa***, Ana C. Duarte*, Ana R. Costa-Brito, Isabel Gonçalves, Cecília R.A. Santos. (2023). Bitter taste signaling in cancer. *Life sciences*, *315*: 121363. https://doi.org/10.1016/j.lfs.2022.121363

Some alterations to the original publication were introduced to further sustain the aim of the thesis.

*These authors contributed equally.

1.1 Taste receptor signalling

In mammals, taste perception is a primordial function triggered by the food ingestion in the oral cavity, and transduced as bitter, sweet, sour, salty, or umami to the central nervous system, before evoking aversion or attracting responses (Chandrashekar *et al.* 2006). There are three major classes of taste bud cells – type I, II, and III. These cells have a limited life span and are replaced every 8–10 days in the oral cavity (Perea-Martinez *et al.* 2013), but presumably not outside of the oral cavity. Type II cells or taste receptor cells are modified epithelial cells that are innervated by afferent nerve fibres, where the taste receptors can be activated by sweet, bitter, and umami tasting compounds (Johnson & Lajtha 2007). Taste receptors are transmembrane proteins that belong to the superfamily of G-protein coupled receptors (GPCRs) and can be divided into type 1 and type 2. There are three taste receptor type 1 (TAS1Rs) subunits (TAS1R1, TAS1R2, and TAS1R3) that by TAS1R2+TAS1R3 and TAS1R1+TAS1R3 heterodimer formation mediate sweet and umami tastes, respectively. On the other hand, there are 25 different receptors type 2 (TAS2Rs) that deploy bitter taste perception when activated by specific bitter tasting compounds (Adler *et al.* 2000; Chaudhari & Roper 2010). Unlike the GPCR-mediated sweet, bitter, and umami flavours, sour and salty are mediated directly by H^+ and Na^+ ion channels, respectively (Chaudhari & Roper 2010).

The bitter, sweet, and umami taste receptor signalling cascades are initiated by the binding of cognate ligands to the taste receptor resulting in the activation of the specific heterotrimeric G-protein, gustducin, composed by α-gustducin, Gβ3, and Gγ13 subunits. Although gustducin appears to be the primary G-protein α in taste transduction, other subunits, like Gαi2 and Gα14, might also be involved (Caicedo *et al.* 2003; Tizzano *et al.* 2008). Moreover, Kim and colleagues showed that gustducin knockdown did not affect the TAS2R signalling in human airway smooth muscle, which indicates that in extraoral tissues TAS2Rs can couple to prevalent G-proteins in a cell type-dependent manner (D. Kim *et al.* 2017). Subsequently, phospholipase C Beta 2 (PLCβ2) is activated and induces the production of inositol 1,4,5-trisphosphate (IP3) causing an increase in intracellular Ca^{2+} levels. Then, the transient receptor potential cation channel subfamily M member 5 (TRPM5) is activated in response to Ca^{2+} elevation and triggers cell depolarization (Chandrashekar *et al.* 2006; Chaudhari & Roper 2010) (Figure 1.1).

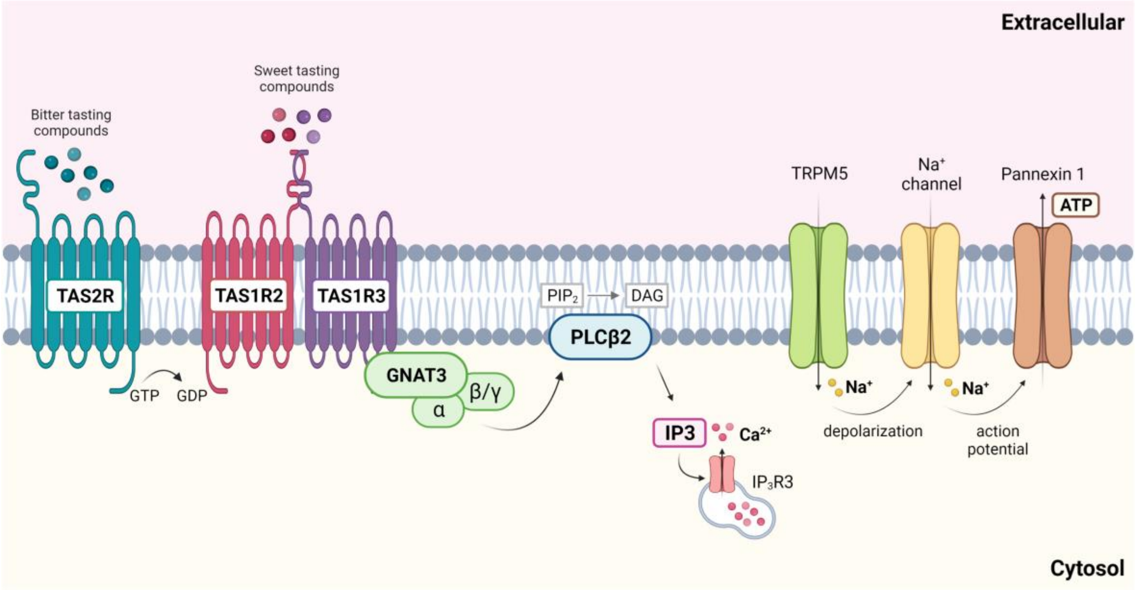


Figure 1.1 – The bitter and sweet taste transduction cascade. The binding of a bitter or sweet tasting compound to a TAS2R or TAS1R2/TAS1R3 heterodimer, respectively, members of the GPCR family, generates a conformational change activating the G-protein gustducin (formed by α- gustducin, Gβ3, and Gγ13 subunits), resulting in PLCβ2 activation that cleaves PIP2 into DAG producing the second messenger IP3 and consequently increasing intracellular Ca^{2+} levels. In turn, Ca^{2+} increasing triggers TRPM5 activation, which eventually depolarizes the cell. [Abbreviations – DAG: diacylglycerol; GNAT3: gustducin; GPCR: G protein-coupled receptors; IP3: inositol 1,4,5-trisphosphate; IP3R3: inositol 1,4,5-trisphosphate receptor type 3; PIP2: phosphatidylinositol 4,5-bisphosphate; PLCβ2: phospholipase C-beta 2; TAS1R: taste receptor type 1; TAS2R: taste receptor type 2; TRPM5: transient receptor potential channel M5].

Bitter taste receptors

In contrast to a single sweet taste receptor and a single umami taste receptor, humans have 25 functional TAS2R that recognize hundreds of bitter tasting compounds (Adler *et al.* 2000; Chandrashekar *et al.* 2000; Jaggupilli *et al.* 2016). This fact, together with several experimental data, suggests a critical physiological role of TAS2Rs in human biology and health.

TAS2R, like the other members of the GPCR superfamily, contain seven transmembrane domains, short amino- and carboxy-termini, and are composed of 291 to 334 amino acids. All TAS2R encoding genes are located on chromosomes 5, 7, and 12 (Conte *et al.* 2002), and do not contain introns (Adler *et al.* 2000; Matsunami *et al.* 2000). Although TAS2R proteins present high sequence conservation between human and rodents, the most conserved amino acids are located in the intracytoplasmic loops and transmembrane domains (Conte *et al.* 2003). In addition, intracytoplasmic loops also share significant sequence conservation between different TAS2R proteins in comparison to the extracellular loops (Conte *et al.* 2002). Interestingly, sequence conservation is also significant in the predicted sites of G-protein interaction, whereas the less conserved regions correspond to the ligand-binding pockets (Adler *et al.* 2000; Gilbertson *et al.* 2000).

Among the 25 human TAS2R, 21 have already been deorphanized. Interestingly, TAS2R present either similarities or dissimilarities regarding their activation potential since a TAS2R might be activated by several ligands, or by only a single bitter tasting compound. Of note, combined responses of broadly tuned TAS2R lead to the detection of about 50% of the bitter tasting compounds because each of these TAS2R recognizes about one-third of bitter tasting agonists (Meyerhof *et al.* 2010). Although many TAS2R bind the same ligand, concentration thresholds that induce receptor activation may differ (Jaggupilli *et al.* 2016). Humans are more sensitive to bitter compounds than other taste modalities since TAS2Rs activation requires lower ligand concentrations (µM range) (Meyerhof *et al.* 2010). Notably, the agonist selectivity of TAS2R differs between species, which compromises the translation of data obtained in rodent models to humans (Lossow *et al.* 2016).

Taste receptors were initially found in the taste buds of the oral cavity, where their activation triggers an aversive response that acts as a warning to avoid the ingestion of poisonous or toxic compounds (Chandrashekar *et al.* 2006). However, TAS2R expression have been reported in many other organs and tissues such as the airways, gastrointestinal tract, pancreas, heart, kidney, thyroid, testis, placenta, and brain (Dalesio *et al.* 2018; S.J. Lee *et al.* 2019). Although their role at extraoral sites is less understood, some studies sustain that they might be involved in the assessment of the chemical composition of body fluids to activate transporter and detoxifying systems or to trigger other biological actions accordingly (Duarte *et al.* 2020). Although many bitter tasting compounds are indeed toxic, this concept is not that straightforward since many bitter tasting molecules, such as flavonoids, have well recognised beneficial health properties (Ballard & Maróstica 2018; Panche *et al.* 2016).

Sweet taste receptor

Sweet taste receptor (STR) is an important sensor of glucose availability and regulator of glucose metabolism in several organs. STR belongs to the GPCR superfamily and is composed by heterodimers of TAS1R2 and TAS1R3 proteins.

The human TAS1Rs are located in chromosome 1, following the order TAS1R2–TAS1R1–TAS1R3, and are also constituted by a secondary structure that comprises seven transmembrane helices, a long extracellular N-terminus composed by a Venus flytrap and a cysteine-rich domain (Fredriksson *et al.* 2003; Liao & Schultz 2003). Most sweeteners bind to the Venus flytrap module, triggering coupled conformational rearrangements of transmembrane, cysteine-rich and intracellular domains, and shifting the receptor from the rest to the active state simultaneously (U.K. Kim *et al.* 2006). Like TAS2Rs, the STR proteins also present high sequence conservation in vertebrates (Shi & Zhang 2006).

STR binds various sugars, artificial sweeteners, and sweet amino acids (Welcome & Mastorakis 2018). Although initially identified in the taste buds of the oral cavity, STR are also expressed throughout the body, including the adipose tissue, airways, bladder, gut, heart, kidney, liver, pancreas, testes, sperm and brain (Santos *et al.* 2019). The STR role at extraoral sites is not completely understood, but some studies sustain that it might control glucose metabolism and be an important glucosensor, with a recognised role in the regulation of glucose uptake in cells. In addition, it is likely that the STR might also be instrumental for glucose metabolism in cancer cells, where metabolic demands are overriding.

1.2 Bitter taste signalling in cancer

The global burden of cancer remains overwhelming and a major healthcare problem worldwide. According to the World Health Organization, in 2020 were estimated about 19.3 million new cancer cases and 9.96 million cancer deaths (Ferlay *et al.* 2021; Sung *et al.* 2021). By 2040, these numbers are estimated to rise to 28.9 million new cases and 16.2 million deaths (Bray & Møller 2006; Ferlay *et al.* 2020), highlighting the need to identify new therapeutic targets to improve prognosis. To date, chemotherapy before or following surgery is still the most promising treatment for metastatic cancer. Radiation and laser therapy, hormone and gene therapy, immunotherapy, combination therapy, and targeted therapy are other options for non-disseminated cancer (Baskar *et al.* 2012; Urruticoechea *et al.* 2010; X. Wang *et al.* 2019). Despite the great advances in cancer treatment, pharmacoresistance has been reported for almost all the drugs used in chemotherapy (Holohan *et al.* 2013). More than 80 % of poor survival outcomes and mortality in cancer patients are directly or indirectly attributed to chemoresistance (Mansoori *et al.* 2017).

Multidrug resistance transporters are a major cause of cancer drug resistance since they are responsible for extruding drug compounds out of the cells. The best-known class of multidrug resistance proteins is ATP-binding cassette (ABC) transporters, which couple drug/lipid efflux with energy derived from ATP hydrolysis to translocate a broad range of substrates across the cell membrane (Higgins 1992). However, the mechanisms underlying the regulation of ABC transporters are still a matter of debate.

One of the potential molecular mechanisms by which ABC transporters may be regulated in cancer cells involves the bitter taste receptors (TAS2Rs), whose activation can trigger the up or downregulation of specific transporters. For example, in pancreatic cancer cell lines, the activation of TAS2R10 and TAS2R38 by specific agonists induced ABCG2 downregulation and ABCB1 upregulation, respectively (Gaida *et al.* 2016; Stern *et al.* 2018). Notably, many anti-cancer drugs are bitter compounds and ligands of TAS2Rs (Banerjee & Preissner 2018).

Herein we review the potential therapeutic role of TAS2Rs and bitter tasting compounds in cancer, specifically the expression of TAS2Rs in different types of cancer (*e.g.*, breast, ovarian, and prostate) and the evidence showing the ability of bitter tasting compounds to overcome chemoresistance mechanisms in cancer.

1.2.1 Expression and function of bitter taste receptors in cancer

TAS2Rs are expressed in extraoral organs with different functional activities according to the type of cell or tissue (reviewed in (Dalesio *et al.* 2018; Duarte *et al.* 2022; S.J. Lee *et al.* 2019)). In addition, several authors already reported differences in TAS2Rs expression in cancer versus normal cell lines and tissues, reinforcing their importance in cancer biology. In Table 1.1, we summarize the gene and protein expression levels of TAS2Rs in cancer versus normal cell lines and tissues. Despite the relevance of these data, it is important to stress that most of these results correspond to very small studies with low number of samples, reinforcing the need to carry out large scale studies to better clarify if the differences are due to individual genetic variation or reflect disease states.

The first evidence for these differences was reported by Singh and colleagues that showed lower levels of TAS2R4 mRNA and protein in breast cancer compared to non-cancer cell lines (Singh *et al.* 2014). Moreover, lower mRNA levels of TAS2R1, TAS2R10, TAS2R39, and TAS2R49/20 compared to commonly used breast cancer marker proteins (estrogen receptor and E-cadherin) were also observed (Singh *et al.* 2014). A study carried out on breast cancer and normal breast tissues showed decreased TAS2R4 and increased TAS2R14 gene and protein expression levels in cancerous tissues, respectively (Singh *et al.* 2020). In addition, TAS2R14 and TAS2R49/20 transcripts were upregulated in highly metastatic cell line MDA-MB-231 in comparison to normal breast epithelial cell line MCF-10 (Jaggupilli *et al.* 2017). However, it is important to mention that the MCF-10A cells is an immortalized cell line derived from benign fibrocystic disease and, although presenting similarities with human breast epithelial cells, may not reflect entirely the characteristics of the normal tissue (Qu *et al.* 2015; Soule *et al.* 1990).

Further studies reinforced that the expression pattern of TAS2Rs is not only altered in breast cancer cells but also in other types of cancer. In endometrial and ovarian cancer cell lines, the levels of TAS2R1, TAS2R4, TAS2R14, and TAS2R38 were lower than in normal uterine and fallopian tube tissues (L. Martin *et al.* 2019). The same study also showed decreased expression levels of TAS2R1, TAS2R4, TAS2R10, TAS2R14, and TAS2R38 in three prostate cancer cell lines (DU145, LNCaP, and PC3) when compared to benign prostate hyperplasia cells (BPH-1 cells) (L. Martin *et al.* 2019). In human neuroblastoma cells (SK-N-BE(2)C), TAS2R8 and TAS2R10 had lower levels of gene expression in comparison to the less malignant and more differentiated cell line SH-SY5Y (Seo *et al.* 2017). The immunohistochemical analysis of pancreatic ductal adenocarcinomas showed that TAS2R10 and TAS2R38 proteins were detectable in more than 75 % of a set of cancerous samples, but not in normal tissue samples (Gaida *et al.* 2016; Stern *et al.* 2018). TAS2R38 was also found in tumour-infiltrating leukocytes (Gaida *et al.* 2016).

Apart from addressing differences in mRNA and protein expression levels, most studies showed that the expressed TAS2Rs are functionally active in cancer cells since they respond to the respective bitter compound. In both cancerous and normal breast cells, TAS2R1 and TAS2R10, TAS2R4, and TAS2R38 were activated by the specific TAS2Rs bitter agonists dextromethorphan, quinine, and phenylthiocarbamide, respectively, eliciting a dose-dependent increase in calcium mobilization, with the highest response in non-cancerous cells (Singh *et al.* 2014). In addition, bitter tasting compounds quinine and apigenin also increased intracellular calcium levels in highly metastatic and non-cancerous breast cell lines through TAS2R4 and TAS2R14 activation, respectively, attenuating MDA-MB-231 cells proliferation and migration and inducing early apoptosis (Singh *et al.* 2020). These effects were not observed in non-metastatic MCF-10A cells. The increased calcium response observed in MDA-MB-231 cells might be related to the upregulation of both TAS2R4 and TAS2R14 in these cells (Table 1.1). In ovarian and prostate cancer cells, exposure to noscapine after TAS2R14-silencing with specific siRNAs led to increased apoptosis in a receptor-dependent manner (L. Martin *et al.* 2019). The stimulation of neuroblastoma cells with the TAS2R agonist denatonium benzoate triggered calcium release into the cells, showing TAS2R8 and TAS2R10 activation (Seo *et al.* 2017). In addition, TAS2R8 and TAS2R10 upregulation was associated with increased neurite elongation, and the reduction of cancer stem cell markers and self-renewal characteristics, as well as cell migration and invasion. *In vivo*, mice overexpressing TAS2R8 and TAS2R10 presented reduced tumour incidence and volume in comparison with control mice. This suggests that these receptors play an important role in suppressing the metastatic potential of neuroblastoma cells (Seo *et al.* 2017). Also, the function of TAS2R38 in pancreatic cancer cells was demonstrated after phenylthiourea and N-acetyl-dodecanoyl homoserine binding (Gaida *et al.* 2016). In addition, the exposure of primary acute myeloid leukemic cells and human leukaemia cell lines to quinine or denatonium benzoate, both TAS2Rs' ligands, increased intracellular calcium levels, suggesting the functionality of the bitter taste pathway (Salvestrini *et al.* 2020).

In four oral cancer cell lines (HSC2, HSC3, HSC4, and SAS) mRNA and protein expression of the TAS2R14 were detected, but TAS2R39 was not expressed (Kon *et al.* 2022). Epigallocatechin gallate treatment induced histamine production and decreased the expression of the histamine H1 receptor. In addition, epigallocatechin gallate also upregulated or downregulated TAS2R14 in the oral cancer cell lines studied, suggesting that the ligand-receptor interaction might regulate TAS2R14 expression and protein stability (Kon *et al.* 2022).

More recently, Carey and colleagues analysed the expression of TAS2Rs and their function in head and neck squamous carcinoma cell lines (SCC4, SCC5, SCC47, SCC90, SCC152, OCT22, and VU147T) in response to some bitter tasting compounds (Carey *et al.* 2022). TAS2R4, TAS2R14, TAS2R19/23/48, TAS2R20/49, TAS2R30/47, TAS2R43, and TAS2R45 were expressed at high levels in the cell lines studied. Tissue samples from head and neck squamous carcinoma patients were also analysed and no significant alterations on TAS2Rs expression were found between control and cancer samples. In cancer cell lines, increasing calcium levels, due to TAS2Rs activation by bitter compounds, induced mitochondrial depolarization and increased plasma membrane permeability. In addition, the bitter tasting compound denatonium benzoate induced cellular apoptosis in these cancer cell lines through caspase activation via TAS2R4. In the same study, the expression of the 25 TAS2Rs was analysed in the biopsies of head and neck squamous cell carcinoma patients (Carey *et al.* 2022). All the TAS2Rs were detected, but TAS2R1, TAS2R13, TAS2R14, TAS2R19/23/48, TAS2R20/49, TAS2R30/47, TAS2R42, TAS2R43, and TAS2R44 presented the highest mRNA relative expression. Interestingly, TCGA analysis of mRNA samples from head and neck squamous cell carcinoma patients indicates a correlation between increased TAS2Rs expression and improved survival (Carey *et al.* 2022).

Recently, Carey and colleagues reported an association between the expression levels of certain TAS2Rs with the survival differences of 12 solid tumour subtypes, like adrenocortical and kidney clear cell carcinomas, oesophageal adenocarcinoma, and non-papillary bladder cancer, in comparison to normal tissue (Carey *et al.* 2022b). TAS2R5, TAS2R19 and TAS2R20 presented negative survival associations in at least one tumour histology, but TAS2R4 and TAS2R14 were associated with opposing survival outcomes in different malignancies. These findings suggest that TAS2Rs might be biomarkers for clinical outcomes or targets in some cancers, although their expression levels and clinical relevance is variable in different cancers (Carey *et al.* 2022b). Despite all these data, only a few of the 25 TAS2Rs were examined, highlighting the need for further studies on other receptors, to identify the most relevant in the context of cancer from different cell types and tissues. Even so, this evidence clearly shows that TAS2Rs are differentially expressed in some types of cancer cells, and can be activated by specific agonists, positioning them as potential targets for cancer therapy.

Table 1.1 –TAS2Rs expression in cancer.

	Findings on receptor	*Cancer type*	*Experimental model*	*References*
TAS2R1	↓ mRNA *versus* breast cancer markers	Breast	MCF7, MCF10, MDA-MB-31cells	Singh *et al*. 2014
	↓ mRNA	Endometrial	HEC-1a cells *versus* normal uterine	L. Martin *et al*. 2019
		Ovarian	OVCAR8 cells *versus* normal fallopian tube tissue	
		Prostate	DU145, LNCaP and PC3 *versus* BPH-1 cells	
TAS2R4	↓ mRNA and protein	Breast	MCF7 and MDA-MB-31 *versus* MCF10 cells	Singh *et al*. 2014
	↓ mRNA		Breast cancer *versus* normal breast tissues	Singh *et al*. 2020
	↓ mRNA	Endometrial	SKOV3 cells *versus* normal uterine	L. Martin *et al*. 2019
		Ovarian	OVCAR8 cells *versus* normal fallopian tube tissue	
		Prostate	DU145, LNCaP and PC3 *versus* BPH-1 cells	
TAS2R8	↓ mRNA in more malignant cells	Neuroblastoma	SK-N-BE(2)C *versus* SH-SY5Y cells	Seo *et al*. 2017
TAS2R10	↓ mRNA *versus* breast cancer markers	Breast	MCF7, MCF10, MDA-MB-31cells	Singh *et al*. 2014
	↓ mRNA	Prostate	DU145, LNCaP and PC3 *versus* BPH-1 cells	L. Martin *et al*. 2019
	↓ mRNA in more malignant cells	Neuroblastoma	SK-N-BE(2)C *versus* SH-SY5Y cells	Seo *et al*. 2017
	75-80% mRNA and protein expression	Pancreatic	Pancreatic ductal adenocarcinoma	Stern *et al*. 2018
TAS2R14	↑ mRNA in highly metastatic	Breast	MDA-MB-231 *versus* MCF10A cells	Jaggupilli *et al*. 2017
	↑ mRNA and protein		Breast cancer *versus* normal breast tissues	Singh *et al*. 2020
	↓ mRNA	Endometrial	HEC-1a and SKOV3 cells *versus* normal uterine	L. Martin *et al*. 2019
		Ovarian	OVCAR8 cells *versus* normal fallopian tube tissue	
		Prostate	DU145, LNCaP and PC3 *versus* BPH-1 cells	
TAS2R38	↓ mRNA *versus* breast cancer markers	Breast	MCF7, MCF10, MDA-MB-31cells	Singh *et al*. 2014
	↓ mRNA	Endometrial	HEC-1a, IGROV1 and SKOV3 cells *versus* normal uterine	L. Martin *et al*. 2019
		Ovarian	OVCAR4 and OVCAR8 cells *versus* normal fallopian tube tissue	
		Prostate	DU145 and LNCaP *versus* BPH-1 cells	
	78% protein expression in pancreatic cancer and in tumour infiltrating leukocytes	Pancreatic	Pancreatic ductal adenocarcinoma	Gaida *et al*. 2016
TAS2R49/20	↓ mRNA *versus* breast cancer markers	Breast	MCF7, MCF10, MDA-MB-31cells	Singh *et al*. 2014
	↑ mRNA in highly metastatic		MDA-MB-231 *versus* MCF10A cells	Jaggupilli *et al*. 2017

1.2.2 Many anticancer compounds taste bitter

Bitter tasting compounds are detected by TAS2Rs at much lower concentrations than sweet or umami compounds are detected by TAS1Rs (Meyerhof 2005; Purves *et al.* 2001). In addition, some TAS2Rs can be activated by several structurally dissimilar molecules, while others have few or no known ligands. This may be due to the existence of binding pocket subsites and different types of ligand interactions, especially due to an extracellular site involved in the binding process (Born *et al.* 2013; Di Pizio & Niv 2015; Levit *et al.* 2014). Moreover, the highly variable individual bitter sensitivity in humans can be partially attributed to TAS2Rs genetic polymorphisms (Roura *et al.* 2015).

Of the hundreds of bitter tasting compounds summarized on the BitterDB, almost 30 % of these appear in the DrugBank. Besides, more than 75 % of the approved pharmacological drugs are predicted to be bitter, reinforcing their potential to be TAS2Rs' ligands (Banerjee & Preissner 2018; Dagan-Wiener *et al.* 2017; Wiener *et al.* 2012; Wishart *et al.* 2008). The therapeutic effects triggered by bitter tasting compounds may be directly mediated by TAS2Rs activation or act, at the same time, on additional targets.

Bitter tasting molecules, natural or synthetic, belong to several chemical classes, including alkaloids, flavonoids, polyphenols, amino acids, simple salts, lipids, peptides, and many low-calorie sweeteners (Behrens *et al.* 2017; Laffitte *et al.* 2016; Meyerhof *et al.* 2010). Although the largest number of bitter tasting molecules belongs to the alkaloid, terpene, and phenolic families, flavonoids (flavones, isoflavones, flavanones, flavonols, flavanols, and anthocyanidins) have been more widely studied, since they are among the most promising anti-cancer drugs (Crozier *et al.* 2006).

1.2.2.1 Anticancer effects of bitter tasting compounds

A growing body of evidence has shown that many bitter tasting compounds display anti-tumoral activity, either *in vitro* or *in vivo* (Table 1.2). Among others, the bitter tasting compounds azathioprine, brucine, noscapine, papaverine, dapsone, and parthenolide have been shown to induce cell cycle arrest, cell apoptosis, and to inhibit angiogenesis and metastasis occurrence in different types of cancer, and cancer models (Table 1.2). Parthenolide, a bitter tasting compound and known ligand of five TAS2Rs (TAS2R4, TAS2R8, TAS2R10, TAS2R14, and TAS2R31/TAS2R44 (Jaggupilli *et al.* 2016), has been the focus of many studies showing anti-tumoral effects in various systemic cancers, including breast (Carlisi *et al.* 2017; Jafari *et al.* 2018), colorectal (Carlisi *et al.* 2017; X. Li *et al.* 2020), lung (S.L. Kim *et al.* 2017; Y.C. Liu *et al.* 2017; Talib & Al Kury 2018), pancreatic (Y.C. Liu *et al.* 2017), and prostate cancers (Marino *et al.* 2019) (Table 1.2). Interestingly, the expression of TAS2R4, TAS2R10, and TAS2R14 is altered in breast and prostate cancers (Table 1.1).

The flavonoids epigallocatechin gallate, kaempferol, and quercetin, and the polyphenol resveratrol, have been widely described as important anti-tumoral compounds in breast (G. Hu *et al.* 2019; X. Li *et al.* 2018; Xu *et al.* 2019; Zan *et al.* 2019), colorectal (Darband *et al.* 2020; Chung *et al.* 2018; W. Zhu *et al.* 2020), and prostate cancers (Da *et al.* 2019; Khusbu *et al.* 2020; X. Lu *et al.* 2020a; Martínez-Martínez *et al.* 2019), as well as in glioblastoma (Grube *et al.*

2018; Xie *et al.* 2018; H.C. Yang *et al.* 2019), among others (Table 1.2). Epigallocatechin gallate, kaempferol, and resveratrol are ligands of TAS2R14 and TAS2R39, and quercetin can bind TAS2R14 (Jaggupilli *et al.* 2016; Levit *et al.* 2014; Roland *et al.* 2013). Indeed, among the TAS2Rs family, TAS2R14 and TAS2R39 are the receptors with more known ligands (Wiener *et al.* 2012). In addition, TAS2R14 is differently expressed in cancer cells (breast, endometrial, ovarian, prostate) in comparison to normal cells or tissues (Table 1.1). Together, this evidence reinforce TAS2R14 has a potential therapeutic target in cancer.

The anticancer effects of bitter tasting compounds comprise the induction of apoptosis through the upregulation of p53 and the activation of caspase-3, −9, and PARP (Poly (ADP-ribose) polymerase) and decreasing the expression of anti-apoptotic proteins, such as Bcl-2 and Bcl-xL (Table 1.2). Moreover, bitter tasting molecules are also involved in the inhibition of angiogenesis and epithelial-mesenchymal transition (EMT) processes, which contributes to reducing critical cancer hallmarks including cell growth, proliferation, and invasiveness (Table 1.2). Other studies also addressed anti-metastatic effects, DNA methylation, inhibition of the proliferation of cancer stem cells, and inhibition of glycolysis in response to bitter tasting compounds (Table 1.2). What remains to be understood is if these actions are to some extent also mediated by their cognate TAS2Rs.

Breast cancer

Brucine is a bioactive and toxic alkaloid found in different plant species with recognized anti-proliferation, anti-migration, anti-invasion, and pro-apoptotic effects in breast cancer cells (K.F. Hu *et al.* 2017; M. Li *et al.* 2018; Xu *et al.* 2019). In a rat model of chemically-induced mammary carcinogenesis, brucine diminished the tumour incidence and led to the restoration of the levels of biochemical markers (H. Ren *et al.* 2019).

The anticancer effects of flavonoids epigallocatechin gallate, kaempferol, and quercetin have already been studied in several types of breast cancer cell lines. In breast cancer MCF7 and MDA-MB-231 cells, epigallocatechin gallate decreased the cell viability, induced apoptosis by increasing PARP and caspases-3 and -9, and led to cell cycle arrest (Hong *et al.* 2017; Schröder *et al.* 2019; Zan *et al.* 2019). Kaempferol also showed anti-proliferative and pro-apoptotic effects in late-stage breast cancer MDA-MB-231 cells (S. Li *et al.* 2017; L. Zhu & Xue 2019). In xenograft mice models of breast cancer, quercetin delayed and decreased tumour growth and inhibited the metastatic ability of cancer stem cells in proliferating and generating mammospheres (Jia *et al.* 2018; X. Li *et al.* 2018).

Resveratrol has been extensively analysed in MCF7 and MDA-MB-231 breast cancer cells, either alone or in combination with other drugs. Several studies showed that resveratrol can modulate epigenetic factors and inhibit mTOR, ERK1/2, PI3K/Akt, and self-renewal signalling pathways, therefore decreasing proliferation, migration, and invasion of breast cancer cells and suppressing stemness properties (Abdel-Latif *et al.* 2015; Alayev *et al.* 2015; Chatterjee *et al.* 2019; K.Y. Chen *et al.* 2019; Cipolletti *et al.* 2019; Izquierdo-Torres *et al.* 2019; Suh *et al.* 2018; Sun *et al.* 2019).

The induction of ERK1/2 phosphorylation was observed in clones of HEK293 cells stably expressing the mouse Tas2r5 and rat Tas2r9, respectively, upon exposure to cycloheximide (Ozeck *et al.* 2004). In addition, the Akt phosphorylation in BxPC-3 cells was inhibited after TAS2R10 knockdown, and ERK and CREB phosphorylation was induced by TAS2R16 activation in the human neuroblastoma SH-SY5Y cell line (Stern *et al.* 2018; Wölfle *et al.* 2015). Although none of these TAS2R is a resveratrol ligand, these studies suggest that the activation of ERK, Akt, and CREB pathways may be partially dependent on TAS2Rs expression.

Colorectal cancer

The effects of bitter tasting compounds, namely epigallocatechin gallate and parthenolide, in colorectal cancer are very similar to those observed for breast cancer. Epigallocatechin gallate has shown anti-tumoral activity by inhibiting the activation of several signalling pathways, inducing chromosomal instability, and blocking drug efflux (Ding *et al.* 2018; La *et al.* 2019; Ni *et al.* 2018; W. Zhu *et al.* 2020). Moreover, epigallocatechin gallate is also involved in decreasing the number of precancerous lesions and solid tumours, and in the inhibition of carcinogenesis and spheroid formation by colorectal cancer stem cells (Y. Chen *et al.* 2017; X. Wang *et al.* 2017).

In several types of colorectal cancer cell lines, parthenolide inhibited deubiquitinating enzymes, Wnt signalling, and transforming growth factor (TGF)-β1-induced cell migration and invasion, as well as hypoxia-inducible factor (HIF)1α activity, thus downregulating proteins associated with glucose metabolism, angiogenesis, and survival (S.L. Kim *et al.* 2017; X. Li *et al.* 2020; Y.C. Liu *et al.* 2017; S.M. Zhu *et al.* 2019). In a nude xenograft mouse model, parthenolide was able to decrease colorectal carcinoma growth by regulating the self-renewal nuclear factor kappa B (NF-κB) pathway, HIF1α, and EMT (S.L. Kim *et al.* 2017). In a study carried out in primary human gingival fibroblasts, the activation of TAS2R16 by salicin repressed NF-κB phosphorylation and nuclear translocation induced by lipopolysaccharide, thus suggesting that TAS2Rs may play a role in reducing or suppressing the inflammatory cytokine expression (Z. Zhou *et al.* 2021).

Glioblastoma

In the brain, gliomas are the most common malignant primary brain tumours and comprise astrocytomas, oligodendrogliomas, and ependymomas. Glioblastoma, a central nervous system astrocytoma classified as a grade 4 by the World Health Organization (WHO), is the most prevalent and aggressive primary brain tumour and is associated with a poor prognosis and low life expectancy (reviewed in (Lapointe *et al.* 2018)). Besides, brain metastasis with origin in peripheric tumours, such as lung, breast, colorectal cancers, melanoma, or renal cell carcinoma contributes to the high mortality associated with these cancers (Achrol *et al.* 2019).

Grube and colleagues showed that epigallocatechin gallate, at achievable CNS concentrations, induced cell stress in primary glioblastoma cells, activating the autophagic cellular response by increasing reactive oxygen species (ROS) levels (Grube *et al.* 2018). More recently, human U-251MG glioblastoma cells treated with epigallocatechin gallate showed increased cell senescence related to the inhibition of telomerase activity (Udroiu *et al.* 2019). Epigallocatechin gallate also reversed temozolomide (TMZ) resistance, in part by decreasing methylguanine DNA methyltransferase (MGMT) expression, in MGMT-positive GBM-XD and T98G cells (Xie *et al.* 2018). Another flavonoid, luteolin, inhibited epidermal growth factor (EGF)-induced glioblastoma U87 and U252 cells proliferation and induced apoptosis by arresting the cell cycle at the S and G2/M phases and increasing cleaved caspase-3 and PARP (Anson *et al.* 2018). Moreover, luteolin prevented the phosphorylation of proteins involved in the Akt/PKB and MAPK pathways.

A combination of quercetin and sodium butyrate was tested in rat C6 and human T98G glioblastoma cells. This combination had a synergistic effect in inhibiting protective autophagy by downregulating Beclin-1 and microtubule-associated protein LC3-II, thus inducing cell apoptosis (Taylor *et al.* 2019).

The effects of resveratrol have been studied in glioblastoma. A recent study showed *in vitro* and *in vivo* that resveratrol reverses TGF-β1 induced EMT via E-cadherin upregulation, and N-cadherin, vimentin, β-catenin, downregulation. Moreover, resveratrol inhibited cell migration, invasion, and stem-cell-like properties of glioblastoma cells (Song *et al.* 2019). TMZ effects also are enhanced by resveratrol in both T98G and U-138MG cells, as well as in a T98G xenograft mice model (H.C. Yang *et al.* 2019).

Prostate cancer

LNCaP and PC3 cell lines are the most studied *in vitro* models of prostate cancer. In these cells, bitter tasting compounds, such as naringenin, parthenolide, quercetin, and resveratrol, showed anti-proliferative and pro-apoptotic effects and capacity to suppress EMT (Erdogan *et al.* 2018; Fonseca *et al.* 2019; Lim *et al.* 2017; X. Lu *et al.* 2020a; Marino *et al.* 2019). In addition, resveratrol sensitized DU145 and PC3 prostate cancer cells to the pro-apoptotic effects of cisplatin (Khusbu *et al.* 2020; Martínez-Martínez *et al.* 2019).

It is well known that androgen and androgen receptor (AR) signalling pathways are crucial for the development of prostate cancer. Da and colleagues recently reported that kaempferol decreased dihydrotestosterone-induced AR activation, by downregulation of AR downstream targets, and decreased AR expression and nuclear accumulation. In addition, the effects of kaempferol on cell growth were significantly more pronounced in LNCaP AR-positive cells than in non-malignant cells or in PC3 AR-negative prostate cancer cells (Da *et al.* 2019).

Overall, several studies in the literature suggest that bitter tasting compounds display anti-cancer properties. However, despite their cognate receptors having been identified, most of these studies failed in addressing the role of TAS2Rs activation in the regulation of the downstream effects of bitter tasting compounds in cancer cells. Therefore, future studies aiming to evaluate the anti-cancer effects of bitter tasting compounds such as resveratrol and quercetin, among others, should address the role of their cognate TAS2R in this and any other types of cancer.

1.2.2.2 Bitter tasting compounds are chemosensitizers

Chemoresistance is an important obstacle for cancer therapy and is responsible for therapy failure, relapse, and death. Different factors contribute to chemoresistance including the tumour cells' heterogeneity, expression of drug efflux transporters, changes in drug targets, inhibition of apoptosis pathways, epigenetic modifications, and enhanced DNA-repair mechanisms (Figure 1.2) (reviewed in (Maleki Dana *et al.* 2022; Mansoori *et al.* 2017)).

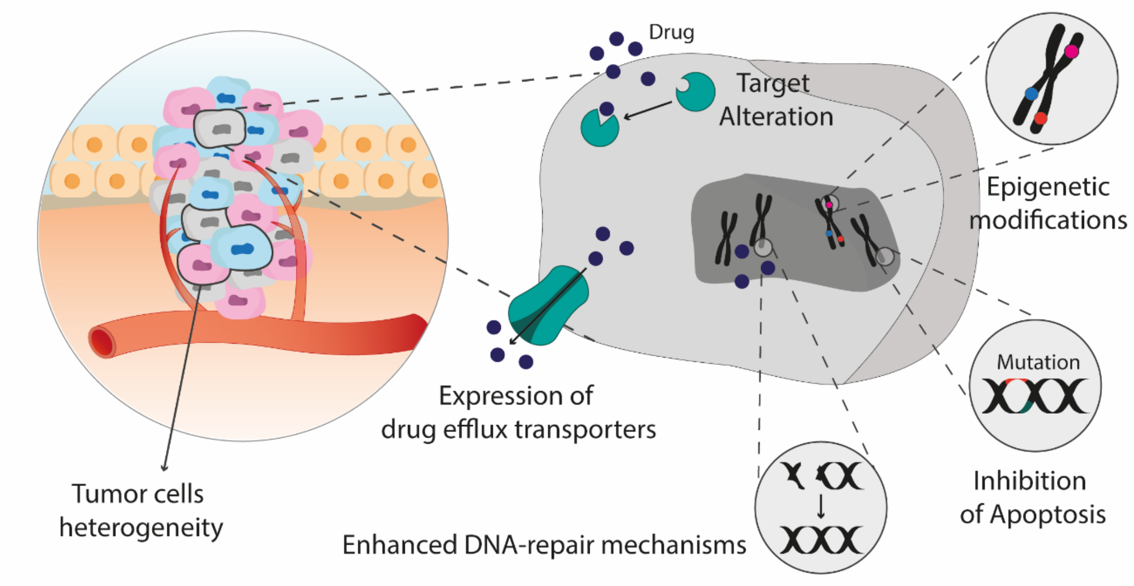


Figure 1.2 – Chemoresistance mechanisms in cancer. Chemoresistance is an important obstacle in cancer therapy and often results in therapy failure, relapse, and death. Cancer cells become resistant to therapeutic drugs by different mechanisms, such as the tumour cells' heterogeneity, expression of drug efflux transporters, changes in the target of drugs, epigenetic modifications, inhibition of apoptosis pathways, and enhanced DNA-repair mechanisms.

Besides interfering with cellular events possibly via TAS2R activation, bitter tasting compounds also seem to enhance the effects of many chemotherapeutic drugs. Among the bitter tasting compounds, epigallocatechin gallate, quercetin, and resveratrol have been extensively studied as co-adjuvant therapies in combination with chemotherapeutic drugs targeting different cancers, including breast, colorectal, lung cancers, and glioblastoma.

Epigallocatechin gallate

The combination of epigallocatechin gallate with different chemotherapeutic drugs has shown good outcomes in different cancers including glioblastoma (Xie *et al.* 2018), lung (Cromie *et al.* 2017; Datta & Sinha 2022), and colorectal cancers (La *et al.* 2019; Toden *et al.* 2016).

Epigallocatechin gallate decreased DNA-methyltransferase (MGMT) mRNA and protein expression, via the WNT/β-catenin pathway, which reversed glioblastoma-XD and T98G cells' resistance to TMZ, both MGMT-positive glioblastoma cell lines (Xie *et al.* 2018). Conversely, in non-tumour glial cells, epigallocatechin gallate enhanced MGMT expression by downregulating DNA (cytosine-5)-methyltransferase 1 and inhibiting the demethylation of MGMT promoter, showing anti-tumoral effects.

Epigallocatechin gallate also sensitizes lung cancer cells to gefitinib, increasing cell death in a model of non-small lung cancer through the regulation of the epidermal growth factor receptor (EGFR) and p53 pathways (Cromie *et al.* 2017). More recently, the effects of epigallocatechin gallate co-adjuvant treatment with doxorubicin were analysed in A549 cells, an *in vitro* model of lung cancer resistant to doxorubicin (Datta & Sinha 2022). Low doses of epigallocatechin gallate in combination with doxorubicin reversed A549 cells' resistance to doxorubicin by modulating the intracellular antioxidant machinery and inhibiting the doxorubicin efflux which increased its cytotoxic potential.

In the treatment of colorectal cancer, 5-fluorouracil is commonly used, but its efficacy decreases after long-term treatment resulting in acquired resistance (La *et al.* 2019). Epigallocatechin gallate sensitized colon cancer cells to 5-fluorouracil (La *et al.* 2019; Toden *et al.* 2016). Toden and colleagues showed that epigallocatechin gallate targets stem cancer cells, suppressing their formation by downregulating self-renewal pathways (Toden *et al.* 2016). In another study, epigallocatechin gallate inhibited ABCB1 expression through the activation of NF-kB/miR155-5p pathway allowing 5-fluorouracil to accumulate in colon cancer cells and enhancing its pro-apoptotic effects (↑ caspase-3, PARP, Bax; ↓ Bcl2) (La *et al.* 2019). Moreover, in the presence of epigallocatechin gallate, the IC50 values of 5-fluorouracil were significantly reduced showing the potential of epigallocatechin gallate to be used as a chemosensitizer in colorectal cancer treatment (La *et al.* 2019). Duarte and colleagues showed that TAS2R14 modulates the expression and function of ABC transporters in a cell line derived from a choroid plexus papilloma (Duarte *et al.* 2020). Since epigallocatechin gallate binds TAS2R14, its effects on ABCB1 expression might be also regulated via TAS2R14 activation.

Quercetin

Quercetin is a bioactive flavonoid, found in several vegetables and fruits, with remarkable anticancer effects (Table 1.2). Besides, quercetin's ability to reverse chemoresistance has been shown in different types of cancer, such as breast, prostate, and liver cancers (Z. Chen *et al.* 2018; Hassan *et al.* 2020; Koren Carmi *et al.* 2020; S. Li *et al.* 2018a; X. Lu *et al.* 2020b; Shu *et al.* 2018).

Ovarian cancer cells' resistance to platinum therapy was reversed by quercetin treatment in combination with platinum by restoring ERK phosphorylation and inducing cancer cells apoptosis (Koren Carmi *et al.* 2020). In another study, treatment with quercetin and the chemotherapeutics gemcitabine or doxorubicin upregulated the expression of p53 and cleaved caspase 3, increasing the apoptosis rate of pancreatic and hepatic cancer cells in comparison with chemotherapeutics alone (Hassan *et al.* 2020). In addition, the combinatory therapy downregulated HIF1α and inhibited the efflux activity of the ABCB1 transporter. Both HIF1α and ABCB1 are associated with resistance to chemotherapy, thus reinforcing the therapeutic potential of quercetin to reduce chemoresistance to gemcitabine and doxorubicin. Accordingly, quercetin sensitized a doxorubicin-resistant prostate cancer cell line (PC3/R) to doxorubicin by downregulating the tyrosine-protein kinase-met (c-met) and PI3K/AKT pathway, increasing cancer cells apoptosis (Shu *et al.* 2018). More recently, docetaxel-resistant prostate cancer cells

(PC3/R and LNCaP/R) treated with a combination of quercetin and docetaxel restored cancer cells' sensibility to docetaxel (X. Lu *et al.* 2020b). Quercetin decreased the activation of the PI3K/Akt pathway, which is upregulated in prostate cancer cells, resulting in decreased proliferation, tumorigenic potential, migration, and invasion, and increased apoptosis. Also, in xenograft tumours, quercetin and docetaxel treatment inhibited tumour growth and Ki67-positive (proliferative) cells.

Resveratrol

Resveratrol can sensitise cancer cells to chemotherapeutic drugs such as adriamycin (Zhang *et al.* 2019), paclitaxel (Cipolletti *et al.* 2019), gemcitabine (Cheng *et al.* 2018; Z. Zhou *et al.* 2019), 5-fluorouracil (Buhrmann *et al.* 2015; Chung *et al.* 2018), rapamycin (Alayev *et al.* 2015; Jin *et al.* 2018), cisplatin (Leon-Galicia *et al.* 2018; Martínez-Martínez *et al.* 2019), and temozolomide (H.C. Yang *et al.* 2019).

In breast cancer cells resistant to cisplatin (MCF-7R), resveratrol reversed the chemoresistance by enhancing the cytotoxic effects of cisplatin and significantly decreasing the IC50 values of this drug (Leon-Galicia *et al.* 2018). Cisplatin acts in cancer cells by inducing DNA damage, which is counteracted by enhanced DNA repair mechanisms in cancer cells contributing to chemoresistance. Interestingly, breast cancer cells treated with resveratrol and cisplatin showed downregulation of Rad51, which is associated with DNA repair. On the other hand, resveratrol potentiated the proapoptotic effects of paclitaxel in estrogen receptor (ER) α-positive breast cancer cells (MCF-7 and T47D), but not in ERα-negative cells, by decreasing the expression of neuroglobin, an antiapoptotic protein upregulated by 17β-estradiol (Cipolletti *et al.* 2019). Also, in breast cancer MCF-7 cells, resveratrol reversed chemoresistance to adriamycin as shown by the decreasing of IC50 values of adriamycin (Zhang *et al.* 2019). Moreover, resveratrol potentiated adriamycin chemotherapeutic effects including cell cycle arrest and apoptosis through the upregulation of miR-122-5p (Zhang *et al.* 2019). In another study with ERα-positive and ERα-negative breast cancer cells, the combination of resveratrol and rapamycin prevented the upregulation of Akt signalling and cellular autophagy, which is usually triggered by rapamycin and another mTOR complex 1 (mTORC1) inhibitors (Alayev *et al.* 2015). In addition, the combination of resveratrol and rapamycin inhibited the proliferation of breast cancer cells and induced their apoptosis.

In androgen-independent prostate cancer cells, the combination of resveratrol and cisplatin upregulated the expression of dual specificity phosphatase (DUSP1) playing an important role in the regulation of cell proliferation and apoptosis processes and is downregulated in prostate cancer (Martínez-Martínez *et al.* 2019). DUSP1 upregulation leads to the inhibition of the NF-κB pathway and decreases cyclooxygenase 2 (COX-2) expression. Moreover, resveratrol potentiated the cytotoxic effects of cisplatin in prostate cancer cells by a DUSP1-dependent mechanism. More recent findings showed that resveratrol sensitizes osteosarcoma cells to both chemotherapeutics cisplatin and doxorubicin, increasing their cytotoxic effects by reducing the viability of osteosarcoma cells (De Luca *et al.* 2022).

Gemcitabine is a chemotherapeutic drug commonly used in the treatment of pancreatic tumours but has been associated with chemoresistance due to the increase of cancer cell stemness (Z. Zhou *et al.* 2019). Resveratrol has been shown to sensitise pancreatic cancer cells to gemcitabine by enhancing gemcitabine apoptotic effects. Moreover, gemcitabine-induced-stemness was reversed by resveratrol through the inhibition of lipid synthesis and downregulation of sterol regulatory element-binding protein SREBP1.

In multiple myeloma cells, resveratrol in combination with rapamycin therapy was able to inhibit both mTORC1 and mTORC2 signalling pathways, while rapamycin alone only inhibited mTORC1 (Jin *et al.* 2018). The synergistic effect of resveratrol and rapamycin increased PARP and caspase-3 cleavage and downregulated retinoblastoma protein (pRb), resulting in enhanced apoptosis and inhibited proliferation of cancer cells.

In colorectal cancer cells, resveratrol potentiated the effects of 5-fluorouracil by showing a significant anti-proliferative and anti-invasiveness synergic effect (Buhrmann *et al.* 2015). Moreover, resveratrol reversed chemoresistance associated EMT in colon cancer cells by upregulating E-cadherin and claudin-2 and downregulating vimentin expression as well as decreasing NF-κB activation. In accordance, in another study, resveratrol and 5-fluorouracil combination increased apoptosis of colorectal cancer cells and inhibited cell proliferation and EMT (Chung *et al.* 2018). Besides, resveratrol effects were related to the inhibition of STAT3 (signal transducer and activator of transcription 3) and Akt signalling activation in colorectal cancer cells, resulting in decreased telomerase activity.

Temozolomide is used in glioma treatment, but patients frequently acquire chemoresistance (H.C. Yang *et al.* 2019). In the glioma cell lines T98G and U138, resveratrol reversed TMZ resistance by downregulating Wnt2 and β-catenin. The inhibition of the Wnt pathway by resveratrol downregulated the expression of MGMT, which enhanced the efficacy of TMZ, by increasing the apoptosis of glioma cancer cells (H.C. Yang *et al.* 2019).

The data from all these studies support the anti-cancer effects of epigallocatechin gallate, quercetin, and resveratrol as well as their potential as co-adjuvant anti-cancer therapies by sensitising cancer cells to several chemotherapeutics, with application in different types of cancer. Also, these bitter tasting compounds regulate many molecular and cellular processes that are deregulated in cancer and are associated with chemoresistance. Despite all the evidence showing bitter tasting compounds potential as chemosensitizers, the role of TAS2Rs in the regulation of these processes remains to be elucidated.

1.2.2.3 Bitter compounds can sensitise cancer cells to chemotherapeutics by regulating efflux transporters

The overexpression of efflux transporters in cancer cells is a common mechanism to deploy chemoresistance (Z. Chen *et al.* 2016; Mansoori *et al.* 2017). In particular, the ABC transporter family plays a crucial role by mediating multidrug resistance to many chemotherapeutics. Moreover, three members of the ABC family, the ABCB1 (or P-gP), the multidrug resistance-associated protein 2 (MRP2 or ABCC2), and the breast cancer resistance protein (BCRP or ABCG2) are frequently associated with chemoresistance. These efflux transporters are expressed in several tissues to protect cells from harmful compounds in circulation, such as xenobiotics, and avoid the accumulation of toxicants in cells. However, ABCB1, ABCC2, and ABCG2 substrates also include several anticancer drugs (e.g., doxorubicin, cisplatin, 5-fluorouracil) which leads to their low bioavailability in cancer cells. In addition, many of the ABCB1, ABCC2, and ABCG2 substrates overlap, contributing to enhanced resistance to cancer chemotherapies. Taking all these data into consideration, specific inhibitors of these transporters can be used as chemosensitizers (Z. Chen *et al.* 2016).

Interestingly, bitter tasting compounds including epigallocatechin gallate, quercetin, and resveratrol, but also others (e.g., genistein, naringenin, kaempferol) can be either substrates or inhibitors of ABC transporters (reviewed in (Y. Li *et al.* 2017)). Thus, bitter tasting compounds might play a critical role in the regulation of the expression and activity of ABC transporters and good targets to reverse chemoresistance.

In doxorubicin-resistant breast cancer MCF-7 cells, quercetin downregulated ABCB1 expression thereby enhancing the effects of the anti-cancer drugs doxorubicin, paclitaxel, and vincristine. Since doxorubicin is a substrate of the ABCB1 transporter, the downregulation of ABCB1 by quercetin enabled doxorubicin accumulation in cells (S. Li *et al.* 2018b). Additionally, quercetin also downregulated ABCC1 and ABCG2, which can also efflux doxorubicin, in breast cancer MCF-7 and MDA-231 cells (S. Li *et al.* 2018b). Similarly, in the multidrug-resistant cell line BEL/5-FU, a human hepatocellular carcinoma model, quercetin downregulated ABCB1, ABCC1, and ABCC2 expression sensitising the cells to chemotherapeutic drugs 5-fluorouracil, mitomycin C, and doxorubicin. The efflux pump activity of these transporters was inhibited by quercetin as demonstrated by the increase of their substrate's rhodamine-123 and doxorubicin intracellular accumulation after quercetin exposure (Z. Chen *et al.* 2018).

In breast cancer cells, resveratrol reduced resistance to doxorubicin by downregulating ABCB1 expression, contributing to the intracellular accumulation of the doxorubicin (Cooray *et al.* 2004; Huang *et al.* 2014). Similarly, in colon adenocarcinoma Caco-2 cells, resveratrol downregulated ABCB1, ABCC1, and ABCG2 also rendering these cancer cells more susceptible to doxorubicin (El-Readi *et al.* 2019).

In gastric carcinoma MGC-803 cells, genistein downregulated ABCC1, ABCC5, and ABCG2 expression (Huang *et al.* 2014), while in HepG2 cells it upregulated ABCB1 and ABCC2 expression (Rigalli *et al.* 2016). After three days of genistein administration in Wistar Han rats, hepatic ABCB1 expression increased as well as the biliary excretion of rhodamine-123 and digoxin, both well-known ABCB1 substrates (Semeniuk *et al.* 2020). In breast MCF-7 cells, genistein induced ABCC1 and ABCG2 expression, but only ABCC1 was upregulated in MDA-MB-231 cells (Rigalli *et al.* 2016).

Despite the individual ability of flavonoids to interact with ABC transporters, some reports indicate that a combined administration of flavonoids can enhance their bioavailability (Ravisankar *et al.* 2019; Redan *et al.* 2017). The permeability of Caco-2 cells to quercetin and apigenin was analysed in the presence of both compounds. It was observed that the extracellular concentration of quercetin and apigenin decreased and their permeability increased. Moreover, quercetin and apigenin acted synergistically to downregulate ABCB1, ABCC2, ABCC3, and

ABCG2 and inhibit ABCB1 ATPase activity (Ravisankar *et al.* 2019). Blackberry extract, containing epicatechin, kaempferol, and quercetin metabolites among other phenolic compounds, altered transport and metabolising systems in Caco-2 cells (Redan *et al.* 2017). Pre-treatment of Caco-2 cell monolayers with blackberry extracts decreased apical to basolateral transport of epicatechin, quercetin-3-O-glucoside, and kaempferol-7-O-glucoside. These results suggest that the regulation of other ABC transporters by their substrates might also depend on the activation of cognate receptors at the cell membranes.

The biological effects of bitter tasting compounds have been extensively studied in the last years, but the role of TAS2Rs as mediators of their actions has been overlooked. Therefore, considering that TAS2Rs are widely expressed in human tissues and are also found in cancer cells (Table 1.1), it is of utmost importance to explore the cellular actions deployed upon their activation. We recently showed that resveratrol upregulated ABCG2 expression and enhanced its efflux activity in a human choroid plexus papilloma cell line, via TAS2R14 activation (Duarte *et al.* 2020). Since ABCG2 is found in the basolateral membrane of the choroid plexus facing the cerebrospinal fluid, ABCG2 upregulation might contribute to the delivery of chemotherapeutics to brain cells. Similar studies using resveratrol and other bitter tasting compounds aiming to evaluate the role of TAS2Rs in the regulation of transport mechanisms, including ABC transporters expression and function, are crucial to develop novel strategies to overcome efflux transporters associated chemoresistance.

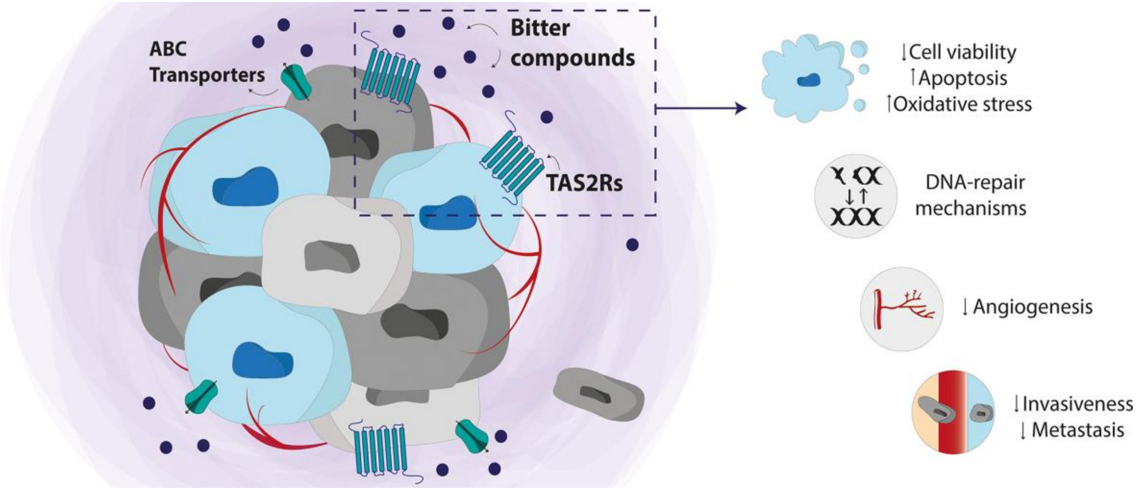
Overall, considering the ability of some bitter tasting compounds to increase drug accumulation on cancer cells and to enhance the effects of conventional anti-cancer drugs, bitter tasting compounds might be used in the future as co-adjuvant therapies in cancer.

Considering that bitter tasting compounds bind and activate TAS2Rs, whose expression has been found in a diversity of cancers, the role of TAS2Rs in cancer must be exploited in future studies. TAS2Rs activation by their bitter ligands might likely deploy cellular responses like the regulation of cell survival, apoptosis, oxidative stress, and other pathways that are imbalanced in cancer cells, and frequently associated with the action of bitter compounds.

Furthermore, the combination of bitter compounds such as epigallocatechin gallate, quercetin, and resveratrol with chemotherapeutics allows the use of lower doses of chemotherapeutic drugs in cancer therapy. Thus, co-adjuvant administration might decrease the side-effects of chemotherapy and reduce chemoresistance.

In the last years, several studies have investigated the molecular mechanisms behind the anti-cancer and chemosensitizer effects of bitter tasting compounds, reinforcing their therapeutic potential. Also, TAS2Rs might play a critical role in cancer treatment management since TAS2Rs polymorphisms have been linked to therapeutics efficacy. Additionally, TAS2Rs expression and functionality upon ligand binding has been addressed in recent studies, which will certainly provide important data to develop new and improved strategies for cancer therapy.

Despite the great advances on the state of the art on TAS2Rs functions in recent years, there are still several constraints limiting our understanding on the role of TAS2Rs in cancer, as well as in other diseases. Loss-of-function studies addressing the deletion of a specific TAS2R in *in vitro* and *in vivo* models are still missing and would be instrumental to enhance our understanding on their function. Alternatively, the identification or development of specific antagonists for each TAS2R would enable further studies elucidating their function in different cellular and animal models. Of note, Probenecid is the only bitter blocker commonly used, but it lacks receptor specificity and blocks TAS2R16, 38, and 43. Moreover, it interferes with ABC transporters activity (Greene *et al.* 2011). Additionally, there are only a few reliable antibodies targeting TAS2Rs, which presents another significant constraint for researchers studying TAS2Rs molecular mechanisms and therapeutic potential.



Graphical abstract

Table 1.2 – Anti-tumoral activity of bitter tasting compounds.

	Bitter agonist ***Human cognate TAS2Rs****	***Biological activity***	***Experimental model***	***References***
Bladder cancer	*Epigallocatechin gallate* *TAS2R39, TAS2R43*	Inhibited tumourspheres ↓ Stem cell markers ↓ Proliferation-associated proteins expression ↑ Apoptosis	Bladder CSCs	Sun *et al.* 2019
		↓ Viability and invasion ↑ Apoptosis	T24 cells	Feng *et al.* 2017
		↓ Proliferation, migration, and invasion ↑ Apoptosis: ↑ caspase-3, -8, -9, Bax, and PARP, and ↓ Bcl-2	SW780 cells	Luo *et al.* 2017
		↓ Tumour volume and weight ↓ NF-κB and MMP-9 mRNA and protein expression	Mice bearing SW780 tumours	
	Genistein *TAS2R14, TAS2R39*	Induced cell cycle arrest in G2/M phase ↓ Cyclin A and B1 ↑ Apoptosis: ↑ Caspase-3, -8, -9, PARP activation, cytosolic release of cytochrome *c*, and ↑ Bax/Bcl-2 ratio ↓ Mitochondria integrity Inactivated PI3K/Akt signalling pathway ↑ ROS accumulation	T24 cells	C. Park *et al.* 2019
	Kaempferol *TAS2R14, TAS2R39*	Promoted DNA methylation Promoted ubiquitin proteasome degradation	Nude mice bearing bladder cancer	Qiu *et al.* 2017
Breast cancer	*Brucine* *TAS2R10, TAS2R46*	↓ Proliferation, migration and invasion ↑ Apoptosis ↓ Angiogenesis and vasculogenic mimicry tube formation Cytoskeleton and microtubule disruption Downregulation of MMP-2 and -9	MDA-MB-231 cells	Xu *et al.* 2019
		↓ Migration, invasion, adhesion ↑ E-cadherin and β-catenin ↓ Vimentin and fibronectin ↓ MMP-2 and -9	Hs578-T and MDA-MB-231 cells	M. Li *et al.* 2018
		↓ Viability and migration ↓ Anchorage-independent growth	MCF7 cells	K.F. Hu *et al.* 2017

Epigallocatechin gallate *TAS2R39, TAS2R43*	↓ Viability ↑ Apoptosis: ↑ PARP, pro-caspase-3 and -9 Induced cell cycle arrest in G2/M phase	MCF7 cells	Zan *et al.* 2019
	Tumour growth suppression Downregulation of proteins associated with apoptosis ↓ Proliferation: ↓ Ki-67 expression	Female CB-17 severe combined immunodeficient mice	
	↓ Viability and proliferation Anticarcinogenic effect	MCF7 and MDA-MB-231 cells	Schröder *et al.* 2019
	↓ Growth ↑ Apoptosis: ↑ caspase-3, -8 and -9 ↑ Autophagy ↑ Mitochondrial depolarization Inhibited glycolytic enzyme activities and mRNA levels ↓ HIF1α and GLUT1 expression	4T1 cells	R. Wei *et al.* 2018
	↓ Tumour weight ↓ Glucose and lactic acid levels ↓ VEGF expression	BABL/C mice injected subcutaneously with 4T1 cells	
	↑ ROS production Changes in nuclear morphology ↓ Viability ↓ Phosphorylation of several proliferation and survival proteins	SK-BR-3 cells	Filippi *et al.* 2018
	↓ Viability ↓ β-catenin, pAkt and cyclin D1 Inactivated β-catenin signalling pathway	MDA-MB-231 cells	Hong *et al.* 2017
	↓ Viability ↑ Caspase-3, and -9, Bax/Bcl-2 ratio ↓ Akt ↓ ERα protein levels	T47D cells	Moradzadeh *et al.* 2017; Hallman *et al.* 2017
Kaempferol *TAS2R14, TAS2R39*	↓ Proliferation ↑ Apoptosis: ↓ Bcl2, and ↑ Bax ↓ p-ERK1/2	BT474 and ZR-75-30 cells	G. Hu *et al.* 2019
	↓ Proliferation	MDA-MB-231 cells	Zhu & Xue

	Induced cell cycle arrest in G2/M phase ↑ Apoptosis: caspase-3 and -9 cleavage ↑ DNA damage		2019
	↓ Migration and invasion Blocked MAPK cascade ↓ MMPs expression and activity	MDA-MB-231 and MDA-MB-453 cells	S. Li *et al.* 2017
Naringenin *TAS2R14*	↓ Growth and ↑ death ↑ pAMPK ↓ Cyclin D1	E0771 cells	Ke *et al.* 2017
	Delayed tumour growth	OVX C57BL/6 mice injected with E0771 cells	Lim *et al.* 2017
Parthenolide *TAS2R1, TAS2R4, TAS2R8, TAS2R10, TAS2R14, TAS2R44, TAS2R46*	Elongation factor1-α and vimentin suppression	MCF7 cells	Jafari *et al.* 2018
	↓ Nrf2 expression ↓ CAT and Bcl-2 ↑ ROS Chemoresistance prevention	MDA-MB-231 cells	Carlisi *et al.* 2017
Quercetin *TAS2R14*	↓ Proliferation ↑ Apoptosis: ↓ ERα, Cyclin D1, Bcl-2, and ↑ Bax Induced cell cycle arrest at G1 phase Inhibited CSCs proliferation, clone formation, and mammosphere generation through PI3K/Akt/mTOR signalling	MCF7 and breast CSCs ($CD44^+/CD24^-$)	X. Li *et al.* 2018
	Inhibited tumour growth and metastatic ability of CSCs	$CD44^+/CD24^-$ CSCs xenograft mice model	
	↓ Proliferation, migration and invasion ↓ MMP-2, -9 and VEGF expression Inhibited glycolysis, ↓ glucose uptake and lactic acid production Induced autophagy via Akt/mTOR	MCF7 and MDA-MB-231 cells	Jia *et al.* 2018
	↓ Tumour growth and metastasis ↓ VEGF Inhibited glycolysis Induced autophagy by inhibiting p-Akt/Akt	MCF7 xenograft mice model	

Resveratrol TAS2R14, TAS2R39	Modulated epigenetic factors ↓ Viability and induces apoptosis ↑ BRCA1, p53 and p21 ↓ Methyl-CpG binding proteins Combined with rapamycin: Prevented Akt upregulation and autophagy Inhibited mTOR signalling Inhibited cell growth and induced apoptosis: ↑ PARP cleavage	MCF7 and MDA-MB-231 cells	Chatterjee *et al.* 2019; Izquierdo-Torres *et al.* 2019
	Induced premature senescence ↑ p21 and p53 ↓ Proliferation, migration, and invasion Supressed EMT: ↑ E-cadherin and γ-catenin, ↓ N-cadherin and vimentin	MCF7 cells	(K. Y. Chen et al. 2019)
	↓ Proliferation and colony formation Enhanced paclitaxel and herceptin apoptotic effects: ↑ PARP cleavage and ↓ Bcl-xL ERK1/2 pathway inhibition and ↓ pAkt Impaired E2/ERα pathway	MCF7 and T47D cells	K.Y. Chen *et al.* 2019; Cipolletti *et al.* 2019
	↓ Migration Reversed TGF-β1 induced EMT through PI3K/Akt signalling: ↓ MMP-2 and -9, p-PI3K, p-Akt, vimentin, and ↑ E-cadherin	MDA-MB-231 cells	Sun *et al.* 2019
	↓ Tumour weight and growth ↓ Lung metastasis	MDA-MB-231 xenograft mice model	
	↓ Proliferation Induced cell cycle arrest at S phase ↑ Apoptosis Induced changes in cell cycle related genes	4T1 cells	Wu *et al.* 2019
	Induced chemosensitivity ↓ Viability ↑ Apoptosis: ↓ Bcl-2 Induced cell cycle arrest at G1 phase	MCF7-ADR	Zhang *et al.* 2019
	↓ Proliferation, migration, and invasion ↓ Cyclin D1, c-Myc, MMP-2 and -9 ↓ pAkt and pSTAT3	MCF7-CAF-CM and MDA-MB-231 cells	Suh *et al.* 2018

		Suppressed stemness properties and ↓ self-renewal signalling		
Cervical cancer	*Kaempferol* *TAS2R14, TAS2R39*	↓ Viability Inhibited telomerase and PI3K/Akt signalling pathway ↑ Apoptosis via p53 and Bax/Bcl-2	HeLa cells	Kashafi *et al.* 2017
	Resveratrol *TAS2R14, TAS2R39*	↓ Proliferation ↑ Apoptosis: ↑ caspase-3, -9, Bax, p53 and ↓ Bcl-2, Bcl-xL ↓ Cyclin B1	HeLa cells	L. Li *et al.* 2018
Colorectal cancer	*Brucine* *TAS2R10, TAS2R46*	↓ Growth and ↑ apoptosis Activated Wnt/β-catenin signalling pathway	DLD1, LoVo and SW480 cells	H. Ren *et al.* 2019
		↓ Tumour volume and weight	DLD1-bearing nude mice	
		↓ Proliferation, migration and colony formation ↑ Apoptosis ↓ MMP-2 and -3 Inhibited Wnt/β-catenin signalling pathway	LoVo cells	X. Shi *et al.* 2018
		↓ Tumour growth	Male immune-deficient BABL/C nude mice	
	Epigallocatechin gallate *TAS2R39, TAS2R43*	↓ Growth Induced cell cycle arrest in G2/M phase ↑ Apoptosis via the mitochondrial pathway ↓ Migration, invasion, and adhesion ↓ MMP-2 and -9 activity Inhibited MAPK and PI3K/Akt signalling pathways activation	Caco-2, HCT116, HT-29, LoVo, and SW480 cells	W. Zhu *et al.* 2020
		↑ Apoptosis: ↑ caspase-3, PARP, Bad, and ↓ Bcl-2 DNA damage ↓ GRP78 expression Activated NF-κB ↓ ABCB1 gene expression and blocked drug efflux	DLD1 and HCT116 cells	La *et al.* 2019
		↓ Growth Induced cell cycle arrest at G1 and G2 phases ↓ Cyclin E and D1 ↑ p21	HCT116 cells	Ding *et al.* 2018
		↑ Chromosomal instability ↑ Apoptosis and inhibited cell division	COLO 205	Ni *et al.* 2018

	↓ Solid tumours Inhibited carcinogenesis ↓ Number of precancerous lesions ↓ Tumour load Histological progression delay	Colon carcinogenesis mouse model	X. Wang *et al.* 2017
	Inhibited spheroid formation ↓ Proliferation and ↑ apoptosis ↓ Wnt/β-catenin pathway activation	Colorectal CSCs	Y. Chen *et al.* 2017
Parthenolide *TAS2R1, TAS2R4, TAS2R8, TAS2R10, TAS2R14, TAS2R44, TAS2R46*	Inhibited deubiquitinating enzymes Inhibited Wnt signalling, partly by destabilizing β-catenin ↓ Proliferation and ↑ apoptosis	Caco2, HCT116, HT-29, SW480 and SW620 cells	X. Li *et al.* 2020
	Inhibited TGF-β1-induced cell migration and invasion ↓ β-catenin, Vimentin, and ↑ E-cadherin	HT-29 and SW480 cells	S.M. Zhu *et al.* 2019
	Inhibited HIF1α activity: ↓ proteins associated with glucose metabolism, angiogenesis and survival ↓ Angiogenesis by preventing NF-κB activation Protected the morphological change from EMT state Inhibited MMP activity ↓ Cell motility	DLD-1, HCT116 and HT-29 cells	S.L. Kim *et al.* 2017
	↓ Colorectal carcinoma xenograft growth Regulated NF-κB, HIF1α and EMT specific marker	Nude mouse tumour xenograft model	
	↓ Proliferation ↑ Apoptosis: ↓ Bcl-2, Bcl-xL, and ↑ activated caspase-3 Suppressed migration/invasion-related protein expression: E-cadherin, β-catenin, vimentin, COX-2, MMP-2, MMP-9	SW620 cells	Y.C. Liu *et al.* 2017
Quercetin *TAS2R14*	↓ Tumour incidence and volume Suppressed DNA damage and induced DNA repair ↓ ROS levels and lipid and protein peroxidation ↑ CAT, SOD expression and activity ↑ Nrf2	Wistar Han colorectal cancer model (dimethylhydrazine-induced)	Darband *et al.* 2020
Resveratrol *TAS2R14, TAS2R39*	↓ Viability ↑ Apoptosis: ↑ p53, ↑ cleaved caspase-3 and PARP	CO115, HCT116 and SW48 cells	Z. Liu *et al.* 2019
	Enhanced 5-flurouracil effects: ↓ Growth and proliferation and ↑ apoptosis	DLD-1 and HCT116 cells	Chung *et al.* 2018

		Induced cell cycle arrest at S phase Supressed EMT: ↓ vimentin ↓ Cell stemness Inhibited Akt and STAT3 activation ↑ Anti-telomerase activity		
Gastric cancer	*Luteolin* *TAS2R14, TAS2R39*	↑ Apoptosis: ↑ caspase-3, -9, cytochrome *c*, Bax/Bcl-2 ratio Suppressed PI3K (↓ p-PI3K, p-Akt and p-mTOR) and MAPK (↓ p-ERK1/2) pathways	BGC-823 cells	X. Lu *et al.* 2017
	Parthenolide *TAS2R1, TAS2R4, TAS2R8, TAS2R10, TAS2R14, TAS2R44, TAS2R46*	↓ Migration and invasion ↑ Apoptosis: ↑ Bax, p53, cleaved caspase-3, -9, and ↓ Bcl-2, Bcl-xL Induced cell cycle arrest in G1 phase ↓ Cyclin D1 Inhibited STAT3 activation	SGC-7901/DDP cisplatin-resistant cells	H. Li *et al.* 2018
	Quercetin *TAS2R14*	↓ Proliferation Alleviated side effects of chemotherapeutic drug SN-38	AGS cells	Lei *et al.* 2018
		Enhanced irinotecan chemotherapeutic effects: ↓ Tumour size Inhibited angiogenesis in tumour tissue ↓ COX-2 and restored E-cadherin expression	AGS xenograft mice model	
Glioblastoma	*Epigallocatechin gallate* *TAS2R39, TAS2R43*	DNA damage ↑ Telomere-shortening-induced senescence ↑ Telomere-independent genotoxicity	U-251 cells	Udroiu *et al.* 2019
		↓ MGMT mRNA and protein expression Reversed TMZ resistance via the Wnt/β-catenin pathway Prevented β-catenin translocation into the nucleus ↓ Transcription factors	GBM-XD and T98G (MGMT-positive)	Xie *et al.* 2018
		↑ Accumulation of autophagic vacuoles ↑ ROS production Sensitized cells to TMZ	Primary cells	Grube *et al.* 2018
	Luteolin *TAS2R14, TAS2R39*	↓ Proliferation Induced cell cycle arrest in S and G2/M phases ↓ pAkt, p-mTOR, p-MAPK ↑ Apoptosis: ↑ caspase, PARP cleavage, and ↓ Bcl-xL	U-251 and U-87 cells	Anson *et al.* 2018

	Quercetin TAS2R14	Inhibited autophagy ↑ Apoptosis: morphological alterations, ↑ Bax, caspase-3, and ↓ Bcl-2, PARP	Rat C6 and human T98G cells	Taylor *et al.* 2019
	Resveratrol TAS2R14, TAS2R39	Reversed TGF-β1 induced EMT: ↑ E-cadherin and ↓ N-cadherin, vimentin, β-catenin ↓ Migration and invasion ↓ MMP-2 and -9 Suppressed stem cell-like properties	LN18 and U-87 cells	Song *et al.* 2019
		Reversed TGF-β1 induced EMT: ↓ N-cadherin, Vimentin	U-87 xenograft mice model	
		Enhanced TMZ effects: ↓ Proliferation and growth ↑ Apoptosis: ↑ cleaved caspase-3, Bax, and ↓ Bcl-2 Inhibited Wnt signalling: ↓ Wnt2, MGMT and β-catenin	T98G and U-138	H.C. Yang *et al.* 2019
		Enhanced TMZ effects: ↓ Tumour volume and growth Inhibited Wnt signalling pathway: ↓ MGMT and β-catenin ↑ Apoptosis	T98G xenograft mice model	
Leukaemia	*Epigallocatechin gallate* TAS2R39, TAS2R43	↓ Proliferation ↑ Apoptosis: ↑ caspase-3 positive cells ↑ Fas expression	Jurkat cells	Ghasemi-Pirbaluti *et al.* 2018
	Genistein TAS2R14, TAS2R39	Induced cell morphological changes ↓ Viability Induced G2/M phase arrest ↑ Apoptosis: ↑ caspase-3, -4, -7, -9, Bax, PARP-cleavage, and ↓ Bcl-2, Bid ↑ ROS and Ca^{2+} production	HL-60 cells	Hsiao *et al.* 2019
		↓ Tumour weight ↑ GRP78, Bax, Bad, Bak	BABL/C nu/nu mice	
	Kaempferol TAS2R14, TAS2R39	↓ Viability ↑ Sub-G1 population ↑ Apoptosis: ↑ caspase-3, Bax/Bcl-2 ratio, and ↓ Bcl-2 ↓ Akt, ABCB1 and ABCC1 genes Inhibited multidrug resistance	HL-60 and NB4 cells	Moradzadeh *et al.* 2018
	Luteolin	Induced PARP cleavage and nuclear fragmentation ↑ Fas and FasL expression	HL-60 cells	S.W. Wang *et*

	TAS2R14, TAS2R39	↑ Caspase-8 and -3 activation ↑ Histone H3 acetylation		*al.* 2018
	Quercetin *TAS2R14*	↓ Proliferation Induced cell cycle arrest at G0/G1 phase ↑ Apoptosis: condensed chromatin and nuclear fragmentation, ↑ PARP cleavage, caspase-3, -8, -9 activation, Bax, Bak, cytochrome *c* release and ↓ Bcl-xL Induced autophagy ↑ ROS levels	HL-60 cells	Chang *et al.* 2017; W.J. Lee *et al.* 2015
		↓ Tumour volume ↑ Apoptosis: ↑ PARP and caspase-3 cleavage ↑ ROS levels ↓ Proliferation: ↓ Ki-67 positive cells	HL-60 xenograft mice model	
	Resveratrol *TAS2R14, TAS2R39*	↓ Proliferation ↑ Apoptosis: ↑ Bax/Bcl-2 ratio, cleaved caspase-3, -8 Induced autophagy Inhibited PI3K/Akt and activated AMPK/mTOR pathway	HL-60 cells	Y. Fan *et al.* 2018
Liver cancer	*Epicatechin* *TAS2R4, TAS2R5, TAS2R39*	↓ Viability and ↑ apoptosis Induced cell cycle arrest MMP dissipation ↓ Fatty acid levels ↑ ROS production	HepG2 cells	Khiewkamrop *et al.* 2018
	Epigallocatechin gallate *TAS2R39, TAS2R43*	Improved serum liver markers ↓ Tumour formation ↓ Expression of genes associated with high cancer risk Inhibited fibrosis progression Induced senescence-associated to secretory phenotype	Male Wistar rats with induced hepatocellular carcinoma	Sojoodi *et al.* 2020
		Radio-sensitized cells ↑ Cell apoptosis: ↓ Bcl-2, and ↑ Bax and caspase-3	H22 cells	Kang *et al.* 2019
		Inhibited migration ↓ Viability ↑ Apoptosis: ↑ Bax/Bcl-2 Induced cell cycle arrest MMP dissipation ↓ Fatty acid levels	HepG2 cells	H. Yang *et al.* 2019; Khiewkamrop *et al.* 2018

		↑ ROS production		
	Kaempferol *TAS2R14, TAS2R39*	Induced selective cytotoxicity Mitochondrial swelling ↑ Apoptosis: release of cytochrome *c*, ↑ caspase-3 activation ↑ ROS production	DEN+2-AAF-induced hepatocellular carcinoma in rats	Seydi *et al.* 2018
	Luteolin *TAS2R14, TAS2R39*	↓ Viability Induced cell cycle arrest in G0/G1 phases ↑ Apoptosis: ↑ caspase-8 and ↓ Bcl-2 ↑ Number of intracellular autophagosomes	SMMC-7721 cells	Cao *et al.* 2018
	Naringenin *TAS2R14*	Induced cell cycle arrest in G0/G1 and G2/M phase ↑ p53 ↑ Apoptosis: nuclei damage, ↑ Bax/Bcl-2 ratio, cytochrome *c* release, caspase-3 activation	HepG2 cells	Hernández-Aquino & Muriel 2018
Lung cancer	*Epigallocatechin gallate* *TAS2R39, TAS2R43*	Inhibition of tumour sphere formation AXL receptor tyrosine kinase highly expression	H1299-sdCSCs	Namiki *et al.* 2020
		↓ Proliferation and ↑ apoptosis ↓ p-PI3K and p-Akt	H1299 cells	Gu *et al.* 2018
		↓ Tumour cells activity Inhibited tumoursphere formation ↓ Cancer stem cells markers ↓ Proliferation and ↑ apoptosis ↓ Wnt/β-catenin activation	A549 and H1299 cells	J. Zhu *et al.* 2017
	Parthenolide *TAS2R1, TAS2R4, TAS2R8, TAS2R10, TAS2R14, TAS2R44, TAS2R46*	Inhibited lung cancer cells ↓ Proliferation stimulating effect of nicotine ↑ Apoptosis: ↓ Bcl-2 and ↑ p53, Bax, caspase-3, -7, -8, -9 VEGF-inhibiting effects	A549 and H526 cells	Talib & Al Kury 2018
		↓ Viability and ↑ apoptosis ↓ Proliferation and invasion Inhibited MAPK/ERK pathway and STAT3 activity	GLC-82 cells	Lin *et al.* 2017
	Resveratrol *TAS2R14, TAS2R39*	Induced premature senescence ↑ p21 and p53 ↓ Proliferation, migration, and invasion Supressed EMT: ↑ E-cadherin and γ-catenin, ↓ N-cadherin and vimentin	A549 cells	K.Y. Chen *et al.* 2019; Fudhaili *et al.* 2019

Ovarian cancer	*Genistein* *TAS2R14, TAS2R39*	↓ Tumour incidence ↓ Number and size of tumours ↓ NF-κB and Bcl-2 expression ↑ Nrf2 and Bax expression ↓ mTOR pathway	Laying hens ovarian cancer model	Sahin *et al.* 2019
	Kaempferol *TAS2R14, TAS2R39*	Induced cell cycle arrest in G2/M phase Stimulated the extrinsic apoptosis ↑ p53	A2780/CP70 cells	Gao *et al.* 2018
		↑ Apoptosis: ↑ caspase-3, -8, -9, Bax, and ↓ Bcl-xL, Bcl-2 ↑ ERK1/2, p38	OVCAR-3 and SKOV-3 cells	Zhao *et al.* 2017
	Quercetin *TAS2R14*	↓ Viability ↑ Apoptosis: ↓ Bcl-2, Bcl-xL, and ↑ caspase-3, -9, Bid, Bad, Bax and cytochrome *c*	PA-1 cells	Teekaraman *et al.* 2019
Pancreatic cancer	*Epigallocatechin gallate* *TAS2R39, TAS2R43*	↓ Cell growth, migration and invasion "Cadherin switch" prevention ↓ Expression level of β-Catenin, and Vimentin Inhibited Akt pathway	BxPC-3, CFPAC-1, HPAF-II, MIA PaCa-2, Panc-1 and Su.86.86 cells	R. Wei *et al.* 2019
	Naringenin *TAS2R14*	↑ Apoptosis: ↑ p38 and p53 ↑ ROS levels	SNU-213 cells	H.J. Park *et al.* 2017
	Parthenolide *TAS2R1, TAS2R4, TAS2R8, TAS2R10, TAS2R14, TAS2R44, TAS2R46*	↓ Growth and ↑ apoptosis ↑ Autophagy	BxPC3 and Panc-1 cells	W. Liu *et al.* 2017
	Quercetin *TAS2R14*	↓ Proliferation, migration, and invasion Inhibited EMT: ↑ E-cadherin and ↓ N-cadherin, Vimentin ↓ MMP-2 and -7 secretion ↓ p-STAT3	Panc-1 and PATU-8988 cells	Yu *et al.* 2017
	Resveratrol *TAS2R14, TAS2R39*	↓ Growth and colony formation ↑ ROS ↑ Nrf2 expression and Nrf2 nuclear translocation ↑ Apoptosis: ↑ Bax and ↓ Bcl-2	MIA PaCa-2 and Panc-1 cells	Cheng *et al.* 2018
		↓ Proliferation	Human pancreatic stellate cells and	Yan *et al.* 2018

		Inhibited ROS-induced cell activation, invasion, migration, and glycolysis	Panc-1	
Prostate cancer	*Kaempferol* *TAS2R14, TAS2R39*	↓ Proliferation and ↑ apoptosis ↓ DHT-induced AR activation ↓ AR downstream targets ↓ AR protein expression and nuclear accumulation Suppressed vasculogenic mimicry of PC3 cells	LNCaP and PC3 cells	Da *et al.* 2019
	Naringenin *TAS2R14*	↓ Proliferation and migration ↑ Apoptosis: ↑ Bax/Bcl-2 ratio (PC3) ↑ ROS production ↓ ERK1/2 ↑ pAkt	LNCaP and PC3 cells	W. Lim *et al.* 2017
	Parthenolide *TAS2R1, TAS2R4, TAS2R8, TAS2R10, TAS2R14, TAS2R44, TAS2R46*	↓ Growth Inhibit NF-κB activation	C4-2B4, DU145, LNCaP, Mat-Ly-Lu, PC3 and RM1-BT cells	Marino *et al.* 2019
	Quercetin *TAS2R14*	↓ Proliferation, migration, and invasion ↑ Apoptosis: ↑ Bax, Bcl-2	PC3 cells	X. Lu *et al.* 2020b
		Inhibited tumour growth, EMT process and PI3K/Akt signalling ↓ Tumour weight and volume ↓ Ki-67 expression ↑ E-cadherin and ↓ N-cadherin	PC3 xenograft mice model	
		↓ Proliferation and stem cells spheroid formation Inhibited PI3K/Akt and MAPK/ERK pathways	CSCs, LNCaP and PC3 cells	Erdogan *et al.* 2018
	Resveratrol *TAS2R14, TAS2R39*	↓ Viability and proliferation Induced lysosomal degradation Inhibited NF-κB pathway Suppressed EMT: ↑ E-cadherin and ↓ vimentin ↓ COX-2 Sensitizes cells to cisplatin pro-apoptotic effects	DU145 and PC3 cells	Khusbu *et al.* 2020; Martínez-Martínez *et al.* 2019
		Inhibited cell growth under hypoxic conditions Cell cycle arrest at G0/G1 phase Prevented HIF1α stabilization	PC3 cells	Fonseca *et al.* 2019

		↓ Glucose uptake		
Skin	*Epigallocatechin gallate* TAS2R39, TAS2R43	↑ ROS production Changes in nuclear morphology ↓ Viability ↓ Phosphorylation of several proteins involved in cell proliferation and survival.	A431 cells	Filippi *et al.* 2018
	Luteolin TAS2R14, TAS2R39	Supressed Src/STAT3 signalling ↓ Migration and invasion ↑ E-cadherin Blocked Akt/mTOR/c-Myc signalling pathway	A431-III cells	J.J. Fan *et al.* 2019; K.C. Chen *et al.* 2018
		↓ Proliferation, migration, invasion, and adhesion Inhibited tube-forming potential Suppressed EMT (↑ E-cadherin, ↓ N-cadherin and vimentin) ↓ p-Akt, HIF1α, VEGF, MMP-2 and -9 protein levels	A375 and B16-F10 cells	C. Li *et al.* 2019
	Quercetin TAS2R14	↓ p-STAT3 ↓ Migration and invasion ↑ E-cadherin Blocked Akt/mTOR/c-Myc signalling pathway	A431-III cells	J.J. Fan *et al.* 2019; K.C. Chen *et al.* 2018
		↓ Viability and proliferation ↑ Apoptosis: chromatin condensation, ↑ Bax, pERK1/2, cleaved PARP, and ↓ Bcl-2	A375SM cells	S.H. Kim *et al.* 2019
		↓ Tumour volume ↑ Apoptosis	A375SM melanoma xenograft	
Thyroid cancer	*Epigallocatechin gallate* TAS2R39, TAS2R43	Suppressed EMT, invasion and migration Inhibited TGF-β1-induced expression of EMT markers (↓ E-cadherin and ↑ vimentin)	8505C cells	T. Li *et al.* 2019

ABC: ATP binding cassette; AMPK: AMP-activated protein kinase; AR: androgen receptor; Bad: Bcl-2-associated death promoter; Bak: Bcl-2 antagonist/killer; Bax: Bcl-2-associated X protein; BRCA: breast cancer gene; CAT: catalase; COX: cyclooxygenase; DEN+2-AAF: diethylnitrosamine (DEN) and 2-acetyl-aminofluorene (2-AAF); CSC: cancer stem cell; DHT: dihydrotestosterone; EMT: epithelial–mesenchymal transition; ER: estrogen receptor; ERK: extracellular signal-regulated kinase; GLUT: glucose transporter; GRP78: glucose-regulated protein 78; HIF: hypoxia-induced factor; MAPK: mitogen-activated protein kinase; MGMT: methylguanine DNA methyltransferase; MMP: matrix metalloproteinase; mTOR: mammalian target of rapamycin; NF-κB: factor nuclear kappa B; Nrf2: nuclear factor erythroid 2–related factor 2; OVX: ovariectomized; PARP: poly(ADP-ribose) polymerase; PI3K: phosphoinositide 3-kinase; ROS: reactive oxygen species; SOD: superoxide dismutase; STAT: signal transducer and activator of transcription; TAS2R: bitter taste receptor; TGF: transforming growth factor; TMZ: temozolomide; VEGF: vascular endothelial growth factor. *Cognate TAS2Rs for the bitter tasting compounds used in the studies according to the BitterDB database (https://bitterdb.agri.huji.ac.il/dbbitter.php); note that the role of the receptors was not analysed in these studies.

1.3 Sweet taste signalling in cancer

During the various stages of the development of a human tumour, cancer cells acquire a set of biological capabilities, called hallmarks of cancer (Figure 1.3), which allow them to meet the biological needs to survive and proliferate (DeBerardinis & Chandel 2016; Hanahan & Weinberg 2011). One of these hallmarks is the metabolic reprogramming which enables tumour cells to absorb abundant nutrients to produce ATP, generate biosynthetic precursors and macromolecules, and tolerate malignancy-associated stress such as redox reactions and hypoxia (DeBerardinis & Chandel 2016).

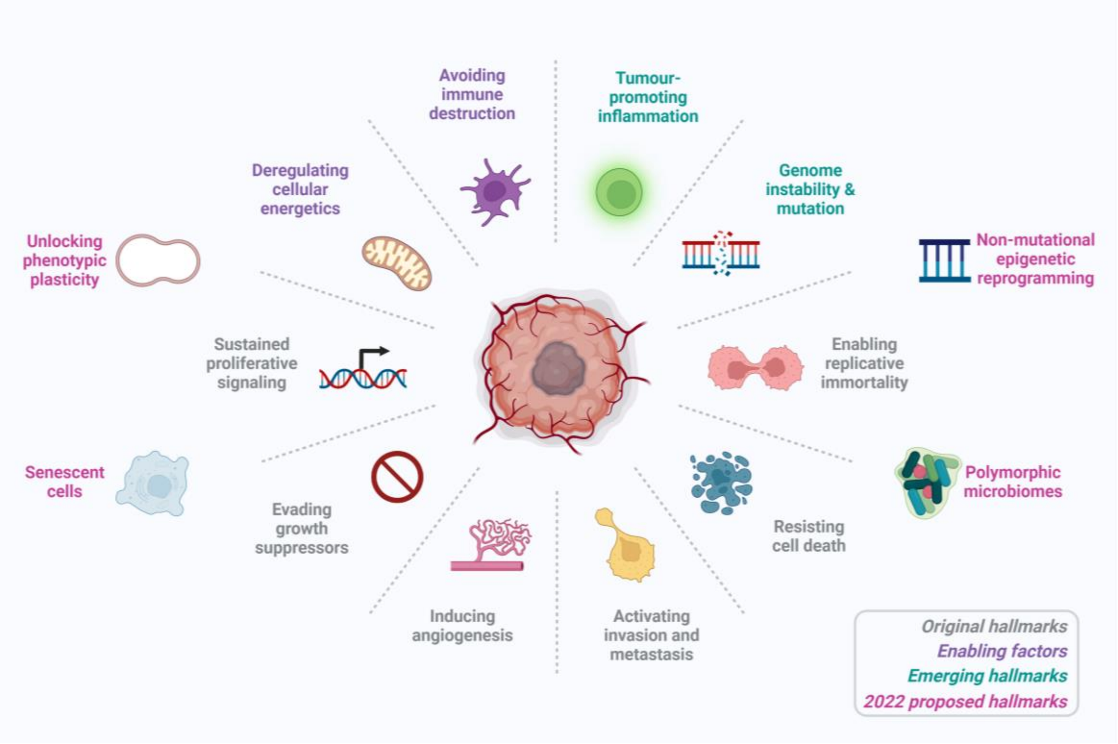


Figure 1.3 – Hallmarks of cancer. During the various stages of tumour development, cancer cells acquire a set of biological capabilities, called hallmarks of cancer, which are in constant updating since new evidence may be added to more fully understanding the complexities, mechanisms, and manifestations of the disease. The first proposal was launched in 2000 and comprised six acquired capabilities. Later, in 2011, two emerging hallmarks and two enabling characteristics were embodied. Recently, new hallmarks and enabling characteristics have been proposed: unlocking phenotypic plasticity, senescent cells, polymorphic microbiomes, and non-mutational epigenetic reprogramming.

In particular, an important hallmark of cancer is the specificity of its energetic metabolism (Hanahan & Weinberg 2011; Pavlova & Thompson 2016). In a subset of cancer cells, glycolysis prevails over oxidative phosphorylation as the main source of energy, even when oxygen is available. This metabolic switch, first observed by Otto Warburg and known as Warburg effect, favours the large demand of nucleotides, amino acids and lipids essential for cell proliferation, and is enhanced by the reduced oxygen and glucose availability in the tumour microenvironment, and largely ensured by increased uptake of glucose and glutamine for cancer cells' survival (Liberti & Locasale 2016). Concurrently, the activation of mitochondrial activity necessary to force glycolytic tumour cells to enter oxidative phosphorylation can effectively sensitize glioblastoma cells to radiotherapy (Pavlova & Thompson 2016). In several types of cancer, reversal of the Warburg effect leads to a reduction in tumour size and aggressiveness (Yuen *et al.* 2016). The inhibition of Warburg effect, by the glycolysis inhibitor 2-deoxyglucose, has also been studied in clinical trials for the treatment of glioblastoma, prostate cancer, and other solid tumours (Mohanti *et al.* 1996; Raez *et al.* 2013; Stein *et al.* 2010). Thus, the identification of potential players mediating the crosstalk between cell proliferation, invasion, hypoxia, and glucose metabolism in cancer is of interest for drug development.

The receptor for sweet compounds (sweet taste receptor; STR) is an important sensor of the availability and regulator of glucose metabolism in several organs, with a recognized role in the regulation of glucose uptake in normal cells of the intestine and brain, where its signalling controls the rate of glucose absorption and metabolism (Smith *et al.* 2018). As mentioned before, this receptor belongs to the GPCR superfamily and is constituted by a heterodimer of two subunits – TAS1R2 and TAS1R3. The STR binds various sugars (glucose, maltose, sucrose and fructose), artificial sweeteners (saccharin, aspartame, cyclamate) and sweet amino acids (D-tryptophan, D-phenylalanine, D-serine) (Santos *et al.* 2019).

Although it is clear that STR is an important glucosensor, with a recognised role in the regulation of glucose uptake in normal cells, it is likely that it might also be instrumental for glucose metabolism in cancer cells, where metabolic demands are overriding. However, to date, there are no reports of a potential role of STR in the regulation of glucose metabolism in cancer cells. In fact, the sweet taste receptor has never been studied in cancer.

1.3.1 Glucose metabolism in cancer

Glucose metabolism is the essential process for sustaining a mammal's life. The process of glycolysis takes place in the cytoplasm by the conversion of glucose to pyruvate. It requires the transport of glucose into cells via glucose transporters (GLUTs) and sodium-glucose transporters (Navale & Paranjape 2016). Thus, through the oxidation of the carbon bonds of glucose, the energy obtained is used in the form of ATP and after the completion of glycolysis, in the following steps, the final product formed can be lactate or CO2, if total glucose oxidation occurs by the mitochondrial respiration process (Liberti & Locasale 2016).

Cancer progression involves an inappropriate proliferation of cells, which have enhanced abilities to produce energy and reprogram their metabolism to promote growth, survival, proliferation, and maintenance over long periods of time, resisting metabolic stress (Vander *et al.* 2009). Additionally, there is also a drastic increase in the rate of glucose uptake and lactate production, even in the presence of oxygen and normal mitochondrial function. This malignancy-associated metabolic shift is known as Warburg effect and triggers an increase in aerobic glycolysis (Figure 1.4). Thus, unlike normal cells that rely primarily on oxidative phosphorylation, tumour cells mostly rely on aerobic glycolysis to produce energy (Liberti & Locasale 2016).

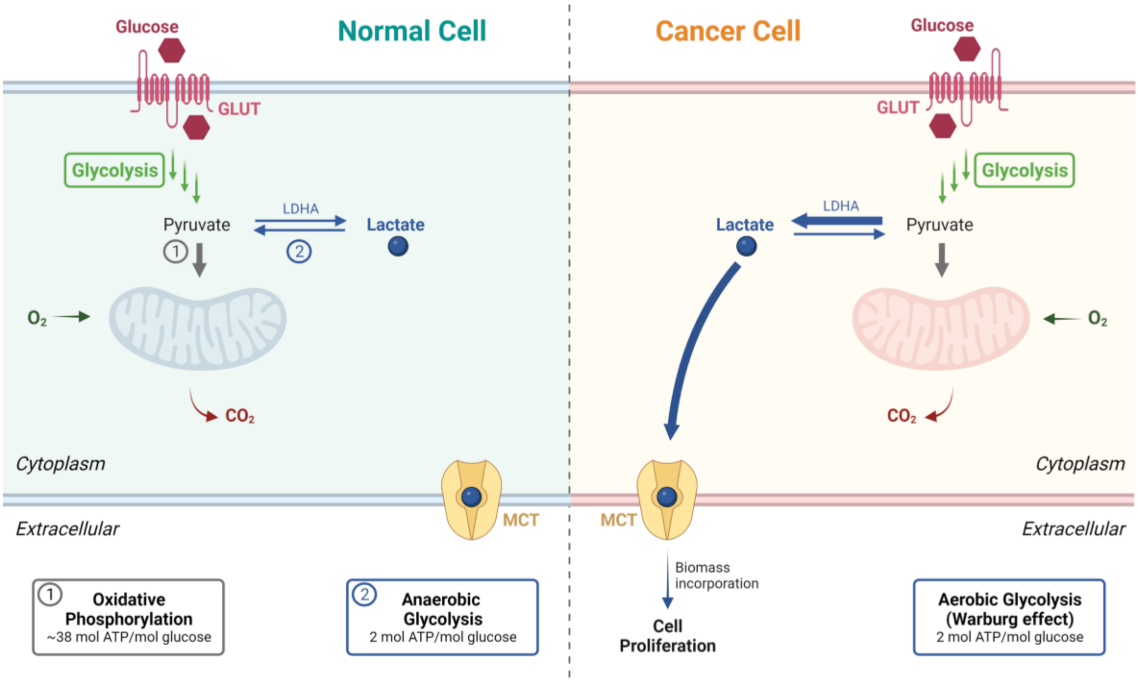


Figure 1.4 – Differences between glucose metabolism in normal and in cancer cells. In normal cells, in the presence of oxygen (O2), glucose is metabolized to pyruvate via glycolysis followed by oxidation of most of the pyruvate obtained to CO2 in the mitochondria, a process called oxidative phosphorylation. In the absence of O2, cells metabolize pyruvate to lactate in a process called anaerobic glycolysis, which results in minimal ATP production compared to oxidative phosphorylation. On the other hand, regardless of the presence or absence of O2 and the normal functioning of the mitochondria, cancer cells tend to convert most of the glucose into lactate, a process called aerobic glycolysis and known as Warburg effect, resulting in less efficient ATP generation than oxidative phosphorylation.

Cancer cells adapt to the low energy yield of glycolysis by increasing glucose uptake to support the higher glycolytic rate (Liberti & Locasale 2016). The high rate of glycolysis is likely to benefit cancer cells as it provides a high rate of ATP production and provides many intermediates, which are used in adjunct metabolic pathways for the *de novo* synthesis of nucleotides, amino acids, lipids and NADPH, necessary for rapid cell proliferation (Lunt & Vander Heiden 2011). However, the presence of aerobic glycolysis under normoxic conditions (normal oxygen levels) and with functionally efficient mitochondria has undoubtedly become a fingerprint of cancer cells, which may contribute to the possibility of tumour glycolysis being used as a potential target for cancer therapy (Abdel-Wahab *et al.* 2019).

Recently, several studies have shown that although aerobic glycolysis is the main energy source for cancer cells, it is also involved in the activation of oncogenes responsible for inducing chemoresistance in cancer through the GLUTs overexpression and important enzymes in glycolysis. On the other hand, aerobic glycolysis acts synergistically to induce changes in the tumour microenvironment (C. Liu *et al.* 2021; Seyfried & Shelton 2010). The hypoxic and acidic microenvironment induces the activation of the HIF1α, that in turns transactivates genes involved in glucose uptake and breakdown, such as GLUTs, hexokinase, phosphofructokinase 1 and aldolase, stimulating glycolysis and angiogenesis by inducing the synthesis of the vascular endothelial growth factor. Concurrently, glycolysis releases lactate to the tumour microenvironment, which decreases the pH, weakening the response of the immune cells surrounding the tumour and providing an additional source of energy for tumour growth (Abdel-Wahab *et al.* 2019; Denko 2008; C. Liu *et al.* 2021). Moreover, cancer chemoresistance might be enhanced by these metabolic features as seen in several types of cancer (Desbats *et al.* 2020).

Although more knowledge is needed to determine the exact mechanism of chemoresistance induced by the Warburg effect, studies have been carried out on inhibitors targeting GLUTs, enzymes and signalling pathways, and have been shown to be effective in suppressing tumour progression. Several of these glycolytic inhibitors are currently under investigation in preclinical and clinical studies with promising results (Abdel-Wahab *et al.* 2019; Ganapathy-Kanniappan & Geschwind 2013; Liberti *et al.* 2017; Liu *et al.* 2012; Velpula *et al.* 2013; Wu *et al.* 2018; M. Zhou *et al.* 2010).

1.3.2 The role of sweet taste receptor in metabolism

Although initially identified in the taste buds of the oral cavity, the STR is expressed throughout the body, including adipose tissue, airways, bladder, intestine, heart, kidney, liver, pancreas, testes, sperm and brain (Santos *et al.* 2019).

In the normal intestine, STR signalling increases the rate of intestinal absorption of glucose by regulating a glucose transporter in enterocytes. In a study performed on a human enteroendocrine cell line with high STR expression, cells released glucagon-like peptide 1 upon stimulation with glucose or sucrose (Jang *et al.* 2007; Park & Song 2019). On the other hand, in wild-type rats, a sucrose-rich diet induced rapid downregulation of the STR in the gut, leading to reduced glucose absorption (Smith et al. 2018).

The STR activation also stimulates insulin secretion by pancreatic β cells through the activation of PLCβ2 and adenylate cyclase, leading to Ca^{2+} accumulation in the cytoplasm and insulin secretion (Henquin 2012; Laffitte *et al.* 2014). Under hyperglycaemic conditions, intestinal expression of the TAS1R2 subunit is mutually regulated by luminal glucose in healthy patients, but not in diabetic patients (Young *et al.* 2013). On the other hand, TAS1R3 inhibition in humans has been shown to have antidiabetic effects without any negative side effects (Teff *et al.* 2010). Additionally, in a gastric cancer cell line, the administration of non-caloric sweeteners induced serotonin secretion dependent on the activation of the TAS1R3 (Zopun *et al.* 2018a).

Of note, sweet and bitter taste receptors do not coexist in extraoral cells, but their coexistence has been demonstrated in solitary chemosensory cells of the respiratory tract, where they appear to act antagonistically in the regulation of innate immunity (R.J. Lee *et al.* 2014). The activation of TAS1R2 and TAS1R3 subunits by glucose inhibits the release of antimicrobial peptides, contrarily to the observed upon TAS2Rs (Maina *et al.* 2018).

In the mammalian brain, the existence of the STR in the hypothalamus, hippocampus and cortex suggests that it functions as a cerebral glucosensor, and that it may be involved in cerebral glucose homeostasis (Herrera Moro Chao *et al.* 2016; X. Ren *et al.* 2009; Shin *et al.* 2010). Recent evidence also suggests that STR controls glucose metabolism in astrocytes, a cell type in the origin of glioblastoma, and in neurons from different nutrient-sensitive regions of the midbrain, including the hypothalamus, hippocampus, and cortex. The STR expression has also been detected in tanycytes specialized glial cells located in the third brain ventricle, a brain region implicated in regulatory responses to hypoglycaemia and consequently highly sensitive to glucose (X. Ren *et al.* 2009; Welcome & Mastorakis 2018). Moreover, the knockout of the TAS1R3 subunit of the STR triggers impairments in the behaviour, learning and memory of these animals (B. Martin *et al.* 2017).

Thus, it becomes clear that the STR is an important glucosensor, with a recognized role in the regulation of glucose uptake in normal cells. This raises the hypothesis that the STR may also regulate glucose uptake and energy metabolism in tumour cells.

1.3.3 Sweet taste receptor expression and putative role in cancer

As referred before, the STR functions as a sensor of the availability and regulator of glucose metabolism in several organs. Moreover, the proliferation of cancer cells is dependent on the metabolic process of aerobic glycolysis known as Warburg effect, and thus dependent of high glucose concentrations. In this context, the STR blocking could play an important role in preventing the glucose uptake by cancer cells and, consequently, limiting the Warburg effect.

The TAS1R2 and TAS1R3 subunits of the sweet taste receptor have been shown to be expressed in tumour cells. The presence of both STR subunits was confirmed in the colorectal carcinoma cell lines NCI-H716 and Caco-2 (Jang *et al.* 2007; Shil *et al.* 2020). The mRNA and protein expression of TAS1R2 and TAS1R3 was also analysed in human gastric parietal tumour cells and in a bile duct carcinoma cell line (Toyono et al. 2007; Zopun *et al.* 2018b), although only the TAS1R3 subunit expression was confirmed in the latter cell line. Gezginci-Oktayoglu and colleagues showed the presence of TAS1R2 and TAS1R3 in the membrane of human pancreatic adenocarcinoma PANC-1 cells (Gezginci-Oktayoglu *et al.* 2021).

Some studies not only addressed the STR expression in cancer cells but also showed that its inhibition may be associated with lower survival rate and lower ability to migrate. The administration of non-caloric sweeteners in a gastric cancer cell line induced serotonin secretion dependent on the TAS1R3 subunit activation (Zopun *et al.* 2018a). In the colorectal cancer cell line Caco-2, the inhibition of the TAS1R3 subunit with lactisole led to a decrease in glucose uptake, suggesting that glucose uptake by Caco-2 cells is regulated by the STR (Andrade *et al.* 2018). In addition, endometrial cancer cells exposed to low concentrations of glucose showed reduced migratory capacity, and inhibition of cell growth and proliferation. This raises the hypothesis that the antiproliferative effects exerted by glucose deprivation can be attributed to the induction cell cycle arrest (senescence) and apoptosis (Han *et al.* 2015).

In the last years, the sweet taste receptor, particularly the TAS1R3 subunit, was shown to have a role in regulating the glycolytic metabolism of different types of normal cells, perceiving the availability of glucose, and triggering cellular mechanisms to increase its uptake and processing along the signalling pathway. However, to date, only few studies addressed the STR expression and functionality in tumours cells. The inhibition of this glucosensor may trigger an illusion in cells that there is no glucose available in the extracellular environment, thus compromising glucose uptake and leading to cell death by starvation.

Whether the STR mediates metabolic reprogramming of cancer cells or its potential involvement in other molecular pathways and other types of cancer remains to be elucidated. Additionally, it will be crucial to understand if the STR inhibition can reverse the Warburg effect, in order to become a potential therapeutic target for cancer.

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Chapter 2
Introduction, Part II

Glioblastoma

Part of this chapter is in the original publication which I co-authored:

> **Ana R. Costa**, Mariana L. Oliveira, Inês Cruz, Isabel Gonçalves, José F. Cascalheira, Cecília R.A. Santos. (2020). The sex bias of cancer. *Trends in endocrinology and metabolism: TEM*, *31*(10), 785–799. https://doi.org/10.1016/j.tem.2020.07.002

Some alterations to the original publication were introduced to further sustain the aim of the thesis.

2.1 Glioblastoma

Unlike other types of cancer for which highly effective treatments exist, CNS tumours, and glioblastoma in particular, still have a very severe prognosis, as anti-cancer drugs hardly reach their targets in the CNS.

Glioblastoma is a grade 4 brain tumour according to the WHO, due to its aggressiveness, invasiveness and poor differentiation (Louis *et al.* 2021). It is the most prevalent among the gliomas and comprises about 16% of the CNS and brain neoplasms, thus being the most common primary brain malignant tumour (Thakkar *et al.* 2014).

Glioblastoma can arise *de nuovo* or derive from low-grade astrocytomas. Primary or *nuovo* tumours arise without evidence of a less malignant precursor and represent about 90% of cases, having worse prognosis, and higher resistance to TMZ (the therapeutic drug of choice for this type of cancer) (Wilson *et al.* 2014). Secondary tumours usually arise from a low-grade tumour and present significantly better prognosis. In addition, glioblastoma occurs almost exclusively in the brain, but can also appear in the brainstem, cerebellum, and spinal cord. Initially, glioblastoma was thought to arise only from glial cells, but according to recent evidence, it may derive from various cell types with neural stem cell-like properties (Phillips *et al.* 2006).

Primary and secondary glioblastoma classification also considers the molecular changes within the cells, namely isocitrate dehydrogenase (IDH) mutations. IDH-wildtype tumours are classified as primary glioblastoma, and IDH-mutant are classified as secondary glioblastoma, and these may have a major role in short- or long-term outcome for patients (Louis *et al.* 2016; Mandel *et al.* 2016; SongTao *et al.* 2012; Yan *et al.* 2009). A possible explanation is that IDH mutations is the increased affinity of IDH enzyme for α-ketoglutarate, and thus the reduced affinity for the standard substrate isocitrate. This leads to impaired DNA and histone demethylation and to HIF1α activation (Fu *et al.* 2012). In addition, secondary glioblastoma is also characterized by mutations in genes encoding p53 and an increased expression of the platelet-derived growth factor receptor PDGFR-A (Ohgaki *et al.* 2004). In contrast, primary glioblastoma is essentially characterized by an amplification and mutation of the EGFR, a decrease in cyclin-dependent kinase inhibitor p164a and a mutated phosphatase and tensin homolog PTEN (Ohgaki *et al.* 2004). These mutations are involved in numerous signalling pathways crucial in tumour development and proliferation.

2.1.1 Incidence, prevalence, and sex-bias

According to the Global Cancer Observatory data from 2020, an estimated 308.000 people worldwide are living with a CNS tumour, and approximately 40% more will be diagnosed by 2040. Primary glioblastoma has higher prevalence in individuals between 55 and 60 years of age, and interestingly, unlike lower-grade gliomas, is 50% more prevalent in males (Ohgaki & Kleihues 2013). Recent reports from the World Health Organization reveal clear gender differences in the incidence and mortality caused by tumours originating in different nonreproductive organs, like the CNS (Sung *et al.* 2021).

The increased male susceptibility to astrocytoma, the most common brain tumour, and particularly to primary glioblastoma (the most aggressive form of astrocytoma), has been well-established since the 1990s. The most recent epidemiological data show that the incidence of glioblastoma in men is 50% higher than in women (Tavares *et al.* 2016; Yu *et al.* 2015). Differences between men and women, possibly related to the different hormone backgrounds, might underlie these differences (McKinley *et al.* 2000). These tumours are known to express estrogen (ERs), progesterone (PRs), and androgen receptors (ARs), and generally higher-grade tumours are associated with decreased expression of ER and increased PR expression (Tavares *et al.* 2016; Yu *et al.* 2015). This is consistent with a study in which higher PR expression was associated with poorly differentiated tumours such as glioblastoma and anaplastic astrocytoma, in contrast to the low levels of PR observed in benign astrocytomas (Altinoz *et al.* 2019; Tavares *et al.* 2016). Moreover, membrane PRs α and β are also expressed in U-251MG and U-87MG glioblastoma cell lines, and their expression in U-87MG cells was regulated after progesterone (P4) and estradiol (E2) treatments (Valadez-Cosmes *et al.* 2015). Expression of other forms of membrane PR directly correlate with patient survival or worse prognosis (Del Moral-Morales *et al.* 2020).

The *in vivo* and *in vitro* studies addressing P4 effects on glioblastoma remain controversial, and some studies report a protumour effect whereas others report an antitumor action of P4 (Altinoz *et al.* 2019; Atif *et al.* 2019; Elmaci *et al.* 2019;Germán-Castelán *et al.* 2014). The differences between these studies might reflect the use of different glioblastoma models or experimental conditions. Regarding E2, some evidence suggests that ERα-mediated effects are oncogenic, whereas ERβ acts as a tumour suppressor. Both receptors are present at low levels in primary glioblastoma (Dueñas Jiménez *et al.* 2014). Moreover, González-Arenas et al. demonstrated *in vitro* that E2 significantly stimulated cell proliferation through ERα (González-Arenas *et al.* 2012). Furthermore, an *in vivo* study reported that E2 increased survival rates in both male and female rats, and that this effect was observed from the early stages of glioblastoma progression, possibly by increasing apoptosis (Barone *et al.* 2009). Again, data on the effects of ovarian hormones and correlates of their cognate receptors with glioblastoma progression often report opposite effects, and further studies are needed.

Although males display the highest incidence of brain tumours, few studies have evaluated the effects of androgens. One study showed clear AR overexpression in glioblastoma compared with adjacent non-tumour tissues. AR expression was also detected in eight glioblastoma cell lines, in particular U-87MG, in which the proapoptotic and antiproliferative effect of transforming growth factor β was reduced by DHT (dihydrotestosterone), suggesting that the AR-mediated signalling pathway promotes glioblastoma tumorigenesis (Yu *et al.* 2015). More recently, an increase in cell migration, invasion, and proliferation was observed in three glioblastoma cell lines incubated with increasing testosterone concentrations (Rodríguez-Lozano *et al.* 2019). Moreover, the upregulation of a membrane AR in glioblastoma cells stimulated cell migration (Münnich *et al.* 2016).

2.1.2 Chemoresistance

Despite the development of new strategies and techniques, the treatment of glioblastoma becomes difficult due to its high proliferative activity and vascularization, location and infiltration in surrounding tissues, the existence of the blood-brain barrier (BBB) and the hypoxic microenvironment, as well as high resistance to radio- and chemotherapy, that often lead to tumour recurrence (Chang *et al.* 2007; Karcher *et al.* 2006; Linkous & Yazlovitskaya 2011; Schröder *et al.* 1991; Wang *et al.* 2019).

Glioblastoma is known by its heterogeneity and proliferative nature that facilitates the selection of resistant populations, leading to rapid resistance development, infiltration into healthy tissue and relapse (Omuro & DeAngelis 2013; Soeda *et al.* 2015). The glioblastoma heterogenous characteristics are not only morphological but also phenotypical, and the existence of four transcriptional cell types within the same tumour were already identified (Omuro & DeAngelis 2013). The genetic expression differences in these cell types contribute to the development of local microenvironments that are involved in the invasive capacity and/or radiochemoresistance of a certain tumour region (Lima *et al.* 2012). Moreover, common genetic mutations modulating several signalling pathways involved in tumour progression towards malignancy could accumulate and lead to extensive inter and intra-patient heterogeneity (Omuro & DeAngelis 2013; Meyer 2008).

The low efficacy of current treatments is compromised by the reduced permeability of brain and tumour barriers (Figure 2.1), due to the overexpression of efflux transporters that prevent the entry of several therapeutic compounds, reducing their absorption and distribution by the brain (Gomez-Zepeda *et al.* 2019; Gullì *et al.* 2022; Gupta *et al.* 2020; Hermann *et al.* 2006; Qosa *et al.* 2015; Saunders *et al.* 2016). The BBB is a highly organized and complex structure constituted by endothelial cells, pericytes, astrocytes, microglial and smooth muscle cells with no fenestrations, conferring an impenetrable barrier (Alyautdin *et al.* 2014; Liu *et al.* 2012; Saraiva *et al.* 2016). Only selective, small, and lipophilic molecules can passively diffuse across the BBB, whereas larger or hydrophilic molecules can only permeate via specific transporters like the ABC (Cardoso *et al.* 2010). The disruption of the BBB induced by the release of proteases enzymes and growth factors by glioblastoma cells leads to the blood-tumour barrier (BTB) formation, typically characterized by heterogenous permeability and irregular structure (Arvanitis *et al.* 2020). Due to the heterogenous nature of BTB, glioblastoma cells typically have hypoxic regions where the drug delivery is restricted, thus exacerbating chemoresistance (Karim *et al.* 2016; Mudassar *et al.* 2020). Like in the BBB, also BTB expresses ABC transporters that promote drug efflux out of the cells, increasing chemoresistance (Gottesman 2002; Munoz *et al.* 2015; Oberoi *et al.* 2016;Sharom 2011). One of these transporters, ABCB1, is expressed in both low- and high-grade gliomas, suggesting an intrinsic resistance of glioblastoma cells to anticancer compounds, including TMZ (de Faria *et al.* 2008; Gomez-Zepeda *et al.* 2019; Karim *et al.* 2016). The failure in drug delivery to all regions of the tumour is a major contributing factor for the success of the therapy, leading to inevitable recurrence and poor patient survival.

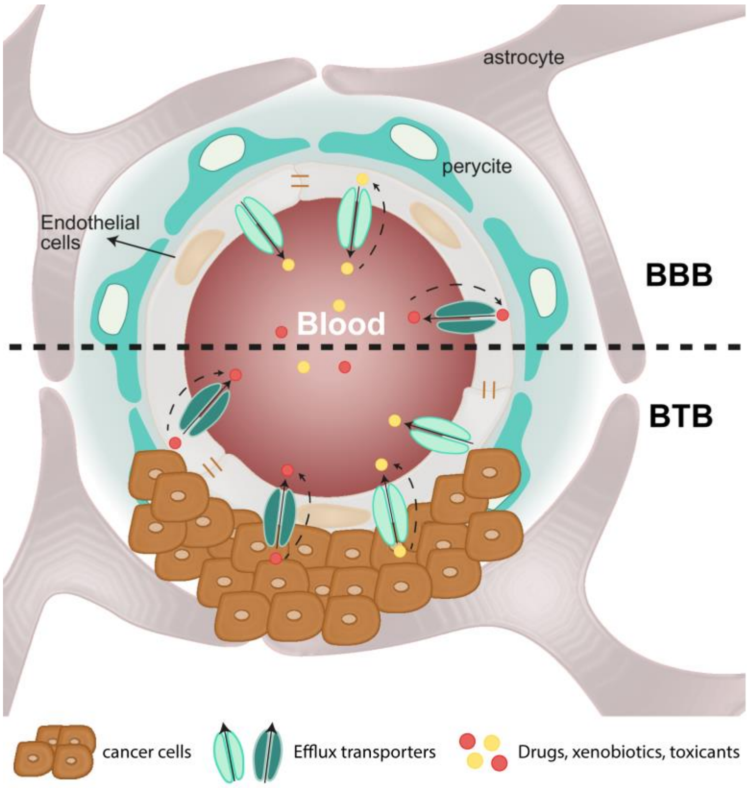

Figure 2.1 – Brain drug delivery is restrained by efflux mechanisms present at blood-brain and blood-tumour barriers. The blood-brain barrier (BBB) is a highly organized and complex structure established by endothelial cells with no fenestrations responsible for controlling the transport of substances into and out of the brain. The blood-tumour barrier (BTB) is formed by brain endothelial cells surrounding brain tumours or metastasis that display particular features that enhance tumour chemoresistance, such as the aberrations in the structure induced by neovascularization and the release of angiogenic and growth factors and extracellular matrix degradation enzymes. In addition, both endothelial and cancer cells express multiple transporters that efflux drugs and other molecules impacting the treatment of many central nervous system disorders.

The methylguanine DNA methyltransferase (MGMT) promoter status is also relevant for TMZ efficacy and overall survival of glioblastoma patients. Up to 50% of glioblastoma patients do not respond favourably to TMZ either due to O6-alkylguanine repairing by increased expression of MGMT or by demethylating the MGMT promoter site (Lee 2016; Rivera *et al.* 2010; Zhang *et al.* 2012). Therefore, investigating agents to overcome TMZ resistance is of a paramount importance. Nowadays, more than 45 ongoing clinical trials are investigating agents to overcome TMZ resistance.

Other contributors to glioblastoma chemoresistance are the immunosuppression induction, that promotes immune invasion and contributes to treatment failure and immunosurveillance evasion, and the fortified tumour location that hinders the therapeutics delivery. The efficacy of emerging immunotherapies in glioblastoma is limited by the overexpression of CD133 which has been linked to worse prognosis, and the recruitment of tumour associated macrophages which promote immune evasion, tumour growth, invasion and angiogenesis (Ardebili *et al.* 2011; Lima *et al.* 2012; Ye *et al.* 2012; Zeppernick *et al.* 2008). On the other hand, the fortified glioblastoma location in the brain generates an inherent challenge for treatment, unlike other tumours. In fact, more than 85% of glioblastomas cases are in the subcortical brain, and patients with tumours in the cerebellum, brainstem or subventricular zone have poorer prognosis, probably due to the limited access in performing surgical resections (Jungk *et al.* 2019; Lam *et al.* 2018; Weinberg *et al.* 2018). Altogether, these glioblastoma features highlight the need of new strategies for drug delivery or efficient ways to circumvent chemoresistance.

2.1.3 Glucose Metabolism

As mentioned before, even in the presence of oxygen or glucose starvation conditions, cancer cells are known for the shift to aerobic glycolysis to selectively extract nutrients from the extracellular space by upregulating GLUT3, while excreting substantial amounts of lactate, to promote tumorigenesis and allowing them to resist to therapies (Alifieris & Trafalis 2015; Flavahan *et al.* 2013; Griguer *et al.* 2005; Warburg 1956). Lactate has been associated with radiotherapy resistance by neutralization of the infrared-produced oxygen species and inhibition of the radiation-induced apoptosis, as well as cell migration, extracellular matrix degradation and remodelling, and tumour angiogenesis (Goetze *et al.* 2011; Hjelmeland *et al.* 2011; Peppicelli *et al.* 2017). In addition, the acidic conditions triggered by lactate could lead to autophagy, which is related to the preservation of glioblastoma stem-like cells phenotype and therapy resistance (Lomonaco *et al.* 2009; Peppicelli *et al.* 2017). Acidosis is also involved in ABCB1 enhanced activity and reduced immune response (Peppicelli *et al.* 2017; Vaupel & Multhoff 2017).

The preference for glycolysis in glioblastoma cells could be explained by the rapid source of ATP compared with other pathways (Marin-Valencia *et al.* 2012). More recently, was showed that the tumour cells preference for glycolysis or oxidative phosphorylation is determined by the tumour microenvironment (Marin-Valencia *et al.* 2012; Talasila *et al.* 2017). Under hypoxic conditions, as the tumour necrotic core, most surrounding cells undergo glycolysis, while those near the vasculature, where oxygen concentration is increased, prefer oxidative phosphorylation (Talasila *et al.* 2017). However, this preference for a specific metabolic mechanism within glioblastoma tumours needs further investigation.

Further evidence that glucose metabolism may play a central role in glioblastoma development and proliferation lies in the fact that it is a type of brain tumour, since the brain is known for the requirement of efficient energy to sustain neuronal activity and synaptic transmission through glucose consumption. In addition, the anabolic needs of gliomas with high mitotic activity, like glioblastoma, are fulfilled by switching on the glucose-dependent biosynthesis (Raichle & Gusnard 2002). Moreover, glucose is a limiting energy resource for both normal and cancer cells in the brain, since glioblastoma cells express a repertoire of glucose transporters, namely GLUT1 and GLUT3, whose expression is regulated by hypoxia conditions and hypoxia-inducible factors (Flavahan *et al.* 2013; Li *et al.* 2009).

Although it is clear that glioblastoma growth, proliferation and therapy resistance are highly favoured by an excellent execution of the metabolic machinery of tumour cells, it could also represent a therapeutic opportunity by changing the availability of nutrients and oxygen of the tumour microenvironment. Therefore, novel therapies that impose metabolic constrains in glioblastoma cancer cells could be a matter of debate.

2.1.4 Treatment approaches

Currently, the standard treatment for glioblastoma consists of surgical removal of the tumour followed by radiotherapy and chemotherapy with TMZ, along with medications to relieve neurological symptoms. Temozolomide is the most used chemotherapeutic agent for the treatment of glioblastoma, since it increases the survival rate of patients, and its use along with radiotherapy has provided an increase in this rate and lower toxicity (Stupp *et al.* 2005; Wang *et al.* 2019). This multimodal therapeutic approach remains the best option available, although it only increases life expectancy from 10% to 26% at 2 years and from 0% to 10% at 5 years, with a median survival of 15 months after diagnosis (Delgado-López & Corrales-García 2016; Stupp *et al.* 2005). This highlights the need to identify new therapeutic targets to improve prognosis for this devastating disease.

The surgical resection of glioblastoma depends on the location and size of the tumour and consists in the tumour mass removal, followed by radio- and chemotherapy. However, the complete tumour resection is very difficult, due to the lack of clear tumour boundaries and to the risk of neurologic deficits in infiltrative tumours (Jackson *et al.* 2001; Lima *et al.* 2012; Marko *et al.* 2014). After surgery, patients undergo radio- and chemotherapy usually with TMZ, the current first-line and most used chemotherapeutic for glioblastoma. TMZ is an oral DNA alkylating agent characterized by small size and lipophilic nature that enables the BBB permeation (Lee 2016; Levin *et al.* 2015; Moody & Wheelhouse 2014; Zhang *et al.* 2012). In its passage into the cytoplasm, TMZ undergoes spontaneous hydrolysis, inducing DNA methylation at three different sites that results in cell damage and cell cycle arrest at G2/M phase, thus promoting cell death and apoptosis (Hirose *et al.* 2001; Karachi *et al.* 2018; Zhang *et al.* 2012). Gliadel® is another therapeutic option currently used for newly diagnosed or recurrent glioblastoma. It consists in the implantation of biodegradable carmustine wafer at the site of the surgery, where the drug is then slowly released (Brem *et al.* 1995; Fleming & Saltzman 2002; Nam *et al.* 2018). However, Gliadel® has limited efficacy, and has been reported to interfere with imaging, and to develop oedema and BBB disruption, leading to the risk of infection (Perry *et al.* 2007; Sabel & Giese 2008). Bevacizumab also promotes glioblastoma regression by reducing tumour growth and cerebral oedema, like in other types of cancer, but it has failed to demonstrate an increase in overall survival of glioblastoma patients (Chinot *et al.* 2014; Gilbert *at al.* 2014; Narita 2015). In addition, bevacizumab is known for commonly hypertension development and venous thrombosis, as well as infections and possible interference with the activity of other agents, like TMZ (Gerstner *et al.* 2020; Narita 2015). For that reason, bevacizumab is reserved for recurrent glioblastoma therapy (Gilbert *at al.* 2014).

More recently, tumour treating fields (TTF) emerged for both newly diagnosed and recurrent glioblastoma as a non-invasive strategy (Anton *et al.* 2012). TTF involves the use of low intensity and intermediate frequency electric fields to the tumour, interfering with mitosis of rapidly dividing glioblastoma cells and triggering cell death (Mittal *et al.* 2018). When used along with radio- and chemotherapy, TTF showed a significant increase in overall survival of glioblastoma patients (Guzauskas *et al.* 2018). TTF is associated with mild dermatitis and local skin irritation, but the major limitation to routine clinical use is the very expensive cost of the treatment (Bernard-Arnoux *et al.* 2016).

Because of the cancer cells preference for glycolysis within the hypoxic tumour microenvironment, other emerging therapeutics to prevent glioblastoma growth and proliferation are related to the reversal of the Warburg effect (Wolf *et al.* 2018). In two resistant glioblastoma cell lines, methylene blue reversed the Warburg effect by shunting pyruvate into the citric acid cycle, and its effects were more pronounced when combined with TMZ (Poteet *et al.* 2013). In another study, the targeting of pyruvate dehydrogenase kinase PDK1 reversed the Warburg effect in two glioblastoma cell lines by decreasing HIF1α, destabilizing the hypoxic core of glioblastoma, and switching the cells preference for oxidative phosphorylation instead of glycolysis, therefore leading to anti-proliferative effects and reducing invasive capacity apoptosis (Velpula *et al.* 2013).

However, to date, none of these therapeutic strategies significantly improved the outcome of glioblastoma patients, highlighting the urgent need for more efficiently therapies.

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Chapter 3

Global Aims

3.1 Global Aims

Glioblastoma is a WHO grade 4 brain tumour, for which standard treatments are still ineffective, and the research for alternative targets for more effective therapies is of paramount importance to enhance the prognosis for this devastating disease. More than 80% of poor survival outcomes and mortality in cancer patients are directly or indirectly attributed to chemoresistance. Cancer chemoresistance might be enhanced by the existence of multidrug resistance transporters and by the metabolic features seen in cancer cells, among other important hallmarks. It is known that many anticancer drugs are bitter tasting compounds and ligands of bitter taste receptors (TAS2Rs), some of them involved in the regulation of multidrug resistance transporters. On the other hand, the metabolism of cancer cells features another important hallmark of cancer which encompasses enhanced glucose uptake and prevalence of aerobic glycolysis over cell respiration.

Based on the available experimental evidences that associate taste receptors with several types of cancer, we hypothesise that some bitter taste receptors and the sweet taste receptor might have a role in glioblastoma drug resistance and glucose metabolism, respectively.

Therefore, this doctoral thesis aimed at addressing two main goals, subdivided in specific goals:

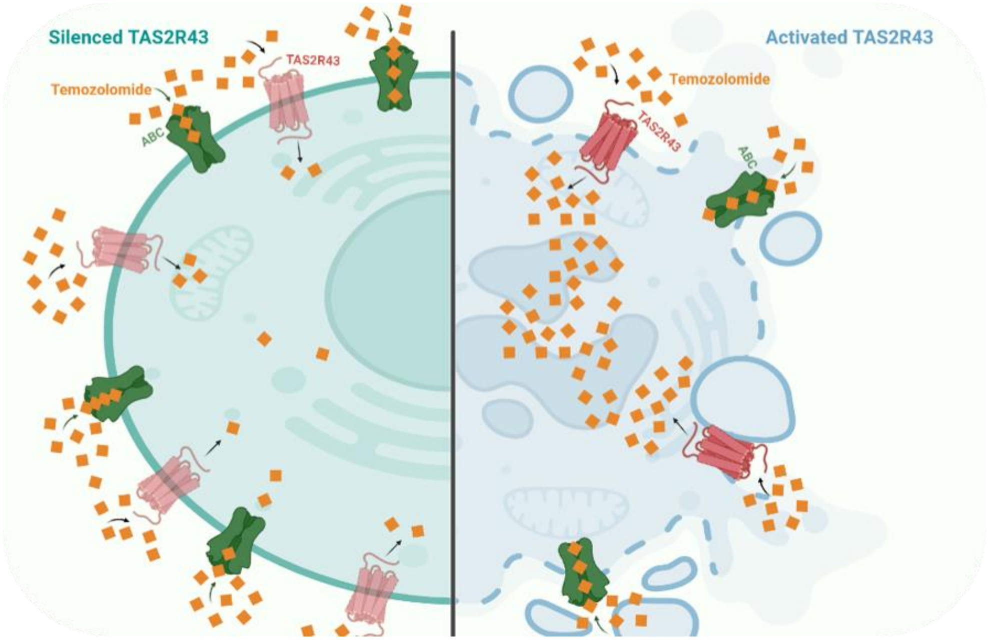
1) Analyse the potential of bitter taste receptors as novel therapeutic target for glioblastoma:
    i) Characterize the expression profile of TAS2Rs in human glioblastoma cell lines and tissue samples, compared with normal cells.
    ii) Analyse the involvement of TAS2Rs and associated pathways in the response of glioblastoma cells to the drug temozolomide.

2) Characterize the role of the sweet taste receptor in the glucose metabolism of glioblastoma cells:
    i) Analyse the STR expression in human glioblastoma cell lines and tissue samples.
    ii) Assess the effect of STR inhibition in cell proliferation and apoptosis in normoxic conditions and in response to glucose/oxygen deprivation in glioblastoma cell lines.
    iii) Determine if STR mediates the metabolic reprogramming of glioblastoma cells by measuring glucose uptake and extracellular L-lactate levels.

Chapter 4

Research Work 1

The anti-cancer effects of temozolomide are partially mediated by activation of the bitter taste receptor TAS2R43 in glioblastoma cells



This chapter corresponds to the original research article:

> **Ana R. Costa**, Ana C. Duarte, Ana R. Costa-Brito, Isabel Gonçalves, Catarina L. Ferreira, Robert Preißner, Isidro Ferrer, Tomomi Furihata, José F. Cascalheira, Helena Marcelino, Cecília R.A. Santos. The anti-cancer effects of temozolomide are partially mediated by activation of the bitter taste receptor TAS2R43 in glioblastoma cells. [Submitted]

Some alterations to the original publication were introduced to improve the appearance and organization of this chapter.

Abstract

Glioblastoma (WHO grade 4) is the most common and aggressive form of primary brain cancer. Despite the great advances in the development of promising drugs for its treatment and/or diagnosis, cancer-targeting drugs often fail to achieve therapeutic levels in the brain, compromising their efficacy. Recent studies show that bitter taste receptors (TAS2Rs), and their ligands, have an important role in cancer progression and metastasis when activated by a range of natural and synthetic compounds. Their activation has been related to drug resistance, apoptosis, and proliferation of malignant tumours. In humans, 25 distinct TAS2Rs are present in several extraoral organs, where they sense the chemical composition of body fluids and trigger biological effects accordingly. In the present study, we analysed the expression and function of bitter taste receptors and the bitter signalling pathway in *in vitro* models of glioblastoma and examined their capacity to recognize the chemotherapeutic drug temozolomide (TMZ), the golden standard treatment for this brain tumour. Herein we demonstrate that the bitter taste machinery is differentially expressed in glioblastoma cells. Strikingly, we demonstrate that the anti-proliferative and pro-apoptotic effects of TMZ in glioblastoma cells were partially mediated by TAS2Rs activation, particularly TAS2R43. These findings highlight the relevance of the taste transduction pathway for the assessment of the chemical composition of the glioblastoma niche and disclose a novel therapeutic target for glioblastoma.

Keywords

Glioblastoma; temozolomide; bitter taste receptors; taste signalling; TAS2R43

4.1 Introduction

Glioblastoma is the most common and aggressive form of primary brain cancer classified grade IV by the World Health Organization, with a 5-year survival of approximately 3% and a median survival of less than 18 months (Louis *et al.* 2016; Reni *et al.* 2017). The current golden standard for glioblastoma treatment is palliative and includes surgery, radiotherapy and temozolomide (TMZ) chemotherapy. TMZ is an alkylating agent known for its ability to alkylate/methylate DNA, resulting in DNA damage and tumour cells death by apoptosis (Strobel *et al.* 2019). However, some tumour cells can repair TMZ-induced DNA damage by expressing the O6-methylguanine DNA methyltransferase (MGMT) gene which repairs the DNA damage thereby abrogating the drug efficacy as well as the efficacy of other cytotoxic alkylating agents (Kaina *et al.* 2007). Moreover, glioblastoma malignancy and relapse are also attributed to the existence of cancer stem-like cells known to be chemo- and radioresistant (Bao *et al.* 2006; J. Chen *et al.* 2012; Eramo *et al.* 2006).

Additionally, despite the great advances in the development of promising drugs for the treatment and/or diagnosis of tumours in the central nervous system (CNS), these remain difficult to treat pharmacologically. This is a consequence of the inability of numerous brain-targeting drugs to achieve therapeutic levels and bioavailability in the brain (Ghersi-Egea *et al.* 2009; Gomez-Zepeda *et al.* 2019; Sanchez-Covarrubias *et al.* 2014), due to their limited capacity to cross brain barriers, or metabolization before reaching their targets. At brain barriers, and the blood-brain tumour barriers (Gomez-Zepeda *et al.* 2019; Hermann *et al.* 2006; Qosa *et al.* 2015; Saunders *et al.* 2016), overexpressed multidrug efflux transporters extrude several CNS-targeting medicines (Gomez-Zepeda *et al.* 2019; Gupta *et al.* 2020), reducing brain drug absorption and distribution (Pitcher & Quevedo 2016). One of these transporters, P-glycoprotein 1, is expressed in low- and high-grade gliomas, suggesting an intrinsic resistance of glioblastoma cells to anticancer drugs, including TMZ (de Faria *et al.* 2008; Gomez-Zepeda *et al.* 2019). In addition to complex enzymatic detoxifying mechanisms, these are the best-known components of the brain chemical surveillance system (Palmer 2010; Sanchez-Covarrubias *et al.* 2014). However, little is known about the mechanisms underlying their regulation.

The human bitter taste receptor (TAS2R) subfamily is composed of 25 chemosensory receptors that mediate signal transduction in response to a wide variety of bitter agonists (Adler *et al.* 2000; Chandrashekar *et al.* 2000). In the canonical pathway, TAS2Rs activation leads to the dissociation of heterotrimeric G protein into α-gustducin (GNAT3) and Gβ3/Gγ13 subunits, which in turn activate downstream phospholipase-C β2 that hydrolyses PIP2 (phosphatidylinositol-4,5-bisphosphate) to inositol-1,4,5-triphosphate (IP3) and diacylglycerol. IP3 triggers an increase in intracellular Ca^{2+} levels that, in turn, activates TRPM5 (transient receptor potential cation channel subfamily M member 5), causing cell depolarization (Behrens & Meyerhof 2006; Chaudhari & Roper 2010). Apart from sensing bitter compounds in the oral cavity, TAS2Rs were also described in several extraoral tissues, like the gastrointestinal tract, airway epithelium, heart, testis and brain (Avau & Depoortere 2016; Duarte *et al.* 2020; Jaggupilli *et al.* 2017; F. Li 2013), and have also been implicated in diverse metabolic and developmental pathologies such as diabetes, schizophrenia, Parkinson's disease and cancer (Jeruzal-Świątecka *et al.* 2020; Lu *et al.* 2017; Shaik *et al.* 2016; Wang *et al.* 2020). Experimental and clinical data revealed that taste receptors, which belong to the large family of G protein-coupled receptors (GPCRs), and their ligands, have a crucial role in cancer progression and metastasis, by controlling many aspects of tumorigenesis, proliferation, migration, cancer cell invasion and cancer-related signalling pathways (Dorsam & Gutkind 2007; O'Hayre *et al.* 2014). Despite their importance as therapeutic targets in cancer treatment, they have rarely been exploited with the exception of certain endocrine and hormone-responsive tumours (Lappano & Maggiolini 2011; Usman *et al.* 2020). Moreover, the TAS2Rs expression and possible chemosensory function(s) in glioblastoma cells were not studied so far.

We hypothesized that the TAS2Rs might be differentially expressed in astrocytes versus glioblastoma cells, and that they could be activated by TMZ and possibly mediate its effects. We first analysed the expression of TAS2Rs and investigated possible expression differences in a human astrocytes cell line and three different glioblastoma cell lines. Bitter taste receptors TAS2R4, R5, R14 and R39 were also analysed in human glioblastoma samples. Next, we investigated if TMZ activates the bitter taste signalling pathway in glioblastoma cells and found that the response was mediated by TAS2R43. The role of TAS2Rs in partially mediating the TMZ cytotoxic effects in glioblastoma was also investigated.

4.2 Materials and methods

4.2.1 Materials

Temozolomide (TMZ; CAS No 85622-93-1) was purchased from Cayman Chemical (#14163). A stock solution was prepared in dimethyl sulfoxide (DMSO) and freshly dissolved in Tyrode's solution or culture medium before the experiments, where the DMSO final concentration did not exceed 1%. A vehicle control was included in all the experiments. Probenecid (CAS No 57-66-9), a known TAS2Rs antagonist, was obtained from Sigma-Aldrich (#P8761), dissolved in 1 N NaOH at 0.17 M and diluted in Tyrode's solution or culture medium. FURA-2AM (#F1221), pluronic acid F-127, Lipofectamine™ 2000 (#11668027), Opti-MEM medium (#11058-021), small interfering RNA (siRNA) targeting GNAT3 (#4392420; ID s51191; sequence: S-GCGAGAUGCAAGAACCGUATt and AS-UACGGUUCUUGCAUCUCGCtc) and TAS2R43 (#AM16708; ID 202331; sequence: S-CCCUACUAUCUUUUAUGCUtt and AS-AGCAUAAAAGAUAGUAGGGtc), and scramble siRNA (#4390843) were purchased in ThermoFisher Scientific. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] was purchased from Gerbu Biotechnik GmbH (#1006).

The RT-PCR and RT-qPCR primers (Tables 4.1 and 4.2) were obtained from STAB VIDA, and the primary and secondary antibodies (Table 4.3), previously validated, were purchased from Santa Cruz Biotechnology, ThermoFisher Scientific and Sigma-Aldrich.

4.2.2 Cell culture

The conditionally immortalized human astrocyte cell line HASTR/ci35 (Furihata *et al.* 2016) was developed by one of the co-authors (T. Furihata) at Chiba University (Japan). HASTR/ci35 cells were cultured at 33°C, 5% CO2 using the recommended Astrocyte Medium (Gibco™ #A1261301) supplemented with 1% N2 supplement, 10% (v/v) fetal bovine serum (FBS), penicillin (100 IU/ml)/streptomycin (100 µg/ml) and 4 µg/mL blasticidin S (Abcam #ab141452).

Human malignant glioblastoma cell lines U-87MG, SNB-19 and U-373MG were grown in Dulbecco's modified Eagle's medium (DMEM) high glucose with stable glutamine (bioWest #L0103) supplemented with 10% (v/v) FBS and penicillin (100 IU/ml)/streptomycin (100 µg/ml), and incubated in a humidified atmosphere containing 5% CO2 at 37°C.

4.2.3 Detection of TAS2R transcripts in human astrocytes and glioblastoma cell lines by RT-PCR

Total RNA was isolated from glioblastoma cells using TRI Reagent® (Sigma-Aldrich #T9424) in accordance with the manufacturer's instructions. The RNA concentration was determined through OD values measurement at 260 nm, and the quality of RNA was evaluated by an agarose gel electrophoresis. cDNA was synthesized using NZY M-MuLV Reverse Transcriptase (NZYTech #MB083) according to the protocol supplied by the manufacturer. For the RT-PCR, cDNA was amplified by HOT FIREPol® DNA Polymerase (Solis BioDyne #01-02) and specific full-length primers (Table 4.1) in a final volume of 10 µL. The RT-PCR protocols comprised a 15-minute initial activation, followed by 40 cycles of 15-seconds denaturation at 95°C, 30-seconds annealing, and 1-minute elongation at 72°C, and 5 minutes of final elongation at 72°C. The PCR products were separated by electrophoresis on 1% agarose gels and visualized using GreenSafe Premium (NZYTech #MB13201) staining. In addition, RT-PCR bands were purified, and Sanger sequenced by STAB VIDA (Portugal) to verify the sequence identity.

From this on, we only analysed the bitter receptors TAS2R4, R5, R14 and 39 based on the following criteria: 1) expression validated by RT-PCR; 2) number and therapeutic relevance of known ligands; 3) primary antibodies commercially available, suitable for both Western blot and immunofluorescence techniques; 4) previous validation of primary antibodies.

Table 4.1 – List of full-length primers used for RT-PCR mRNA expression for the bitter taste receptors in human astrocytes and glioblastoma cell lines.

RefSeq	Gene Name	Gene Symbol	Primer Sequence 5' – 3'	Size (bp)	A.T. (ºC)
NM_019599.3	Taste receptor, type 2, member 1	**TAS2R1**	Fw: TCCCAGCTGTCTGAAGGTGT Rv: GCCTGAAGGGGACATGTTGTA	1334	60
NM_016943.2	Taste receptor, type 2, member 3	**TAS2R3**	Fw: ATCAGGGCTGCCTAATTGCT Rv: GTCCTGTAGTCTTGAGCCAGG	1035	60
NM_016944.2	Taste receptor, type 2, member 4	**TAS2R4**	Fw: TGTTTCGGTTATTCTATTTCTCTGC Rv: CCTGGAGAGTAAAGGGTGGC	823	58
NM_018980.3	Taste receptor, type 2, member 5	**TAS2R5**	Fw: ACTACCAGGGGATCTGACCTC Rv: CCGAGCACACACTGTCTTCC	937	60
NM_023919.2	Taste receptor, type 2, member 7	**TAS2R7**	Fw: GCAGGTGTGGATGTCAAACTC Rv: ATGACTTGAGGGGTAGATTAGAGC	900	60
NM_023918.3	Taste receptor, type 2, member 8	**TAS2R8**	Fw: TGTTCAGTCCTGCAGATAACATC Rv: GCATTCTGACAAATGTCTGCC	897	58
NM_023917.2	Taste receptor, type 2, member 9	**TAS2R9**	Fw: GGCATGCCAAGTGCAATAGAG Rv: AGGGGTCTCTATGGAACAAAAGG	950	58
NM_023921.1	Taste receptor, type 2, member 10	**TAS2R10**	Fw: GCTACGTGTAGTGGAAGGCA Rv: TGCAGTACCCTCAAAGAGGC	876	60
NM_023920.2	Taste receptor, type 2, member 13	**TAS2R13**	Fw: GCTAGGGCTCAGCAGAGAAAT Rv: GGCAAGTCCAAACTTCCCTAAT	1607	58
NM_023922.1	Taste receptor, type 2, member 14	**TAS2R14**	Fw: TGGGTGGTGTCATAAAGAGCAT Rv: CTGAGGGCTCCCCATCTTTG	924	58
NM_016945.3	Taste receptor, type 2, member 16	**TAS2R16**	Fw: GTCCAGGAAGACACTTTGGAGT Rv: TAGGCCTAGCACTTTCCCTT	909	60
NM_176817.5	Taste receptor, type 2, member 38	**TAS2R38**	Fw: TTTCTGCACTGGGTGGCAA Rv: GGCATATTTATGAAGACTCACAGGC	1141	60
NM_176881.2	Taste receptor, type 2, member 39	**TAS2R39**	Fw: TCTGCGATCCTGCAGAAAGT Rv: GATGAAGTCGAAGCTGAAGCC	930	58
NM_176882.2	Taste receptor, type 2, member 40	**TAS2R40**	Fw: TCTTGGCGCAGAAACCTGAA Rv: TTCCAGTCACAGAGTCTGCC	1015	58
NM_176883.2	Taste receptor, type 2, member 41	**TAS2R41**	Fw: GCAGCGAATGGCTTCATTGT Rv: AACAGGAGCTGCGAGAACAC	833	60
NM_181429.2	Taste receptor, type 2, member 42	**TAS2R42**	Fw: ATGGCCACCGAATTGGACA Rv: CTACAAAGGTAAAGGGTTTGGTGT	945	58
NM_176885.2	Taste receptor, type 2, member 44	**TAS2R44**	Fw: TTTTTCCAGTGTGGTAGTGGTTCT Rv: GATGAAGGCTTCTCTCCTTTCACC	900	58
NM_176888.2	Taste receptor, type 2, member 48	**TAS2R48**	Fw: GAACAAGTGTTACTAAGCCTGC Rv: CTTCTTTCACTCAGCGTGTCA	952	58
NM_176890.2	Taste receptor, type 2, member 50	**TAS2R50**	Fw: ACAACCAGTGATATTAGGCTTGC Rv: TCAGGTCTTTTACTCAGCACCT	963	58
NM_177437.1	Taste receptor, type 2, member 60	**TAS2R60**	Fw: TCCTTTTACGCCTGGTAGCAA Rv: AGGAACGACGACTCTTCAGC	864	60

A.T. – Annealing temperature; bp – Base pair; Fw – Forward; Rv – Reverse

4.2.4 Analysis of the mRNA expression levels of TAS2R by real-time quantitative RT-PCR

Analysis of the mRNA expression levels of TAS2R4, R5, R14 and R39 and the housekeeping gene Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were performed by RT-qPCR using the NZYSpeedy qPCR Green Master Mix (NZYTech #MB224). Cycling conditions were the following: 95°C for 2 minutes, followed by 40 cycles of 95°C for 5 seconds, 58°C for 10 seconds, and 72°C for 3 seconds. Primers sequences for TAS2Rs and GAPDH used for RT-qPCR are shown in Table 4.2. A validation assay was performed with cDNA serial dilutions for both genes. The ΔCt was calculated using GAPDH mRNA as the reference gene, and the ΔΔCt was calculated between the normalized ΔCt values from each time point and the average Ct value at all time points tested. Relative amounts of all mRNAs were calculated according to the $2^{-\Delta\Delta Ct}$ method (Pfaffl 2001). A melting curve analysis was performed after the final cycle to ensure that a single product was obtained.

Table 4.2 – List of primers used for real-time quantitative RT-PCR for the bitter taste receptors in human astrocytes and glioblastoma cell lines.

RefSeq	Gene Name	Gene Symbol	Primer Sequence 5’ – 3’	Size (bp)	A.T. (°C)
NM_016944.2	Taste receptor, type 2, member 4	**TAS2R4**	Fw: CTGGAATCCCCAGACGGAAG Rv: CTGGACCAGGGTAGCAACTG	97	60
NM_018980.3	Taste receptor, type 2, member 5	**TAS2R5**	Fw: CTTTTCCAGAGCAGCCGTTG Rv: GTGGCAAACCATAAGCTGGC	80	60
NM_023922.1	Taste receptor, type 2, member 14	**TAS2R14**	Fw: GGTGAACTGTATTGACTGGGTC Rv: GCTGGGAAAAACACAGACACAC	135	58
NM_176881.2	Taste receptor, type 2, member 39	**TAS2R39**	Fw: ATGTGGTCGGTCTGGCTTTT Rv: TGCTTCCCATGTGTAGGGTG	120	58
NM_001357943.2	Glyceraldehyde 3-phosphate dehydrogenase	**GAPDH**	Fw: ATGGGGAAGGTGAAGGTCG Rv: GGGGTCATTGATGGCAACAATA	108	58 / 60

A.T. – Annealing temperature; bp – Base pair; Fw – Forward; Rv – Reverse

4.2.5 Detection of TAS2R and GNAT3 protein in human astrocytes and glioblastoma cell lines

4.2.5.1 Western Blot analysis

Suspensions of human astrocytes and glioblastoma cells in phosphate-buffered saline (PBS) 1x were centrifuged at 10 000 g for 7 min at 4°C, and the pellets homogenized in RIPA buffer [150 mM NaCl, 0.5% Sodium deoxycholate, 0.1% SDS, 1% Triton X-100, 50 mM Tris pH 8.0, 1 mM PMSF and 10 µL/mL Complete EDTA Free protease inhibitor cocktail (Roche #11873580001)]. Total protein measurement was performed with Pierce BCA Protein Assay Kit (ThermoFisher Scientific #23227) following the manufacturer’s instructions. An amount of 50 µg of total protein was mixed with a loading buffer containing 4% β-mercaptoethanol, followed by denaturation for 5 min at 100°C, and then loaded in a 12.5% SDS-PAGE. Proteins were transferred to a PVDF membrane 0.45 µm (GE Healthcare #10600023), which was blocked with 5% non-fat dry milk in Tris-buffered saline (TBS) 1x, for 1h at room temperature (RT). After overnight incubation at 4°C with the primary antibodies GNAT3, TAS2R4, R5, R14 and R39 (Table 4.3), membranes were rinsed three times with TBS containing 0.1% of Tween-20 (TBS-T) and incubated with the respective secondary antibody HRP-linked for 1h at RT. Moreover, GNAT3 primary antibody specificity was assessed through parallel incubation with the respective peptide. After this, signal detection was performed with SuperSignal™ West Pico PLUS Chemiluminescent Substrate (ThermoFisher Scientific #34577) and images were acquired with the Image Lab software in a ChemiDoc™ MP (Bio-Rad). This experiment was done with at least three different cell passages. Additionally, expression of GNAT3, TAS2R4, R5, R14 and R39 was normalized with β-actin incubated for 1h at RT before incubation for 1h with HRP-conjugated goat anti-mouse secondary antibody. After blotting, images were acquired, and protein bands were quantified using the Image Lab software (Bio-Rad).

Table 4.3 – List of antibodies used for Western blot, immunohistochemistry, and immunofluorescence of bitter taste receptors protein detection.

	Host	Catalog Number	WB	IHC	IF
Primary Antibodies					
TAS2R4	Rabbit	ThermoFisher Scientific #OSR00153W	1:500	1:1500	1:500
TAS2R5	Rabbit	ThermoFisher Scientific #OSR00154W	1:500	1:1500	1:500
TAS2R14	Rabbit	ThermoFisher Scientific #PA5-39710	1:300	1:750	1:300
TAS2R39	Rabbit	ThermoFisher Scientific #PA5-39711	1:300	1:500	1:300
TAS2R43	Rabbit	ThermoFisher Scientific #PA5-103257	–	–	1:250
β-Actin	Mouse	Sigma-Aldrich #A1978	1:30000	–	–
GNAT3	Rabbit	SantaCruz Biotechnology #sc-395	1:250	–	1:100
GNAT3 Peptide	Rabbit	SantaCruz Biotechnology #sc-395 P	1:250	–	–
Secondary Antibodies					
Alexa Fluor® 488 Anti-Rabbit	Goat	Molecular Probes #A-11008	–	–	1:1000
Anti-rabbit IgG-HRP	Goat	Invitrogen #31466	1:20000	–	–
Anti-mouse IgG-HRP	Goat	SantaCruz Biotechnology #sc-2005	1:30000	–	–

IF – Immunofluorescence; IHC – Immunohistochemistry; WB – Western blot

4.2.5.2 Immunocytochemistry

The presence of GNAT3, TAS2R4, R5, R14 and R39 was investigated in the human astrocytes and glioblastoma cell lines by fluorescence immunocytochemistry. Briefly, cells were seeded and grown on glass coverslips until 60-70% confluence. After removing the medium, cells were fixed with paraformaldehyde (PFA) 4% for 10 min followed by 1h blocking in PBS containing 3%

bovine serum albumin (BSA) and 0.2% Triton X-100 at RT. Cells were incubated overnight at 4°C with the primary antibodies (Table 4.3) in blocking solution. In negative controls, the primary antibody was omitted. Cells were then incubated for 1h with Alexa Fluor® 488 goat anti-rabbit IgG conjugate. Finally, coverslips were incubated for 10 min with the fluorescent dye Hoechst 33342 to visualize their nuclei. After several washes, cells were mounted onto microscope slides and visualized under a confocal microscope LSM 710 (Carl Zeiss) using a magnification of 63x (Plan-Apochromat 63x/1.4 Oil DIC M27).

4.2.6 Detection of TAS2R in human samples of glioblastoma by immunohistochemistry

Cases for the immunohistochemistry study were obtained from the Institute of Neuropathology Brain Bank (HUB-ICO-IDIBELL Biobank) following the guidelines of the Spanish legislation on this matter (Real Decreto 1716/2011) and the approval of the local ethics committee of the Bellvitge University Hospital-IDIBELL. Human glioblastoma samples were fixed in buffered formalin for no less than 3 weeks and then embedded in paraffin. Paraffin-embedded human glioblastoma slices, from men (n=4) and women (n=6) were pre-treated with Trilogy™ (Cell Marque™ #920P) which combines deparaffinization, rehydration and unmasking, following manufacturer recommendations. After washing with TBS-T, endogenous peroxidases activity was blocked with 3% H2O2 for 10 min at RT. Slices were then washed twice with TBS-T. Next, slices were incubated for 1h at RT with the following primary antibodies TAS2R4, R5, R14 or R39 (Table 4.3). Slices were washed twice with TBS-T and treated with HiDef Detection™ HRP Polymer System (Cell Marque™ #954D). First, HiDef Detection™ Amplifier was applied in the human glioblastoma slices for 10 min RT, washed twice with TBS-T, followed by HiDef Detection™ HRP Polymer Detector also for 10 min at RT. After slices washing with TBS-T, immunoreactivity was detected with diaminobenzidine (DAB) for 10 min RT. Slices were washed twice with TBS-T. Next, tissue sections were stained with Mayer's Hematoxylin for 3 min RT to allow nuclei visualization. Negative control slices were treated under the same conditions without primary antibody. No immunostaining was seen in these sections. After dehydration, the slices were mounted with Q Path® Coverquick 2000 (VWR #805547530) and the images were acquired in a Zeiss Axio Imager Z2 (Carl Zeiss) using a magnification of 20x (Plan-Apochromat 20x/0.8 M27).

4.2.7 Functional assays

4.2.7.1 Effects of TMZ in intracellular Ca^{2+} responses of glioblastoma cells

The effects of TMZ on Ca^{2+} cells responses, in the presence or absence of some TAS2Rs antagonist probenecid, were assessed by single-cell Ca^{2+} imaging assays in glioblastoma SNB-19 and U-373MG cells. Briefly, approximately 3.5×10^4 glioblastoma cells were seeded in µ-slide 8 well ibiTreat (Ibidi # 80826) and grown until 60-70% confluency, followed by measurement of changes in intracellular Ca^{2+} levels after stimulation. Glioblastoma cells were loaded with 5 µM of FURA-2 AM and 0.02% pluronic acid F-127 in culture medium for 45 min. Next, cells were washed twice with Tyrode's solution pH 7.4 [NaCl 140 mM, KCl 5 mM, MgCl2 1.0 mM, CaCl2 2.0 mM, Na-pyruvate 10 mM, glucose 10 mM, HEPES 10 mM, NaHCO3 5.0 mM] and loaded with Tyrode's for 30 min. After that, dose-response experiments were performed with a range of TMZ concentrations (50-200 µM), in the presence or absence of 30 min incubation with probenecid (1 mM). The µ-slide plates were placed on a Widefield Axio Observer Z1 inverted microscope (Carl Zeiss). Stock solution of TMZ and probenecid was freshly prepared in Tyrode's solution before the experiments. The stimulus was applied manually with a micropipette after baseline was recorded. The intracellular Ca^{2+} levels were evaluated by quantifying the ratio of the fluorescence emitted at 520 nm following alternate excitation at 340 nm and 380 nm, using a Lambda DG4 apparatus (Sutter Instruments, Novato) and a 520 nm bandpass filter (Carl Zeiss) under a Fluar 40x/1.30 Oil M27 objective (Carl Zeiss) with an AxioVision camera and software (Carl Zeiss). Data were processed using the Fiji software (MediaWiki). Changes in fluorescence ratio (F=F340/F380) were measured in at least 20 cells, in three or more independent experiments. Response intensity, or intracellular Ca^{2+} variation, (ΔF/F0), was calculated in the following way: ΔF/F0=(F-F0)/F0, where F0 corresponds to fluorescence ratio average at baseline (2 min acquisition before stimulus) and F correspond to maximum peak of fluorescence ratio evoked by the stimulus applied to the cells.

4.2.7.2 Evaluation of TMZ cytotoxicity

After Ca^{2+} imaging experiments, the cytotoxicity of TMZ was assessed in glioblastoma cells in the presence or absence of the TAS2Rs antagonist probenecid, using the MTT assay. Additionally, the specific activation of bitter taste signalling by TMZ was assessed in glioblastoma cells after GNAT3 knockdown with specific siRNA. Briefly, glioblastoma cells were grown until 60% confluency or transfected for 24h with a mixture of GNAT3 siRNA (10 nM) and Lipofectamine™ 2000 in Opti-MEM medium, following the manufacturer's instructions. A scramble siRNA (10 nM) was also used as negative control for GNAT3 specific targeting. Then, cells were incubated for 72h with TMZ (500 µM) or vehicle (DMSO 1%), in the presence or absence of 1 mM probenecid diluted in culture medium. Then, 100 µL culture medium was removed and 10 µL of MTT solution (5 mg/mL in PBS) was added for approximately 45 min at 37°C in a humidified atmosphere containing 5% CO2. Untreated cells and ethanol 70% treated cells were used as negative and positive controls, respectively. Following MTT incubation, formazan crystals were dissolved in DMSO for 15 min, and absorbance was read at 570 nm in a microplate spectrophotometer xMark™ (Bio-Rad). The viability of glioblastoma cells was expressed as a percentage of the absorbance determined in the vehicle control.

The apoptotic effect of TMZ, in the presence or absence of probenecid, was also evaluated in glioblastoma cells by Hoechst 33342 nuclei staining. Glioblastoma cells were seeded in a coverslip and the stimuli were carried out as described above in section 4.2.7.2. After removing the medium, cells were fixed with PFA 4% for 10 min followed by incubation for 10 min with Hoechst 33342 (diluted 1:1000). After several washes, cells were mounted onto microscope slides and visualized under a confocal microscope LSM 710 (Carl Zeiss) using a magnification of 63x (Plan-Apochromat 63x/1.4 Oil DIC M27). Apoptotic cells were distinguished from healthy or necrotic cells by the observation of condensed DNA and fragmented nuclei. The apoptotic rate was calculated using the ratio between the number of apoptotic cells and the total number of cells.

4.2.8 Analysis of the role of TAS2R43 on the glioblastoma cells' response to TMZ

Following the demonstration that the bitter taste signalling pathway was involved in the mediation of the effects of TMZ, we assessed *in silico* which TAS2R could have a higher likelihood of binding to TMZ according to the webserver VirtualTaste method (Fritz *et al.* 2016). Using this approach, six TAS2Rs, namely TAS2R38 and TAS2R43, were identified as possible target-receptors for TMZ. Then, we confirmed TAS2R38 and TAS2R43 expression in glioblastoma cells (U-87MG, SNB-19, and U-373MG) by RT-PCR (TAS2R38: Forward-*TGTGGTCTGTGCCTCCTTTC*; Reverse-*AGGGCTTTAATGTGGGCCTC*; TAS2R43: Fw-*TATCTGGGCAGTGATCAACCA*; Rv-*CCCCAACAACATCACCAGAATG*), and TAS2R43 (Table 4.3) protein detection in SNB-19 and U-373MG cells by immunocytochemistry, as respectively described in sections 4.2.3 and 4.2.5.2. Finally, the role of TAS2R43 on the response to TMZ was assessed in glioblastoma cells after TAS2R43 knockdown with specific siRNA, as described in section 4.2.7.2 for GNAT3.

4.2.9 Data analysis

Statistical analysis and dataset comparisons were performed using GraphPad Prism 7.0 (GraphPad Software). Statistical significance was determined by One-Way ANOVA followed by the software's recommended multiple comparisons post-hoc test. Results are presented as mean ± SEM of at least three independent experiments, and data were considered statistically different for a p-value < 0.05.

4.3 Results

4.3.1 Human astrocytes and glioblastoma cells differentially express 19 TAS2Rs transcripts

In humans, 25 members of the bitter taste receptor gene family were described (Adler *et al.* 2000). The expression profile of bitter taste receptors in a human astrocytes cell line and glioblastoma cells was assessed by RT-PCR using specific full-length primers (Table 4.1). The results demonstrated the mRNA expression of 19 TASR2s (1, 3, 4, 5, 7, 8, 9, 10, 14, 16, 38, 39, 40, 41, 42, 44, 48, 50 and 60) (Table 4.4). No TAS2R13 mRNA was detected in all the cells studied. On the other hand, TAS2R20/49, 30/47, 43, 45 and 46 mRNA expression was not analysed because of the great homology between them, which difficult the design of specific primers. Moreover, it was possible to observe that not all TAS2Rs were expressed in all the cell lines studied. TAS2R1 and 38 were only detected in SNB-19, whereas TAS2R16 was not detected in U-87MG, and TAS2R50 was not detected in U-87MG and U-373MG cells. Interestingly, TAS2R60 was only detected in glioblastoma cells (Table 4.4).

Table 4.4 – mRNA expression profile of bitter taste receptors in human astrocytes (HA) and glioblastoma (U-87MG, SNB-19, U-373MG) cell lines. RT-PCR was performed with cDNA synthetized from total RNA. The identities of the amplified products were confirmed by Sanger sequencing.

Gene Symbol	HA	U-87MG	SNB-19	U-373MG
TAS2R1	-	-	+	-
TAS2R3	+	+	+	+
TAS2R4	+	+	+	+
TAS2R5	+	+	+	+
TAS2R7	+	+	+	+
TAS2R8	+	+	+	+
TAS2R9	+	+	+	+
TAS2R10	+	+	+	+
TAS2R13	-	-	-	-
TAS2R14	+	+	+	+
TAS2R16	+	-	+	+
TAS2R20/49	n/a	n/a	n/a	n/a
TAS2R30/47	n/a	n/a	n/a	n/a
TAS2R38	-	-	+	-
TAS2R39	+	+	+	+
TAS2R40	+	+	+	+
TAS2R41	+	+	+	+
TAS2R42	+	+	+	+
TAS2R43	n/a	n/a	n/a	n/a
TAS2R44	+	+	+	+
TAS2R45	n/a	n/a	n/a	n/a
TAS2R46	n/a	n/a	n/a	n/a
TAS2R48	+	+	+	+
TAS2R50	+	-	+	-
TAS2R60	-	+	+	+

(+) – detected; (-) – not detected; n/a – not analysed

To assess the mRNA relative expression differences between human astrocytes and glioblastoma cells we performed RT-qPCR for TAS2R4, R5, R14 and R39. Compared to human astrocytes, TAS2R4 expression (Figure 4.1A) is significantly increased in SNB-19 and U-373MG cells (2.92-

and 2.89-fold, respectively), and TAS2R5 expression (Figure 4.1B) is significantly increased in U-373MG cells (6.36-fold). TAS2R39 mRNA expression is significantly increased in U-87MG cells (Figure 4.1D) in comparison to human astrocytes (2.11-fold) and SNB-19 (1.81-fold) and U-373MG (3.38-fold) glioblastoma cells. No significant differences were observed for TAS2R14 mRNA expression between both astrocytes and cancer cell lines (Figure 4.1C).

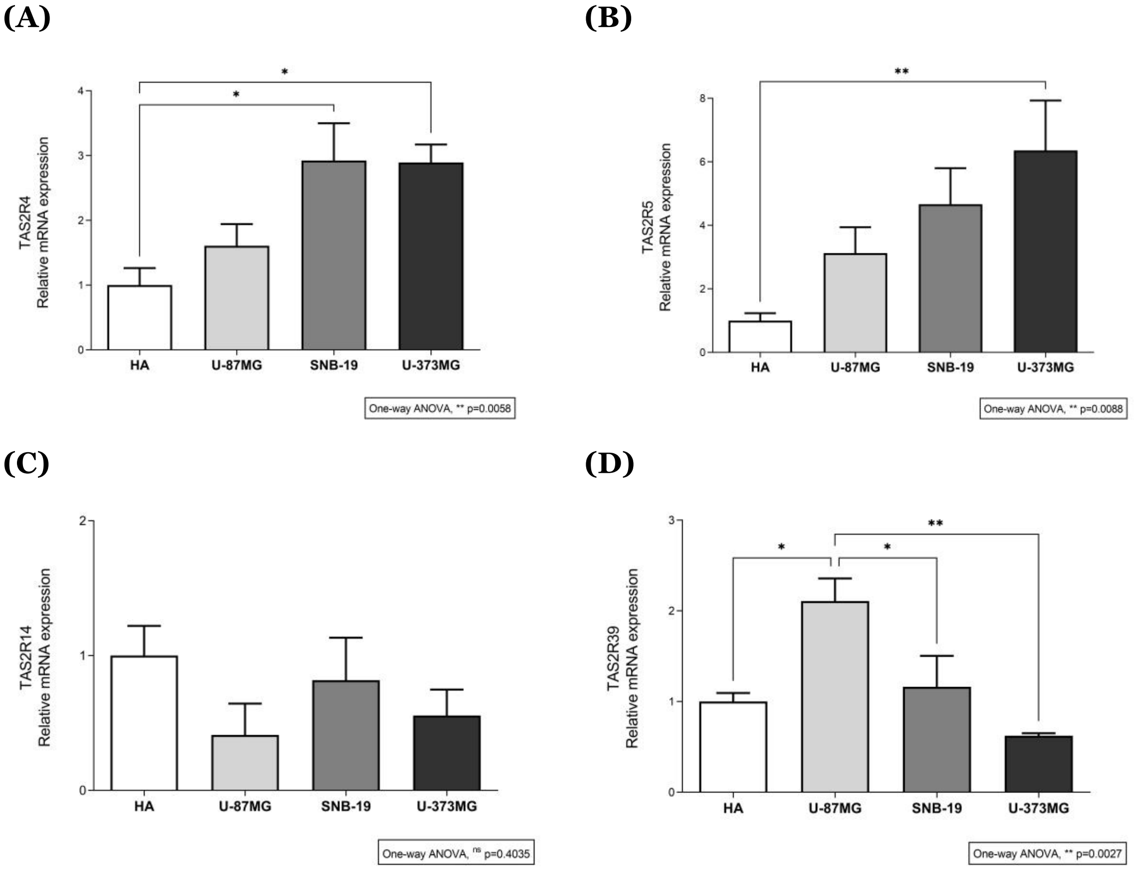


Figure 4.1 – The mRNA of bitter taste receptors is differentially expressed in human astrocytes and in human glioblastoma cell lines. The mRNA expression levels of bitter taste receptors (A) TAS2R4, (B) R5, (C) R14 and (D) R39 normalized to the expression of the housekeeping gene GAPDH, were compared between human astrocytes (HA) and glioblastoma (U-87MG, SNB-19, U-373MG) cell lines by RT-qPCR. Results are presented as mean ± SEM and analysed by one-way ANOVA followed by Tukey's multiple comparisons test. [N≥4 independent experiments; ^{ns} no significant; *p<0.05 and **p<0.01].

4.3.2 Human astrocytes and glioblastoma cells differentially express TAS2Rs proteins

We assessed the taste-related downstream effector protein GNAT3 and the four selected TAS2Rs in protein extracts of human astrocytes and glioblastoma cells by Western blot (Figure 4.2) and immunofluorescence (Figure 4.3) using available antibodies, previously validated in our research group (Duarte *et al.* 2020). The pre-incubation of the GNAT3 antibody with the respective peptide reduced the signal obtained, demonstrating the antibody specificity (Figure

4.2A). In addition, all the taste-related proteins were detected at the expected size (Figure 4.2B). Of notice, TAS2R39 protein in SNB-19 cells was only detected once. The analysis of the TAS2Rs relative protein expression revealed a significant decrease (3.27-fold) of TAS2R4 expression in U-87MG relatively to human astrocytes (Figure 4.2C). Moreover, a significant decrease in TAS2R14 expression was observed for human astrocytes (7.61-fold), U-87MG (8.52-fold) and SNB-19 (7.76-fold) compared to U-373MG glioblastoma cells (Figure 4.2E). No statistical differences were observed for TAS2R5 and TAS2R39 protein levels (Figure 4.2D and 4.2F).

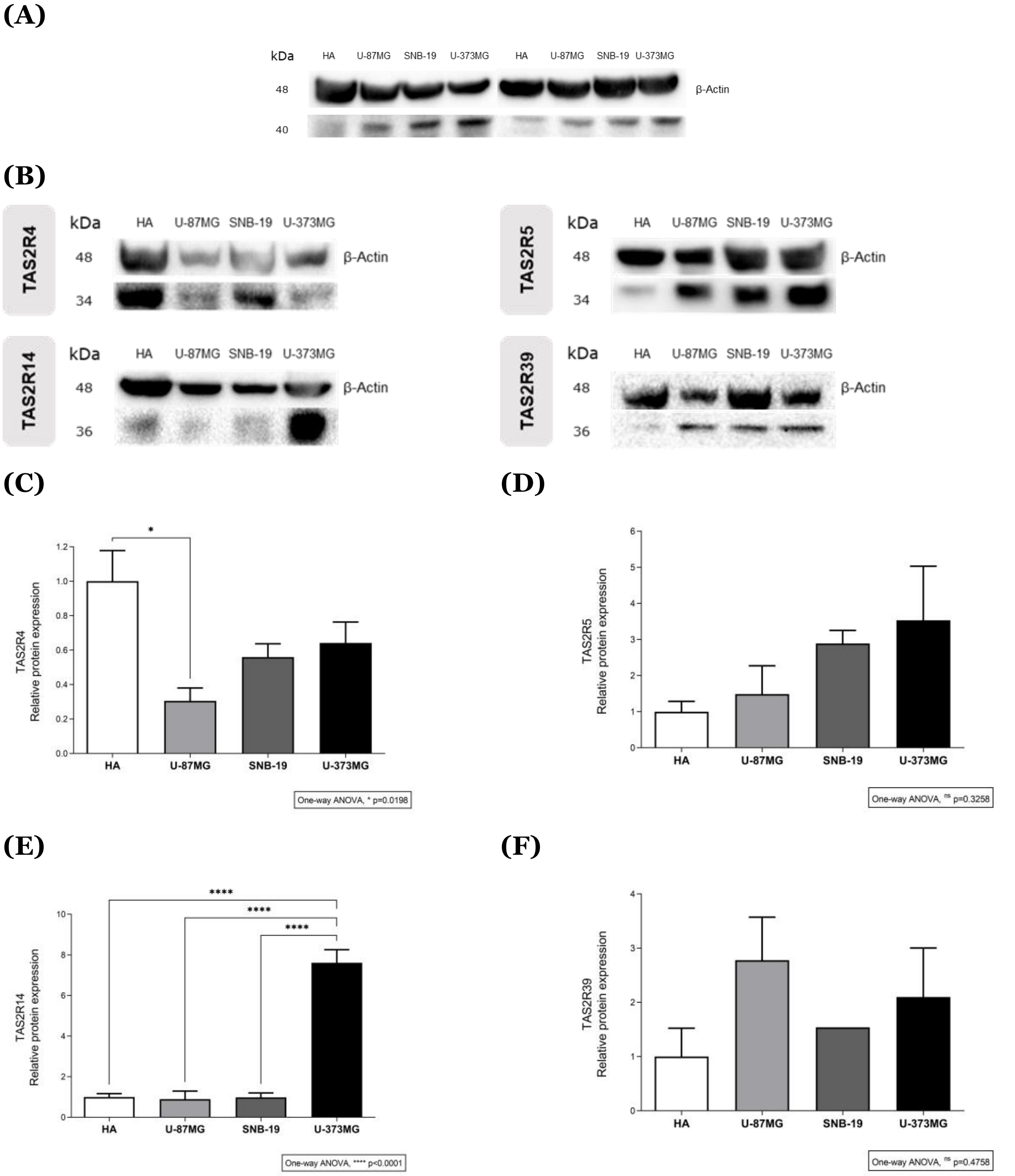


Figure 4.2 – Bitter taste receptors and taste signalling pathway effector GNAT3 are differentially expressed in human astrocytes and glioblastoma cell lines. (A) GNAT3 expression in all cell lines analysed (left panel). When the GNAT3 antibody was pre-incubated with the respective peptide, the intensity of the immune

reaction was strongly diminished showing the specificity of the antibody (right panel). (B) Western blot detection of bitter taste receptors TAS2R4, R5, R14 and R39 in protein extracts of human astrocytes (HA) and glioblastoma (U-87MG, SNB-19, U-373MG) cell lines (representative images). (C–F) Relative quantification of TAS2R4, R5, R14 and R39 protein levels in HA and glioblastoma cells, normalized to β-actin levels. Results are presented as mean ± SEM and analyzed by one-way ANOVA followed by Tukey's multiple comparisons test. [N≥3 independent experiments; ^{ns} no significant; *p<0.05 and ****p<0.0001]. kDa – kilo Dalton.

On the other hand, taste receptors TAS2R4, R5 and R39 were detected in the cytoplasm and plasma cell membrane. Interestingly, TAS2R14 was located exclusively at the nucleus of glioblastoma cells, contrary to its cytoplasmic location in human astrocytes (Figure 4.3). The downstream effector GNAT3 was predominantly found in the cytoplasm of both human astrocytes and glioblastoma cells but was also detected in the nucleus (Figure 4.3).

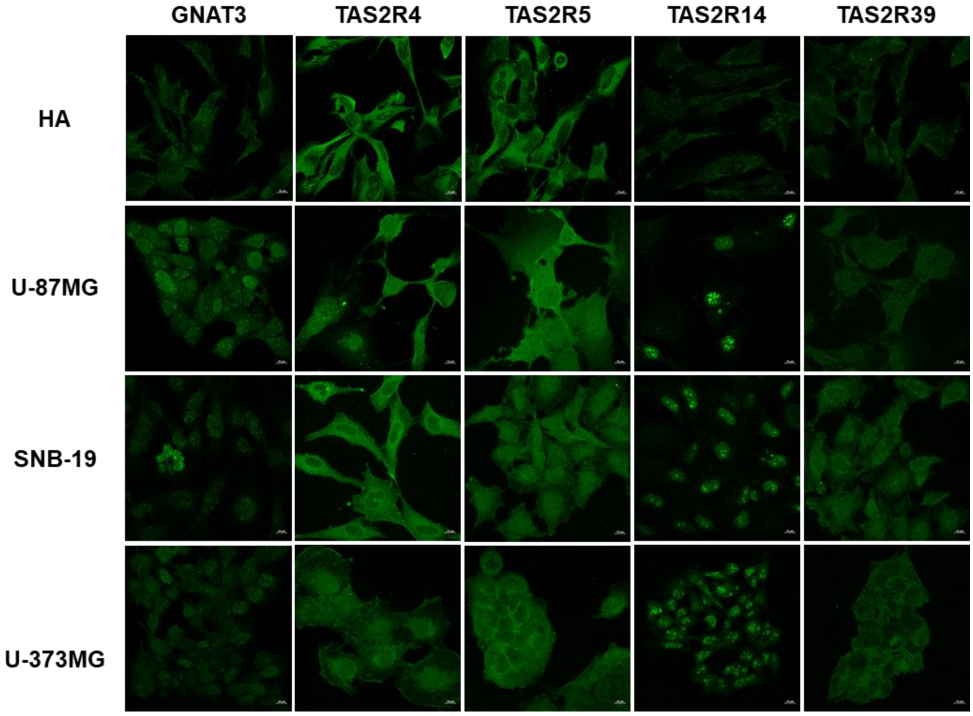


Figure 4.3 – Immunofluorescence detection of bitter taste receptors and GNAT3 in human astrocytes and glioblastoma cell lines. Confocal microscopy images of GNAT3 and bitter taste receptors TAS2R4, R5, R14 and R39 (green) in human astrocytes (HA) and glioblastoma cell lines (U-87MG, SNB-19, U-373MG). Nuclei were stained with Hoechst 33342 (not shown). Scale bar: 10 µm.

4.3.3 TAS2Rs are expressed in human glioblastoma

We analysed the expression of the four selected TAS2Rs in paraffin-embedded sections of human glioblastoma samples obtained by surgical resection of the tumour. TAS2Rs immunoreactivity was observed in all cases, although with TAS2R-dependent, individual-dependent, and region-dependent variations. In general terms, TAS2R4, R5 and R14 immunoreactivity was found in the cytoplasm of large and medium-size glioblastoma cells in areas of high cellular density. Less differentiated areas with small cells showed weak or no immunoreactivity. Areas with loose cellular density showed weak or moderate immunoreactivity when compared with areas with high cellular density. Immunostaining of glioblastoma cells was in striking contrast with the negativity of fibrous regions with predominant fibroblast-like cells. Necrosis areas were negative. TAS2R39 immunoreactivity was lower and more disperse when compared with the other assessed TAS2Rs (Figure 4.4). Due to the regional and individual variations, further densitometric quantifications were not carried out. Similarly, no attempt was made to categorize gender differences.

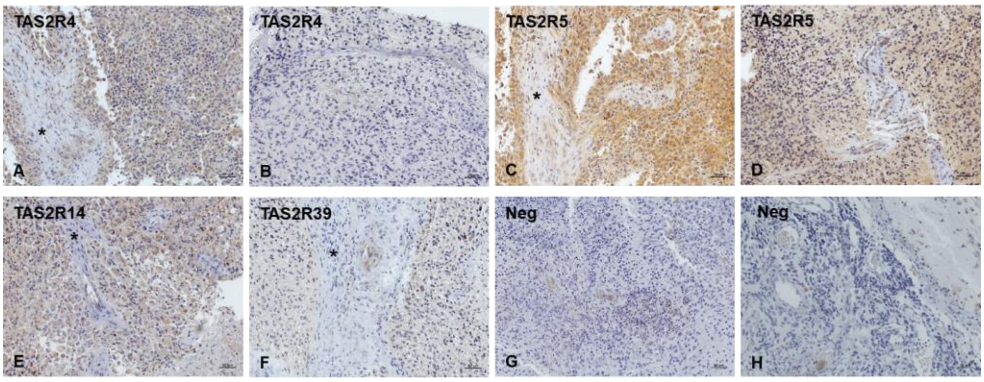


Figure 4.4 – Bitter taste receptors TAS2R4, R5, R14, and R39 immunostaining of tumour samples of human glioblastomas. Anti-TAS2R4, R5, and R14 antibodies decorate the cytoplasm of large and medium size glioblastoma cells in areas with high cellular density (A, C, D, E). In the same sections, fibrous areas (asterisks) are negative. However, TAS2R immunoreactivity was reduced in some areas with loose cellularity (B). In contrast, TAS2R39 immunoreactivity was lower in glioblastoma cells (F). No immunostaining was seen in negative control slices where the primary antibodies were ommited (G, H). Representative images of paraffin sections, lightly counterstained with Mayer's hematoxylin. Scale bar: 50 µm.

4.3.4 TMZ effects in glioblastoma cells depend on bitter taste signalling pathway activation

After confirming taste-related mRNA and protein expression in glioblastoma cells, we assessed SNB-19 and U-373MG response to TMZ (10–200 µM) using a Ca^{2+} functional assay (Figure 4.5). Ca^{2+} imaging assays were carried out in the presence of vehicle control (DMSO ≤ 0.2%) and compared to untreated cells to establish that Ca^{2+} variations observed were not due to DMSO (data not shown). Moreover, Ca^{2+} variations were collected for 2 min before the stimuli to obtain a baseline (F0) that was used to normalize the responses obtained with the compounds, and only assays showing a uniform baseline were evaluated. Moreover, assays were carried out in the presence or absence of probenecid, a blocker of TAS2R16, R38, and R43, to elucidate the role of TAS2Rs in glioblastoma cells response to TMZ (Greene *et al.* 2011; Wölfle *et al.* 2015).

Our results showed that TMZ was able to trigger a functional response in glioblastoma cells. TMZ at 100 µM ($\Delta F/F0=0.220\pm0.039$) triggered a significant increase in intracellular Ca^{2+}

levels in SNB-19 cells in comparison to the vehicle (ΔF/F0=0.025±0.008), abolished in the presence of probenecid (ΔF/F0=0.025±0.006). U-373MG cells stimulated with 50 and 100 µM TMZ showed higher Ca^{2+} levels (ΔF/F0=0.437±0.137 and ΔF/F0=0.718±0.155) in comparison with vehicle control (ΔF/F0=0.052±0.007). In addition, the TMZ effects on intracellular Ca^{2+} levels in the presence of probenecid were reverted (ΔF/F0=0.034±0.012). Neither 10 µM nor 200 µM TMZ triggered significant Ca^{2+} responses in both SNB-19 and U-373MG glioblastoma cells.

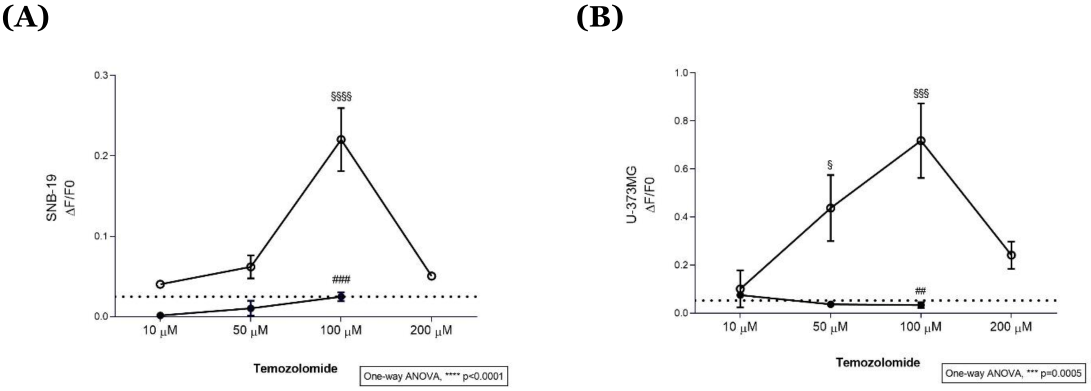


Figure 4.5 – The effect of temozolomide in the Ca^{2+} dose-response curves of glioblastoma cells requires the activation of the bitter taste signalling pathway. Calcium dose-response curves of (A) SNB-19 and (B) U-373MG glioblastoma cells to different concentrations of TMZ (10-200 µM), in the presence (●) or absence (○) of 1 mM probenecid, a known TAS2R inhibitor. Dot line: Ca^{2+} levels measured in cells with vehicle only (DMSO≤0.2%). Results are presented as mean ± SEM. Statistical analysis was performed by one-way ANOVA followed by Tukey's multiple comparisons test. [N≥3 independent experiments; § versus vehicle; $^{\#}$ versus TMZ].

Next, we proceeded to dose-response (50-500 µM) cytotoxicity assays (Figure 4.6). We found that 500 µM TMZ reduced the viability of U-87MG (49.08%±1.92), SNB-19 (44.46%±0.97) and U-373MG (50.92%±0.45) cells.

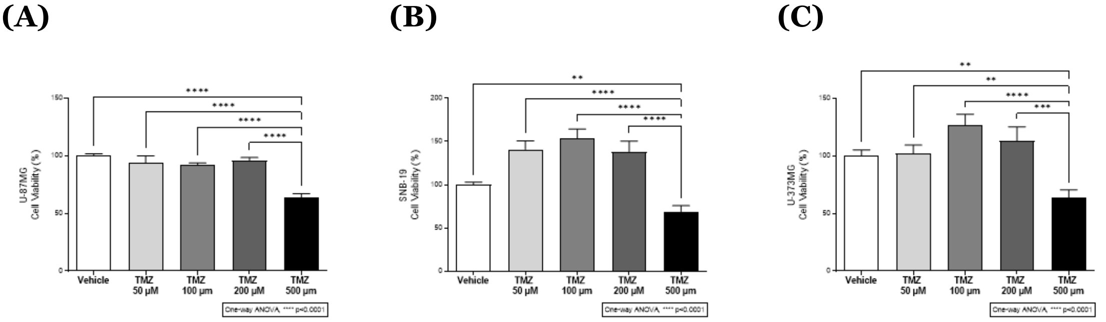


Figure 4.6 – Dose-response cytotoxicity assay of glioblastoma cells exposed to different concentrations of TMZ. The cytotoxic effect of different concentrations of TMZ (50-500 µM) for 72 hours was assessed in (A) U-87MG, (B) SNB-19, and (C) U-373MG glioblastoma cells by MTT assay. Results are presented as mean ± SEM. Statistical analysis was performed by one-way ANOVA followed by Tukey's multiple comparisons test. [N≥3 independent experiments; $^{**}p<0.01$, $^{***}p<0.001$ and $^{****}p<0.0001$].

Then, we evaluated the effects of TMZ on the apoptosis of glioblastoma cells in the presence of probenecid, a known TAS2R16, R38, and R43 inhibitor (Greene *et al.* 2011; Wölfle *et al.* 2015), by staining the cell nuclei with Hoechst 33342 and counting the nuclei with apoptotic vesicles (Figure 4.7). As expected, TMZ-induced apoptosis in these cells.

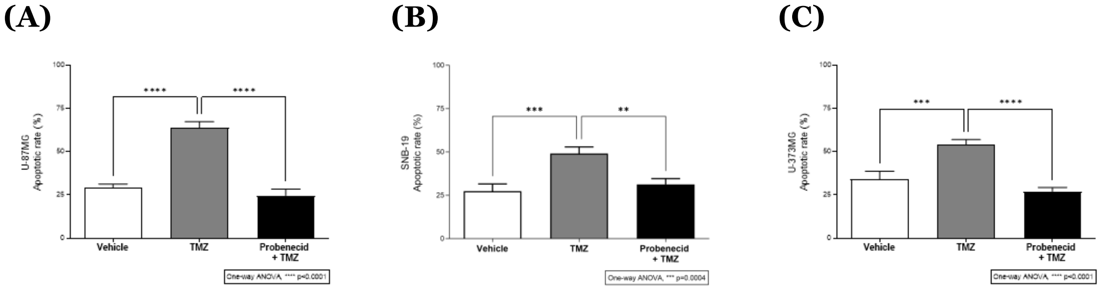
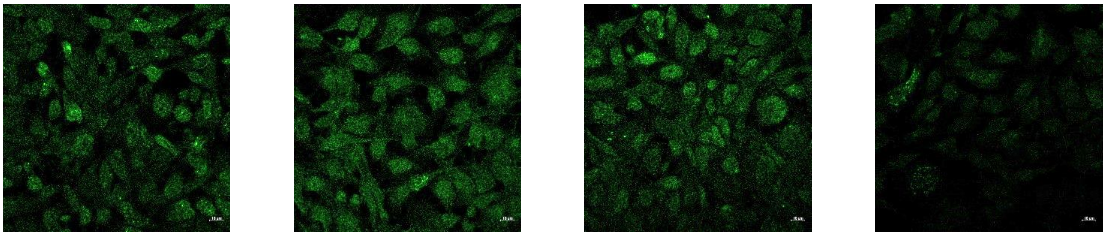


Figure 4.7 – The effect of temozolomide in the apoptosis of glioblastoma cells requires the activation of the bitter taste signalling pathway. The apoptotic rate of (A) U-87MG, (B) SNB-19, and (C) U-373MG glioblastoma cells incubated with 500 μM TMZ for 72h, in the presence or absence of 1 mM probenecid, was carried out by Hoechst 33342 staining. Bar graphs represent mean ± SEM. Statistical analysis was performed by one-way ANOVA followed by Tukey's multiple comparisons test. [N≥3 independent experiments; **p<0.01, ***p<0.001 and ****p<0.0001].

Our observations put in evidence that TMZ may be a TAS2Rs ligand, therefore exerting its cytotoxic effects, at least in part through the taste signalling activation. To test this hypothesis, we proceeded with GNAT3 knockdown experiments (Figure 4.8), since GNAT3 is the specific guanine nucleotide binding protein that transmits bitter, sweet and umami taste signals from taste receptors.



Non-transfected **Mock** **siScramble** **siGNAT3**

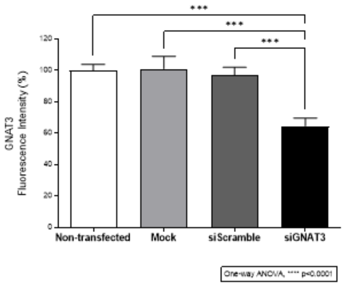


Figure 4.8 – Immunofluorescence analysis of GNAT3 expression after siRNA transfection in SNB-19 glioblastoma cells. Protein levels of siRNA GNAT3-transfected cells are decreased in comparison with non-, mock-, and siRNA scramble-transfected cells. The quantification of GNAT3 fluorescence intensity (green) was performed in different regions of interest (ROIs) of confocal microscopy images obtained from three independent experiments. Nuclei

were stained with Hoechst 33342 (not shown). Scale bar: 10 μm. Bar graphs represent mean ± SEM. Statistical analysis was performed by one-way ANOVA followed by Tukey's multiple comparisons test. [N=3 independent experiments; ***p<0.001].

TMZ induced a reduction of approximately 47.28±3.08% in both U-87MG, SNB-19 and U-373MG cell viability in comparison with non-, mock- and siRNA scramble-transfected cells (Figure 4.9). Notably, the effects of TMZ on the viability of glioblastoma cells were also decreased in the presence of probenecid (37.4% in U-87MG, 41.1% in SNB-19, and 45.9% in U-373MG) (Figure 4.9). On the other hand, no differences were observed in GNAT3-silenced cell viability (Figure 4.9), reinforcing the results obtained in the experiments carried out with probenecid and TMZ.

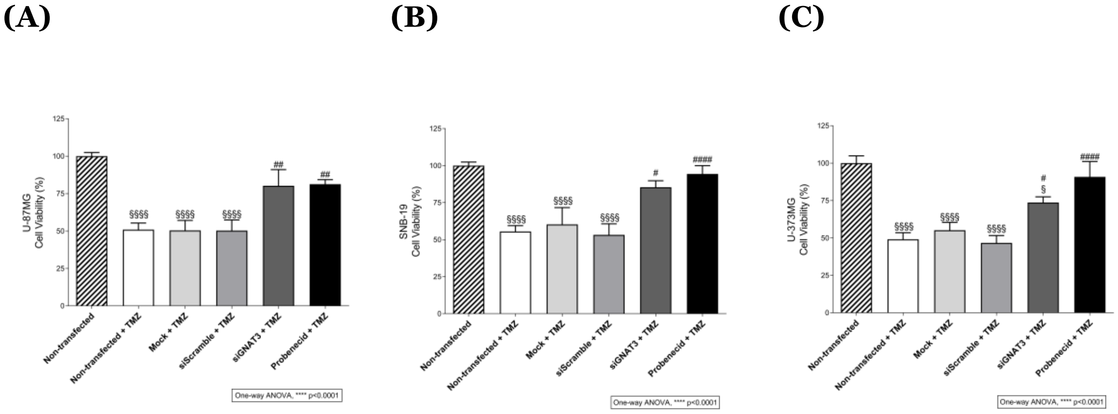


Figure 4.9 – The effect of temozolomide on the viability of glioblastoma cells requires the activation of the bitter taste signalling pathway. Effects of TMZ in the viability of (A) U-87MG, (B) SNB-19 and (C) U-373MG glioblastoma cells transfected or mock-transfected for 24h with GNAT3 or a scramble siRNA, and incubated with 500 μM TMZ for 72h, in the presence or absence of 1 mM probenecid. Bar graphs represent mean ± SEM. Statistical analysis was performed by one-way ANOVA followed by Tukey's multiple comparisons test. [N≥3 independent experiments; § versus non-transfected; $^{\#}$ versus non-transfected+TMZ].

Overall, TMZ elicited Ca^{2+} responses in a dose-dependent manner and showed cytotoxic effects in glioblastoma cells, which were abolished in the presence of probenecid or with GNAT3 silencing, highlighting that TMZ effects depend on activation of the bitter taste signalling pathway.

4.3.5 TAS2R43 mediates the effects of temozolomide in the viability of glioblastoma cells

After confirming the role of the bitter taste signalling pathway in the glioblastoma cells response to TMZ, we first predicted which of TAS2R(s) TMZ could bind, based on the webserver VirtualTaste method (Figure 4.10A). Six TAS2Rs were shown to be possible TMZ ligands: R38

(0.72) > R10 (0.68) > R43 (0.65) > R45 (0.62) > R44 (0.60) > R14 (0.59). Since only TAS2R38 and R43 bind probenecid, TAS2R10, R14, R44 and R45 were discarded for further experimentation. So, we analysed TAS2R38 and R43 mRNA expression in glioblastoma cells by RT-PCR and observed that only TAS2R43 was expressed in all the glioblastoma cell lines (Figure 4.10B). The presence of TAS2R43 protein in U-87MG, SNB-19 and U-373MG cells was also confirmed by immunocytochemistry (Figure 4.10C).

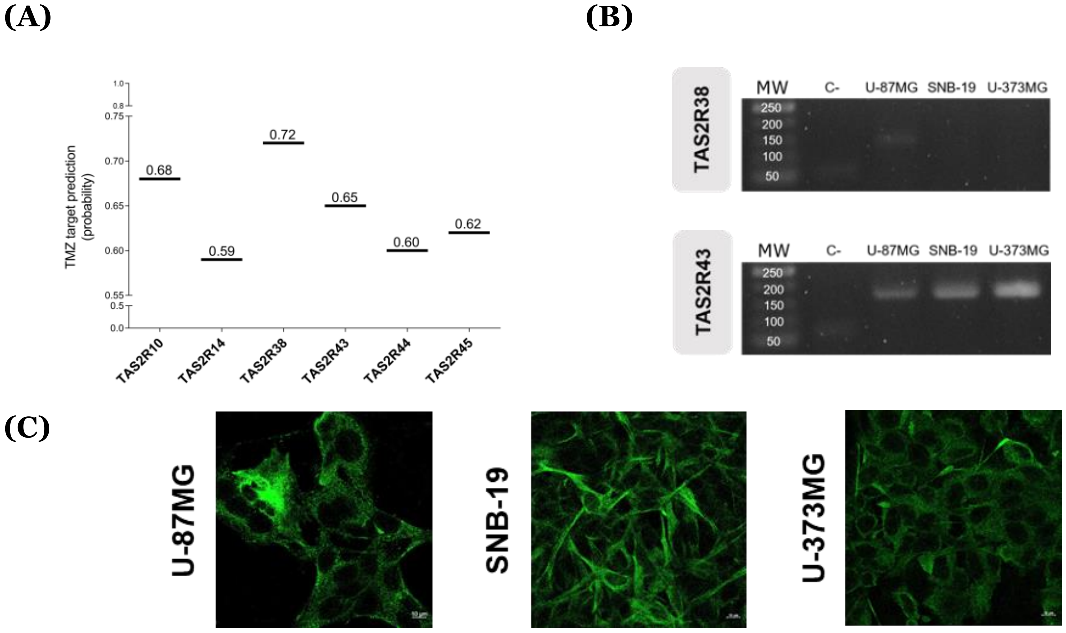


Figure 4.10 – Temozolomide is predicted to bind TAS2R43 in glioblastoma cells. (A) TMZ is predicted to bind TAS2R38 > R10 > R43 > R45 > R44 > R14 according to the webserver VirtualTaste algorithm. Results are presented as probability. (B) mRNA expression profile of TAS2R38 and R43 in glioblastoma cell lines (U-87MG, SNB-19, U-373MG). Only TAS2R43 mRNA and protein were detected in all cell lines. The identities of the amplified products were confirmed by Sanger sequencing. MW: molecular weight (base pair); C–: negative control. (C) Immunofluorescence detection of TAS2R43 (green) in U-87MG, SNB-19 and U-373MG glioblastoma cell lines. Nuclei were stained with Hoechst 33342 (not shown). Scale bar: 10 μm.

These results put in evidence that TMZ may exert its cytotoxic effects in glioblastoma cells through TAS2R43 activation. To test this hypothesis, we proceeded with TAS2R43 knockdown experiments (Figure 4.11) and observed that TMZ induced a reduction of approximately 41.78±2.44% in the cell viability of U-87MG, SNB-19 and U-373MG in comparison with non-, mock- and siRNA scramble-transfected cells. On the other hand, no differences were observed in the viability of TAS2R43-silenced cells (Figure 4.11), suggesting that the reduction of cell viability induced by TMZ is partially mediated by the TAS2R43 activation.

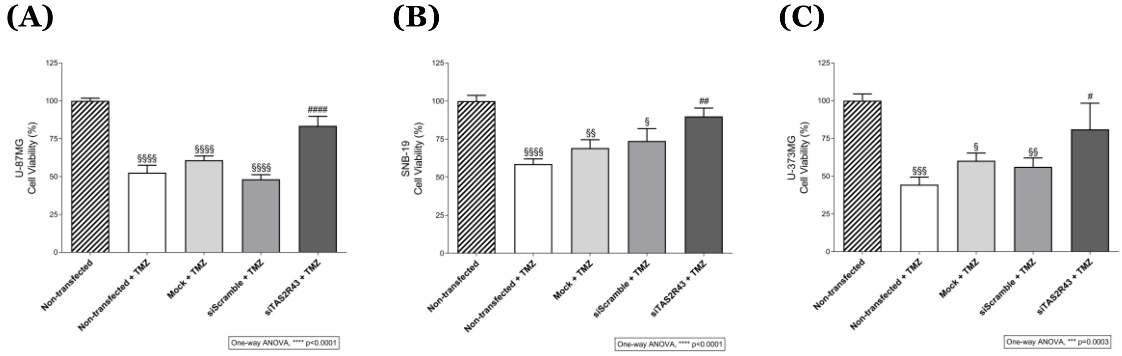


Figure 4.11 – The anti-proliferative effects of temozolomide in glioblastoma cells depend on the activation of TAS2R43. Effects of TMZ in the viability of (A) U-87MG, (B) SNB-19 and (C) U-373MG glioblastoma cells transfected or mock-transfected for 24h with TAS2R43 or a scramble siRNA and incubated with 500 µM TMZ for 72h. Bar graphs represent mean ± SEM. Statistical analysis was performed by one-way ANOVA followed by Tukey's multiple comparisons test. [N=3 independent experiments; § versus non-transfected; # versus non-transfected+TMZ].

4.4 Discussion

Nowadays, an estimated 300.000 people worldwide are living with a CNS tumour, and it is estimated that 45% more will be diagnosed by 2040 (Ferlay *et al.* 2019). Therefore, it is of utmost importance the search for new therapeutic drugs or targets to overcome chemoresistance and relapse, and to improve patient outcomes. Recently, the expression and functionality of the taste transduction pathway, particularly TAS2Rs, has been studied in several types of cancer, especially in breast and pancreatic cancer cells (Jeruzal-Świątecka *et al.* 2020). However, to date, bitter taste signalling in glioblastoma cells has not been studied. Therefore, we aimed to investigate whether TAS2Rs could be involved in the response of human glioblastoma cells to TMZ, the golden standard treatment for these tumours, and predictably, a bitter taste compound.

Herein, we validated and confirmed the mRNA expression of 19 of the 25 human TAS2Rs in both astrocytes and in three different glioblastoma cell lines which differ in terms of morphology and proliferation rates. U-87MG are cluster invasion cells, whereas most chemoresistant SNB-19 and U-373MG (similar to U-251MG) are individual invasion and expansive-growth cells, respectively (Diao *et al.* 2019; Memmel *et al.* 2014). Among the TAS2R expressed in these cell lines, TAS2R4, R5, R14 and R39 were selected based on criteria described in section 2.3 to carry out further experiments. The presence and functionality of the taste signalling pathway in human astrocytes and glioblastoma cells were further supported by analysing the expression of the downstream effector protein GNAT3, an important and specific component of the taste signalling pathway (McLaughlin *et al.* 1992).

Our findings not only confirmed the presence of the bitter taste signalling machinery in the human glioblastoma but also highlighted the differential expression of several TAS2R between the cell lines analysed, and in human glioblastoma samples. Among the TAS2Rs studied, R4 and R5 bind fewer agonists than many other TAS2Rs. Both are located on the same chromosomal position 7q31 (Bachmanov & Beauchamp 2007; Dagan-Wiener *et al.* 2019; Wiener *et al.* 2012). We show that TAS2R4 presented higher mRNA levels in SNB-19 and U-373MG glioblastoma cells, contrary to the highest protein levels found in human astrocytes. These results contrast with results described in two previous studies performed by Singh et al., where TAS2R4 exhibits significantly lower mRNA expression levels in breast cancer cell lines and clinical samples compared to their respective non-cancerous controls (Singh *et al.* 2014; Singh *et al.* 2020). TAS2R5 also presented higher mRNA levels in the U-373MG glioblastoma cell line in comparison to human astrocytes.

Contrarily to TAS2R4 and R5, that are moderately and narrowly tuned, respectively, TAS2R14 and R39 are broadly tuned to detect many bitter compounds of different chemical classes (Beckett *et al.* 2014; Behrens *et al.* 2004; Dagan-Wiener *et al.* 2019; Wiener *et al.* 2012), including compounds that exhibit anticancer or chemotherapy enhancing activities (Kawano et al. 2012; Lyn-Cook et al. 1999), suggesting their potential to be used as therapeutic targets in the cells or tissues where they are expressed. In the present study, TAS2R14 presented higher protein levels in one glioblastoma cell line relatively to human astrocytes. Jaggupilli *et al.* already reported that transcript levels of TAS2R14 were significantly elevated in highly invasive breast cancer MDA-MB-231 cells when compared to non-cancerous MCF-10A cells (Jaggupilli *et al.* 2017). Similar results were recently reported in breast cancer clinical samples compared to non-cancerous controls (Singh *et al.* 2020). We also analysed the subcellular location of these TAS2Rs in the glioblastoma cell lines. Notably, TAS2R14 location in glioblastoma cells was exclusively nuclear, contrary to its cytoplasmic and/or membrane location in human astrocytes. Prior studies already reported nuclear localization of some TAS2Rs in non-ciliated or inflamed airway tissues (Carey *et al.* 2022; McMahon *et al.* 2022; Zborowska-Piskadło *et al.* 2020). One of these studies showed the nuclear localization of TAS2R13 and R42 in head and neck squamous cell carcinomas (Carey *et al.* 2022). To our knowledge, there are no studies addressing the cellular location of any TAS2Rs in cancer compared to non-cancer cells.

Although TAS2R39 is poorly studied in cancer, it resides in the same chromosomal position (chromosome 7q34) as TAS2R38, indicating shared regulation by the same control elements and a possible correlation in their expression (Bachmanov & Beauchamp 2007). Interestingly, TAS2R38 is upregulated in tumour cells from pancreatic cancer patients and tumour-derived cell lines compared to RLT pancreatic stellate cell line (Gaida *et al.* 2016). In the present study, TAS2R39 mRNA, but not protein, levels were higher in one glioblastoma cell line, than in human astrocytes. Other studies reported that mRNA expression of TAS2R14 and R38 is decreased in both prostate and ovarian cancer cells (Martin *et al.* 2019). In addition, TAS2Rs polymorphic variants, including those of TAS2R4 and R14, have been studied, but their association with increased cancer risk remains controversial (Campa *et al.* 2010; Choi *et al.* 2018).

We also investigated if TAS2Rs may be differentially expressed in human glioblastoma tissue samples. TAS2R4, R5 and R14 immunoreactivity was higher and less dispersed than TAS2R39. Moreover, TAS2Rs were found in high cellular density areas, contrarily to fibrous regions.

Similar results were observed by Gaida *et al.* for TAS2R38 in pancreatic ductal adenocarcinoma tissue samples (Gaida *et al.* 2016).

In the last years, a growing body of evidence show anti-proliferative, anti-invasiveness, pro-apoptotic, anti-angiogenic, and anti-metastasis effects of flavonoids, alkaloids, immunosuppressors, antibiotics, cannabinoids and lactones in several types of cancer, including glioblastoma (reviewed in (Duarte *et al.* 2022; Luís *et al.* 2020)). Most of these compounds are known, or at least predicted, to be TAS2Rs ligands and have also been reported as potential adjuvants in glioblastoma therapy with TMZ (Banerjee & Preissner 2018). Moreover, in a study carried out by Seo *et al.*, TAS2R8 and R10 overexpression induced neurite elongation, decreased the expression of cancer stem cell markers, and inhibited self-renewal characteristics, as well as cell migration and invasion in human neuroblastoma cells SH-SY5Y, and reduced tumour incidence and volume in mice. This suggests that these receptors could suppress the metastatic potential of neuroblastoma cells (Seo *et al.* 2017).

The TASR14 and R39 ligand resveratrol (Roland *et al.* 2013) have been extensively analysed in different cancers, including glioblastoma, where its ability to restrain cell proliferation, suppress tumour growth and induce apoptosis in combination with TMZ was shown. Moreover, resveratrol downregulates TMZ-induced MGMT overexpression and represses the activation of the Wnt signalling pathway in several human glioblastoma cells (Dionigi *et al.* 2020; H. Li *et al.* 2016; Liu *et al.* 2020; Song *et al.* 2019; Yang *et al.* 2019).

Several flavonoids which bind TAS2Rs are also considered promising anticancer candidates. An example is the TAS2R39 ligand epigallocatechin-gallate, which induced cell stress in primary glioblastoma cells via autophagy induction and reactive oxygen species production and was also able to sensitize glioblastoma cells to TMZ and inhibit MGMT expression, decreasing cell viability and TMZ chemoresistance (Grube *et al.* 2018; McCubrey *et al.* 2017; Udroiu *et al.* 2019). Although the effects of TAS2R5 ligands, such as epicatechin and procyanidins, were not analysed in the frame of glioblastoma nor as TMZ adjuvants, their antitumoral properties were reported in other types of cancer (Choi *et al.* 2018; Khiewkamrop *et al.* 2018; Koteswari *et al.* 2020; Way *et al.* 2015).

Although alkylating agents, like TMZ, are known for their strong bitterness and astringency, little is known about their potential to bind and activate specific TAS2Rs. To ascertain the capacity of TMZ to activate the bitter taste signalling in glioblastoma cells, we performed functional assays with TMZ in the presence or absence of probenecid, a known TAS2R16, R38, and R43 antagonist (Greene *et al.* 2011; Wölfle *et al.* 2015), or with the taste signalling pathway silenced by GNAT3 knockdown. Our findings showed that TMZ reduced the cell viability, increased cell apoptosis, and elicited intracellular Ca2+ levels in a dose-dependent manner in both U-87MG, SNB-19 and U-373MG glioblastoma cells. Interestingly, these TMZ effects were abolished in the presence of probenecid. Wölfle et al. showed that probenecid inhibited salicin-induced ERK and CREB phosphorylation, the key transcription factor of neuronal differentiation, in the human neuroblastoma cell line SH-SY5Y via TAS2R16 blockage (Wölfle *et al.* 2015). In addition, our results demonstrated that the decrease of cell viability induced by

TMZ in glioblastoma cells was abrogated by GNAT3 silencing, suggesting that TMZ may exert cytotoxic effects through the activation of the bitter taste signalling pathway. The imposing question arising from these data was which TAS2R could be mediating the cytotoxic effects of TMZ. So, we used an *in-silico* analysis to predict the potential target-receptors for TMZ. Of the six possible TAS2Rs to which TMZ could bind, probenecid is known to bind only two of them, TAS2R38 and R43 (Greene *et al.* 2011). In fact, Greene and colleagues showed that probenecid completely attenuated HEK-293T cells response to bitter compounds salicin, phenylthiocarbamide and 6-propyl-2-thiouracil, and aloin via TAS2R16, R38 and R43 inhibition, respectively (Greene *et al.* 2011). However, in our results, RT-PCR showed that only TAS2R43 is expressed in all the glioblastoma cell lines analysed. Given the likelihood that TAS2R43 could be binding TMZ and mediating, at least in part, the anti-proliferative and proapoptotic effects of the drug, we knocked it down and observed that TMZ was unable to reduce the viability of TAS2R43-silenced glioblastoma cells, suggesting that TMZ may exert cytotoxic effects also via TAS2R43 activation. Interestingly, TAS2R43 is also a target of the bitter compound epigallocatechin gallate, which in turn has been widely used as an adjuvant agent in the treatment of gliomas, especially in glioblastoma, enhancing the therapeutic efficacy of TMZ (T.C. Chen *et al.* 2011; Grube *et al.* 2018; Xie *et al.* 2018; Zhang *et al.* 2015).

In summary, herein we demonstrated the expression and function of the bitter taste signalling pathway in human glioblastoma samples and cell lines, and that TAS2R43 is activated by the chemotherapeutic alkylating drug TMZ, thereby contributing to the anti-proliferative and anti-apoptotic effects of the drug. These data suggest that the anti-cancer TMZ acts two-fold, by its alkylating effects on DNA eliciting DNA damage and cell death, and by activating TAS2R43 and eliciting downstream cascades leading to reduced proliferation and apoptosis.

4.5 References

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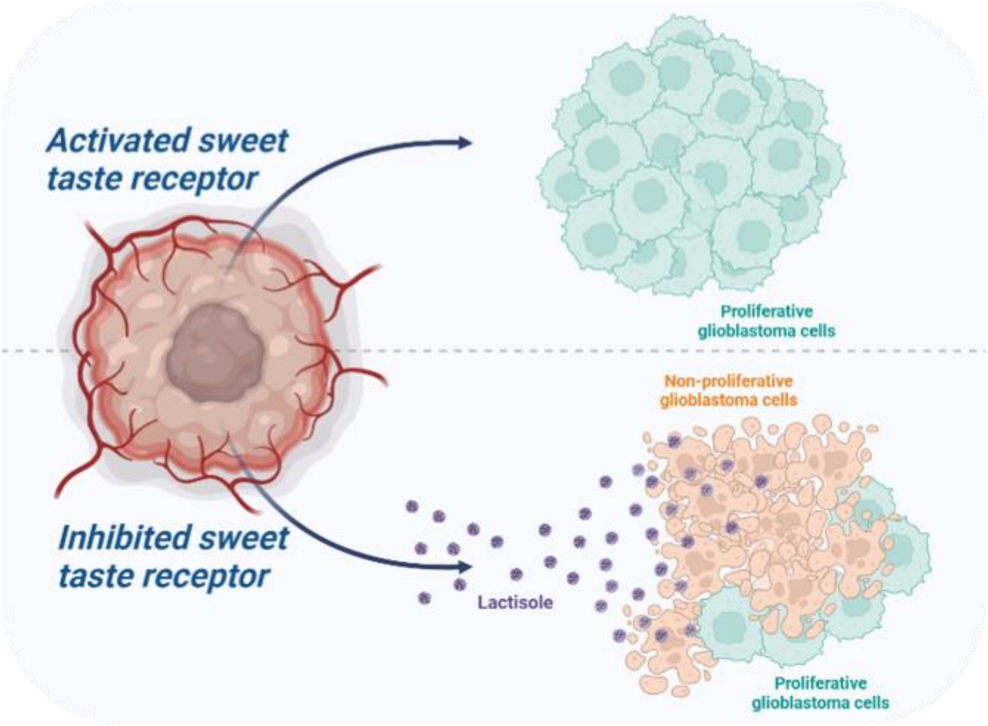
Chapter 5
Research Work 2

The sweet taste receptor controls cell proliferation, migration, and glucose uptake in glioblastoma cells



This chapter is part of a national patent application:

> **Ana Raquel Costa**, Cecília Santos, Isabel Gonçalves, Carla Cruz. (2022). Composto de fórmula (I) para a utilização no tratamento e/ou na prevenção do cancro. (PT Patent – Application No. PAT 118208).

Abstract

Glioblastoma is a brain tumour classified by the World Health Organization as grade 4 due to its aggressiveness, invasiveness, and poor differentiation. Currently, the standard treatments for glioblastoma are still ineffective, and the research for alternative targets for more effective therapies is of paramount importance to enhance the prognosis for this devastating disease. Reprogramming of cellular metabolism is an important hallmark of cancer, leading to the prevalence of glycolysis over oxidative phosphorylation as the main source of energy. This metabolic switch, known as Warburg effect, is enhanced by the reduced oxygen and glucose availability in the tumour microenvironment. Sweet taste receptor (STR) is an important glucosensor with recognised role in the regulation of glucose uptake in several organs and in astrocytes, the glioblastoma precursor cells. We hypothesised that STR may also act as potential regulator of glucose uptake and metabolism in glioblastoma enabling its use as an anticancer target. Thus, we analysed the STR expression in three glioblastoma cell lines (U-87MG, SNB19 and U-373MG), and found that both subunits TAS1R2 and TAS1R3 colocalize in glioblastoma cell lines and found that STR expression was clearly lower in human astrocytes than in U-87MG cells. We provided evidence that STR inhibition with lactisole, a specific TAS1R3 inhibitor, consistently reduced the viability and migration of the glioblastoma cell lines, particularly under oxygen and glucose withdrawal, and triggered cell starvation by impairing glucose uptake in glioblastoma cells.

Keywords

Glioblastoma; sweet taste receptor; glucose metabolism; Warburg effect; TAS1R3

5.1 Introduction

Glioblastoma is a highly aggressive astrocytoma classified by the World Health Organization as a grade 4 brain tumour (Louis *et al.* 2021). Currently, the standard treatment procedure for glioblastoma is surgical resection of the tumour followed by radiation and temozolomide (TMZ) therapy, along with medication to relief neurological symptoms. Unfortunately, although this multimodal therapeutic approach is the best option available, it only enhances life expectancy from 10% to 26% at 2 years and from 0% to 10% at 5 years after diagnosis (Ferlay *et al.* 2021; Reni *et al.* 2017). The inefficacy of the treatment approaches is further compromised by the low permeability of the blood brain barrier to TMZ (Da Ros *et al.* 2018). In this scenario, the research for more effective therapies against glioblastoma is timely and of paramount importance, and novel therapeutic targets must be identified to enhance the prognosis for this devastating disease.

An important hallmark of cancer, including glioblastoma, is the specificity of its energetic metabolism (Hanahan & Weinberg 2011; Pavlova & Thompson 2016; Taylor *et al.* 2019). In a subset of cancer cells, glycolysis prevails over oxidative phosphorylation as the main source of energy, even when oxygen is available. This metabolic switch, first observed by Otto Warburg, favours the large demand of nucleotides, amino acids, and lipids essential for cell proliferation, and is enhanced by the reduced oxygen and glucose availability in the tumour microenvironment (Liberti & Locasale 2016; Mohanti *et al.* 1996; Yuen *et al.* 2016). Under hypoxic conditions, the hypoxia-inducible factor HIF1α is activated, which in turn transactivates several genes involved in glucose uptake, like glucose transporters, and glucose breakdown, such as hexokinase, phosphofructokinase, and aldolase, stimulating glycolysis and angiogenesis. Concurrently, glycolysis releases lactate to the tumour microenvironment, which decreases the pH, weakening the immune response of the tumour surrounding cells and providing an additional energy source for tumour growth (Denko 2008). Moreover, glioblastoma chemoresistance might be enhanced by these metabolic features, as seen in other types of cancer (Desbats *et al.* 2020). Hence, the identification of metabolic targets to treat glioblastoma is promising. The reversal of the Warburg effect in glioblastoma was shown to reduce tumour size and aggressiveness, but only the glycolysis inhibitor 2-deoxyglucose is undergoing clinical trials to prevent the Warburg effect in glioblastoma (Raez *et al.* 2013; Sborov *et al.* 2015; Stein *et al.* 2010). Therefore, the identification of other potential players mediating the crosstalk between cell proliferation, invasion, hypoxia, and glucose metabolism in glioblastoma is of interest for the drug development against this particular type of cancer.

The taste receptor for sweet compounds (sweet taste receptor – STR) is an important glucosensor, with a recognised role in the regulation of glucose uptake in several organs, including the brain, by controlling the rate of glucose absorption and metabolism (Ren *et al.* 2009; Santos *et al.* 2019; Smith *et al.* 2018). The STR is composed by a heterodimer of TAS1R2 and TAS1R3 subunits that bind various sugars, artificial sweeteners, and sweet amino acids

(Welcome & Mastorakis 2018). Although initially identified in the taste buds of the oral cavity, STR subunits are expressed throughout the body (Santos *et al.* 2019). Recent findings suggest that STR controls the glucose metabolism in astrocytes, the glioblastoma precursor cells, and in neurons of different nutrient-sensing forebrain regions (Ren *et al.* 2009; Smith *et al.* 2018). STR expression was also detected in cells in the third ventricle, which are sensitive to high concentrations of glucose (Ren *et al.* 2009; Welcome & Mastorakis 2018). This raises the hypothesis that STR might be instrumental for glucose uptake and energetic metabolism regulation in tumour cells, where metabolic demands are overriding, a possibility that has not been addressed before.

Therefore, we hypothesize that STR is expressed in glioblastoma cell lines and in human tumour samples of glioblastoma patients, where we propose it might function as sensor of the glucose availability in the tumour microenvironment. STR inhibition with lactisole, a specific TAS1R3 inhibitor, reduced cell viability and migration, but did not induce apoptosis, in glioblastoma cell lines, particularly under oxygen and glycose deprivation, highlighting its potential as therapeutic target in glioblastoma.

5.2 Materials and methods

5.2.1 Materials

Glucose Uptake Cell-Based (#600470) and L-Lactate (#700510) assay kits, and Lactisole (CAS No 150436-68-3; #18657) were purchased from Cayman Chemical. A stock solution of lactisole 0.8 M was prepared in dimethyl sulfoxide (DMSO) and freshly dissolved in culture medium before the experiments, where the DMSO final concentration did not exceed 0.625%. A vehicle control was included in all the experiments. CellEvent™ Caspase-3/7 Green Detection Reagent (#C10423), and MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (#1006) were purchased in ThermoFisher Scientific and Gerbu Biotechnik GmbH, respectively.

The RT-PCR primers (Table 5.1) were obtained from STAB VIDA, and the primary and secondary antibodies (Table 5.2), previously validated, were purchased from Santa Cruz Biotechnology, ThermoFisher Scientific and Sigma-Aldrich.

5.2.2 Cell culture

The experimental procedures were performed using three commercial glioblastoma cell lines (U-87MG, SNB-19 and U-373MG), that present different characteristics and proliferation rates, in order the attempt to mimic glioblastoma heterogenicity. Human malignant glioblastoma cell lines grown in Dulbecco's modified Eagle's medium (DMEM) with 4.5 g/L glucose (approximately 25 mM; high glucose) with stable glutamine (bioWest #L0103) supplemented with 10% (v/v) fetal bovine serum (FBS) and penicillin (100 IU/ml)/streptomycin (100 µg/ml), and incubated at 37°C in a humidified atmosphere containing 5% CO2.

5.2.3 Detection of TAS1R2 and TAS1R3 transcripts in glioblastoma cell lines by RT-PCR

Total RNA was isolated from glioblastoma cell lines using TRI Reagent® (Sigma-Aldrich #T9424) according to the manufacturer's instructions. The RNA concentration was determined through OD values measurement at 260 nm, and the RNA quality was evaluated by agarose gel electrophoresis. cDNA was synthesized using NZY M-MuLV Reverse Transcriptase (NZYTech #MB083) in accordance with the manufacturer's protocol. For the RT-PCR, cDNA was amplified by NZYTaq II 2x Green Master Mix (NZYTech #MB358) and specific primers (Table 5.1) in a final volume reaction of 10 µL. The RT-PCR protocols comprised a 3-minute initial activation at 95ºC, followed by 40 cycles of 30-seconds denaturation at 94ºC, 30-seconds annealing, and 30-seconds elongation at 72ºC, and 5 minutes of final elongation at 72ºC. The PCR products were separated through 1.5% agarose gel electrophoresis and visualized using GreenSafe Premium (NZYTech #MB13201) staining. In addition, RT-PCR bands were purified, and Sanger sequenced by STAB VIDA (Portugal) to verify the sequence identity.

Table 5.1 – List of primers used for RT-PCR mRNA expression for the sweet taste receptor subunits in glioblastoma cell lines.

RefSeq	Gene Name	Gene Symbol	Primer Sequence 5' – 3'	Size (bp)	A.T. (ºC)
NM_152232.2	Taste receptor, type 1, member 2	**TAS1R2**	Fw: CTCGGCTGTGACAAAAGCAC Rv: CCTTGCGGGTCGAAGAAGAT	119	60
NM_152228.3	Taste receptor, type 1, member 3	**TAS1R3**	Fw: GACAGAGCGCCTGAAGATCC Rv: CGATGTCGTCTGGGTTTTGC	173	60

A.T. – Annealing temperature; bp – Base pair; Fw – Forward; Rv – Reverse

5.2.4 Detection of TAS1R2 and TAS1R3 proteins in glioblastoma cell lines

5.2.4.1 Western Blot analysis

Suspensions of glioblastoma cells in phosphate-buffered saline (PBS) 1x were centrifuged for 7 min at 10 000 g at 4°C, and the pellets homogenized in RIPA buffer [150 mM NaCl, 0.5% Sodium deoxycholate, 0.1% SDS, 1% Triton X-100, 50 mM Tris pH 8.0, 1 mM PMSF and 10 µL/mL of complete EDTA-free protease inhibitor cocktail (Roche #11873580001)]. Total protein quantification was performed with Pierce BCA Protein Assay Kit (ThermoFisher Scientific #23227) according to the manufacturer's protocol. An amount of 30 µg of total protein was mixed with a loading buffer containing 4% β-mercaptoethanol, followed by denaturation for 10 min at 95ºC, and then loaded in a 12.5% SDS-PAGE. Proteins were transferred to a PVDF membrane 0.45 µm (GE Healthcare #10600023) in a Trans-Blot® Turbo™ transfer system and blocked with 5% non-fat dry milk in Tris-buffered saline (TBS) 1x, for 1h at room temperature

(RT). After overnight incubation at 4ºC with the primary antibody TAS1R2 (Table 5.2), membranes were rinsed three times with TBS containing 0.1% of Tween-20 (TBS-T) and incubated with the respective secondary antibody HRP-linked for 1h at RT. Signal detection was performed with SuperSignal™ West Pico PLUS Chemiluminescent Substrate (ThermoFisher Scientific #34577) and images were acquired with the Image Lab software in a ChemiDoc™ MP (Bio-Rad). This experiment was done with at least three different cell passages. Additionally, expression of TAS1R2 was normalized with β-actin incubated for 1h at RT before incubation for 1h with HRP-conjugated goat anti-mouse secondary antibody. After blotting, images were acquired, and protein bands were quantified using the Image Lab software (Bio-Rad).

Table 5.2 – List of antibodies used for Western blot, immunohistochemistry, and immunofluorescence of bitter taste receptors protein detection.

	Host	Catalog Number	WB	IHC	IF
Primary Antibodies					
TAS1R2	Rabbit	SantaCruz Biotechnology #sc-50306	1:250		1:100
TAS1R3	Goat	SantaCruz Biotechnology #sc-22458	–	–	1:100
β-Actin	Mouse	Sigma-Aldrich #A1978	1:30000	–	–
Secondary Antibodies					
Biotin-conjugated Anti-Rabbit	Donkey	Jackson Immunoresearch #711-065-152	–	–	1:800
Streptavidin, Alexa Fluor® 488	–	Molecular Probes #S-32354	–	–	1:800
Alexa Fluor® 647 Anti-Goat	Chicken	Molecular Probes #A-21469	–	–	1:1000
Anti-rabbit IgG-HRP	Goat	Invitrogen #31466	1:20000	–	–
Anti-mouse IgG-HRP	Goat	SantaCruz Biotechnology #sc-2005	1:30000	–	–

IF – Immunofluorescence; IHC – Immunohistochemistry; WB – Western blot

5.2.4.2 Immunocytochemistry

The presence of TAS1R2 and TAS1R3 was investigated in the glioblastoma cell lines by fluorescence immunocytochemistry. Briefly, cells were seeded and grown on glass coverslips until 60-70% confluence. After removing the medium, cells were fixed with paraformaldehyde (PFA) 4% for 10 min followed by 1h blocking in PBS containing 3% bovine serum albumin (BSA) and 0.2% Triton X-100 at RT. Cells were incubated overnight at 4ºC with the primary antibodies (Table 5.2) in blocking solution. In negative controls, the primary antibody was omitted. Glioblastoma cells were then incubated at RT with secondary biotinylated antibody donkey anti-rabbit for 90 minutes followed by streptavidin Alexa Fluor® 488 for 1 hour, or with Alexa Fluor® 647 chicken anti-goat for 1 hour. Finally, coverslips were incubated for 10 min with the fluorescent dye Hoechst 33342 to visualize the nuclei. After several washes, cells were mounted onto microscope slides and visualized under a confocal microscope LSM 710 (Carl Zeiss) using a magnification of 63x (Plan-Apochromat 63x/1.4 Oil DIC M27).

5.2.5 Detection of TAS1R2 in human samples of glioblastoma by immunohistochemistry

Human glioblastoma samples for the immunohistochemistry study were obtained from the Institute of Neuropathology Brain Bank (HUB-ICO-IDIBELL Biobank) following the guidelines of the Spanish legislation on this matter (Real Decreto 1716/2011) and the approval of the local ethics committee of the Bellvitge University Hospital-IDIBELL. Samples were fixed in buffered formalin for no less than 3 weeks and then embedded in paraffin. Paraffin-embedded human glioblastoma slices from men (n=29) and women (n=16) were pre-treated with Trilogy™ (Cell Marque™ #920P) which combines deparaffinization, rehydration and unmasking, according to the manufacturer's instructions. After washing with TBS-T, endogenous peroxidases activity was blocked for 10 min at RT with 3% H_2O_2, and then incubated for 1h at RT with TAS1R2 primary antibody (Table 5.2). Human glioblastoma slices were washed twice with TBS-T and treated with HiDef Detection™ HRP Polymer System (Cell Marque™ #954D), by incubation with HiDef Detection™ Amplifier for 10 min RT, followed by a washing step with TBS-T, and incubation with HiDef Detection™ HRP Polymer Detector for 10 min at RT. The immunoreactivity was detected with diaminobenzidine (DAB) for 10 min RT, followed by nuclei staining with Mayer's Hematoxylin for 3 min RT. Negative control slices were treated under the same conditions without primary antibody. No immunostaining was seen in these sections. After dehydration, the slices were mounted with Q Path® Coverquick 2000 (VWR #805547530) and the images were acquired in a Zeiss Axio Imager Z2 (Carl Zeiss) using a magnification of 20x (Plan-Apochromat 20x/0.8 M27).

5.2.6 Effect of TAS1R3 inhibition in glioblastoma cells proliferation

5.2.6.1 Cell viability

The glioblastoma cells' viability was carried out using MTT assay. Briefly, glioblastoma cells were grown until 60% confluency and incubated for 48h at 37ºC and 5% CO_2 with DMEM high glucose (4.5 g/L; HG), in the presence or absence of 5 mM lactisole, a known inhibitor of the sweet taste receptor TAS1R3 subunit. Glioblastoma cells incubated with DMEM HG without lactisole represent the control condition. After 48 hours, 100 µL culture medium were removed and 10 µL of MTT solution (5 mg/mL in PBS) were added for approximately 45 min at 37ºC in a humidified atmosphere containing 5% CO_2. Ethanol 70% treated cells were used as positive control. Following MTT incubation, formazan crystals were dissolved in DMSO for 15 min, and absorbance was read at 570 nm in a microplate spectrophotometer xMark™ (Bio-Rad). The viability of glioblastoma cells was expressed as a percentage of the absorbance determined in the vehicle control.

5.2.6.2 Migration

Migration assays were performed to evaluate the cells capacity to migrate upon STR inhibition. Glioblastoma cell lines were grown until 90-100% confluency, and then a scratch was created in each well by scraping a straight line with a micropipette tip. The debris were removed, and the edge of the scratch was smoothed by washing the cells once with PBS 1x. The culture medium was added to each well, in the presence or absence of 5 mM lactisole, as described above. After that, each well was photographed at 0, 24 and 48 hours under an inverted microscope Axio Observer Z1 (Carl Zeiss) using a magnification of 5x (A-Plan 5x/0.12 Ph0 M27). For each image, the area of the scratch was measured using Fiji software (Schindelin *et al.* 2012), by individually comparing the images from the 24 and 48 hours timepoints to the measurements at the ground state (0 hours).

5.2.6.3 Cell death and apoptosis

Cell apoptosis is a form of cell death, that unlike necrosis, is characterized by cell shrinkage, loss of membrane integrity and nuclear fragmentation without triggering inflammatory processes. Therefore, this assay was carried out to investigate whether inhibition of the sweet taste receptor TAS1R3 subunit with lactisole deploys apoptosis or necrosis. To perform this assay, glioblastoma cells were stained with CellEvent™ Caspase-3/7 Green kit to evaluate apoptosis and propidium iodide to evaluate cell death by necrosis. Briefly, the three glioblastoma cell lines were seeded on 10 mm coverslips and incubated at 37°C and 5% CO2 until reaching 60-70% confluence. When the cells reached the desired confluence, the culture medium HG was added in the presence or absence of 5 mM lactisole, or 1 µM staurosporine (apoptosis positive control), followed by incubation for 48 hours. After incubation, CellEvent™ (5 µM) and propidium iodide (1:1000) were added and the cells were incubated at 37°C and 5% CO 2 for 30 minutes. The culture medium was discarded, the cells were washed with PBS 1x and fixed with 4% PFA for 10 minutes, followed by a 10-minute incubation at room temperature with the Hoechst 33342 to nuclei staining. After washing, cells were mounted onto microscope slides and visualized under an Axio Imager Z2 (Carl Zeiss) microscope using a magnification of 40x (Plan-Apochromat 40x/1.3 Oil DIC M27).

5.2.6.4 Glucose uptake

The glucose consumption assay was performed to determine the effect of STR inhibition in the amount of glucose absorbed by SNB-19 glioblastoma cells, using a deoxyglucose analogue (2-NBDG) fluorescent marker. To perform this assay, the commercial Glucose Uptake Cell-Based Assay Kit was used. Briefly, SNB-19 glioblastoma cells were seeded in black clear-bottom 96-well plates and incubated overnight at 37°C and 5% CO2 in HG culture medium. Thereafter, the culture medium was discarded, and the cells were subjected to glucose starvation, in the presence or absence of 5 mM lactisole. After 4 hours of incubation at 37°C and 5% CO2, 100 µg/mL of 2-NBDG was added to each well and incubated for 30 minutes. Then, the cells were washed with cell-based assay buffer and centrifuged at 400 g for 5 minutes at RT. Subsequently, a solution of Hoechst 33342 diluted 1:1000 in cell-based assay buffer was added to label the cell nuclei, followed by centrifugation at 400 g for 5 minutes at RT. The supernatant was discarded, and 100 µL cell-based assay buffer were added to each well. Finally, the fluorescence of the Hoechst 33342 was read at excitation/emission wavelengths 361/497 nm and the fluorescence of glucose 2-NBDG at 485/535 nm on a SpectraMax Gemini spectrofluorometer (Molecular Devices). Glucose uptake was given by the ratio between 2-NBDG and Hoechst 33342 fluorescence.

5.2.7 Effect of sweet taste receptor inhibition in SNB-19 glioblastoma cells deprived of glucose and oxygen

To mimic tumour microenvironment, the effect of STR inhibition in SNB-19 glioblastoma cells upon glucose and oxygen withdrawal was evaluated. To assess the effects of glucose deprivation, SNB-19 glioblastoma cells were subjected to two different concentrations of glucose, high glucose (4.5 g/L; HG) and low glucose (1 g/L; LG). In addition, to mimic hypoxic intra-tumoral conditions, SNB-19 cells were subjected to oxygen deprivation in a hypoxia chamber (STEMCELL Tecnhologies) under a unidirectional flux of 95% N2 and 5% CO2 mixture (20 L/min) for 4 minutes. Then, tubing clamps were closed, valves shut down and the chamber incubated at 37°C for 48 hours. In addition, the specific STR inhibition was assessed by TAS1R2 knockdown with specific siRNA, or TAS1R3 subunit inhibition with 2.5 mM lactisole incubation. Cells subjected to normoxic and HG conditions and non-inhibited STR were considered the experiments' control.

5.2.7.1 Cell proliferation

The SNB-19 glioblastoma cells' viability was carried out using MTT assay as described in section 5.2.6.1. Briefly, SNB-19 cells were grown until 60% confluency or transfected for 24 hours with a mixture of TAS1R2 siRNA (10 nM) and Lipofectamine™ 2000 in Opti-MEM medium, following the manufacturer's recommendations. A scramble siRNA (10 nM) was used as negative control for TAS1R2 specific targeting. Then, cells were incubated for 48h at 37°C and 5% CO2 with DMEM HG or LG and subjected to normoxia or hypoxia, in the presence or absence of 2.5 mM lactisole diluted in culture medium. After 48 hours, the culture media was removed and the MTT solution was added for approximately 45 min at 37°C and 5% CO2. Ethanol 70% treated cells were used as positive control. Following MTT incubation, formazan crystals were dissolved in DMSO for 15 min, and absorbance was read at 570 nm in a microplate spectrophotometer xMark™ (Bio-Rad). The viability of glioblastoma cells was expressed as a percentage of the absorbance determined in the vehicle control.

5.2.7.2 Extracellular L-lactate production

The extracellular L-lactate is mainly generated from glycolysis. Culture media from the MTT experiments with lactisole described in section 5.2.7.1 were collected and used for the determination of extracellular L-lactate, following the manufacturer's instructions. This assay allowed the detection of L-Lactate in a fluorescence-based method, in which lactate dehydrogenase catalyses the oxidation of L-lactate to pyruvate, with a consequent reduction of NAD+ to NADH, which in turn reacts with the fluorescent substrate and results in a fluorescent product. Due to the presence of lactate dehydrogenase in the samples, and to prevent the conversion of lactate to pyruvate, the samples were deproteinized with 0.5 M metaphosphoric acid and centrifuged at 10 000 g during 5 minutes at 4°C. Then, the supernatant was discarded and 50 µL of potassium carbonate were added to maintain the pH. The samples were centrifuged again under the same conditions, the supernatant was collected, and samples were diluted 1:2 in an assay buffer solution. In an opaque black 96-well plate, 20 µL of L-lactate standard samples with known concentrations (0-1000 µM) and 20 µL of the samples of interest were added in duplicate. Then, 100 µL of buffer solution, 20 µL of cofactor mix and 20 µL of fluorescent substrate were added to each well. Reactions were initiated by the addition of 40 µL of enzyme mix, and the plates were incubated, protected from light, for 20 minutes at RT. Fluorescence values were read in a SpectraMax Gemini spectrofluorometer at 530-540 nm excitation and 585-595 nm emission, and the concentration of L-Lactate was calculated as recommended by the manufacturer.

5.2.8 Data analysis

Statistical analysis and dataset comparisons were performed using GraphPad Prism 7.0 (GraphPad Software). Statistical significance between two or more groups was determined by Unpaired *t* test or ANOVA followed by the software's recommended multiple comparisons post-hoc test, respectively. Results are presented as mean ± SEM of at least three independent experiments, and data were considered statistically different for a p-value < 0.05.

5.3 Results

5.3.1 The sweet taste receptor is expressed in glioblastoma cells

The expression of the sweet taste receptor in glioblastoma cells was assessed by RT-PCR using specific primers (Table 5.1). The results demonstrated the mRNA expression of both TAS1R2 and TAS1R3 subunits in all the glioblastoma cell lines with predicted sizes of 119 bp and 173 bp, respectively (Figure 5.1). The amplified products were sequenced by STAB VIDA (Portugal), and sequences were identified by comparison with *Homo sapiens* sequence from NCBI-Blast database, with homology percentages between 93% and 100%.

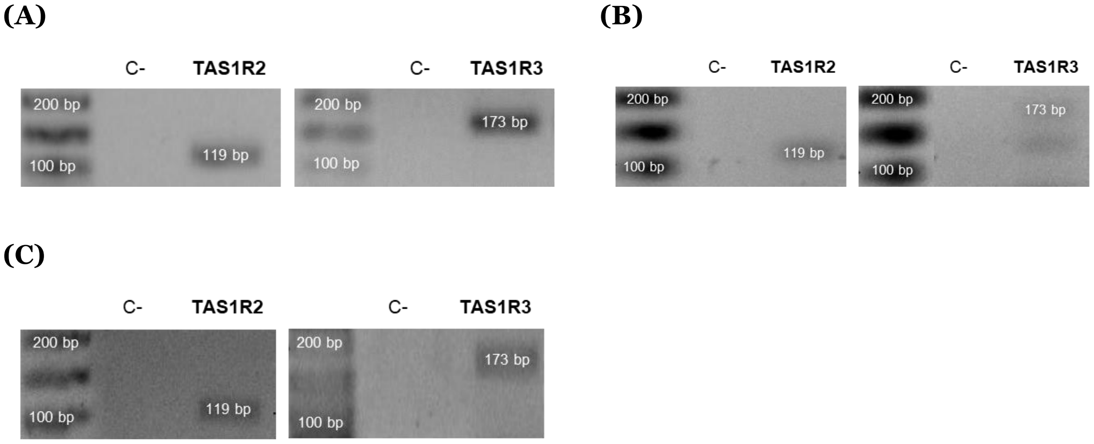


Figure 5.1 – mRNA expression profile of the sweet taste receptor in glioblastoma cells. RT-PCR was performed with cDNA synthetized from total RNA. The identities of the amplified products were confirmed by Sanger sequencing. (A) U-87MG, (B) SNB-19, and (C) U-373MG. bp: base pair; C-: negative control.

5.3.2 Glioblastoma cell lines differentially express TAS1R2 protein

We detected the STR subunits in protein extracts of human astrocytes and glioblastoma cells by Western blot (Figure 5.2) and immunofluorescence (Figure 5.3). The TAS1R2 protein was detected at approximately 100 kDa (Figure 5.2A), and the analysis of its relative protein expression revealed significant higher levels in U-87MG relatively to human astrocytes (3.02-fold), and glioblastoma cells SNB-19 (2.26-fold) and U-373MG (2.44-fold) (Figure 5.2B).

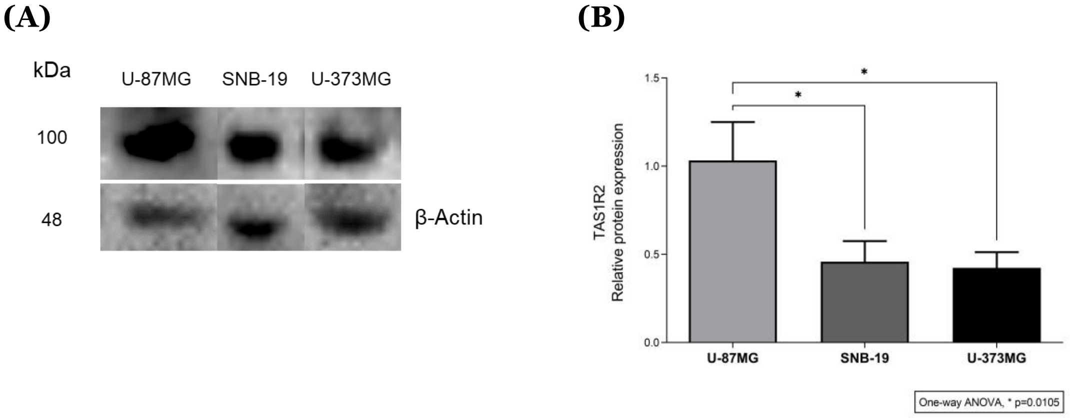


Figure 5.2 – TAS1R2 subunit of the sweet taste receptor is differentially expressed in glioblastoma cell lines. (A) Representative images from Western blot detection of TAS1R2 subunit in glioblastoma cell lines (U-87MG, SNB-19, U-373MG) protein extracts. (B) Relative quantification of TAS1R2 protein levels in glioblastoma cells, normalized to β-actin levels. Results are presented as mean ± SEM and analysed by one-way ANOVA followed by Tukey's multiple comparisons test. [N≥3 independent experiments; *p<0.05]. kDa: kilo Dalton.

5.3.3 The sweet taste receptor is expressed in human glioblastoma cell lines and tumour samples

We proceeded by analysing the expression of the sweet taste receptor in glioblastoma cell lines and in paraffin-embedded sections of human glioblastoma samples obtained from surgical resection of tumours. The sweet taste receptor subunits TAS1R2 and TAS1R3 co-localized were predominantly in the cytoplasm and plasma cell membrane of glioblastoma cell lines U-87MG, SNB-19 and U-373MG (Figure 5.3).

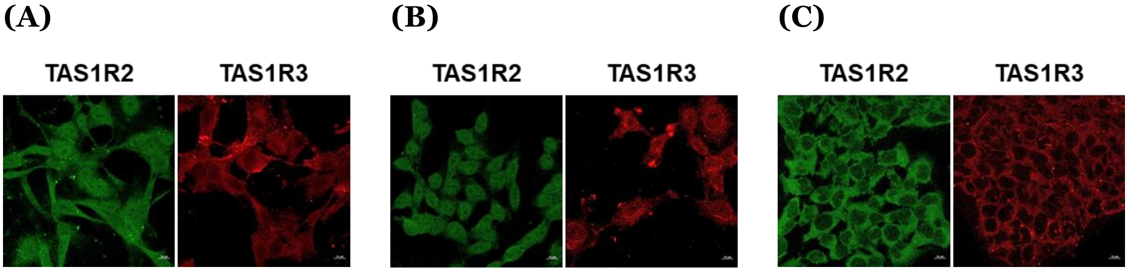


Figure 5.3 – Immunofluorescence detection of the sweet taste receptor subunits TAS1R2 and TAS1R3 in glioblastoma cell lines. Confocal microscopy images of TAS1R2 (green) and TAS1R3 (red) in glioblastoma cell lines (A) U-87MG, (B) SNB-19 and (C) U-373MG. Nuclei were stained with Hoechst 33342 (not shown). Scale bar: 10 μm.

TAS1R2 immunoreactivity was also observed in all cases of glioblastoma samples, although with region-dependent variations. TAS1R2 immunoreactivity was found in areas of high cellular density and in the cytoplasm of large and medium-size glioblastoma cells. Less differentiated areas or areas with loose cellular density showed weak or no immunoreactivity. Necrosis and fibrous regions with predominant fibroblast-like cells areas were negative (Figure 5.4). Further densitometric quantifications and gender differences categorization were not carried out.

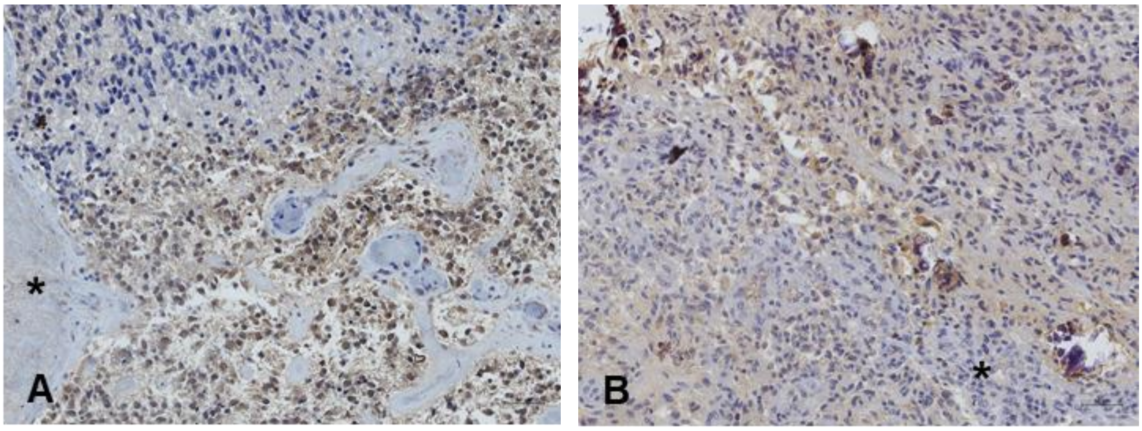


Figure 5.4 – Sweet taste receptor subunit TAS1R2 immunostaining of human glioblastoma tumour samples. Anti-TAS1R2 antibody decorates the cytoplasm of large and medium size glioblastoma cells in areas with high cellular density (A and B), contrarily to areas with loose cellularity or less differentiated areas. In the same sections, fibrous areas (asterisks) are negative. Representative images of paraffin sections, lightly counterstained with Mayer's hematoxylin. Scale bar: 50 μm.

5.3.4 TAS1R3 inhibition impairs the proliferative capacity but does not trigger cell death in glioblastoma cells

After confirming STR-related mRNA and protein expression in glioblastoma cells, we assessed the effects of TAS1R3 inhibition with lactisole in the viability and migration of glioblastoma cell lines U-87MG, SNB-19 and U-373MG (Figure 5.5).

We found that TAS1R3 inhibition reduced the viability of U-87MG (22.48%±1.32), SNB-19 (24.35%±3.43) and U-373MG (33.60%±1.25) glioblastoma cell lines.

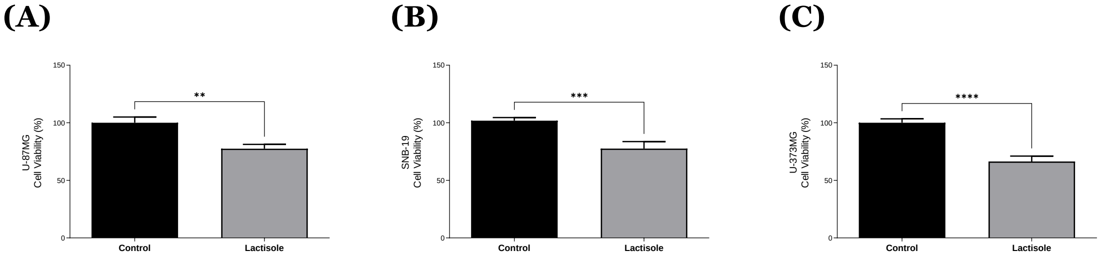
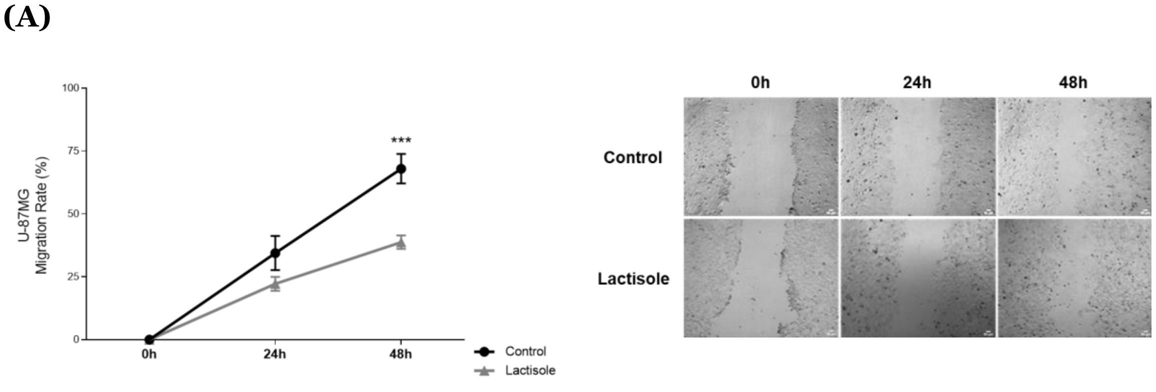


Figure 5.5 – Effect of TAS1R3 inhibition with lactisole on glioblastoma cell lines’ viability. Effect of TAS1R3 inhibition in the viability of (A) U-87MG, (B) SNB-19 and (C) U-373MG glioblastoma cells grown in high glucose media (control), in the presence or absence of 5 mM lactisole, for 48 hours. Bar graphs represent mean ± SEM. Statistical analysis was performed by unpaired *t* test. [N≥3 independent experiments; **p<0.01, ***p<0.001 and ****p<0.0001].

Then, we evaluated the effects of TAS1R3 inhibition with lactisole on the migratory capacity of glioblastoma cells (Figure 5.6). Overall, we observed that the migration and proliferation rates of the control group is faster when compared to the group of cells where the STR was inhibited. The migration rate of U-87MG and U-373MG cells was statistically different between the control and lactisole groups only after 48 hours of incubation (Figure 5.6A and 5.6C). On the other hand, the TAS1R3 inhibition was shown to be affective in decreasing the ability of SNB-19 cells to migrate after 24 and 48 hours of incubation with lactisole (Figure 5.6B).



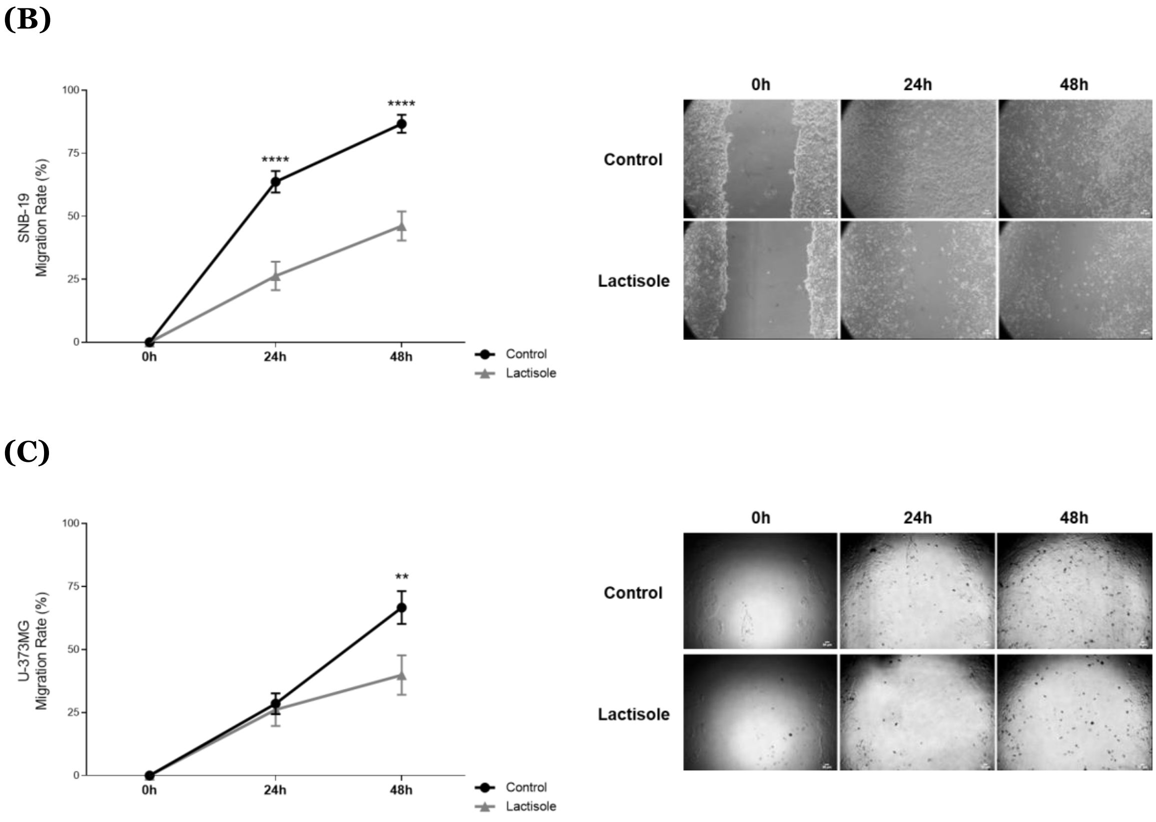
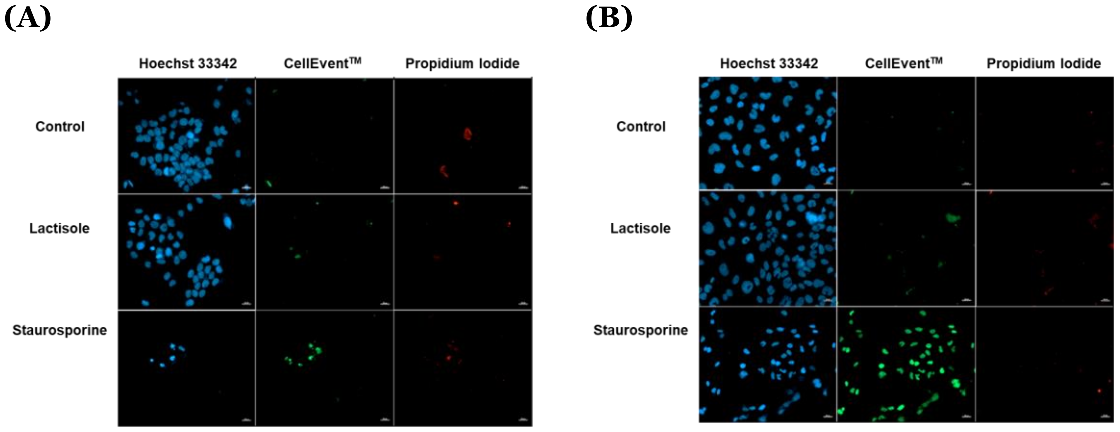


Figure 5.6 – Effect of TAS1R3 inhibition with lactisole on glioblastoma cell lines' migration. Effect of TAS1R3 inhibition in the migratory capacity at 0, 24, and 48 hours of glioblastoma cells (A) U-87MG, (B) SNB-19 and (C) U-373MG grown in high glucose media (control), in the presence or absence of 5 mM lactisole. Results are presented as mean ± SEM and analysed by two-way ANOVA followed by Sidak's multiple comparisons test (left panel). Representative images of cell migration (right panel). [N≥3 independent experiments; **$p<0.01$, ***$p<0.001$ and ****$p<0.0001$].

Our observations put in evidence that STR inhibition, via blockage of the TAS1R3 subunit, impairs the proliferative capacity of glioblastoma cells. To elucidate the mechanism by which STR inhibition leads to decreased proliferation and migration of glioblastoma cells, we proceeded with the assessment of cell death by staining apoptotic or necrotic cells (Figure 5.7).



(C)

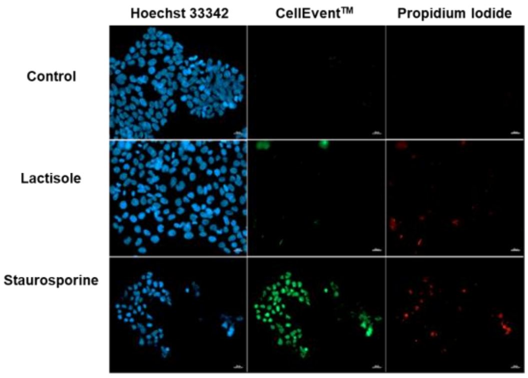


Figure 5.7 –TAS1R3 inhibition did not induced cell death in glioblastoma cells. Cell death assessment was performed by staining apoptotic (CellEvent™; green) or necrotic (propidium iodide; red) glioblastoma cells. (A) U-87MG, (B) SNB-19 and (C) U-373MG grown in high glucose media (control), in the presence or absence of 5 mM lactisole, for 48 hours. A positive control for apoptosis was used by incubating the cells with 1 µM staurosporine for 48 hours. Nuclei were stained with Hoechst 33342 (blue). Scale bar: 20 µm.

To assess the effect of TAS1R3 inhibition on glucose uptake by glioblastoma cells, a fluorometric glucose uptake assay was performed using the 2-NBDG marker. As seen in figure 5.8, blocking TAS1R3 reduced the glucose consumption of SNB-19 glioblastoma cells (Figure 5.8).

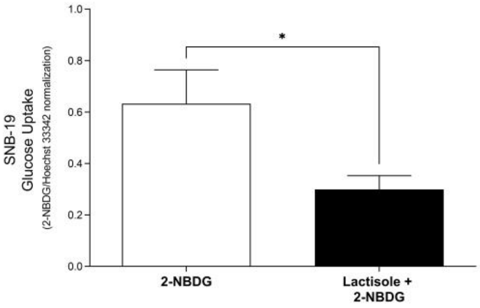


Figure 5.8 – Effect of TAS1R3 inhibition on SNB-19 glioblastoma cells' glucose consumption. Effect of TAS1R3 inhibition in the glucose uptake by SNB-19 glioblastoma cells glucose-starved during 4 hours, in the presence or absence of 5 mM lactisole. Bar graphs represent mean ± SEM. Statistical analysis was performed by unpaired *t* test. [N≥3 independent experiments; **p<0.01, ***p<0.001 and ****p<0.0001].

Overall, TAS1R3 inhibition impaired the proliferative and migratory capacity, but did not trigger cell death in glioblastoma cells, highlighting that glucose metabolism may be, at least in part, dependent on the sweet taste receptor normal functioning.

5.3.5 TAS1R3 blocking reduces the proliferative capacity of SNB-19 cells deprived of oxygen and glucose

Our previous observations put in evidence that inhibition of the TAS1R3 subunit of the STR by itself impairs the proliferative and migratory capacities of glioblastoma cells under normal conditions of oxygen and glucose. The next question was if these evidences would be similar under oxygen and glucose withdrawal.

Since the STR is a heterodimer of TAS1R2 and TAS1R3 subunits, and TAS1R3 is common to the sweet and umami receptors, we analysed the individual effect of inhibiting these two subunits in SNB-19 glioblastoma cells. To test this hypothesis, we proceeded with TAS1R2 knockdown experiments (Figure 5.9).

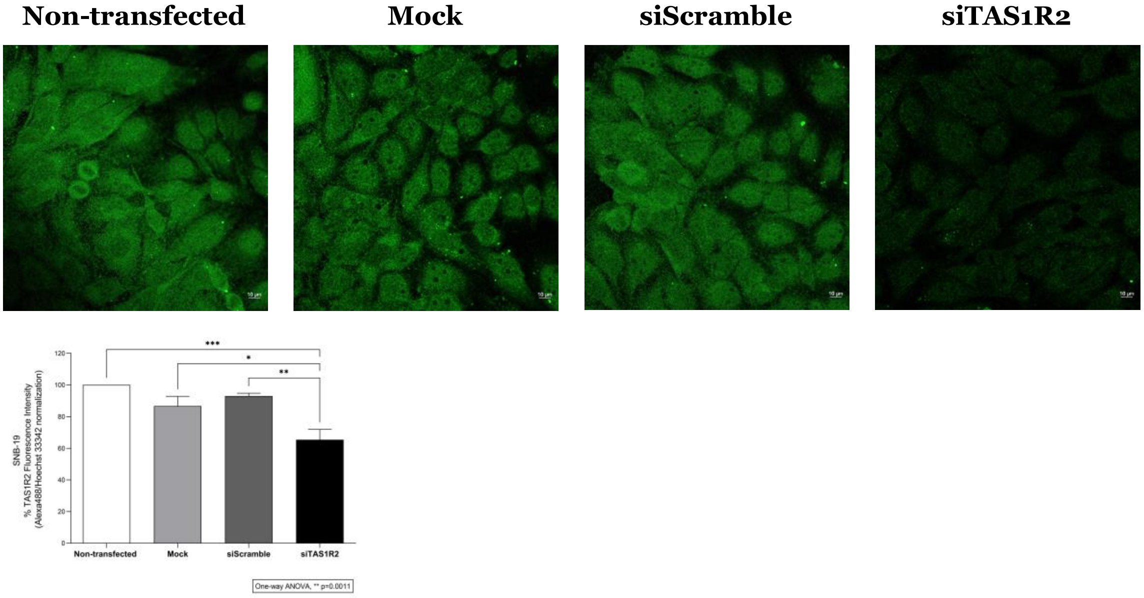


Figure 5.9 – Immunofluorescence analysis of TAS1R2 in SNB-19 glioblastoma cells after specific siRNA knockdown. Protein levels of siRNA TAS1R2-transfected cells are decreased in comparison with non-, mock-, and siRNA scramble-transfected cells. The quantification of TAS1R2 fluorescence intensity (green) was performed in different regions of interest (ROIs) of confocal microscopy images obtained from four independent experiments. Nuclei were stained with Hoechst 33342 (not shown). Scale bar: 10 µm. Bar graphs represent mean ± SEM. Statistical analysis was performed by one-way ANOVA followed by Tukey’s multiple comparisons test. [N=4 independent experiments; *$p<0.05$, **$p<0.01$ and ***$p<0.001$].

Overall, the SNB-19 cell viability was decreased in both transfected or mock-transfected cells with TAS1R2 or scramble siRNAs compared to non-transfected (Figure 5.10). In normoxic conditions, glucose deprivation exerted a global effect in decreasing SNB-19 cell viability (Figure 5.10A), whereas under hypoxic conditions, a similar effect was only observed in TAS1R2-silenced cells non-deprived of glucose compared to mock-transfected (Figure 5.10B). However, the TAS1R2 knockdown did not affected the viability of SNB-19 cells deprived of oxygen and glucose (Figure 5.10).

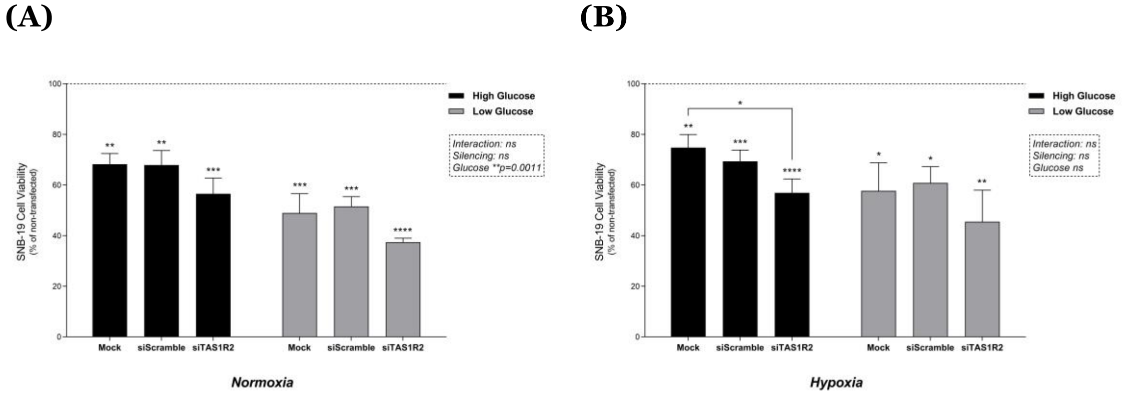


Figure 5.10 – Effect of TAS1R2 knockdown in the viability of SNB-19 glioblastoma cells deprived of oxygen and glucose. Effect of TAS1R2 knockdown in the viability of SNB-19 glioblastoma cells grown in (A) normoxic or (B) hypoxic conditions, transfected or mock-transfected for 24h with TAS1R2 or a scramble siRNA, and incubated with high (control) or low glucose media, for 48 hours. Non-transfected cells (100% viability) are represented by the dashed line. Bar graphs represent mean ± SEM. Statistical analysis was performed by one- or two-way ANOVA followed by recommended multiple comparisons tests. [N≥3 independent experiments; ^{ns} no significant, *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001].

These results suggest that the proliferation of glioblastoma cells is partially dependent on the correct functioning of the sweet taste receptor, particularly by TAS1R3 subunit activation. Therefore, we proceeded with the inhibition of TAS1R3 and tested its effect in the viability of SNB-19 cells deprived of oxygen and glucose.

Overall, oxygen and glucose withdrawal affect the SNB-19 cell viability (Figure 5.11). As expected, under normoxic conditions, TAS1R3 inhibition reduced approximately 28% and 39% the viability of SNB-19 glioblastoma cells non- or deprived of glucose, respectively (Figure 5.11A). In addition, TAS1R3 inhibition led to a decrease of about 42% in glucose-deprived SNB-19 cells (Figure 5.11A). In hypoxic conditions, the effect of TAS1R3 inhibition with lactisole in SNB-19 cells non- or deprived of glucose was similar, by decreasing cell viability of about 30% and 32%, respectively (Figure 5.11B). Moreover, TAS1R3 inhibition led to a decrease of about 32% in glucose-deprived SNB-19 cells (Figure 5.11A). Overall, it became clear that the anti-proliferative effect of TAS1R3 inhibition is exacerbated by oxygen and glucose withdrawal in SNB-19 glioblastoma cells (Figure 5.11C).

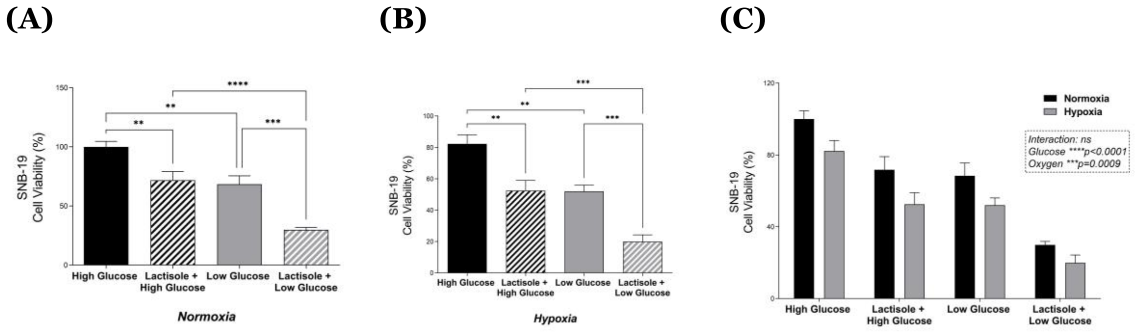


Figure 5.11 – Effect of TAS1R3 inhibition in the viability of SNB-19 glioblastoma cells deprived of oxygen and glucose. Effect of TAS1R3 inhibition in the viability of SNB-19 glioblastoma cells grown in normoxic or hypoxic conditions with high (control) or low glucose media, in the presence or absence of 2.5 mM lactisole, for 48 hours. Bar graphs represent mean ± SEM. Statistical analysis was performed by one- or two-way ANOVA followed by recommended multiple comparisons tests. [N≥3 independent experiments; $^{**}p<0.01$, $^{***}p<0.001$ and $^{****}p<0.0001$].

Finally, considering that under hypoxic environment the cancer cells majorly rely on glycolysis to proliferate, we aimed at investigating the effect of TAS1R3 inhibition in L-lactate production by SNB-19 glioblastoma cells. Although TAS1R3 inhibition with lactisole reduced glucose uptake by glioblastoma cells, both in normal or oxygen and glucose deprivation states, it did not affect the concentration of extracellular L-lactate (Figure 5.12). In fact, only glucose deprivation decreased L-lactate production by SNB-19 cells (Figure 5.12C).

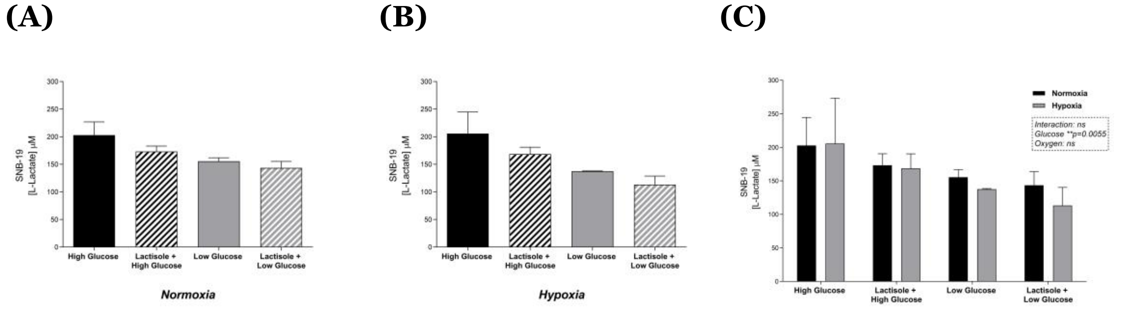


Figure 5.12 – Effect of TAS1R3 inhibition in the L-lactate production of SNB-19 glioblastoma cells deprived of glucose and oxygen. Effect of TAS1R3 inhibition in the extracellular L-lactate produced by SNB-19 glioblastoma cells grown in normoxic or hypoxic conditions with high (control) or low glucose media, in the presence or absence of 2.5 mM lactisole, for 48 hours. Bar graphs represent mean ± SEM. Statistical analysis was performed by one- or two-way ANOVA followed by recommended multiple comparisons tests. [N≥3 independent experiments].

5.4 Discussion

Glioblastoma is characterized by high proliferative activity and vascularization, whose cells are extremely dependent on the metabolic process of aerobic glycolysis, also known as Warburg effect, that implies high glucose consumption (Liberti & Locasale 2016; Linkous & Yazlovitskaya 2011; Schröder *et al.* 1991). Our experiments aimed at assessing whether STR could function as a sensor of the glucose availability and regulator of glucose metabolism and uptake in glioblastoma, as reported in several organs and tissues (Smith *et al.* 2018). For example, in the intestine, STR signalling increases the rate of glucose absorption rate by regulating a glucose transporter in enterocytes. The administration of non-caloric sweeteners in a gastric cancer cell line was shown to induce serotonin secretion dependent on the activation of the TAS1R3 subunit (Zopun *et al.* 2018). In wild-type rats, a sucrose-rich diet reduced the glucose uptake by rapidly inducing STR downregulation in the gut (Smith *et al.* 2018). In the rat brain, Tas1r1, Tas1r2 and Tas1r3, and G protein-associated genes, are expressed in different nutrient-sensitive regions of the forebrain, including the hypothalamus, hippocampus, habenula, cortex, and in the choroid plexus (Ren *et al.* 2009). In this way, G protein-coupled taste receptors can function as membrane-bound chemosensors, particularly the STR that is a major candidate for brain glucosensing. A recent study carried out in astrocytes and in neurons from several nutrient-sensitive regions of the midbrain, suggest that STR has a role in controlling glucose metabolism. In addition, STR expression was detected in tanycytes specialized glial cells of the third brain ventricle, a brain region highly sensitive to glucose (Ren *et al.* 2009; Welcome & Mastorakis 2018). Moreover, in the hypothalamus, the taste-related gene expression levels were shown to be dependent on the nutritional status (Ren *et al.* 2009), suggesting that taste signalling mechanisms in the brain could be involved in the assessment and regulation of nutrient uptake, particularly glucose.

All these data led us to analyse the expression of the STR in glioblastoma cells. Of note, STR has never been never studied before in any type of cancer, despite the higher demand for nutrient supply essential for cancer grow. We demonstrated the mRNA and protein expression of both TAS1R2 and TAS1R3 subunits in three different glioblastoma cell lines. Interestingly, we also observed differential TAS1R2 protein expression levels between the cell lines analysed. In addition, we also detected TAS1R2 in human glioblastoma tissue samples, particularly in high cellular density areas. In cancer cells, including glioblastoma, higher STR expression is likely to be an advantage to the tumour development since it could enhance glucose sensing and uptake from the tumour microenvironment (Jiang 2017). As seen in other types of cancer, glioblastoma growth and chemoresistance is highly enhanced by the metabolic switch to aerobic glycolysis, which favours the lactate release to tumour microenvironment and the weakening of the immune response in the tumour vicinity (Denko 2008; Desbats *et al.* 2020). Thus, we also investigated the effect of TAS1R3 subunit inhibition, with lactisole, in the proliferative capacity, migration, and apoptosis of the three glioblastoma cell lines. Interestingly, STR inhibition consistently reduced the viability and migration of the glioblastoma cells. However, the STR inhibition did not induce apoptosis or cell death in any of the cell lines under study, suggesting that these cancer cells might undergo senescence when the TAS1R3 subunit is blocked, as a consequence of reduced glucose uptake. A similar effect was described in endometrial cancer cells exposed to low glucose concentrations. In these cells, the migratory capacity was reduced, and the cell growth and proliferation were inhibited, indicating that the antiproliferative effects exerted by glucose deprivation could be attributed to cell cycle arrest (senescence) (Han *et al.*

2015). Recently, it was showed that astrocytes subjected to low glucose conditions underwent senescence (Gao *et al.* 2022). As described before, cancer cells adapt to the low energy yield of glycolysis by increasing glucose uptake to support the high glycolytic rate (Liberti & Locasale 2016). This can benefit cancer cells by providing a high rate of ATP and many intermediates production necessary for rapid cell proliferation but depends on high glucose concentrations (Liberti & Locasale 2016; Lunt & Vander Heiden 2011). The inhibition of the TAS1R3 subunit of the sweet taste receptor in SNB-19 glioblastoma cells led to a decrease in glucose uptake, similarly to what was observed in a previous study in a colorectal cancer cell line (Andrade *et al.* 2018). Therefore, herein we showed that STR inhibition in glioblastoma cells is associated with lower proliferative and migration rates, possibly by a reduction in glucose uptake and induction of cell cycle arrest. These data support that glucose uptake by glioblastoma cells may be regulated by the STR correct functioning.

Upon these findings, we wondered if the STR could also regulate the viability of glioblastoma cells under oxygen and glucose withdrawal, which usually occur in the core of the tumours, and in the tumour microenvironment (Liberti & Locasale 2016). We first analysed the individual effect of the inhibition of the two STR subunits and observed that only TAS1R3, but not TAS1R2, may have a crucial role in the anti-proliferative effect exacerbated by oxygen and glucose withdrawal in SNB-19 glioblastoma cells. In fact, in a colorectal cancer cell line (Caco-2), only TAS1R3 knockdown, but not TAS1R2, was shown to attenuate the pro-apoptotic and barrier permeabilization effects of artificial sweeteners (Shil *et al.* 2020).

Under hypoxic environment, and to prevent intracellular acidification, the cancer cells release lactate to the extracellular medium, allowing aerobic glycolysis and lactate production to continue, while simultaneously increasing extracellular lactic acid concentrations, whose accumulation is associated with poor prognosis (Brown & Ganapathy 2020; S. Sun *et al.* 2017). While physiological lactate levels in blood and normal tissue are in the range of 1.5–3 mM, the release of lactate from tumour cells can result in extracellular concentrations up to 40 mM, as observed by Fisher and colleagues in the serum of patients with different types of cancer (Fischer *et al.* 2007; Pérez-Tomás & Pérez-Guillén 2020). Therefore, we investigated the effect of TAS1R3 inhibition in L-lactate production by glioblastoma cells. Although TAS1R3 inhibition with lactisole prevented glucose uptake by glioblastoma cells, it did not reduce extracellular L-lactate levels. Likewise, in the lung carcinoma cell line A549, glucose deprivation did not impact the extracellular lactate concentrations (Huang *et al.* 2015). In addition, glycolysis inhibition with 2-deoxyglucose and 3-bromopyruvate in ovarian cancer cells decreased extracellular lactate production while the extracellular glucose levels increased (L. Sun *et al.* 2017). These evidences suggest that lactate could not be used as a substrate for energy metabolism.

In summary, herein we demonstrate the expression and function of the sweet taste receptor in human glioblastoma samples and cell lines, particularly the TAS1R3 subunit. It seems to play an important role in the normal functioning of cancer cell metabolism as an important glucosensor of glucose availability. In line with this, the sweet taste signalling, appears as a promising way to treat glioblastoma by inhibiting the TAS1R3 subunit to preventing the entry of glucose and, consequently, starving the cell and concurrently reducing cell proliferation without causing cell death. In addition, no negative side effects have been reported in humans for blockage of STR with lactisole. On the contrary, blocking STR has antidiabetic effects in humans without negative side effects (Teff *et al.* 2010). These are promising features for targeting this receptor as an anti-cancer therapeutic strategy.

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Chapter 6

Concluding Remarks and Future Perspectives

6.1 Concluding remarks

Although efforts and advances have been made to improve the outcome of glioblastoma patients, the chemoresistant and metabolic features of this type of brain cancer are still a great challenge to uncover more efficient therapies.

Several studies have been investigating the molecular mechanisms behind the anticancer and chemosensitizer effects of bitter tasting compounds, reinforcing their therapeutic potential, but the involvement of bitter taste receptors (TAS2Rs) in the mediation of their effects have been largely overlooked. Considering that many of these bitter compounds with anticancer properties bind and activate TAS2Rs, expressed in a diversity of cancers, the role of TAS2Rs in glioblastoma was exploited in this study. The starting point for this doctoral thesis was to characterize the potential of the bitter taste signalling pathway to consider its potential as novel therapeutic targets for glioblastoma.

In the chapter 4, corresponding to the submitted original article "The anti-cancer effects of temozolomide are partially mediated by activation of the bitter taste receptor TAS2R43 in glioblastoma cells", the mRNA expression of 19 TAS2Rs in both human astrocytes and glioblastoma cells was confirmed. Among them, TAS2R4, R5, R14 and R39 were chosen for protein analysis in human glioblastoma samples and glioblastoma cell lines. In addition, we observed the expression of the downstream effector protein GNAT3, an important and specific component of the taste signalling pathway, and differential expression levels of TAS2Rs between human astrocytes and glioblastoma cells. Contrasting with previous studies that reported lower TAS2Rs expression levels in cancer cells and clinical samples compared to their respective non-cancerous controls, we observed higher levels of TAS2R4 and R5, which bind fewer agonists than many other TAS2Rs, in glioblastoma cells relatively to human astrocytes. On the other hand, TAS2R14 and R39, which are broadly tuned to detect many bitter compounds of different chemical classes, presented higher levels in glioblastoma cells than human astrocytes, like previously reported by other authors in breast, pancreatic, ovarian, and prostate cancers. We also observed that TAS2R4, R5 and R14 immunoreactivity was higher and less dispersed than TAS2R39 in human glioblastoma tissue samples. Moreover, TAS2Rs were found in high cellular density areas, contrarily to fibrous regions, as shown by other authors in pancreatic adenocarcinoma tissue samples.

In the last few years, it became accepted that TAS2Rs activation by their bitter ligands deploy cellular responses involved in the regulation of cell survival, apoptosis, oxidative stress, and other pathways that are imbalanced in cancer cells. Furthermore, the combination of bitter compounds such as epigallocatechin gallate, quercetin, and resveratrol with chemotherapeutics allows the use of lower doses of chemotherapeutic drugs in cancer therapy. Although alkylating agents, like temozolomide (TMZ), are known for their strong bitterness and astringency, little is known about their potential to bind and activate specific TAS2Rs. Additionally, co-adjuvant administration might increase therapies specificity, decreasing the side-effects, and avoiding the development of chemoresistance. The known mode of action of TMZ is the induction of DNA methylation and consequently leading to cell damage and cell cycle arrest, promoting cell death and apoptosis (Hirose et al. 2001; Karachi et al. 2018; Zhang et al. 2012). In spite of that, we aimed at elucidating the capacity of TMZ to specifically activate the bitter taste signalling in glioblastoma cells.

Our findings show that TMZ elicited intracellular Ca2+ levels in a dose-dependent manner and reduced the cell viability by increasing cell apoptosis in the glioblastoma cell lines under study. Interestingly, TMZ effects were reduced in the presence of probenecid, a known TAS2Rs antagonist, or with the taste signalling pathway silenced by GNAT3 knockdown. These results suggest that TMZ may also exert cytotoxic effects through the activation of the bitter taste signalling pathway. However, these data imposed the question of which TAS2R could be mediating the cytotoxic effects of TMZ. We firstly predicted, by *in silico* analysis, six potential target-receptors for TMZ, of which only TAS2R38 and R43 are known to bind probenecid. In addition, only TAS2R43 was expressed in all the glioblastoma cells in study. Given the likelihood that TAS2R43 could be binding TMZ and mediating, at least in part, the anti-proliferative and proapoptotic effects of the drug, we knocked it down and observed that TMZ was unable to reduce the viability of TAS2R43-silenced glioblastoma cells, suggesting that TMZ may exert cytotoxic effects also via TAS2R43 activation. Interestingly, TAS2R43 is also a target of the bitter tasting compound epigallocatechin gallate, which in turn has been widely used as an adjuvant agent in the treatment of gliomas, especially in glioblastoma, enhancing the therapeutic efficacy of TMZ.

In summary, we demonstrated the expression and function of the bitter taste signalling pathway in human glioblastoma samples and cell lines, and that TAS2R43 is activated by the chemotherapeutic alkylating drug TMZ, suggesting that it acts two-fold by eliciting DNA damage and cell death, and by activating TAS2R43 and eliciting downstream cascades leading to reduced proliferation and apoptosis.

Mounting evidence supports the role of the sweet taste receptor (STR) in the regulation of the glycolytic metabolism of different types of normal cells, conveying information on the availability of glucose, and triggering cellular mechanisms to increase its uptake and processing along the signalling pathway. However, to date, only few studies addressed the STR expression and function in tumours cells. The inhibition of this glucosensor may trigger an illusion in cells that there is no glucose available in the extracellular environment, thus compromising glucose uptake and leading to cell death by starvation. Whether the STR mediates metabolic reprogramming of cancer cells or its potential involvement in other molecular pathways and cancer remains to be elucidated. In addition, it is crucial to understand if the STR inhibition can reverse the Warburg effect, to become a potential therapeutic target for cancer.

In the chapter 5, we aimed at characterizing the role of the sweet taste signalling pathway in the glucose metabolism of glioblastoma cells. Firstly, we confirmed the expression of STR subunits TAS1R2 and TAS1R3 in three glioblastoma cell lines. In addition, TAS1R2 presented differential protein expression in the glioblastoma cell lines and was also immunodetected in high cellular density areas of human glioblastoma tissue samples. This suggests that the higher STR expression in glioblastoma is likely to be an advantage to the tumour development since it could enhance glucose sensing at the tumour microenvironment.

In line with this, we next investigated the TAS1R3 subunit inhibition with lactisole in the proliferative capacity and apoptosis of glioblastoma cells. Interestingly, STR inhibition consistently reduced the viability, migration and glucose uptake by glioblastoma cells but did not induce apoptosis or cell death in any of the cell lines under study, suggesting that these cancer cells might undergo senescence, as previously reported in other studies. Therefore, STR inhibition in glioblastoma cells may induce cell cycle arrest, and its correct functioning may be crucial for glucose metabolism of glioblastoma cells.

Since cancer cells are known for their metabolic switch to glycolysis, enhanced by the reduced oxygen and glucose availability in the tumour microenvironment, the imposing question was if STR could regulate the viability of glioblastoma cells under oxygen and glucose withdrawal. We observed that only TAS1R3 activation, but not TAS1R2, may play a crucial role in the anti-proliferative effect exacerbated by oxygen and glucose withdrawal in glioblastoma cells. We also assessed the effect of TAS1R3 inhibition in the L-lactate production by glioblastoma cells, since under hypoxic environments, the cancer cells release lactate into the extracellular medium, allowing aerobic glycolysis and lactate production to continue to guarantee cell proliferation. However, our study did not reflect any alterations on extracellular L-lactate levels upon TAS1R3 inhibition with lactisole.

In summary, herein we demonstrated the expression and function of the sweet taste signalling pathway in human glioblastoma samples and cell lines, and that the sweet taste receptor, particularly the TAS1R3 subunit, plays an important role in the normal functioning of cancer cell metabolism and that it is an important glucosensor of glucose availability.

Overall, we provided evidence that the taste signalling pathway, particularly the bitter and sweet taste receptors, may be used as viable alternatives to the glioblastoma challenging treatment, either as a way to tackle chemoresistance or by reversing the Warburg effect, thus enhancing the prognosis for this devastating disease.

6.2 Future perspectives

Beyond the scientific advances achieved with the experimental work carried out in this doctoral thesis, several novel research questions arose:

The effect of bitter taste receptors silencing on the transport of anticancer drugs across brain barriers and on the regulation of efflux transporters in glioblastoma.

Chemoresistance imposed by brain barriers is the main obstacle for the proper delivery of therapeutic agents to treat neurological diseases, especially brain cancer and metastasis from peripheral primary cancers. Despite the advances in the field, brain barriers limit the efficacy of many, otherwise effective therapeutic agents. Brain pharmacoresistance is essentially attributed to the overexpression of multidrug efflux transporters (ABC transporters) and often responsible for the recurrence of tumours. Therefore, it would be of interest to investigate if TAS2Rs affect the permeability of the blood-cerebrospinal fluid and the blood-brain barriers, and of glioblastoma cells to anticancer drugs. In addition, it is hypothesized that TAS2Rs could be used as therapeutic targets to manipulate the flux of compounds across brain barriers, and enhance the efficacy of brain-directed therapies, to assess the impact of TAS2Rs in the regulation of ABC transporter substrates. In addition, it would also be important to assess the potential of manipulating TAS2Rs to enable the entrance of therapeutic compounds into the brain and demonstrate the efficacy of this approach in glioblastoma.

The reposition of anticancer drugs or other chemicals as agonists or antagonists of taste receptors for treatment of glioblastoma.

Taste receptors, particularly bitter and sweet, are attractive druggable targets, given their location in the cell membrane and the variety of downstream pathways they can activate. In addition, more than 50% of the drugs on the market, target receptors of the G protein-coupled receptor family. In the last few years, a wide diversity of pharmacological and natural compounds, including flavonoids found in edible foods, have been known for their anticancer and anti-inflammatory properties. Most of these chemicals are tasting compounds, and consequently taste receptors ligands.

The effect of the sweet taste receptor, particularly TAS1R3 subunit, inhibition in glioblastoma in response to glucose and oxygen withdrawal.

This doctoral thesis showed that TAS1R3 inhibition with lactisole diminishes the cell viability and migration of SNB-19 glioblastoma cells in normoxic and hypoxic conditions. To better recapitulate the heterogeneity of glioblastoma, it would be important to extend these preliminary studies to other glioblastoma cell lines and patient-derived glioblastoma cells, as well as human astrocytes from which glioblastoma is originated. In addition, the effect of blocking STR with lactisole in the major hallmarks of these cancer cells is of utmost importance and could be addressed by analysing the expression of proliferation, angiogenesis, senescence, invasion, and apoptosis markers.

The effect of the sweet taste receptor, particularly TAS1R3, inhibition in glucose uptake.

An important hallmark of cancer is metabolism, particularly glucose metabolism. High glucose environments promote cell survival and cell adhesion whereas low glucose levels accelerate epithelial-mesenchymal-transition and regulate several genes, favouring more aggressive and invasive cell phenotypes. Upstream regulators of glucose uptake are potential therapeutic targets to starve cancer cells to death. So far, PI3K/Akt signalling, Ras, and HIF1α are known positive regulators of glucose uptake which promote the expression of glucose transporters and the activity of hexokinase, which phosphorylates glucose molecules, thereby preventing their efflux back into the extracellular space. Also, the phosphofructokinase enzyme, which catalyses the conversion of fructose 6-phosphate to fructose 1,6-biphosphate, is an important step of glycolysis. Therefore, the glycolytic flux and metabolism, and how glucose and oxygen availability will impact on these pathways and on the Warburg effect, should be studied by analysing the expression profiling of key transporters and enzymes associated with the glycolytic flux and the lactate efflux (e.g. glucose transporters, hexokinase, phosphofructokinase, lactate dehydrogenase and monocarboxylate transporters).

The identification of the subsets of cells expressing the taste receptors in patient-derived cells and in tumour samples.
Patient-derived cells and human glioblastoma tumour samples could be doubled labelled with anti-taste receptors, either bitter or sweet, and known markers of subsets of cells like astrocytes, neurons, cancer stem cells, microglia, and tumour associated macrophages.

The translational potential of the work.
Previous CNS drug programs in which pharmacokinetic parameters and CNS toxicity of candidate compounds have all been assessed in rodent's brain barriers failed due to differences between humans and rodents. Thus, *in vivo* rodent models should only be used when human data is consolidated and similarities and differences between species thoroughly evaluated. To increase the translational potential of this work into the clinics, it would be important to use human-derived cell lines and brain sections for *in vitro* studies, and patient-derived tumour organoids and human tumour grafts in mouse models for *ex-vivo* and *in vivo* analysis, respectively.