ANA LÍDIA ALVES DOS SANTOS

EFFECTS OF OSELTAMIVIR TREATMENT IN THE BIOLOGICAL BEHAVIOUR AND GLYCOSYLATION PATTERN OF MAMMARY TUMOUR CELL LINES

Dissertação de candidatura ao grau de Mestre em Oncologia - Especialização em Oncologia Molecular submetida ao Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto.

Orientadora - Professora Doutora Joana Tavares de Oliveira
Categoria - Professora Auxiliar
Afiliação - Faculdade de Medicina Veterinária da Universidade Lusófona de Humanidades e Tecnologias.

Co-orientadora – Professora Doutora Maria de Fátima Moutinho Gärtner
Categoria - Professora catedrática
Afiliação - Instituto de Ciências Biomédicas Abel Salazar Universidade do Porto.

ACKNOWLEDGMENTS

I would like to sincerely thank to:

Professor Joana de Oliveira and Professor Fátima Gärtner, for having accepted me and for all the support during this past year.

Professor Manuel Sobrinho-Simões, head of IPATIMUP, for hosting me for my thesis work.

Professor Celso Reis, for having accepted me in the Glycobiology in Cancer group.

To all the members of Glycobiology in Cancer and Differentiation in Cancer, two nice groups of which I'm grateful to be part of. Particularly to Catarina, for all the strength and support she gave me.

To all of my friends, for making me know that friends are the family we choose.

Finally, the most important and deepest thank. To my parents, my brother and my godmother, thank you for making of me all that I am.

ABREVIATTIONS

IARC, *International Agency for Research on Cancer*

TMC, tumores mamários de cadela

TMMC, tumores mamários malignos de cadela

CMT, canine mammary tumours

CMMT, canine mammary malignant tumour

TACA, tumour-associated carbohydrate antigens

T antigen, Thomsen-Friedenreich antigen

sT antigen, sialylated form of T antigen / sialylated T antigen

Tn antigen, Thomsen-nouvelle antigen

sTn antigen, sialylated form of Tn antigen / sialylated Tn antigen

sLe^x antigen, sialyl-Lewis antigen x

sLe^a antigen, sialyl-Lewis antigen a

Le^a antigen, Lewis antigen a

Le^b antigen, Lewis antigen b

Le^x antigen, Lewis antigen x

Le^y antigen, Lewis antigen y

MUC1, Mucin-1

ST6Gal-I, Beta-galactoside alpha-2,6-sialyltransferase 1

Mgat5, Alpha-1,6-mannosylglycoprotein 6-beta-N-acetylglucosaminyltransferase A

SNA, *Sambucus nigra* agglutinin

MAL / MAL-I / MAA / MAA-1, *Maackia amurensis* leukoagglutinin

MAH / MAL-II / MAA / MAA-2, *Maackia amurensis* hemagglutinin

Neu1, neuraminidase/sialidase 1

Neu2, neuraminidase/sialidase 2

Neu3, neuraminidase/sialidase 3

Neu4, neuraminidase/sialidase 4

EGFR, epidermal growth factor receptor

EGF, epidermal growth factor

GM3, monosialoganglioside

GD3, trisialoganglioside

LPS, lipopolysaccharide

PTNM, Pathological Tumour-Node Metastasis

IDC, invasive ductal carcinomas

RESUMO

Os tumores mamários de cadela (TMC) partilham muitas semelhanças com o cancro de mama na mulher, no entanto, apresentam uma incidência 3 vezes maior, e um tempo de doença mais curto. Deste modo, os TMC constituem um excelente modelo espontâneo da carcinogénese mamária. Relativamente à mulher, o cancro de mama é o tipo de cancro mais comum, no entanto, a taxa de mortalidade é baixa. Por ano, em cada 100.000 mulheres cerca de 6 a 19 morrem por cancro de mama. Este facto deve-se à melhoria dos diagnósticos e terapêutica. Assim sendo, o tumor mamário maligno de cadela, considerado um excelente modelo espontâneo da carcinogénese mamária, poderá melhorar o conhecimento sobre o processo metastático em humanos e trazer vantagens no que diz respeito ao tratamento tanto na cadela como na mulher.

Os glicoconjugados na superfície celular desempenham papéis importantes numa variedade de funções biológicas, tais como interação célula-a-célula e célula-substrato, adesão de bactérias, organização da membrana e imunogenicidade. Alterações à estrutura dos glicoconjugados desempenham um papel relevante durante o processo de carcinogénese. Geralmente estas alterações estão associadas à expressão de antigénios característicos de tumor em glicoproteínas e glicolípidos que decoram a superfície celular. Por exemplo, um aumento em estruturas sialiladas é frequentemente observado em carcinomas e está associado ao estadio da doença, capacidade de invasão, metástases e mau prognóstico.

Devido à função preponderante na tumorigénese mamária de várias glicoproteínas, e dos glicanos que estas transportam, o presente estudo teve como objetivo investigar o papel do oseltamivir como inibidor de sialidases a fim de compreender o seu modo de ação em glicoproteínas durante a progressão do tumor mamário, recorrendo a um modelo celular de TMC. Para isso, procedeu-se à avaliação da viabilidade celular, da capacidade de proliferação, migração e invasão, e a possível alteração no perfil de glicoconjugados em duas linhas celulares CMA07 e CMT-U27, após inibição das sialidases por tratamento com oseltamivir.

Os resultados mostraram que o oseltamivir desempenha um papel importante no comportamento biológico e leva à alteração do padrão de glicosilação de glicoconjugados nas linhas celulares de TMC. A inibição das sialidases demonstrou a sua importante função durante a progressão do tumor mamário tendo-se verificado um aumento na capacidade de invasão e também na migração das células após tratamento. Além disso, e tendo em consideração o padrão de glicosilação, células malignas CMT-U27 tratadas com oseltamivir apresentam um aumento em estruturas sialiladas, com uma

concomitante diminuição de ligandos de galectina-3. Essas observações apontam para um papel dinâmico e importante das sialidases em TMMC (tumores mamários malignos de cadela). Assim, os resultados deste estudo demonstram o papel importante da expressão e atividade de sialidases na capacidade invasiva de células de carcinoma mamário possivelmente graças à sua capacidade de remoção de ácidos siálicos.

ABSTRACT

Canine mammary tumours (CMT) are considered an excellent spontaneous model of mammary carcinogenesis because they share many similarities with human breast cancer. However, CMT presents a 3 times increased incidence than that of humans, and a shorter disease time course. Regarding women, breast cancer is the most common type of cancer. In women, breast cancer mortality rate is much lower than incidence rate. CMT can be used to improve our understanding about the metastatic process in humans bringing advantages for cancer treatment both for female dog and women.

Cell surface glycoconjugates play important roles in a variety of biological functions, such as cell-cell and cell-substrate interactions, membrane organization and cell immunogenicity. Thus, alterations in glycosylation are considered a hallmark during carcinogenesis, and these alterations usually lead to the expression of tumour-associated carbohydrate antigens on glycoproteins and glycolipids decorating cell surfaces. Increased sialylation is commonly observed in carcinoma cells and is associated with tumour grade, invasion, metastasis and poor prognosis of patients. Sialic acids removal from carbohydrate chains of glycolipids and glycoproteins by sialidases can affect the conformation of glycoproteins.

Due to the preponderant function of several glycoproteins and their carried glycans in mammary tumourigenesis, the present study aimed to investigate the role of oseltamivir, a sialidase inhibitor, in order to understand how sialidases are putatively able to act on glycoproteins during mammary tumour progression using CMT derived cell lines. To achieve this, we assessed cellular viability, proliferation capacity, migration and invasion in two cell lines CMA07 and CMT-U27, and evaluated a possible alteration in the glycoprofile of glycoconjugates in CMT cell lines upon sialidase inhibition.

The results showed that oseltamivir plays an important role in the biological behaviour of cancer cells and leads to alterations in the glycosylation pattern of glycoconjugates in CMT cell lines. Sialidase inhibition results highlight their important role during mammary tumour progression since we found an increase in invasion capacity and also in migration of cells upon treatment. Moreover, regarding the glycosylation pattern, highly malignant CMT-U27 cells treated with oseltamivir, showed an increase in sialylated structures, with concomitant decrease in galectin-3-ligands. These observations point to an important dynamic role of sialidases expression in mammary tumours. Overall, these results imply an important role of sialidases expression and activity in the invasive capacity of mammary cancer cells, possibly due to their ability to remove sialic acid.

INTRODUCTION

Canine mammary tumours

Canine mammary tumours (CMT) are the second most common type of canine neoplasia (25-50% of all diagnosed cases) (Moulton 1990). CMT are the most frequent type of female dog tumours being up to 53% of all tumours (Rutteman 2001). CMT are mainly diagnosed in elderly females, being relatively rare before 2 to 4 years of age and presenting a higher incidence between 10 and 11 years of age (Schneider 1970); (Johnston 1993).

Mammary tumour initiation and progression are influenced by similar factors in both human and canine cancers, including age, nutrition, gender, reproductive status and environmental exposure (Lana 2007). In addition, several major aspects of metastasis-associated gene expression are also similar between human and canine mammary tumours (Rutteman 2001, Lindblad-Toh *et al.,* 2005). However, the incidence of mammary tumours is 3 times higher in female dogs than in women and the time course of the disease much shorter, approximately 2 years (Sorenmo 2003). As such, it is an excellent comparative model to understand various aspects of not only breast carcinogenesis but also and perhaps most importantly metastasis in women. Also, the relatively rapid cancer progression rates, compared with those in humans, provide the opportunity to observe therapeutic effects in a shorter period of time than trials conducted in human patients (Leahy *et al.,* 2006). In addition, a great advantage of spontaneous tumours is the cross-sectional value of genetic diversity background provided, which similar to that seen in human populations as opposed to experimental animal studies (Lindblad-Toh *et al.,* 2005).

Thus, the pet dog might support the transition between mouse models and human patients (Paoloni *et al.,* 2008).

Human breast cancer

Cancer is a major public health problem in the world. Breast cancer is one of the most prevalent cancers (Figure 1), and metastatic breast cancer accounts for the highest number of cancer-related deaths among women worldwide. In 2008, breast cancer incidence was higher in Western Europe, Australia/New Zealand, Northern Europe and Northern America (Ferlay *et al.,* 2010).

Currently, about 1 in 3 women in the United States develop cancer in her lifetime (Siegel *et al.,* 2012), and it is estimated that there are nearly 3 million people with a invasive breast cancer history. Overall, 60% of these breast cancer cases are diagnosed

in an early stage (Howlader *et al*., 2011). Despite more than 1.38 million of new cases diagnosed each year and over 458.000 deaths recorded, our knowledge on systemic cancer cell dissemination is scarce (Ferlay *et al.,* 2010).

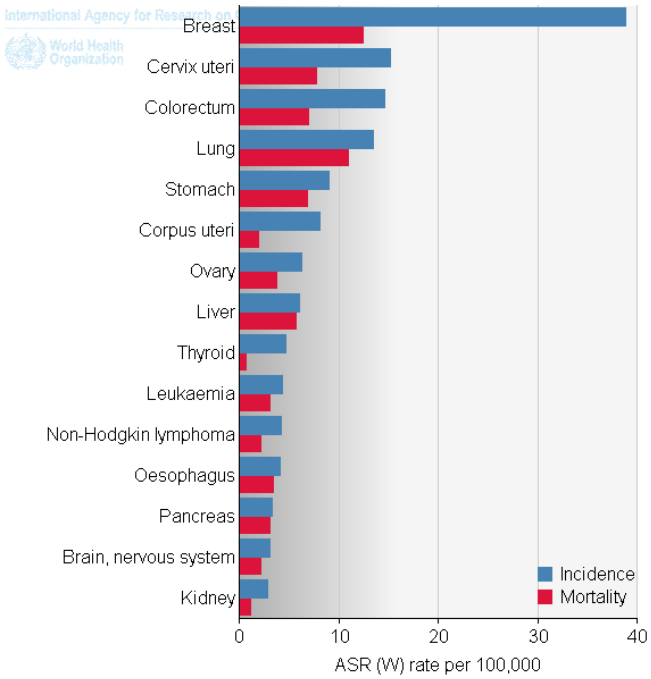


Figure1. Incidence and mortality rates of the different types of cancer affecting women worldwide, in 2008. Breast cancer presented both the higher incidence and mortality rates in women worldwide. (Data from GLOBOCAN - IARC).

Glycosylation

Glycosylation is one of the most important modifications in proteins and lipids, originating several glycoconjugates: glycoproteins, glycosaminoglycans, proteoglycans and glycolipids. Vital interactions between cells and the microenvironment surrounding them are mediated by the glycocalyx, a glycan layer which covers the external cell surface (Malagolini *et al.,* 2009). In addition, cellular glycosylation has also been shown to coordinate key biological processes including cell-cell communication, signal transduction, protein folding and stability (Varki 1993); (Dwek 1995); (Wells *et al.,* 2001).

In proteins, oligosaccharide chains can be attached to the peptide backbone via two chief types of linkages: (a) aglycosidic bond of N-acetylgalactosamine (GalNAc) to the hydroxyl group of threonine or serine on the polypeptide chain, the *O*-linked glycans which are predominantly membrane bound or secreted on proteins; (b) a glycosidic bond of *N*-

acetylglucosamine (GlcNAc) to asparagine occurring on the consensus sequence Asn-X-Ser/Thr of a protein chain, the to *N*-linked glycan chains (Opdenakker *et al.,* 1993). Mammalian cells have a specific enzymatic machinery that includes glycosyltransferases and glycosidases, which is required for biosynthesis of these diverse glycan structures, in the endoplasmic reticulum and Golgi apparatus. The glycan structures found are further dependent on the cell type, developmental stage and cell differentiation, and are fundamentally altered in many pathologic states, including cancer (Opdenakker *et al.,* 1993). In cancer, glycosylation changes have been often associated to a deregulation of glycosyltransferase genes expression. Glycosyltransferase genes ST6Gal-I (N-acetyllactosaminide α-2,6 sialyltransferase) and Mgat5 (Alpha-1,6-mannosylglycoprotein 6-beta-N-acetylglucosaminyltransferase A) are, for instance, regulated by oncogenes. However more recently, a role played by glycosidases such as sialidases in neoplasia has been increasingly recognized.

Sialidases

Sialidases are enzymes that exist widely in vertebrates and in a variety of microorganisms (viruses, bacteria, fungi, mycoplasma and protozoa) with evidence of great importance in various cellular functions (Schengrund *et al.,* 1976); (Corfield *et al.,* 1981); (Corfield *et al.,* 1982); (Saito *et al.,* 1995). The enzymes also are present in a large variety of cells and tissues from mammalians (Carubelli *et al.,* 1962).

Four types of mammalian sialidases have been identified and characterized to date, and designated as Neu1, Neu2, Neu3 and Neu4. The first three were classically described to localize predominantly in the lysosomes, cytosol and plasma membranes (Miyagi *et al.,* 1999) respectively, and Neu4, the most recently identified sialidase, is often found in lysosomes, mitochondria and the endoplasmic reticulum (Roggentin *et al.,* 1989); (Saito *et al.,* 1996). However, their subcellular localization can vary with particular cell stimuli. Neu1, Neu2 and Neu4 show mobilization to the cell surface in certanin conditions (Saito *et al.,* 1996). The overall amino acid identity between the four sialidases is variable. Amino acid identity between Neu1 and the other sialidases is relatively low (19-24%), while Neu2, Neu3 and Neu4 present a higher 34-40% homology between each other. Regarding comparative expression levels of sialidases in different human tissues, Neu1 usually presents the highest expression, on the opposite to Neu2, which presents extremely low expression levels (Yamaguchi *et al.,* 2006). Sialidases have been implicated in lysosomal catabolism, and regulation of important cellular functions such as cell differentiation, cell growth and apoptosis (Thomas *et al.,* 2001); (Miyagi *et al.,* 2012).

The function of each sialidase is linked to its glycan specificity. Neu1 hydrolyzes glycoproteins and *in vitro* sialidase activity assays showed that Neu1 reacts with high specificity with oligosaccharides and glycopeptides (Saito *et al.,* 1996). Moereover, it acts preferentially on oligosaccharide substrates with α2-3 sialyl linkage when comparing to those with α2-6 (Miyagi *et al.,* 1984). Neu2 hydrolyzes native glycoproteins, oligosaccharides and also gangliosides (Miyagi *et al.,* 1985). It acts in the cytosol preferentially on α2-8 sialyl linkages (Miyagi *et al.,* 1985). Despite glycoconjugates being not usually present in the cytosol (Ishizuka *et al.,* 2008), glycoproteins, oligosaccharides, gangliosides, glycosidases and lectins have been reported to occur in cytosol (Funakoshi *et al.,* 2009). Neu3, hydrolyzes specifically gangliosides with α2-3 (GM3, monosialoganglioside) and α2-8 (GD3, trisialoganglioside) and α2-6 linkages (synthetic GM3) (Hata *et al.,* 1998). The murine enzyme acts on oligosaccharides and glycoproteins to a certain extent (Li *et al.,* 2001). Neu4 it is the only sialidase known toacts on mucins with high efficiency (Shiozaki *et al.,* 2011). NEU4 was found to *in vitro* hydrolyze sLe^a and sLe^x antigens and to decrease their cell surface levels much more effectively than other sialidases (Shiozaki *et al.,* 2011).

Sialidases and cancer

It is known that alterations in glycosylation such as increased sialylation are a common feature of malignancy (Reis *et al.,* 2010). Sialic acids are terminal acidic monosaccharides usually found in the terminal position of the carbohydrate groups of glycoproteins and glycolipids. Their removal catalysed by sialidases may affect the conformation of glycoproteins, and therefore contribute to recognition or masking of biological sites in molecules and cells (Yogeeswaran *et al.,* 1981); (Dennis *et al.,* 1987); (Schauer 2000). This may influence the malignant phenotype of cancer cells, including the metastatic potential and invasiveness (Hakomori 2002). Sialidases have been found to be differentially expressed in several types of cancer, specifically in highly metastatic cells (Miyagi *et al.,* 2008).

NEU1 expression is decreased in several types of cancer. Its expression levels present an inverse relation with the metastatic cancer capacity. In different clones of mouse colon adenocarcinoma cells, lower expression of NEU1 sialidase where seen in those with higher metastatic rates when comparing to less invasive ones. There were concomitantly higher levels of sialyl Lex and GM3 in these cells (Sawada *et al.,* 2002). Furthermore, transfection of Neu1 sialidase into mouse melanoma cells resulted in suppression of experimental pulmonary metastasis due to concomitant reduction in

anchorage-independent growth and increased sensitivity to apoptosis (Kato *et al.,* 2001). Overexpression of human NEU1 with the PPCA gene resulted in similar alterations in human colonic adenocarcinoma cells with suppressed cell migration and invasion being shown. On the other hand, Neu1 knockdown resulted in the opposed effect (Galjart *et al.,* 1988); (D'Azzo *et al.,* 1982). NEU1-expressing cells present reduced *in vivo* liver metastatic potential in mice (Uemura *et al.,* 2009).

Regarding NEU2, its up-regulation also affects cancer cell behaviour. Transfection of rat gene Neu2 into highly invasive and metastatic B16-BL6 mouse melanoma cells, lead to a decrease in pulmonary metastasis, possibly related to the GM3 ganglioside decrease (Tokuyama *et al.,* 1997). Within addition, highly metastatic mouse colon adenocarcinoma cells transfected with Neu2 showed a marked reduction in lung metastasis, invasion and cell motility, with concomitant decrease in sLe^x and GM3 levels. This was suggested to be related with changes in cell adhesion and/or cell motility (Sawada *et al.,* 2002), pointing to desialylation of these and other molecules, as targets of sialidase, involvement in the suppression of metastasis. Furthermore, a human epidermoid carcinoma cell line transfected with Neu2 gene, showed reduced GM3 levels, and a concomitantly increased cell growth and tyrosine autophosphorylation of epidermal growth factor receptors (EGFR) at low EGF (epidermal growth factor)concentration levels (Meuillet *et al.,* 1999). Moreover, human NEU2 overexpressing leukemic cells presented marked decrease in anti-apoptotic factors Bcl-XL and Bcl-2, resulting in increased sensitivity to apoptotic stimuli (Tringali *et al.,* 2007). NEU2 overexpression in the cells reduced gene expression and activity of Bcr-Abl, together with a decrease in Bcr-Abl dependent Src and Lyn kinase activity probably through desialylation of cytosolic glycoproteins.

Neu3 is up-regulated in several types of human cancer, such as colon, renal, ovarian and prostate cancers. However, Neu3 down-regulation was also observed in acute lymphoblastic leukemia and associated to disease progression (Mandal *et al.,* 2010). NEU3 mRNA levels were found to be 3–100-fold increased in human colon cancers, compared with adjacent normal mucosa (Kakugawa *et al.,* 2002). Furthermore, in renal cell carcinomas NEU3 mRNA levels were observed to be significantly increased (Ueno *et al.,* 2006). In the case of prostate cancer, Neu3 is also up-regulated and it's correlated with malignancy (Kawamura *et al.,* 2012). Prostate cancer cells revealed a significant decrease in invasion and migration capacity after NEU3 knockdown *in vitro*.NEU3 activates molecules such as EGFR, FAK, ILK, Shc and integrin β4, frequently up-regulated in carcinogenesis, being possibly the reason for the development of a malignant phenotype. In addition, this sialidase was found to increase azoxymethane-induced aberrant crypt foci formation in colon mucosa by suppression of apoptosis

possibly due to the activation of EGF signalling. NEU3 is thus involved in the regulation of transmembrane signalling at the cell surface through both modulation of gangliosides as the result of enzyme reactions and by interaction with other signal molecules, including caveolin-1, Rac-1, integrin β4, Grb-2 and EGFR (Miyagi *et al.,* 2008).

In what regardsNEU4 sialidase, its levels are markedly decreased in human colon cancer (Yamanami *et al.,* 2007). In addition, human colon adenocarcinoma cell lines transfected with NEU4 showed increased apoptosis and decreased invasiveness capacity and cellular motility (Shiozaki *et al.,* 2011). To elucidate the significance of NEU4 down-regulation in colon cancer, sialyl-Lewis antigens, sLe^a and sLe^x, were investigated, and Neu4 was found to hydrolyze these antigens *in vitro* and decrease their cell surface levels farly more than the other sialidases (Shiozaki *et al.,* 2011).

These multiple features suggest that each type of sialidase may play a unique role according to its properties. The role of sialidases in cancer is probably closely related to the available substrate which poses several therapeutic implications.

Sialylation and cancer

A general increase in sialylation is often found in cell surface glycoproteins of malignant cells (Neufeld *et al.,* 2001). Altered sialylation of glycolipids is also observed to be a ubiquitous phenotype (Achyuthan *et al.,* 2001). In fact, tumour-associated antigens are often sialylated.

Tn antigen is the precursor of the T antigen, also known as Core 1. The Tn antigen does not occur in abundance in normal cells and tissues of adult animals, however it can be detected in almost all kinds of carcinomas (Julien *et al.,* 2001). Almost 90% of breast cancers express Tn antigen while it is scarcely detected in normal mammary tissue (Springer 1997). This antigen was found to be associated to with the Pathological Tumour-Node Metastasis (PTNM staging) tumour stage in Invasive Ductal Carcinoma of the breast (Wang *et al.,* 1997). Tn antigen can be sialylated at the C6 position of GalNAc residue, resulting in the dissacharide Neu5Acα2-6GalNAc-R, sTn antigen (sialylated Tn antigen). sTn is suggested to have an important role in carcinogenesis. Its expression is rarely observed in normal tissues but highly expressed in most several carcinomas such as gastric (David *et al.,* 1992); (Victorzon *et al.,* 1996); (Baldus *et al.,* 2000), colorectal (Itzkowitz *et al.,* 1990), ovarian (Kobayashi *et al.,* 1992), breast (Leivonen *et al.,* 2001); (Yonezawa *et al.,* 1992), and pancreatic (Kim *et al.,* 2002). Thus, sTn has been associated with carcinoma aggressiveness and poor prognosis.

T antigen is an oncofetal glycan antigen. It is originated by a non-elongated form of Core 1. In normal epithelium, T antigen is masked by sialic acids, sulphates or by addition of other sugar chains to form branched and complex *O*-glycans (Springer 1984); (Hanisch *et al.,* 1997). T antigen is a *pancarcinoma* antigen expressed in several types of cancer including breast (Kumar *et al.,* 2005), colon (Baldus *et al.,* 2000), bladder (Coon *et al.,* 1982), prostate (Janssen *et al.,* 1996), liver (Cao *et al.,* 1996), ovary (Ghazizadeh *et al.,* 1990) and stomach (Baldus *et al.,* 2001), suggesting that changes in *O*-glycosylation provide some advantage to tumour development (Cazet *et al.,* 2010). *In vitro* and *in vivo* studies show that T antigen facilitates metastases by binding galectin-3 which clusters MUC1 mucin at the cell surface thereby facilitating not only heterotypic adhesion between tumour and endothelial cells (Yu *et al.,* 2007), but also homotypic adhesion between tumour cells essential for their survival in the blood stream (Zhao *et al.,* 2010). Inhibition of galectin-3 with T antigen interaction was shown to reduce metastatic capacity (Glinskii *et al.,* 2012). Core 1 was found to be mostly sialylated (sT antigen) in CMT, however its non-sialylated form (T antigen) is expressed in tumour emboli (de Oliveira *et al.,* 2011).

The histoblood group Lewis antigens are found in most human epithelial tissues, where they are expressed at the terminal part of glycolipid and glycoprotein carbohydrate chains (Ravn *et al.,* 2000). These antigens derive from the substitution of type 1 (Galβ1-3GlcNAc) or type 2 (Galβ1-4GlcNAc) dissacharide sequences by fucose and sialic acid residues. Le^a, Le^b and sLe^a antigens derive from type 1 sequences, and Le^x Le^y and sLe^x antigens derive from type 2 (Cazet *et al.,* 2010). In a normal context, sLe^x antigen expression is restricted to the immune system cells contributing to leukocyte function in the inflammatory response event *via* interaction with E-selectin expressed on endothelial cells (Cazet *et al.,* 2010). On the other hand, sialyl-Lewis antigens are usually altered in cancer cells and show a good correlation with the metastatic risk in breast cancer patients. sLe^a and sLe^x antigens expression are found to be increased in breast cancer tissues, including primary breast carcinoma lesions (Renkonen *et al.,* 1997). Levels of expression of sLe^x antigen are higher in breast cancer patients who had distant metastasis, when compared with patients presenting non-metastatic lesions (Matsuura *et al.,* 1997); (Jeschke *et al.,* 2005).

Sialidase inhibition

Oseltamivir phosphate (Tamiflu®, Roche) is an effective sialidase inhibitor, extensively used as an anti-influenza virus drug. It is a sialic acid analogue which interacts with the active sites of the influenza sialidase enzymes. Once in the liver, after oral

administration, oseltamivir is processed by esterases into the active form, oseltamivir carboxylate, which can inhibit the sialidase of influenza virus with high affinity (Varghese *et al.,* 1998); (Wilson *et al.,* 2003); (Zaccai *et al.,* 2003). Other sialidase inhibitors are other oseltamivir carboxylate and zanamivir.

Since this drug is targeted against viral sialidases, it is of the most importance to evaluate if it may also affect the activity of endogenous sialidases in humans. The four human sialidases are homologous of that of virus. For example, the structure of human cytosolic sialidase NEU2 showed to be similar to that of viral sialidases, containing exactly the same active site residues (Chavas *et al.,* 2005). Some observations pointed to a possible inhibitory effect of oseltamivir on endogenous sialidases in rats and mice (Crain *et al.,* 2004); (Izumi *et al.,* 2007); (Moore *et al.,* 2007); (Woronowicz *et al.,* 2007). Oseltamivir and zanamivir are known to be effective in human sialidases at 1.2 and 0.05–0.43 μM, respectively, by administration in patient's plasma. Regarding viral sialidases, only low nanomolar concentrations are suficient to block viral sialidases activity, because viral and bacterial sialidases are 10 fold more sensitive than that of mammalian (Hata *et al.,* 1998); (Chavas *et al.,* 2010). In addition, 2-deoxy-2,3dehydro-N-acetylneuraminic acid (DANA), Neu5Ac2en, is a sialic acid analog and well-known inhibitor of almost all members of sialidases (Chavas *et al.,* 2005).

Thus, the role of oseltamivir in cancer cells has not been determined and information about its effects on cancer cells is scarce. It is necessary to perform studies in order to assess the role of oseltamivir in the biological behaviour of cells, and also its effect on living organisms.

Aims

Malignant CMT is considered an excellent spontaneous model of mammary carcinogenesis, and comparative studies could greatly improve our understanding about key steps of the metastatic process in humans bringing mutual advantages in what concerns treatment (Soremno 2003) (Paoloni *et al.,* 2008).

The present study aimed to investigate the effects of a sialidase inhibitor, oseltamivir, in order to better understand whether sialidases play a putative role during mammary tumour progression. Thus, our specific objectives in order to evaluate the effect of sialidases inhibition in the biological behaviour and glycosylation pattern of CMT cell lines were:

a) To assess the effect of sialidases inhibition, using oseltamivir, in cellular viability, proliferation capacity, migration and invasion capacity;
b) To evaluate a possible glycoprofile alteration upon sialidase inhibition with oseltamivir.

MATERIAL AND METHODS

Cell lines and culture conditions

Two distinct cell lines were used: 1) a benign cell line - CMA-07 - established at our laboratory from a primary complex adenoma excised from a 6-year-old female dog; and 2) a highly metastatic CMT cell line - CMT-U27 - established from a spontaneous canine mammary tumour, excised from a 14-year-old female dog through fine-needle aspiration, kindly provided by Professor Eva Héllmen, from Sweden (Hellmen 1992). The two cell lines were cultured at 37ºC in a humidified 5% CO_2 incubator (Thermo Scientific, U.S.A.) and maintained in RPMI 1640 medium with Glutamax and 25 mM Hepes (Gibco Life Technologies, U.K.), supplemented with 10% of fetal bovine serum, FBS (Gibco Life Technologies, U.K.) and 1% Penicillin Streptomycin (10.000 units/ml penicillin: 10.000 µg/ml streptomycin; Gibco Life Technologies, U.K.).

Whenever necessary, re-plating of the cells was done by cell dethatching using tripsin, followed by a centrifugation (Eppendorf AG, Germany) at 37° C, 1200 rpm, for 5 minutes. The resulting cell pellets were ressuspended in 3 ml of RPMI 1640 and cells were counted in a Newbauer's chamber in a 1:2 dilution of cells in 0.4% trypan blue and cell count was done using the volume conversion factor for 1 mm^3, which is $1x10^4$. Cells were then prepared to be re-plated.

Cell morphology analysis

CMA07 and CMT-U27 cells were plated in a density of $1x10^4$ cells per well, in triplicate. Three different oseltamivir doses were studied: 125 ng/mL, 1.25 µg/mL, and 12.5 µg/mL oseltamivir, and PBS was used as control. Analysis of cell confluence and morphology were performed using a contrast inverted microscope during 7 days. Photographs were taken in days 1, 3 and 7 in two different magnifications (x4, x20).

Cell proliferation assay

CMA07 and CMT-U27 cells were cultured in 24 wells plates in triplicate for each condition: 125 ng/ml, 1.25 µg/ml, 12.5 µg/ml and 125 µg/ml oseltamivir and PBS as control. Cell number was measured every day, in triplicate, during 7 days. Results obtained were recorded, the growth curves traced and statistical analysis was performed.

Cell viability assay

Cell viability was determined using a commercial available kit CellTiter 96® AQ_{ueous} One Solution reagent (Promega Corporation, U. S. A.), and performed according to manufacturer's instructions.

Briefly, cells were plated in triplicate in a 96 wells plate (Orange Scientific, Belgium), with a density of $5x10^3$ cells per well. After cell adherence to the wells, oseltamivir was added in 125 ng/ml, 1.25 µg/ml, 12.5 µg/ml and 125 µg/ml oseltamivir concentrations, and PBS was used as control. Cell viability was measured by adding MTS tetrazolium reagent and absorbance was recorded at 490nm 2 hours after reagent addition. The assays were performed in triplicate or both CMA07 and CMT-U27 cell lines, during 48 hours, with time-points at 0, 2, 4, 6, 8, 10, 12, 24 and 48 hours. An additional control measurement was performed at time-point 0, in a culture well without cells.

Wound-healing

The wound-healing assay was performed in a *time-lapse* microscope, and wound image acquisition was done with 5 minutes intervals during 48 hours, using the program Axio Vision Release 4.8.2. and converted in video.

Briefly, $20x10^4$ cells were plated into a 24 wells culture plate (Falcon by Becton Dickinson Labware, U. S. A.) and after reaching high confluence an artificial "wound" was made with a pipette tip. Culture medium was replaced with the different oseltamivir doses: 125 ng/ml, 1.25 µg/mL and 12.5 µg/ml oseltamivir and PBS as control. The migration was evaluated considering the "healing capacity" of cells during 48 hours.

Matrigel Invasion Assay

A matrigel invasion assay was performed to evaluate the invasive capacity of CMT-U27 cells. Briefly, inserts were re-hydrated with RPMI 1640 and maintained for 1 hour at 37º in a humidified 5% CO_2 incubator (Thermo Scientific, U.S.A.) to complete the hydration process. After insert rehydration, $1x10^5$ cells were seeded on Matrigel-coated chambers in the presence of different oseltamivir doses (125 ng/ml, 1.25 µg/ml, 12.5 µg/ml oseltamivir), and PBS as control, and cultured during 6 hours. After that, the content of each insert was removed and washed twice with PBS. Then, invasive cells were fixed with cold methanol for 20 minutes. After fixation, inserts were transferred to slides (Industrial Quality, Germany) and mounted with Vectashield mounting medium with DAPI

(Vector Laboratories, U.S.A.). Cell invading capacity was measured by counting the number of cells that passed through the Matrigel-coated filter.

Fluorescence

Cells were cultured in glass coverslips in 24 wells culture plates, and the culture medium was supplemented with 125 ng/ml, 1.25 µg/ml and 12.5 µg/ml oseltamivir and PBS as control, during 24 hours. After 24 hours of treatment, cells were washed with PBS and fixed with cold methanol for 20 minutes. Following methanol fixation, cells were re-hydrated with PBS and then blocked with 10% BSA for 20 min. BSA was replaced with plant lectins SNA (Biotinylated *Ederberry bark* lectin, B-1305, Vector Laboratories, U.S.A.), MAL I (Biotinylated *Maackia amurensis* lectin I, B-1315, Vector Laboratories, U.S.A.), and MAL II (Biotinylated *Maackia amurensis* lectin II, B-1265, Vector Laboratories, U.S.A.) 1:300 diluted in PBS, incubated for 1 hour, at room temperature. For galectin-3-ligands fluorescence, BSA was replaced with biotinylated galectin-3 1:100 diluted in PBS, and slides were incubated for 1 hour, at room temperature. After two washes with PBS, Streptavidin FITC-conjugated was incubated for 1 hour at room temperature in a 1:1000 dilution in PBS, protected from the light. For galectin-3 fluorescence, BSA was replaced with anti-gal-3 antibody, 1:100 diluted in PBS, and slides were incubated overnight, at 4ºC. After two washes with PBS, slides were incubated with FITC-conjugated anti-rat secondary antibody diluted 1:200 in 5% BSA for 1 hour, protected from light, at room temperature.

After two washes with PBS, slides were incubated for 10 minutes with DAPI (Sigma-Aldrich, U.S.A.) in PBS and slides were mounted in Vectashield mounting medium for fluorescence (Vector Laboratories, U.S.A.). Slides were analysed and images were taken in a Carl Zeiss fluorescent microscope (Carl Zeiss Microscopy, Germany).

Western Blot analysis

Cells from CMA07 and CMT-U27 cell lines were grown to confluence in 6 well-plates. Then, oseltamivir was added (125 ng/ml, 1.25 µg/ml and 12.5 µg/ml oseltamivir), and cells were incubated for 24 hours. Then, cells were washed three times with PBS, and 200µl of a pre-prepared mixture of 1000µl of RIPA lysis buffer (50 mM Tris HCl, pH 8; 150 mM NaCl; 1% NP-40; 0,5% sodium desoxicolate; 0,1% SDS) containing 40µl of complete protease inhibitor cocktail (Roche, Switzerland), 10µl of PMSF (phenylmethyl sulfonyl fluoride), and 10µl Na_3VO_4 (sodium orthovanadate) were added to each well.

After 10 minutes incubation, cells were scrapped, collected to tubes and centrifuged (Eppendorf AG, Germany) at 13000 rpm, for 10 minutes, at 4°C. After centrifugation, supernatants were collected and protein amount was quantified using the biocinchoninic acid method, Pierce™ BCA Protein Assay Kit (Pierce/ Thermo Scientific, U.S.A.), according to the manufacturer's instructions.

The total extracts were boiled for 5 min at 95°C in Laemmli sampling buffer, and runned in 10% SDS-PAGE. After electrophoresis, gels were transferred to a nitrocellulose membrane (Amersham Biosciences/GE Healthcare Life Sciences, U.K.) and incubated with lectins: Biotinylated *Maackia amurensis* lectin I (B-1315, Vector Laboratories, U.S.A.), Biotinylated *Maackia amurensis* lectin II (B-1265, Vector Laboratories, U.S.A.) and Biotinylated *Ederberry bark* lectin, SNA (B-1305, Vector Laboratories, U.S.A.) diluted 1:500 in 5% BSA (Sigma-Aldrich, U.S.A.) in 1x PBS with 0.05% Tween-20 (Sigma-Aldrich, U.S.A.). After lectins incubation, three washes with PBS 0,05% Tween-20 were performed and membranes were incubated with avidin-biotin complex kit (Vectastain ABC kit Standard, Vector Laboratories, U.S.A.) for 1 hour at room temperature. For galectin-3-ligand analysis, membranes were incubated with galectin-3 biotinylated 1:200 diluted. After three washes with PBS 0,05% Tween-20 were performed, membranes were incubated with avidin-biotin complex kit for 1 hour at room temperature. For galectin-3 analysis membranes were incubated with anti-galectin-3 antibody 1:200 diluted (eBioscience, San Diego, CA) followed by HRP-conjugated anti-rat secondary antibody for 1 hour incubation. Analysis were done by chemiluminescence using the ECL Western blotting detection reagent and films (both from GE Healthcare, U.K.). Western blot for actin diluted 1:4000 (Santa Cruz Biotechnology) was used as loading control.

Statistical analysis

Whenever adequate, the results were presented as mean ± standard deviation. Statistical analysis was performed using One Way ANOVA (Analysis of variance) test and for multiple comparisons Dunnett and Tukey's tests with $p<0,05$ as the level of significance, in GraphPad Prism 5.02 version.

RESULTS

CMT cell morphology assessment in the absence and presence of oseltamivir

Cell morphology assay was performed in CMA07 and CMT-U27 cell lines during 7 days in order to determine if oseltamivir leads to morphologic alterations of cells. Different concentrations of oseltamivir were tested, 125 ng/mL, 1.25 µg/mL, 12.5 µg/mL, and PBS was used as control. Cell morphology was observed in an inverted microscope in days 1, 3 and 7, and photographs were taken.

No differences in cell clustering were observed in both CMA 07 and CMT- U27 cells in the different doses of oseltamivir, when compared with control cells. CMA07 and CMT-U27 cell lines showed no differences in cell morphology for both cell lines with the different oseltamivir doses, when compared to non-treated cells (Figure 1).

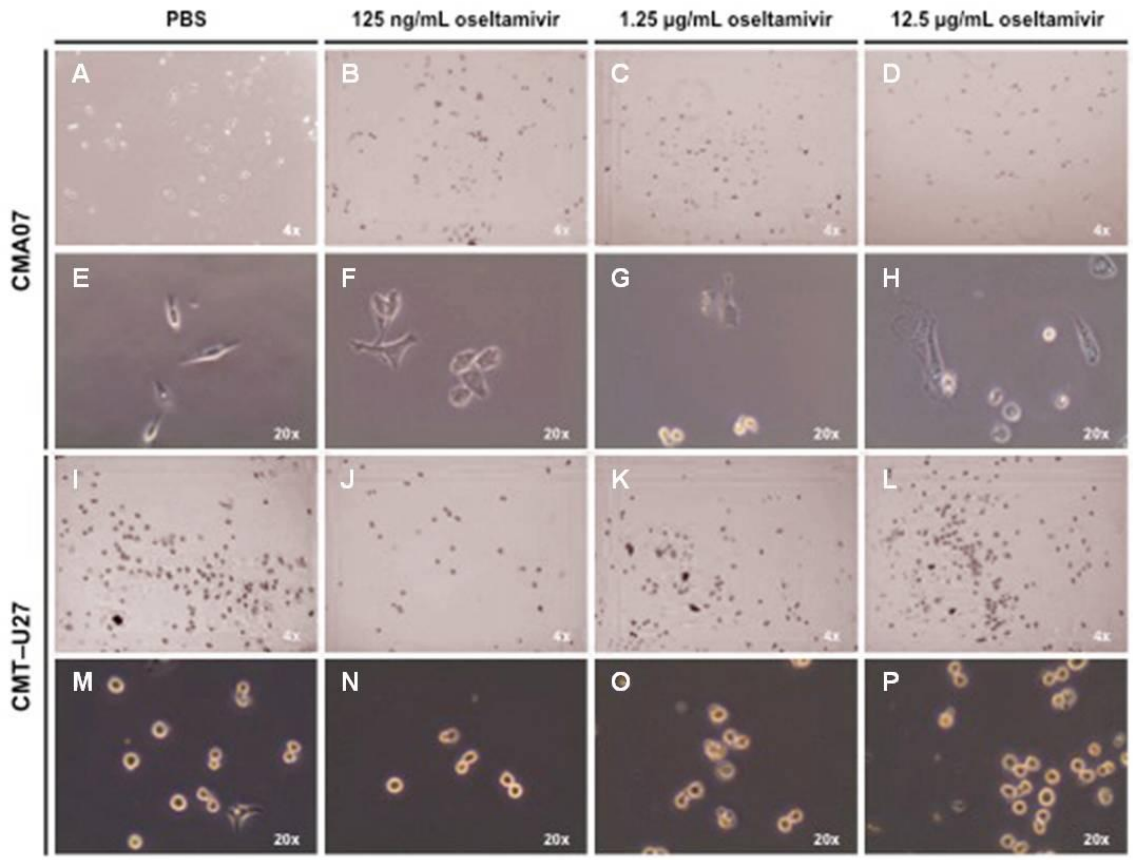


Figure 1. Evaluation of CMA07 and CMT-U27 cell morphology upon oseltamivir treatment. Morphology of cells was evaluated after oseltamivir addition. Compared with control cells (**A**, **I** respectively, x4 magnification), no differences in cell clustering were observed in both CMA 07 (**B** – **D**, x4 magnification) and CMT- U27 cells (**J** – **L**, x4 magnification) in the different doses of oseltamivir. Compared with non-treated cells (**E**, **M** x20 magnification), no differences were observed in both CMA07 (**F**-**H**, x20 magnification) and CMT-U27 cells (**N**-**P**, x20 magnification) in the different doses of oseltamivir.

In day 3, CMA07 cells treated with 1.25 and 12.5 µg/mL oseltamivir presented less cell clustering, compared with non-treated and 125 ng/mL oseltamivir treated cells. Concerning CMT-U27 cells treated with 12.5 ng/mL oseltamivir, less cell clustering was observed, compared with non-treated, 125 ng/mL oseltamivir, and 1.25 ng/mL oseltamivir treated cells.

CMA07 cells showed no alterations concerning cell morphology in the different oseltamivir doses, when compared to non-treated cells. However, CMT-U27 cells treated with 12.5 µg/mL oseltamivir present larger and irregular cytoplasm and protrusions, when compared to non-treated cells (Figure 2).

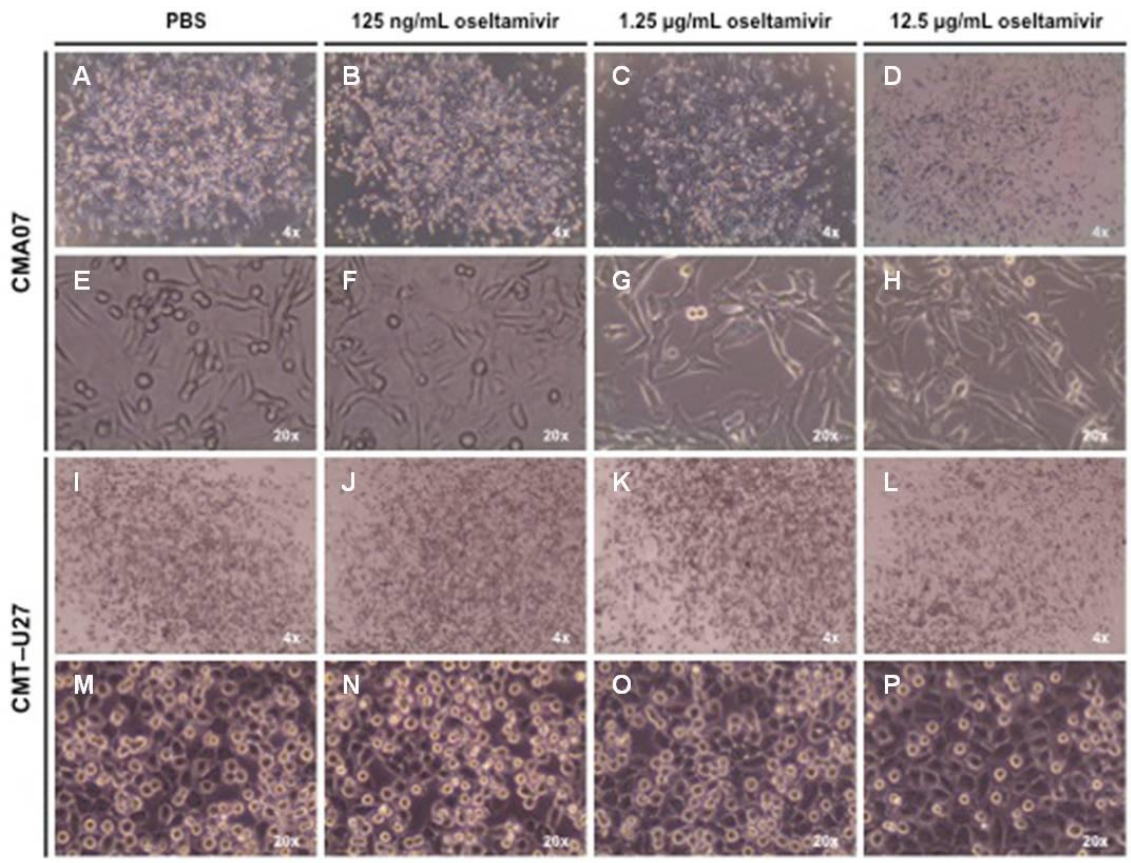


Figure 2. Evaluation of CMA07 and CMT-U27 cell morphology three days after oseltamivir treatment. After three days of oseltamivir treatment, cell morphology was evaluated. Compared with non-treated and 125 ng/mL oseltamivir treated cells (**A**, **B**, x4 magnification),CMA07 cells treated with 1.25 and 12.5 µg/mL oseltamivir (**C**, **D**, x4 magnification) presented less cell clustering. Compared with non-treated, 125 ng/mL oseltamivir, and 1.25 ng/mL oseltamivir treated cells (**I** - **K**, x4 magnification), CMT-U27 cells treated with 12.5 ng/mL oseltamivir (**L**, x4 magnification) showed less cell clustering. Compared with non-treated cells (**E**, x20 magnification), CMA07 cells treated with oseltamivir (**F** - **H**, x20 magnification) showed larger and irregular cytoplasm, and protrusions. Compared with non-treated cells (**M**, x20 magnification), CMT-U27 cells treated with 12.5 µg/mL oseltamivir (**P**, x20 magnification) showed larger and irregular cytoplasm, and protrusions.

In day 7, no differences in cell clustering were observed in both CMA 07 and CMT-U27 cells in the different doses of oseltamivir, when compared with control cells. Differences in CMA07 cell morphology were observed in both 125 ng/mL and 12.5 µg/mL oseltamivir doses, presenting more round and shiny cells, when compared to non-treated cells. Concerning CMT-U27 cell line, no differences were observed in cell morphology in the different oseltamivir doses, when compared with non-treated cells (Figure 3).

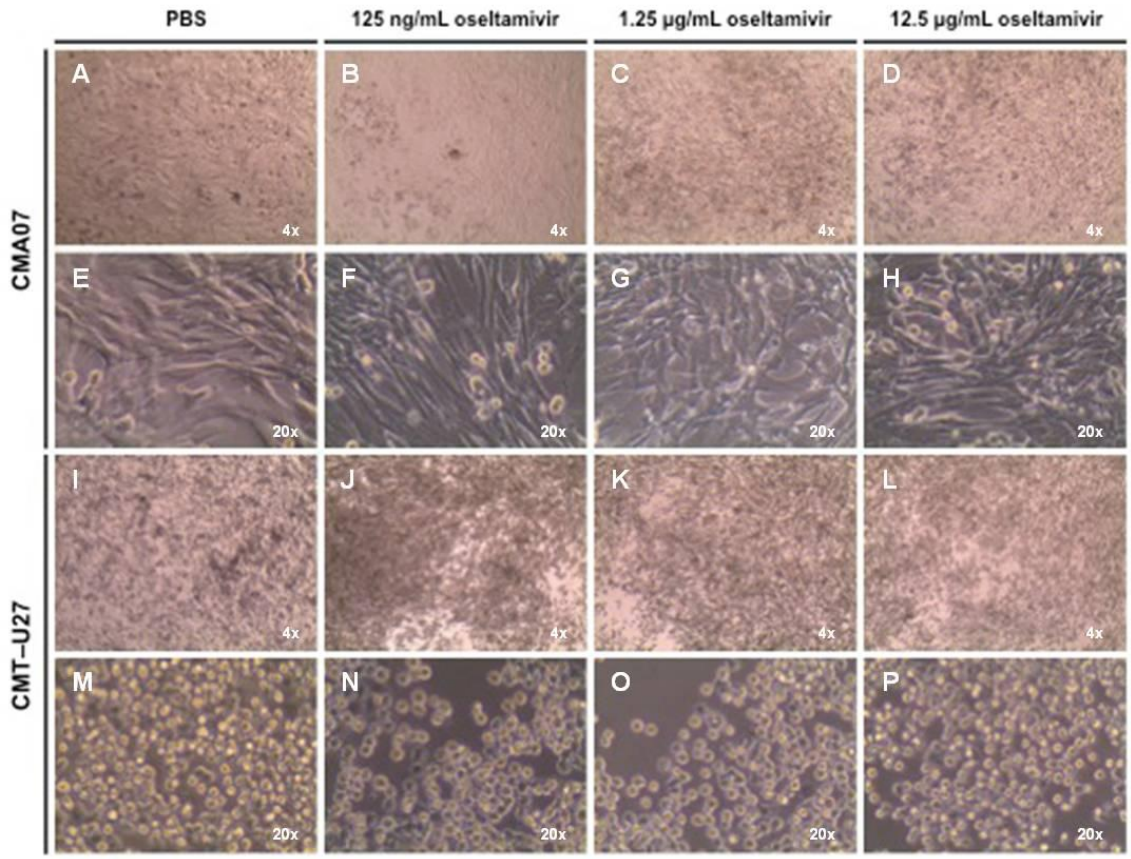


Figure 3. Evaluation of CMA07 and CMT-U27 cell morphology seven days after oseltamivir treatment. Compared with control cells (**A** and **I** respectively, x4 magnification), no differences in cell clustering were observed in both CMA 07 (**B** – **D**, x4 magnification) and CMT- U27 cells (**J** – **L**, x4 magnification) in the different doses of oseltamivir. After seven days of oseltamivir treatment, compared to non-treated cells (**E**, x20 magnification), differences in CMA07 cell morphology where observed in both 125 ng/mL (**F**, x20 magnification) and 12.5 µg/mL oseltamivir treatment (**H**, x20 magnification), presenting more round and shiny cells. Compared with non-treated cells (**M**, x20 magnification), CMT-U27 cells treated with treated with oseltamivir (**N**-**P**, x20 magnification) showed no differences in cell morphology.

Cell proliferation assay in CMT cell lines in the absence and presence of oseltamivir

In this study, cell proliferation assay was performed both with CMA07 and CMT-U27 cell lines, using oseltamivir in order to determine if cell proliferation is affected by its presence. Oseltamivir was tested in different doses (125 ng/mL, 1.25 µg/mL, 12.5 µg/ml and 125 µg/mL oseltamivir) and PBS was added to the non-treated cells group. Cell growth was monitored during 7 days by counting cells every day (Figure 4).

Proliferation rates of CMA07 and CMT-U27 cell lines were not significantly affected by treatment with different oseltamivir doses, when compared to the proliferation curve of non-treated cells. However, an exception was observed for the highest dose tested, 125 µg/mL of oseltamivir, which seemed to impair CMA07 cell growth, though with no statistical significance was found when compared to non-treated cells.

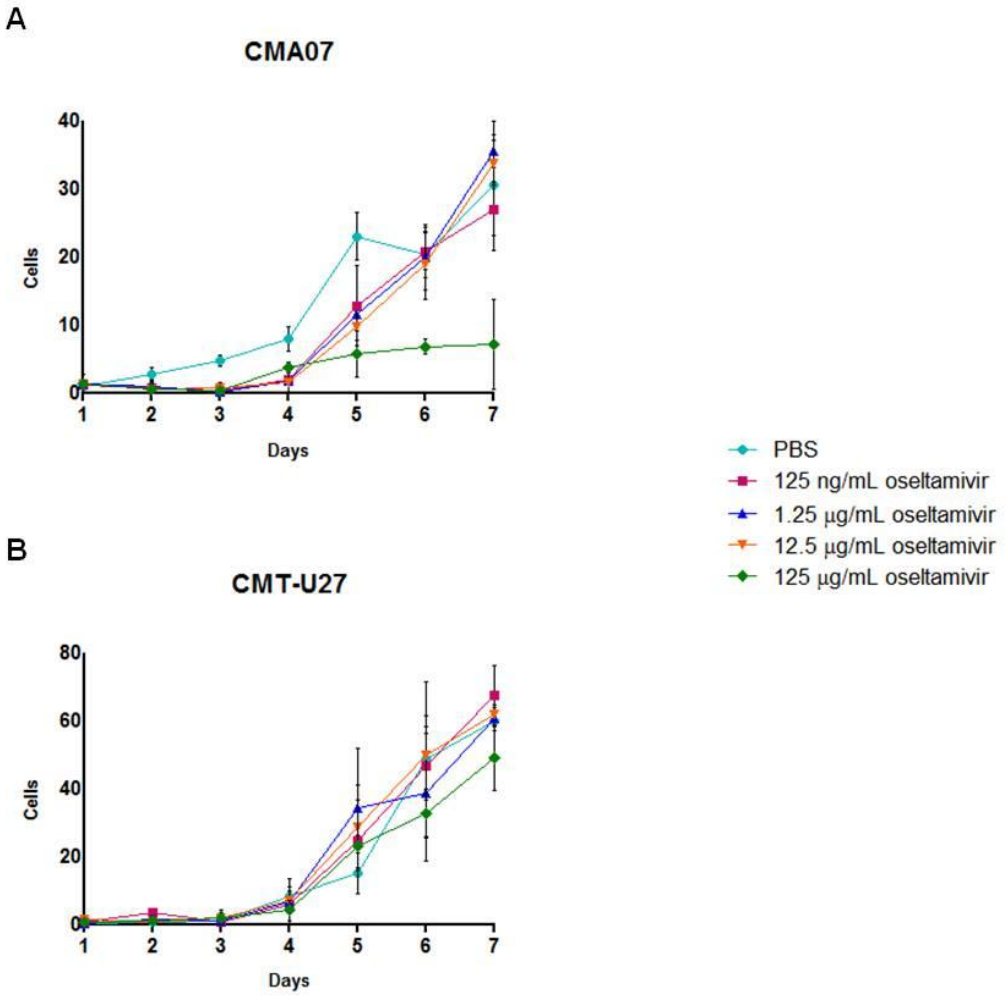


Figure 4. *In vitro* proliferation analysis of CMA07 and CMT-U27 cell lines upon oseltamivir treatment. Cells were cultured in 24-well dishes in the presence of different oseltamivir doses, and cell growth was monitored during 7 days by counting cells every single day. CMA07 and CMT-U27 cell lines in the presence of

different doses of oseltamivir did not present significantly different proliferation rates when compared with non-treated cells. The highest dose administered, 125 µg/mL oseltamivir, impairs CMA07 cell growth (although with no statistical significant difference, *ns p* = 0.3492).

Cell viability assay of CMT cell lines in the absence and presence of oseltamivir

Cell viability assay performed with the CellTiter 96® AQ_{ueous} One Solution reagent (Promega Corporation, U. S. A.) is a colorimetric method for determining the number of viable cells in proliferation or cytotoxicity assays. The MTS tetrazolium compound in the reagent is bioreduced by cells into a colored formazan product and analysed after recording the absorbance at 490nm. The quantity of formazan product as measured by the absorbance at 490nm is directly proportional to the number of living cells in culture.

With the purpose of evaluate the effect of oseltamivir treatment in both CMA07 and CMT-U27 cells' viability, CellTiter 96® AQueous One Solution Cell Proliferation Assay was performed in both CMA 07 and CMT- U27 cell lines, during 48 hours, with time-points at 0, 2, 4, 6, 8, 10, 12, 24 and 48 hours (Figure 5).

For CMA07 and CMT-U27 cell lines treated with 125 ng/mL, 1.25 µg/mL and 12.5 µg/mL oseltamivir doses, no significant differences (*ns* $p>0.05$) were observed in cells' viability, when compared to non-treated cells. However, the highest tested dose, 125 µg/ml oseltamivir, severely impaired cell viability both in CMA07, ***$p=0.0005$, and CMT-U27, ****$p<0.0001$, cell lines, since the number of viable cells decreased over time. For this reason, this dose was excluded from the further studies.

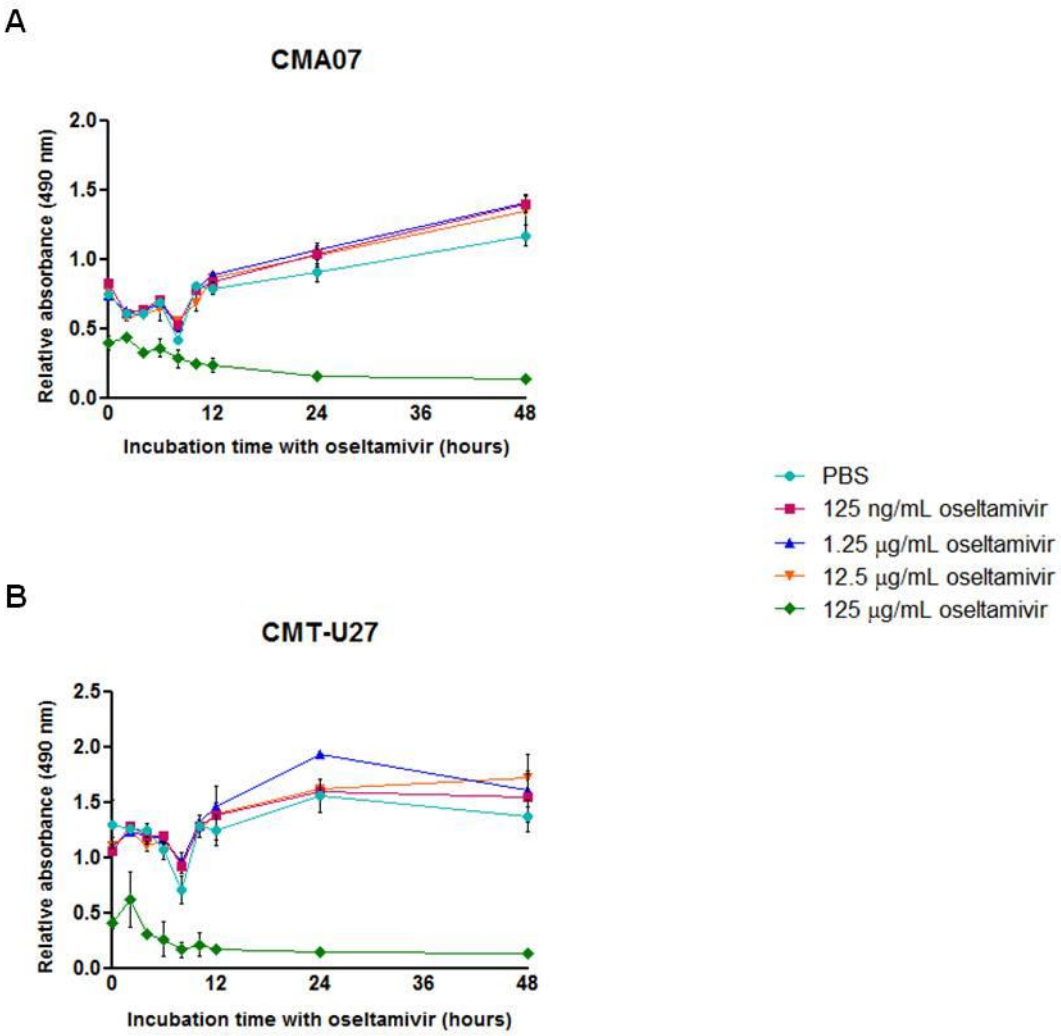


Figure 5. Evaluation of CMA07 and CMT-U27 cell viability upon oseltamivir treatment. Cells were cultured in 96-well plates in the presence of the different oseltamivir doses, and cell viability was monitored in a time course manner during 48 hours. Cell viability from CMA07 and CMT-U27 cell lines was significantly affected by treatment with 125 µg/mL oseltamivir dose when compared to non-treated cells, which severely impairs CMA07 (***p=0.0005) and CMT-U27 (****p<0.0001) cell growth.

CMT cell lines migration capacity evaluation by wound-healing in the absence and presence of oseltamivir

Wound-healing assay is a simple and inexpensive method that mimics cell migration to close an artificial wound. In order to determine if oseltamivir treated cells from both CMA07 and CMT-U27 cell lines showed alterations in their migration rate, when compared to non-treated cells and between different oseltamivir doses, it was created an artificial "wound" in a cell monolayer. Results obtained by *time-lapse* acquisition allowed observing the cell migration of CMA07 and CMT-U27 cell lines during wound healing for 40h. Oseltamivir was tested in different doses (125 ng/ml, 1.25 µg/ml, 12.5 µg/ml) and PBS was added to the non-treated cells group.

Oseltamivir treated cells from both CMA07 and CMT-U27 cell lines showed evident alterations in their migration rates, when compared to non-treated cells and between doses. Regarding CMA07 cell line (Figure 6), it was observed that cells treated with 125ng/mL and 1.25 µg/mL oseltamivir migrated in order to close the wound in a larger extent and moved faster, when compared to non-treated cells and cells treated with 12.5 µg/mL oseltamivir. Concerning CMT-U27 cell line (Figure 7), it was also observed that cells migrated to close the wound in a dose-dependent manner.

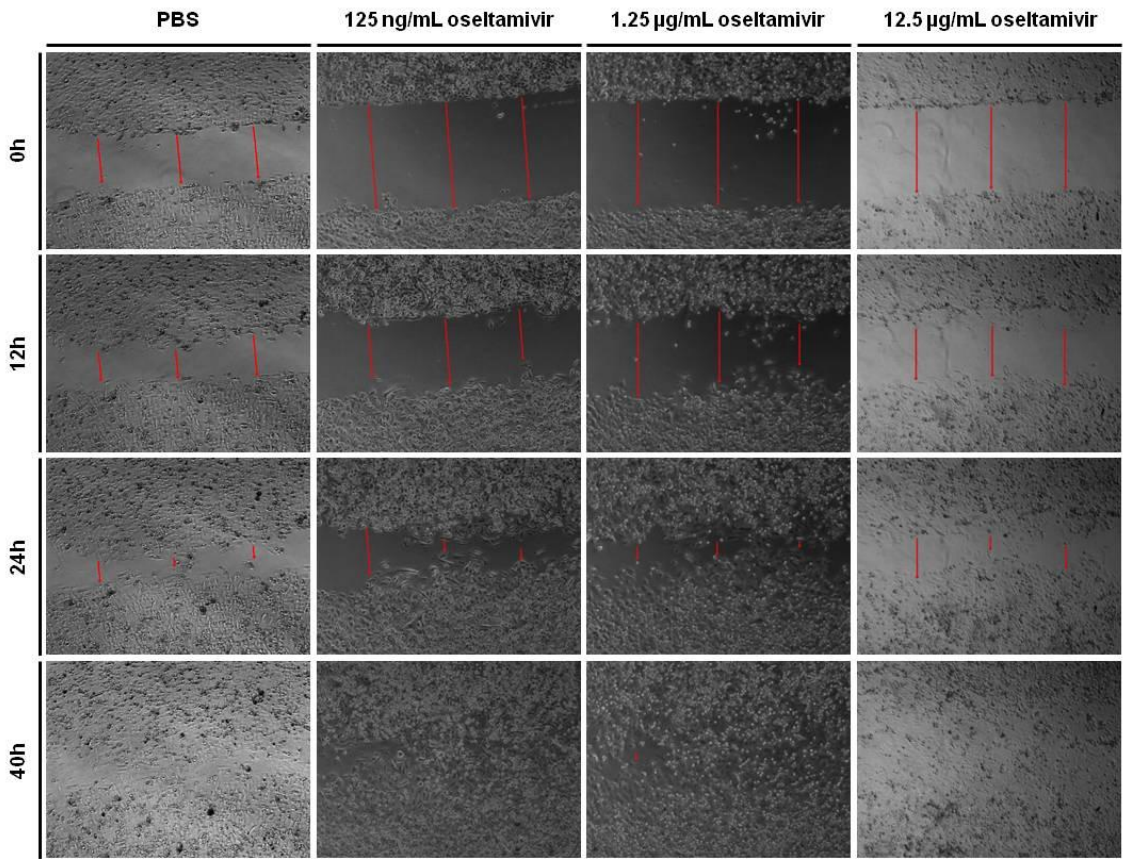


Figure 6. Wound-healing assay in CMA07 cells treated with oseltamivir. Results obtained by *time-lapse* acquisition allowed the observation of cell migration of CMA07 cell line during *wound* healing for 40 hours. Cell migration was evaluated in each oseltamivir dose tested, and alterations were observed in the migration rate when compared to non-treated cells. CMA07 cells treated with 125ng/mL and 1.25 µg/mL oseltamivir migrated in order to close the wound in a larger extent and moved faster when compared to non-treated cells and cells treated with 12.5 µg/mL oseltamivir.

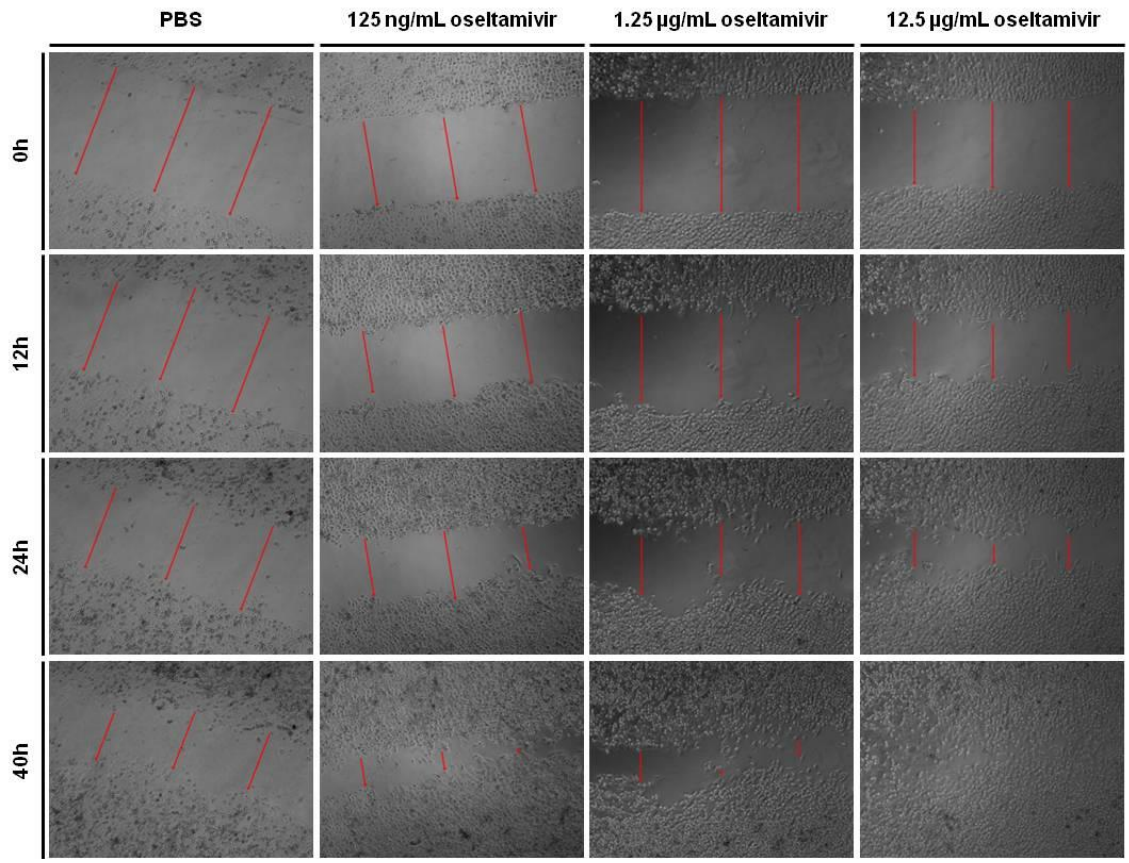


Figure 7. Wound-healing assay in CMT-U27 cells treated with oseltamivir. Results obtained by *time-lapse* acquisition allowed the observation of cell migration of CMT-U27 cell line during wound-healing for 40 hours. Cell migration was evaluated in each oseltamivir dose tested, and alterations in the migration rate were observed when compared to non-treated cells. It was observed that cells migrated to close the wound in a dose-dependent manner.

Assessment of invasion capacity of CMT-U27 cell line in the absence and presence of oseltamivir

The BD BioCoat™ Matrigel™ Invasion Chamber (BD Biosciences, U.S.A.) is useful to evaluate the capacity of malignant and normal cells to invade basement membranes. In addition, it also can be used to study the effect of specific drugs or altered protein expression on the invasive capacity of cells *in vitro*. The matrigel matrix works as a reconstituted basement membrane *in vitro*. The layer occludes the pores of the membrane, blocking non-invasive cells from migrating through the membrane. In contrast, invasive cells are able to detach and invade through the matrigel matrix and the 8 micron membrane pores.

CMT-U27 cell line is described to be a highly metastatic canine mammary tumour cell line (Hellmen 1992). Thus, the Matrigel invasion assay was performed using this cell line. CMT-U27 cell invasion capacity was evaluated by counting the number of invasive cells on Matrigel invasion chambers in the presence and absence of oseltamivir treatment (Figure 8).

Cells treated with 125 ng/mL oseltamivir showed no significant differences in the invasion capacity when compared with non-treated cells (*ns* $p<0.05$). A significantly increase in cell invasion was observed in cells treated both with 1.25 µg/mL and 12.5 µg/mL oseltamivir, compared to control cells (* $p<0.05$ 1.25 µg/mL oseltamivir treated cells versus control cells; ** $p<0.01$ 12.5 µg/mL oseltamivir treated cells versus control cells). Furthermore, significant differences in cell invasion capacity were also observed between different oseltamivir doses tested (**$p<0.01$ 125 ng/mL oseltamivir *versus* 12.5 µg/mL oseltamivir; *$p<0.05$ 1.25 µg/mL oseltamivir *versus* 12.5 µg/mL oseltamivir).

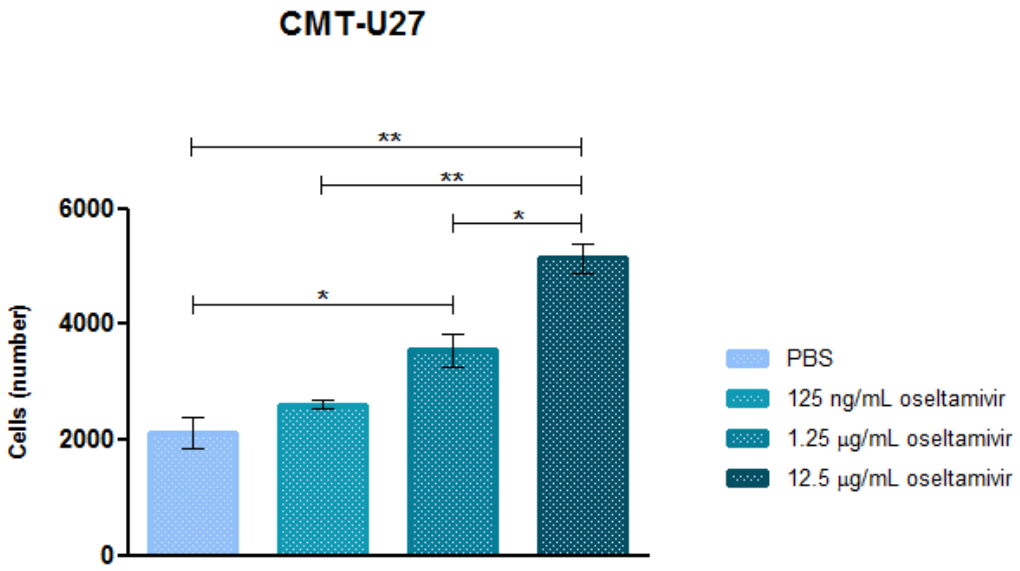


Figure 8. *In vitro* evaluation of CMTU-27 invasive capacity upon oseltamivir treatment. Cell invasion was assessed on Matrigel chambers through 6 hours of oseltamivir treatment. Cells treated with 125 ng/mL oseltamivir show no significant differences in invasion capacity when compared to with non-treated cells (*ns* $p>0.005$). Cells with 1.25 µg/mL oseltamivir treatment present a significant increase in their invasive capacity when compared to non-treated cells (*$p<0.05$), as well as cells with 12.5 µg/mL oseltamivir treatment (**$p<0.01$). Differences in cell invasion capacity was also observed between the different oseltamivir treatments, indicating a dose-dependent invasion capacity (*$p<0.05$ 1.25 µg/mL oseltamivir *versus* 12.5 µg/mL oseltamivir, and **$p<0.01$ 125 ng/mL oseltamivir *versus* 12.5 µg/mL oseltamivir).

Evaluation of terminal α2-6 and 2-3 sialic acid structures expression in CMT cell lines in the absence and presence of oseltamivir

In order to evaluate the effect of oseltamivir treatment in the glycosylation pattern of CMA07 and CMT-U27 cell lines, namely, alterations in terminal α2-3 and α2-6 sialic acid structures expression, fluorescence analysis were performed with SNA (*Sambucus nigra* agglutinin), MAL I (*Maackia amurensis* leukoagglutinin) and MAL II (*Maackia amurensis* hemagglutinin) plant lectins.

Terminal α2-6 sialic acid structures expression in CMA07 and CMT U27 cell lines was assessed using the SNA plant lectin (Figure 9). CMA07 cells treated with oseltamivir showed an increased expression in terminal α2-6 sialic acids structures, when compared to non-treated cells. CMT-U27 cells treated with different doses of oseltamivir did not present an increased in the percentage of cells expressing α2-6 sialic acids structures, however cells presented a much more intense pattern of expression is displayed when compared to non-treated cells.

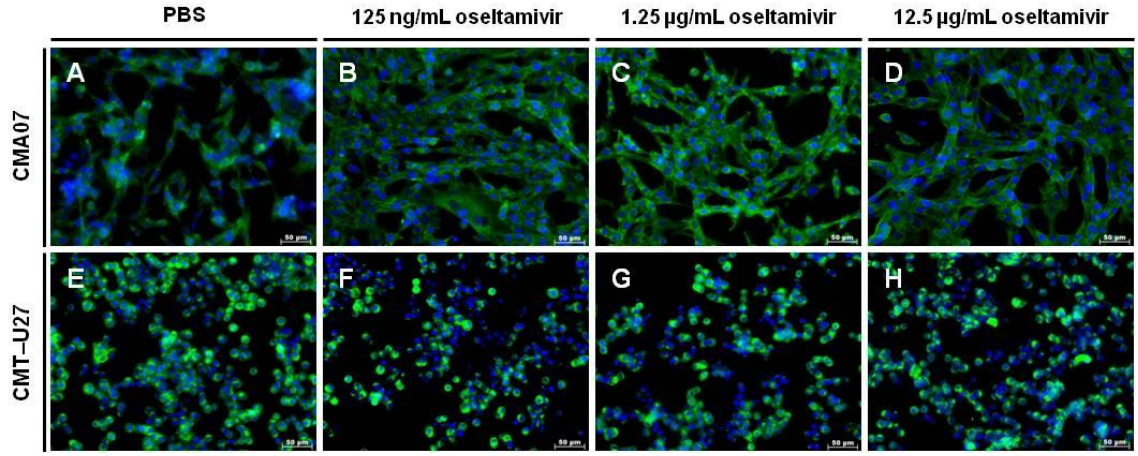


Figure 9. Expression of terminal α2-6 sialic acids structures in CMA07 and CMT-U27 cell lines upon oseltamivir treatment, evaluated by SNA lectin labelling. Compared to non-treated cells (**A**, x20 magnification), CMA07 cells treated with oseltamivir (**B**-**D**, x20 magnification), show an increased expression in terminal α2-6 sialic acids structures. Compared to non-treated cells (**E**, x20 magnification), CMT-U27 cells treated with different doses of oseltamivir (**F**-**H**, x20 magnification) did not present an increased in the percentage of cells expressing α2-6 sialic acids structures, however cells displays a much more intense pattern of expression.

Regarding Siaα2-3Galβ1-4GlcNAc terminal structures, their expression in CMA07 and CMT-U27 cell lines was assessed by MAL I lectin labelling (Figure 10). CMA07 cells treated with 12.5 µg/mL oseltamivir showed an increased in Siaα2-3Galβ1-4GlcNAc structures, when compared to non-treated cells. CMT-U27 cells treated with oseltamivir in different doses, showed an increased in Siaα2-3Galβ1-4GlcNAc structures when compared to non-treated cells.

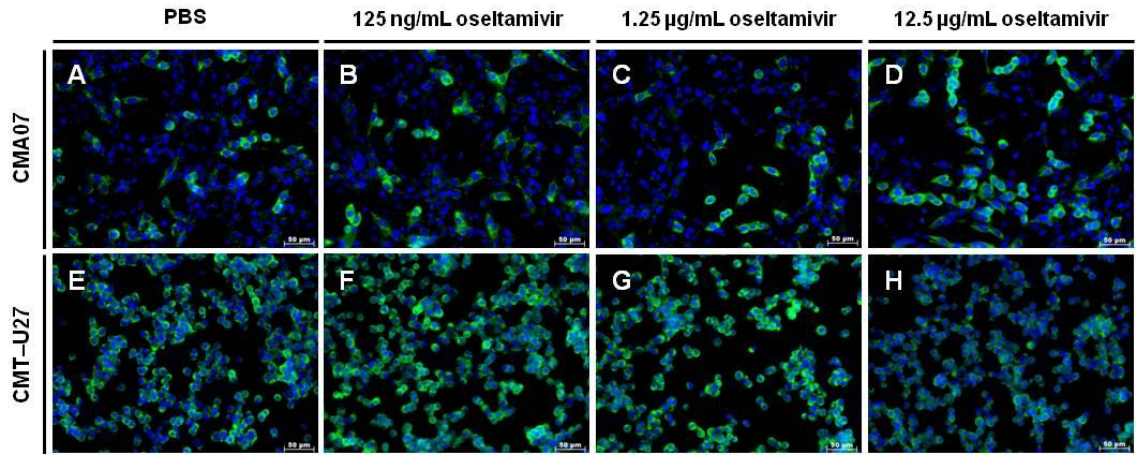


Figure 10. Expression of Siaα2-3Galβ1-4GlcNAc terminal structures in CMA07 and CMT-U27 cell lines upon oseltamivir treatment, assessed by MAL I lectin labelling. Compared to non-treated cells (**A**, x20 magnification), CMA07 cells treated with 12.5 µg/mL oseltamivir (**D**, x20 magnification) show an increased in Siaα2-3Galβ1-4GlcNAc structures. Compared to non-treated cells (**E**, x20 magnification), CMT-U27 cells treated with oseltamivir in different doses (**F**-**H**, x20 magnification), show an increased in Siaα2-3Galβ1-4GlcNAc structures.

Concerning Siaα2-3Galβ1-3GlcNAc terminal structures, expression in CMA07 and CMT-U27 cell lines was accessed by MAL II lectin labelling (Figure 11). CMA07 cells treated with different doses of oseltamivir did not present alteration in Siaα2-3Galβ1-3GlcNAc expression, when compared to non-treated cells. CMT-U27 cells treated with 1.25 µg/mL oseltamivir showed increased expression of Siaα2-3Galβ1-3GlcNAc, when compared to non-treated cells.

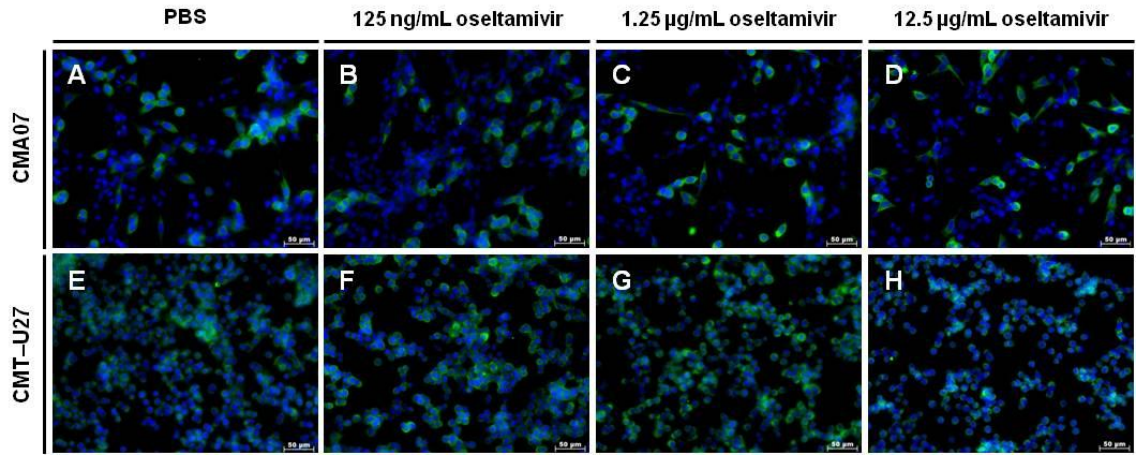


Figure 11. Expression of Siaα2-3Galβ1-3GlcNAc terminal structures in CMA07 and CMT-U27 cell lines upon oseltamivir treatment, assessed by MAL II lectin labelling. Compared to non-treated cells (**A**, x20 magnification), CMA07 cells treated with different doses of oseltamivir (**B**-**D**, x20 magnification) do not present alteration in Siaα2-3Galβ1-3GlcNAc expression. Compared to non-treated cells (**E**, x20 magnification), CMT-U27 cells treated with 1.25 µg/mL oseltamivir (**F**, x20 magnification) show increased expression of Siaα2-3Galβ1-3GlcNAc.

Evaluation of galectin-3-ligands expression in CMT cell lines in the absence and presence of oseltamivir

Fluorescence assay was performed in order to evaluate galectin-3-ligands expression in CMA07 and CMT-U27 cell lines treated with oseltamivir, when compared to non-treated cells (Figure 12).

CMA07 cells treated with oseltamivir showed no differences in galectins-3-ligands expression, when compared with control cells.

Treatment of CMT-U27 cells with 1.25 µg/mL and 12.5 µg/mL oseltamivir resulted in a decreased expression of galectin-3-ligands when compared to non-treated cells.

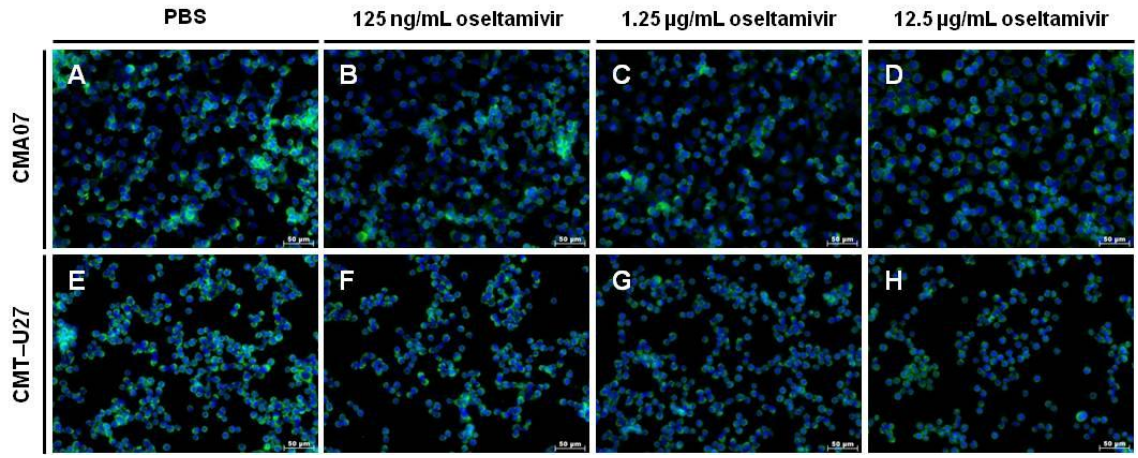


Figure 12. Evaluation of galectin-3 ligands expression in both CMA07 and CMT-U27 cell lines upon oseltamivir treatment. Compared to non-treated cells (**A**, x20 magnification), treatments of CMA07 cells (**B**-**D**, x20 magnification) showed no differences in galectin-3-ligands expression. Compared to non-treated cells (**E**, x20 magnification), treatment of CMT-U27 cells with 1.25 µg/mL and 12.5 µg/mL oseltamivir (**G**,**H**, x20 magnification), show a decreased in expression of galectin-3 ligands.

Evaluation of galectin-3 expression in CMT cell lines in the absence and presence of oseltamivir

Immunofluorescence assay was performed in order to evaluate galectin-3 expression in CMA07 and CMT-U27 cell lines upon oseltamivir treatment, when compared to non-treated cells (Figure 13).

Results showed that galectin-3 is increased in both CMA07 and CMT-U27 cells upon treatment with 1.25 µg/mL and 12.5 µg/mL oseltamivir, when compared to both non-treated cells and to cells treated with 125 ng/mL oseltamivir. Moreover, differences in galectin-3 expression were also observed between the two cell lines, with more intense galectin-3 expression pattern in CMT-U27, when compared with CMA07.

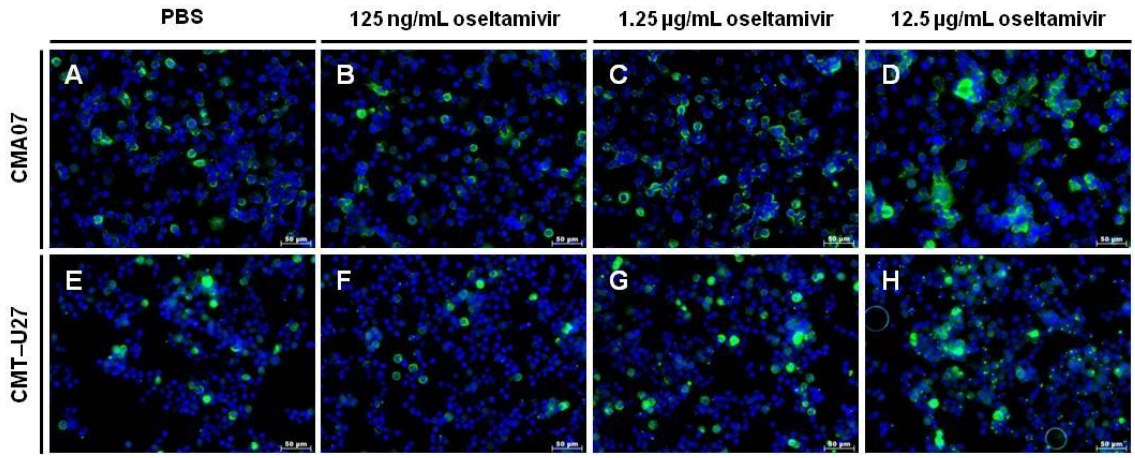


Figure 13. Expression of galectin-3 in CMA07 and CMT-U27 cell lines upon oseltamivir treatment. Compared to both non-treated cells (**A**,**E**, x20 magnification) and to cells treated with 125ng/mL of oseltamivir (**E**,**F**, x20 magnification), there is increased expression of galectin-3 in both CMA07 (**C**-**D**, x20 magnification) and CMT-U27 cells (**G**-**H**, x20 magnification) upon treatment with 1.25 µg/mL and 12.5 µg/mL oseltamivir. Furthermore, galectin-3 expression pattern in CMT-U27 is more intense when compared with CMA07.

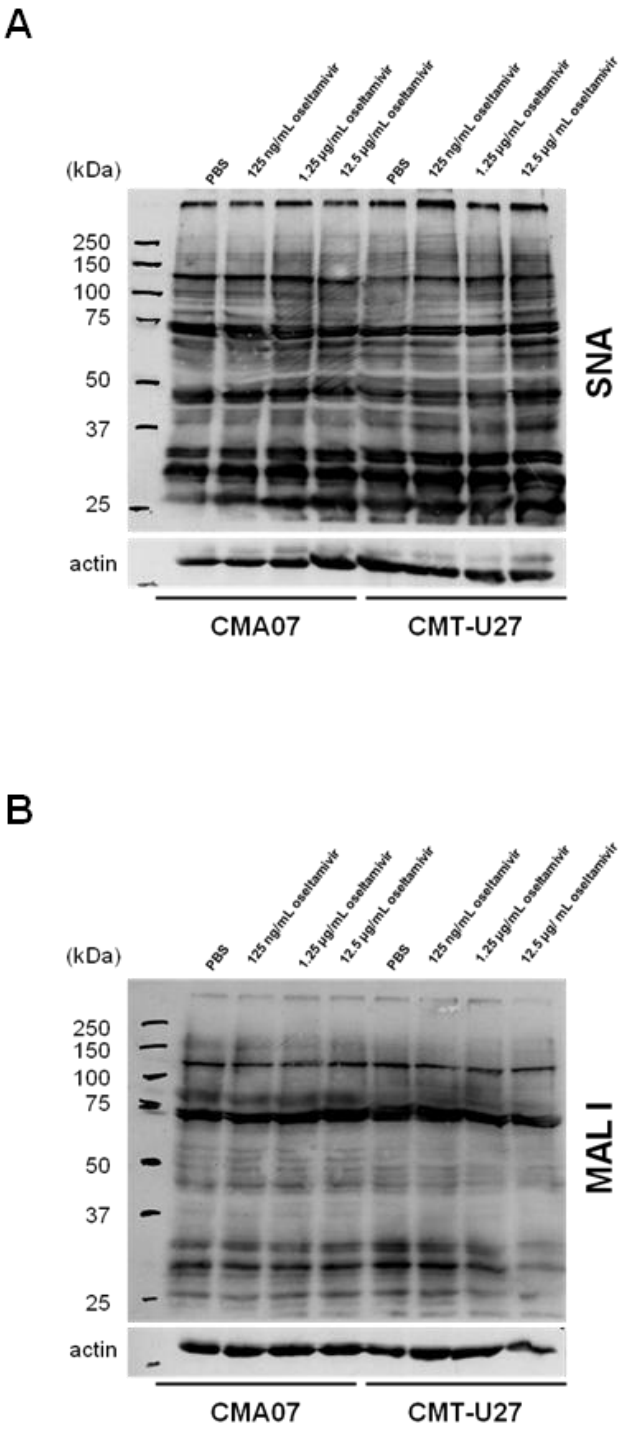
Evaluation of terminal α2-6 and 2-3 sialic acid structures expression in proteins from total cell lysates of CMT cell lines in the absence and presence of oseltamivir

Expression of terminal α2-6 and α2-3 sialylated structures were evaluated by lectin blot analysis with SNA, MAL I and MAL II lectins. The results demonstrated an increased expression of sialylated proteins upon oseltamivir treatment (Figure 14).

Both CMA07 and CMT-U27 cells presented differences in the expression of terminal α2-6 and α2-3 sialic acid structures in some proteins upon oseltamivir treatment. These results demonstrated the effect of oseltamivir in sialidase inhibition, and consequent inhibition of sialic acid cleavage, since it was observed an increased α2-6 and α2-3 sialic acid terminal structures in cells treated with oseltamivir.

The results from SNA lectin blot demonstrated an increase in the expression of terminal α2,6 sialic acid structures in proteins from CMT-U27 cells treated with oseltamivir (125 ng/mL, 1.25 µg/mL and 12.5 µg/mL oseltamivir) when compared to proteins from non-treated cells (PBS), especially in a molecular weight around 120 kDa. Regarding MAL I lectin blot, no differences were observed in α2,3 sialic acid structures in proteins from both CMA07 and CMT-U27 cells treated with oseltamivir (125 ng/mL, 1.25 µg/mL and 12.5 µg/mL oseltamivir), when compared to proteins from non-treated cells (PBS). The

results from MAL II lectin blot demonstrated an increase in terminal α2,3 sialic acid structures expression in proteins from lysates of cells treated with 125 ng/mL, 1.25 µg/mL oseltamivir, in a molecular weight around 120 kDa, when compared to proteins from non-treated cells (PBS) and cells treated with 12.5 µg/mL oseltamivir.



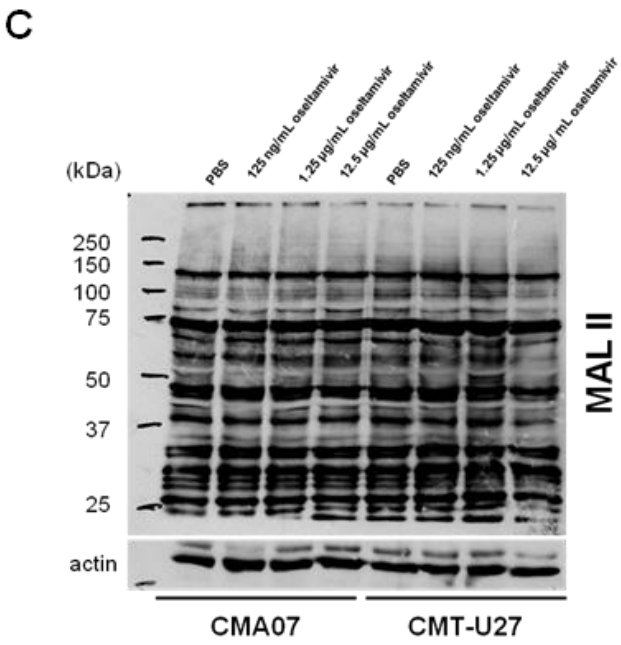


Figure 14. Western analysis of SNA, MAL I and MAL II lectins in proteins from total cell lysates of both CMA07 and CMT-U27 cell lines upon oseltamivir treatment. Expression of terminal sialylated structures were evaluated by western blot analysis with SNA (**A**), MAL I (**B**) and MAL II (**C**) plant lectins. The results from SNA lectin blot (**A**) demonstrated an increase in the expression of terminal α2,6 sialic acid structures in proteins from CMT-U27 cells treated with oseltamivir (125 ng/mL, 1.25 µg/mL and 12.5 µg/mL oseltamivir) when compared to proteins from non-treated cells (PBS), especially in a molecular weight around 120 kDa. Regarding MAL I lectin blot (**B**), no differences were observed in α2,3 sialic acid structures in proteins from both CMA07 and CMT-U27 cells treated with oseltamivir (125 ng/mL, 1.25 µg/mL and 12.5 µg/mL oseltamivir), when compared to proteins from non-treated cells (PBS). The results from MAL II lectin blot (**C**) CMT-U27 cells demonstrated an increase in terminal α2,3 sialic acid structures expression in proteins from lysates of cells treated with 125 ng/mL, 1.25 µg/mL oseltamivir, in a molecular weight of about 120 kDa, when compared to proteins from lysate correspondent to non-treated cells (PBS) and cells treated with 12.5 µg/mL oseltamivir.

Evaluation of galectin-3-ligands expression in proteins from total cell lysates of CMT cell lines in the absence and presence of oseltamivir

Proteins from CMA07 and CMT-U27 cell lines treated with oseltamivir were labelled with biotinylated galectin-3 in order to evaluate galectin-3-ligands expression in proteins from total cell lysates of both cell lines (Figure 15).

Regarding galectin-3-ligands expression in proteins from CMA07 cell line treated with oseltamivir, no differences were observed for when compared to proteins from non-treated cells (PBS). Concerning galectin-3-ligands expression in proteins from CMT-U27 cell line treated with oseltamivir, we observed a decrease in galectin-3 ligands in proteins when compared to proteins from non-treated cells, especially at the molecular weight around 120 kDa.

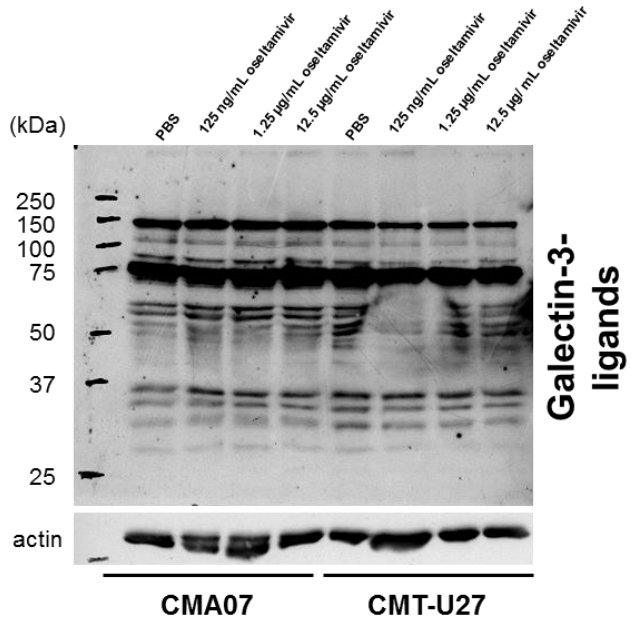


Figure 15. Western blot analysis of galectin-3-ligands in proteins from total cell lysates of both CMA07 and CMT-U27, upon oseltamivir treatment. Expression of galectin-3-ligands was evaluated by western blot analysis with a biotinylated galectin-3. Regarding galectin-3-ligands expression in proteins from CMA07 cell line treated with oseltamivir, no differences were observed for when compared to proteins from non-treated cells (PBS). Concerning galectin-3-ligands expression in proteins from CMT-U27 cell line treated with oseltamivir, we observed a decrease in galectin-3 ligands in proteins when compared to proteins from non-treated cells, principally at the molecular weight around 120 kDa.

Evaluation of galectin-3 expression in proteins from total cell lysates of CMT cell lines in the absence and presence of oseltamivir

CMA07 cells treated with oseltamivir showed no differences in galectin-3 expression, when compared to non-treated cells (Figure 16). CMT-U27 cells treated with 1.25 µg/mL and 12.5 µg/mL oseltamivir treatments, showed increased galectin-3 expression, when compared to non-treated cells. These results are in accordance with those observed for galectin-3 immunofluorescence.

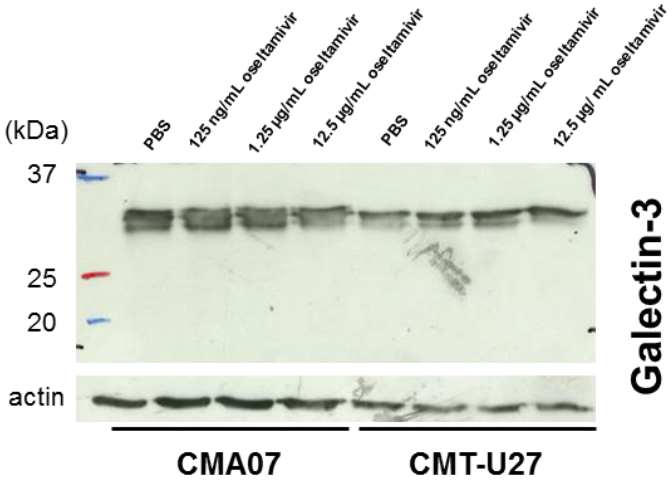


Figure 16. Western blot analysis of galectin-3 in proteins from total cell lysates of both CMA07 and CMT-U27, upon oseltamivir treatment. CMA07 cells treated with oseltamivir, showed no differences in galectin-3 expression, when compared to non-treated cells. CMT-U27 cells treated with 1.25 µg/mL and 12.5 µg/mL oseltamivir treatments, showed increased galectin-3 expression, when compared to non-treated cells.

DISCUSSION

Discussion

Canine mammary tumours (CMT) affect female dogs with higher incidence between 10 and 11 years of age (Johnston, 1993), being the second most common type of canine neoplasia, 25-50% of all diagnosed cases (Moulton, 1990). CMT present many similarities with human breast cancer, namely, histological appearance, tumour genetics, molecular targets, biological behaviour and response to conventional therapies (Paoloni *et al.,* 2008). However, CMT show a higher incidence compared to humans, about 3 folds, and a shorter disease time course of approximately 2 years (Sorenmo 2003). Regarding breast cancer, it is one of the most prevalent cancers worldwide, being the leading cause of cancer-related death in women (Ferlay *et al.,* 2010). Hence, the study of CMT as an animal model of human breast cancer could greatly improve our knowledge about the metastatic process in humans and bring mutual advantages for cancer treatment (Paoloni *et al.,* 2008).

Glycoconjugates present in the cell surface play important roles in a variety of biological functions, and alterations in glycosylation are considered a hallmark during carcinogenesis. These alterations lead to the expression of tumour-associated carbohydrate antigens (TACA) on glycoproteins and glycolipids which decorate the cell surfaces (Reis *et al.,* 2010). T antigen, its sialylated form sT, Tn antigen and sTn, and sialyl Lewis antigens: sLe^x and sLe^a are among these TACA (Baldus *et al.,* 2004); (Cazet *et al.,* 2010). Geseneral sialylation increaare commonly found in glycans of carcinoma cells, being associated with tumour grade, invasion, metastasis and poor prognosis of patients (Cazet *et al.,* 2010). Removal of α-glycosidically linked sialic acid residues form carbohydrate groups of glycoproteins and glycolipids is catalized by sialidases. This event affects the conformation of glycoproteins (Yogeeswaran *et al.,* 1981).

In the present work we took advantage of a well-known sialidase inhibitor, oseltamivir, to assess its effect both on the biological behaviour and glycosylation pattern of canine mammary tumour cells.

Breast cancer cells express shorter *O*-glycans due to blocking of further extension by premature sialylation (Cazet *et al.,* 2010). Sialidases are glycosidases which catalyse the removal of α-glycosidically linked sialic acid residues from carbohydrate groups of glycoproteins and glycolipids (Miyagi *et al.,* 2012). In order to assess the effect of sialidases inhibition by treatment with oseltamivir in the biological behaviour of CMA07 and CMT-U27 cell lines, we proceeded to several *in vitro* studies. We performed a cell morphology assay during 7 days and it was observed that treatment with oseltamivir affected cell morphology. At day 3, CMT-U27 cells treated with 12.5 µg/mL oseltamivir showed larger and irregular cytoplasm, in contrast with small and round control cells. And

at day 7 these differences were no longer visible. Regarding CMA07 cells, at day 7 cells treated with 125 ng/ml and 12.5 µg/mL oseltamivir depicted a more round morphology, comparing with control cells. Some studies evaluated the effect of sialidases in cancer cell morphology. In accordance with our observations, HEK293 cell line transfected with TLR4 gene when treated with oseltamivir and LPS (TLR4 ligand lipopolysaccharide) depicted a circular morphology compared with controls (Amith *et al.,* 2010). On the other hand, in a sialidase overexpressing cell model, Kato et al. and Sawada et al. did not find any alterations in cell morphology (Kato *et al.,* 2001, Sawada *et al.,* 2002). Furthermore, we evaluated cell proliferation and cell viability both in CMA07 and CMT-U27 cell lines. Cell proliferation and viability were not significantly affected with oseltamivir treatment. This result lead us to assume that 1) alterations in sialic acid content at cell surface by sialidase inhibition with oseltamivir treatment do not affect cell cycle machinery, or 2) oseltamivir treated cells acquire an adaptive capacity, thereby maintaining a normal proliferation rate and viability. However, the highest oseltamivir dose tested was an exception to the general results since it impaired cell proliferation and viability of both cell lines. Nevertheless, it seems that this dose was cytotoxic since we observed more death cells in the culture medium, but without performing further cell death assays, we cannot conclude about its cytotoxicity. Some studies evaluated the influence of sialidases in tumour growth. Kato el al., reported that overexpression of sialidase markedly reduced cell growth of murine melanoma cells both *in vitro* (by MTT assay) and *in vivo* (Kato *et al.,* 2001). In addition, Fluorinated sialic acid analogue (P-3Fax-Neu5Ac), a highly potent inhibitor of sialylation, blocked sialylation in murine melanoma B16F10 cells and reduced their growth *in vivo* (Bull *et al.,* 2013).

The metastatic process is the main cause of tumour-related death (Chambers *et al.,* 2002). Distant metastases occurrence is a complex process dependent on several crucial anti/pro-adhesive steps among which detachment of tumour cells from primary sites, by loss of homotypic and heterotypic adhesion and homotypic aggregation of vessel-invading tumour cells is required for their survival in the blood stream (Orr *et al.,* 2001). Also, increased sialylation has been highly associated with tumour invasion (Cazet *et al.,* 2010); (Gomes *et al.,* 2013). Taking these findings into consideration, we decided to perform *in vitro* wound-healing and Matrigel invasion assays, in order to determine if oseltamivir treatment, and consequent increase in terminal sialic acid structures, would affect the capacity of CMT cells to invade and migrate. Results obtained by *time-lapse* acquisition allowed the observation of cell migration in both cell lines during wound healing for 40 hours. Cell migration was evaluated for each oseltamivir dose tested, and both cell lines showed evident alterations in migration rate, when compared to non-treated cells. Regarding CMA07 cell line, cells treated with 125ng/mL and 1.25 µg/mL oseltamivir

migrated in order to close the wound in a larger extent and moved faster, when compared to non-treated cells and cells treated with 12.5 µg/mL oseltamivir. In addition, in CMT-U27 cell line, it was clearly observed that cells migrated to close the *wound* in a dose-dependent manner. These observations suggest that the increase in terminal sialic acid structures, due to inhibition of sialidases by oseltamivir treatment, promoted increased cell migration, resulting in a higher ability to close the wound. These results are in agreement with some other reports that showed the importance of sialylated structures for cancer cells migration by wound healing experiments. In particularly, migration ability of human pancreatic carcinoma SW1990 cells treated with 1,3,4-O-Bu_3 ManNAc, analog precursor for sialic acid biosynthesis, was evaluated by wound-healing assay and showed increased migration capacity in surface coated with fibronectin and collagen type (Almaraz *et al.,* 2012). In addition, B16F10 cancer cells treated with P-3Fax-Neu5Ac, which blocked sialylation, exhibited diminished migratory capacity (Bull *et al.,* 2013). The results are in accordance with our observations, since the increase in sialylation increased migration capacity of cells. Concerning the results on Matrigel invasion assay, differences were observed in cell invasion capacity of CMT-U27 cells in a dose-dependent manner. The results evidenced the importance of sialic acid contents in cell invasive capacity. The association between sialylation and invasion have been claimed for many years. For instance, the expression of sialyl-Lewis antigens in breast cancer patients as been described as weak in carcinoma in situ, moderate in invasive carcinomas without metastases and high in primary carcinoma with lymph node metastases, showing a good correlation with the metastatic risk (Jeschke *et al.,* 2005). In agreement with our results, invasion capacity of sialidase overexpressing colon carcinoma cells showed an inhibition of invasiveness around 30 to 40% (Sawada *et al.,* 2002). Also, highly metastatic NL17 mouse melanoma cell line overexpressing sialidase showed a marked suppression of pulmonary metastasis (Sawada *et al.,* 1998). In addition, experimental pulmonary metastasis of a mouse colon adenocarcinoma cell line was inhibited by a sialyltransferase inhibitor, KI-8110 (Kijima-Suda *et al.,* 1986); (Kijima-Suda *et al.,* 1988). These results reinforce our observations that invasion capacity is enhanced by increase in sialylation.

Our group has reported that sialylation is a predominant type of glycosylation in CMT (Pinho *et al.,* 2009). Sialyl-Lewis antigens such as sLe^x, sLe^a and sTn are TACA normally found in breast cancer (Narita *et al.,* 1993); (Soares *et al.,* 1996). Normal breast tissue commonly expresses Le^x, mainly in the apical part of cell ducts, together with MUC1 (Croce *et al.,* 2007). On the contrary to unsyalylated structures, sLe^a and sLe^x expression is found to be increased in breast cancer tissues, including primary breast carcinoma lesions (Renkonen *et al.,* 1997) (Julien *et al.,* 2001). Tn antigen can be sialylated resulting in the synthesis of the disaccharide Neu5Acα2-6GalNAc-R, sTn, which

is absent in normal healthy tissues but detected in almost all kinds of carcinomas. In order to verify if inhibition of sialidases by oseltamivir can caused an increase in sialic acid contents and consequently affected cell glycosylation pattern, we performed fluorescence analysis of both CMA07 and CMT-U27 cell lines with plant lectins SNA (*Sambucus nigra* agglutinin), MAL I (*Maackia amurensis* leukoagglutinin) and MAL II (*Maackia amurensis* hemagglutinin). SNA is used to recognize specifically terminal α2-6 sialic acids structures. MAL I recognize not only terminal sialic acids α2-3 linked to galactose, but preferably Siaα2-3Galβ1-4GlcNAc structures. MAL II recognize preferably O-linked disialylated tetrassacharides Siaα2-3Galβ1-3(Neu5Ac α2-6)GlcNAc. Both cell lines showed an increase in α2-6 and α2-3 terminal sialylated structures upon treatment with oseltamivir. This result demonstrated that oseltamivir functions as a sialidase inhibitor in our cell line models, and confirms that inhibition of sialidases leads to an increase in sialic acid structures. It is described that increased β 1,6-branching, that leads to increases in sLe^x or sLe^a antigens, or a general increase in sialylation are commonly observed in *N*-linked and *O*-linked glycans in carcinoma cells and are associated with grade, invasion, metastasis and poor prognosis (Cazet *et al.,* 2010). In our study, and according to the recognition specificity of the different plant lectins used, the results suggested that we have an increase in sialylated structures, possibly sLe^x and sLe^a as well as sTn. In addition, and as described previously, we also observed that highly metastatic CMT-U27 cell line showed increased capacity to invade Matrigel invasion chambers in a dose-dependent manner when treated with oseltamivir. These results are in line with previous reported ones that showed that expression of sialyl-Lewis antigens is weak in carcinoma in situ, is moderate in invasive carcinomas without metastases and is high in primary carcinoma with lymph node metastases, showing a good correlation with the metastatic risk in breast cancer patients (Jeschke *et al.,* 2005). Furthermore, levels of expression of sLe^x are higher in patients who had distant metastasis compared with patients presenting non-metastatic cancers (Matsuura *et al.,* 1997).Regarding sTn antigen expression, it has been associated with carcinoma aggressiveness and poor prognosis, being highly expressed in most gastric (Baldus *et al.,* 2000); (David *et al.,* 1992); (Victorzon *et al.,* 1996), colorectal (Itzkowitz *et al.,* 1990), ovarian (Kobayashi *et al.,* 1992), breast (Yonezawa *et al.,* 1992); (Leivonen *et al.,* 2001), and pancreatic (Kim *et al.,* 2002) carcinomas.

MUC1 is a highly sialylated glycoprotein and a natural ligand of galectin-3 in human cancer cells, and usually its increased expression is associated with high metastatic potential and poor prognosis (Bresalier *et al.,* 1991); (Nakamori *et al.,* 1994). Cancer associated MUC1 expression has increased short oligosaccharides such as Tn, sTn, and T antigens (Lloyd *et al.,* 1996). In our study, we performed fluorescence assay in

order to assess the presence of galectin-3-ligands both in CMA07 and CMT-U27 cell lines upon treatment with oseltamivir. Results obtained showed a decrease in galectin-3 ligands expression in CMT-U27 cells treated with 1.25 µg/mL and 12.5 µg/mL oseltamivir. Galectin-3 recognition was fund to be particularly impaired in a molecular weight which corresponds to that of MUC1. The results are in accordance with what was expected in which increased terminal sialic acid structures, as a consequence of sialidase inhibition by oseltamivir treatment, mask the common recognition structures of galectin-3. Our observations are in accordance with Powell *et al*. who reported that presence of sialic acid mask carbohydrate ligands of galectin-3 (Powell *et al.,* 1987). de Oliveira JT *et al.,* suggested that loss of galectin-3 and sialylation-related masking of its ligands is crucial in regulating adhesive/de-adhesive events in the progression and invasive capacity of metastatic CMT (de Oliveira *et al.,* 2011). The presence of sialic acid can inhibit cell-cell interactions by masking carbohydrate ligands blocking receptor recognition (Powell *et al.,* 1987). This data is confirmed by our results in which highly metastatic CMT cells expressing increased terminal sialylated structures due to oseltamivir treatment, present decreased galectin-3-ligand expression, and a more aggressive phenotype as well as and an increased invasion capacity. Galectin-3 expression levels are increased in a variety of primary tumours (Liu *et al.,* 2005). It is up-regulated in gastric, liver and thyroid cancer, compared to normal tissues. However, its downregulation was also described in some carcinomas such as the case of prostate cancer (Ellerhorst *et al.,* 1999), ovarian cancer (van den Brule *et al.,* 1994), colon cancer (Lotz *et al.,* 1993), head and neck squamous cell carcinoma (Choufani *et al.,* 1999), and breast (Castronovo *et al.,* 1996) cancers. Cancer patients with metastatic dissemination have circulating galectin-3 concentrations markedly increased than those with localized tumours (Iurisci *et al.,* 2000); (Vereecken *et al.,* 2006); (Saussez *et al.,* 2008). In order to assess galectin-3 expression, we performed immunofluorescence both in CMA07 and CMT-U27 cells upon treatment with oseltamivir. Results showed that galectin-3 expression is increased in both cell lines treated with 1.25 µg/mL and 12.5 µg/mL oseltamivir. In addition, highly metastatic CMT-U27 cell line presented increased galectin-3 expression when compared to benign CMA07 cell line.

Since protein glycosylation has been reported as important in the process of galectin3-protein interactions and also invasion, we decided to analyse the glycosylation pattern of glycoproteins in cell lysates of both CMA07 and CMT-U27 cell lines upon oseltamivir treatment. We assessed the expression of terminal sialylated structures by lectin blot analysis using plant lectins SNA, MAL I and MAL II. Interestingly, the results from lectin blot analysis with SNA lectin demonstrated an increase in terminal α2,6 sialic acid structures expression in proteins from CMT-U27 cells upon oseltamivir treatment. At least a very distinct glycosylation pattern was observed at the molecular weight of 120

kDa. Regarding the results of western blot analysis with MAL I and MAL II lectins, differences in the terminal α2,3 sialic acid structures were observed in few proteins from MAL II blot in CMT-U27 cells treated with oseltamivir. Apparently, MAL II showed increased protein recognition in the same molecular weight of proteins that had increased terminal α2,6 sialic acid structures in SNA blot. In addition, and regarding galectin-3-ligands expression in proteins from CMT-U27 cells upon oseltamivir treatment, it was observed a decrease in galectin-3-ligands in proteins in a molecular weight around 120 kDa. This observation strengthened the result of the western blot analysis with SNA lectin that demonstrated an increase in terminal α2,6 sialic acid structures expression in proteins from CMT-U27 cells treated with oseltamivir (125 ng/mL, 1.25 µg/mL and 12.5 µg/mL oseltamivir), more specifically, in a molecular weight a around 120 kDa. We conclude that, when we have increase in terminal α 2,6 sialic acid structures, galectin-3-ligands decrease, suggesting a masking of these ligands by α2,6 sialylated structures. This observation was reinforced by our fluorescence results where we observed a decrease in galectin-3-ligands in CMT-U27 cells treated with 1.25 µg/mL and 12.5 µg/mL oseltamivir with a concomitant increase expression of terminal α2,6 sialic acid structures. Regarding galectin-3 expression in proteins from total cell lysates of CMT-U27 cells, upon treatment with oseltamivir we observed an increase in galectin-3 expression. This observation was strengthened by the immunofluorescence galectin-3 expression in CMT-U27 cell line showed an increase in galectin-3 when cells were treated with 1.25 µg/mL and 12.5 µg/mL oseltamivir.

In summary, these results lead us to highlight the role of sialylation during CMT progression, since upon oseltamivir treatment we observed an increase in sialylated structures accompanied by an increase in invasion capacity and migration of cells, which are common features of enhanced malignancy. Sialidase inhibition is possibly a bottom line for further studies and anti-cancer therapy.

CONCLUSIONS

The present study showed that oseltamivir plays an important role in the biological behaviour and in glycosylation pattern of CMT (canine mammary tumour) cell lines. Sialidases inhibition by treatment with oseltamivir demonstrated that they act on glycoproteins during mammary tumour progression since we found an increase in invasion capacity and also in migration of cells. Moreover, in what regards glycosylation pattern, highly malignant CMT-U27 cells treated with oseltamivir, showed an increase in sialylated structures, with concomitant decrease in galectin-3-ligands. These observations point to an important dynamic expression of sialidases in CMMT (canine mammary malignant tumour). Thus, the results of this study are of the utmost importance suggesting in what concerns about differential/dynamic expression and activity of sialidases in invading cells in CMMT, possibly involved in sialic acid capping and uncapping of glycans during tumour progression and invasion.

These results indicate that the discovery of a powerful inhibitor capable of inhibit aberrant sialylation could potentially be used for anti-cancer therapy.

REFERENCES

Achyuthan K. E. and Achyuthan A. M. (2001). *Comparative enzymology, biochemistry and pathophysiology of human exo-alpha-sialidases (neuraminidases).* Comp Biochem Physiol B Biochem Mol Biol. 129. (1): 29-64.

Almaraz R. T., Tian Y., Bhattarcharya R., Tan E., Chen S. H., Dallas M. R., . . . Yarema K. J. (2012). *Metabolic flux increases glycoprotein sialylation: implications for cell adhesion and cancer metastasis.* Mol Cell Proteomics. 11. (7): M112 017558.

Amith S. R., Jayanth P., Franchuk S., Finlay T., Seyrantepe V., Beyaert R., . . . Szewczuk M. R. (2010). *Neu1 desialylation of sialyl alpha-2,3-linked beta-galactosyl residues of TOLL-like receptor 4 is essential for receptor activation and cellular signaling.* Cell Signal. 22. (2): 314-324.

Baldus S. E., Engelmann K. and Hanisch F. G. (2004). *MUC1 and the MUCs: a family of human mucins with impact in cancer biology.* Crit Rev Clin Lab Sci. 41. (2): 189-231.

Baldus S. E., Zirbes T. K., Glossmann J., Fromm S., Hanisch F. G., Monig S. P., . . . Dienes H. P. (2001). *Immunoreactivity of monoclonal antibody BW835 represents a marker of progression and prognosis in early gastric cancer.* Oncology. 61. (2): 147-155.

Baldus S. E., Zirbes T. K., Hanisch F. G., Kunze D., Shafizadeh S. T., Nolden S., . . . Dienes H. P. (2000). *Thomsen-Friedenreich antigen presents as a prognostic factor in colorectal carcinoma: A clinicopathologic study of 264 patients.* Cancer. 88. (7): 1536-1543.

Bresalier R. S., Niv Y., Byrd J. C., Duh Q. Y., Toribara N. W., Rockwell R. W., . . . Kim Y. S. (1991). *Mucin production by human colonic carcinoma cells correlates with their metastatic potential in animal models of colon cancer metastasis.* J Clin Invest. 87. (3): 1037-1045.

Bull C., Boltje T. J., Wassink M., de Graaf A. M., van Delft F. L., den Brok M. H. and Adema G. J. (2013). *Targeting Aberrant Sialylation in Cancer Cells Using a Fluorinated Sialic Acid Analog Impairs Adhesion, Migration, and In Vivo Tumor Growth.* Mol Cancer Ther.

Cao Y., Linden P., Shima D., Browne F. and Folkman J. (1996). *In vivo angiogenic activity and hypoxia induction of heterodimers of placenta growth factor/vascular endothelial growth factor.* J Clin Invest. 98. (11): 2507-2511.

Carubelli R., Trucco R. E. and Caputto R. (1962). *Neuraminidase activity in mammalian organs.* Biochim Biophys Acta. 60. 196-197.

Castronovo V., Van Den Brule F. A., Jackers P., Clausse N., Liu F. T., Gillet C. and Sobel M. E. (1996). *Decreased expression of galectin-3 is associated with progression of human breast cancer.* J Pathol. 179. (1): 43-48.

Cazet A., Julien S., Bobowski M., Burchell J. and Delannoy P. (2010). *Tumour-associated carbohydrate antigens in breast cancer.* Breast Cancer Res. 12. (3): 204.

Chambers A. F., Groom A. C. and MacDonald I. C. (2002). *Dissemination and growth of cancer cells in metastatic sites.* Nat Rev Cancer. 2. (8): 563-572.

Chavas L. M., Kato R., Suzuki N., von Itzstein M., Mann M. C., Thomson R. J., . . . Wakatsuki S. (2010). *Complexity in influenza virus targeted drug design: interaction with human sialidases.* J Med Chem. 53. (7): 2998-3002.

Chavas L. M., Tringali C., Fusi P., Venerando B., Tettamanti G., Kato R., . . . Wakatsuki S. (2005). *Crystal structure of the human cytosolic sialidase Neu2. Evidence for the dynamic nature of substrate recognition.* J Biol Chem. 280. (1): 469-475.

Choufani G., Nagy N., Saussez S., Marchant H., Bisschop P., Burchert M., . . . Hassid S. (1999). *The levels of expression of galectin-1, galectin-3, and the Thomsen-Friedenreich antigen and their binding sites decrease as clinical aggressiveness increases in head and neck cancers.* Cancer. 86. (11): 2353-2363.

Coon J. S., Weinstein R. S. and Summers J. L. (1982). *Blood group precursor T-antigen expression in human urinary bladder carcinoma.* Am J Clin Pathol. 77. (6): 692-699.

Corfield A. P., Veh R. W., Wember M., Michalski J. C. and Schauer R. (1981). *The release of N-acetyl- and N-glycolloyl-neuraminic acid from soluble complex carbohydrates and erythrocytes by bacterial, viral and mammalian sialidases.* Biochem J. 197. (2): 293-299.

Corfield A. P., Wember M., Schauer R. and Rott R. (1982). *The specificity of viral sialidases. The use of oligosaccharide substrates to probe enzymic characteristics and strain-specific differences.* Eur J Biochem. 124. (3): 521-525.

Crain S. M. and Shen K. F. (2004). *Neuraminidase inhibitor, oseltamivir blocks GM1 ganglioside-regulated excitatory opioid receptor-mediated hyperalgesia, enhances opioid analgesia and attenuates tolerance in mice.* Brain Res. 995. (2): 260-266.

Croce M. V., Isla-Larrain M., Rabassa M. E., Demichelis S., Colussi A. G., Crespo M., . . . Segal-Eiras A. (2007). *Lewis x is highly expressed in normal tissues: a comparative immunohistochemical study and literature revision.* Pathol Oncol Res. 13. (2): 130-138.

D'Azzo A., Hoogeveen A., Reuser A. J., Robinson D. and Galjaard H. (1982). *Molecular defect in combined beta-galactosidase and neuraminidase deficiency in man.* Proc Natl Acad Sci U S A. 79. (15): 4535-4539.

David L., Nesland J. M., Clausen H., Carneiro F. and Sobrinho-Simoes M. (1992). *Simple mucin-type carbohydrate antigens (Tn, sialosyl-Tn and T) in gastric mucosa, carcinomas and metastases.* APMIS Suppl. 27. 162-172.

de Oliveira J. T., de Matos A. J., Santos A. L., Pinto R., Gomes J., Hespanhol V., . . . Gartner F. (2011). *Sialylation regulates galectin-3/ligand interplay during mammary tumour progression--a case of targeted uncloaking.* Int J Dev Biol. 55. (7-9): 823-834.

Dennis J. W. and Laferte S. (1987). *Tumor cell surface carbohydrate and the metastatic phenotype.* Cancer Metastasis Rev. 5. (3): 185-204.

Dwek R. A. (1995). *Glycobiology: "towards understanding the function of sugars".* Biochem Soc Trans. 23. (1): 1-25.

Ellerhorst J., Troncoso P., Xu X. C., Lee J. and Lotan R. (1999). *Galectin-1 and galectin-3 expression in human prostate tissue and prostate cancer.* Urol Res. 27. (5): 362-367.

Ferlay J., Shin H. R., Bray F., Forman D., Mathers C. and Parkin D. M. (2010). *Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008.* Int J Cancer. 127. (12): 2893-2917.

Funakoshi Y. and Suzuki T. (2009). *Glycobiology in the cytosol: the bitter side of a sweet world.* Biochim Biophys Acta. 1790. (2): 81-94.

Galjart N. J., Gillemans N., Harris A., van der Horst G. T., Verheijen F. W., Galjaard H. and d'Azzo A. (1988). *Expression of cDNA encoding the human "protective protein" associated with lysosomal beta-galactosidase and neuraminidase: homology to yeast proteases.* Cell. 54. (6): 755-764.

Ghazizadeh M., Oguro T., Sasaki Y., Aihara K., Araki T. and Springer G. F. (1990). *Immunohistochemical and ultrastructural localization of T antigen in ovarian tumors.* Am J Clin Pathol. 93. (3): 315-321.

Glinskii O. V., Sud S., Mossine V. V., Mawhinney T. P., Anthony D. C., Glinsky G. V., . . . Glinsky V. V. (2012). *Inhibition of prostate cancer bone metastasis by synthetic TF antigen mimic/galectin-3 inhibitor lactulose-L-leucine.* Neoplasia. 14. (1): 65-73.

Gomes C., Osorio H., Pinto M. T., Campos D., Oliveira M. J. and Reis C. A. (2013). *Expression of ST3GAL4 leads to SLe(x) expression and induces c-Met activation and an invasive phenotype in gastric carcinoma cells.* PLoS One. 8. (6): e66737.

Hakomori S. (2002). *Glycosylation defining cancer malignancy: new wine in an old bottle.* Proc Natl Acad Sci U S A. 99. (16): 10231-10233.

Hanisch F. G. and Baldus S. E. (1997). *The Thomsen-Friedenreich (TF) antigen: a critical review on the structural, biosynthetic and histochemical aspects of a pancarcinoma-associated antigen.* Histol Histopathol. 12. (1): 263-281.

Hata K., Wada T., Hasegawa A., Kiso M. and Miyagi T. (1998). *Purification and characterization of a membrane-associated ganglioside sialidase from bovine brain.* J Biochem. 123. (5): 899-905.

Hellmen E. (1992). *Characterization of four in vitro established canine mammary carcinoma and one atypical benign mixed tumor cell lines.* In Vitro Cell Dev Biol. 28A. (5): 309-319.

Ishizuka A., Hashimto Y., Naka R., Kinoshita M., Kakehi K., Seino J., . . . Narimatsu H. (2008). *Accumulation of free complex-type N-glycans in MKN7 and MKN45 stomach cancer cells.* Biochem J. 413. (2): 227-237.

Itzkowitz S. H., Bloom E. J., Kokal W. A., Modin G., Hakomori S. and Kim Y. S. (1990). *Sialosyl-Tn. A novel mucin antigen associated with prognosis in colorectal cancer patients.* Cancer. 66. (9): 1960-1966.

Iurisci I., Tinari N., Natoli C., Angelucci D., Cianchetti E. and Iacobelli S. (2000). *Concentrations of galectin-3 in the sera of normal controls and cancer patients.* Clin Cancer Res. 6. (4): 1389-1393.

Izumi Y., Tokuda K., O'Dell K A., Zorumski C. F. and Narahashi T. (2007). *Neuroexcitatory actions of Tamiflu and its carboxylate metabolite.* Neurosci Lett. 426. (1): 54-58.

Janssen T., Petein M., Van Velthoven R., Van Leer P., Fourmarier M., Vanegas J. P., . . . Kiss R. (1996). *Differential histochemical peanut agglutinin stain in benign and malignant human prostate tumors: relationship with prostatic specific antigen immunostain and nuclear DNA content.* Hum Pathol. 27. (12): 1341-1347.

Jeschke U., Mylonas I., Shabani N., Kunert-Keil C., Schindlbeck C., Gerber B. and Friese K. (2005). *Expression of sialyl lewis X, sialyl Lewis A, E-cadherin and cathepsin-D in human breast cancer: immunohistochemical analysis in mammary carcinoma in situ, invasive carcinomas and their lymph node metastasis.* Anticancer Res. 25. (3A): 1615-1622.

Johnston SD (1993). Reproductive systems. Surgery ToSA, W B Saunders. **2:** 2185-2192.

Julien S., Krzewinski-Recchi M. A., Harduin-Lepers A., Gouyer V., Huet G., Le Bourhis X. and Delannoy P. (2001). *Expression of sialyl-Tn antigen in breast cancer cells transfected with the human CMP-Neu5Ac: GalNAc alpha2,6-sialyltransferase (ST6GalNac I) cDNA.* Glycoconj J. 18. (11-12): 883-893.

Kakugawa Y., Wada T., Yamaguchi K., Yamanami H., Ouchi K., Sato I. and Miyagi T. (2002). *Up-regulation of plasma membrane-associated ganglioside sialidase (Neu3) in human colon cancer and its involvement in apoptosis suppression.* Proc Natl Acad Sci U S A. 99. (16): 10718-10723.

Kato T., Wang Y., Yamaguchi K., Milner C. M., Shineha R., Satomi S. and Miyagi T. (2001). *Overexpression of lysosomal-type sialidase leads to suppression of metastasis associated with reversion of malignant phenotype in murine B16 melanoma cells.* Int J Cancer. 92. (6): 797-804.

Kawamura S., Sato I., Wada T., Yamaguchi K., Li Y., Li D., . . . Miyagi T. (2012). *Plasma membrane-associated sialidase (NEU3) regulates progression of prostate cancer to androgen-independent growth through modulation of androgen receptor signaling.* Cell Death Differ. 19. (1): 170-179.

Kijima-Suda I., Miyamoto Y., Toyoshima S., Itoh M. and Osawa T. (1986). *Inhibition of experimental pulmonary metastasis of mouse colon adenocarcinoma 26 sublines by a sialic acid:nucleoside conjugate having sialyltransferase inhibiting activity.* Cancer Res. 46. (2): 858-862.

Kijima-Suda I., Miyazawa T., Itoh M., Toyoshima S. and Osawa T. (1988). *Possible mechanism of inhibition of experimental pulmonary metastasis of mouse colon adenocarcinoma 26 sublines by a sialic acid: nucleoside conjugate.* Cancer Res. 48. (13): 3728-3732.

Kim G. E., Bae H. I., Park H. U., Kuan S. F., Crawley S. C., Ho J. J. and Kim Y. S. (2002). *Aberrant expression of MUC5AC and MUC6 gastric mucins and sialyl Tn antigen in intraepithelial neoplasms of the pancreas.* Gastroenterology. 123. (4): 1052-1060.

Kobayashi H., Terao T. and Kawashima Y. (1992). *Serum sialyl Tn as an independent predictor of poor prognosis in patients with epithelial ovarian cancer.* J Clin Oncol. 10. (1): 95-101.

Kumar S. R., Sauter E. R., Quinn T. P. and Deutscher S. L. (2005). *Thomsen-Friedenreich and Tn antigens in nipple fluid: carbohydrate biomarkers for breast cancer detection.* Clin Cancer Res. 11. (19 Pt 1): 6868-6871.

Lana SE (2007). Tumors of the Mammary Gland. Oncology WMsSAC, Saunders Company**:** 619-636.

Leahy M. F., Seymour J. F., Hicks R. J. and Turner J. H. (2006). *Multicenter phase II clinical study of iodine-131-rituximab radioimmunotherapy in relapsed or refractory indolent non-Hodgkin's lymphoma.* J Clin Oncol. 24. (27): 4418-4425.

Leivonen M., Nordling S., Lundin J., von Boguslawski K. and Haglund C. (2001). *STn and prognosis in breast cancer.* Oncology. 61. (4): 299-305.

Li S. C., Li Y. T., Moriya S. and Miyagi T. (2001). *Degradation of G(M1) and G(M2) by mammalian sialidases.* Biochem J. 360. (Pt 1): 233-237.

Lindblad-Toh K., Wade C. M., Mikkelsen T. S., Karlsson E. K., Jaffe D. B., Kamal M., . . . Lander E. S. (2005). *Genome sequence, comparative analysis and haplotype structure of the domestic dog.* Nature. 438. (7069): 803-819.

Liu F. T. and Rabinovich G. A. (2005). *Galectins as modulators of tumour progression.* Nat Rev Cancer. 5. (1): 29-41.

Lloyd K. O., Burchell J., Kudryashov V., Yin B. W. and Taylor-Papadimitriou J. (1996). *Comparison of O-linked carbohydrate chains in MUC-1 mucin from normal breast epithelial cell lines and breast carcinoma cell lines. Demonstration of simpler and fewer glycan chains in tumor cells.* J Biol Chem. 271. (52): 33325-33334.

Lotz M. M., Andrews C. W., Jr., Korzelius C. A., Lee E. C., Steele G. D., Jr., Clarke A. and Mercurio A. M. (1993). *Decreased expression of Mac-2 (carbohydrate binding protein 35) and loss of its nuclear localization are associated with the neoplastic progression of colon carcinoma.* Proc Natl Acad Sci U S A. 90. (8): 3466-3470.

Malagolini N., Chiricolo M., Marini M. and Dall'Olio F. (2009). *Exposure of alpha2,6-sialylated lactosaminic chains marks apoptotic and necrotic death in different cell types.* Glycobiology. 19. (2): 172-181.

Mandal C., Tringali C., Mondal S., Anastasia L., Chandra S., Venerando B. and Mandal C. (2010). *Down regulation of membrane-bound Neu3 constitutes a new potential marker for childhood acute* (2): 337-349.

Matsuura N., Narita T., Mitsuoka C., Kimura N., Kannagi R., Imai T., . . . Takagi H. (1997). *Increased level of circulating adhesion molecules in the sera of breast cancer patients with distant metastases.* Jpn J Clin Oncol. 27. (3): 135-139.

Meuillet E. J., Kroes R., Yamamoto H., Warner T. G., Ferrari J., Mania-Farnell B., . . . Bremer E. G. (1999). *Sialidase gene transfection enhances epidermal growth factor receptor activity in an epidermoid carcinoma cell line, A431.* Cancer Res. 59. (1): 234-240.

Miyagi T., Takahashi K., Hata K., Shiozaki K. and Yamaguchi K. (2012). *Sialidase significance for cancer progression.* Glycoconj J. 29. (8-9): 567-577.

Miyagi T. and Tsuiki S. (1984). *Rat-liver lysosomal sialidase. Solubilization, substrate specificity and comparison with the cytosolic sialidase.* Eur J Biochem. 141. (1): 75-81.

Miyagi T. and Tsuiki S. (1985). *Purification and characterization of cytosolic sialidase from rat liver.* J Biol Chem. 260. (11): 6710-6716.

Miyagi T., Wada T., Iwamatsu A., Hata K., Yoshikawa Y., Tokuyama S. and Sawada M. (1999). *Molecular cloning and characterization of a plasma membrane-associated sialidase specific for gangliosides.* J Biol Chem. 274. (8): 5004-5011.

Miyagi T., Wada T. and Yamaguchi K. (2008). *Roles of plasma membrane-associated sialidase NEU3 in human cancers.* Biochim Biophys Acta. 1780. (3): 532-537.

Miyagi T. and Yamaguchi K. (2012). *Mammalian sialidases: physiological and pathological roles in cellular functions.* Glycobiology. 22. (7): 880-896.

Moore M. L., Chi M. H., Zhou W., Goleniewska K., O'Neal J. F., Higginbotham J. N. and Peebles R. S., Jr. (2007). *Cutting Edge: Oseltamivir decreases T cell GM1 expression and inhibits clearance of respiratory syncytial virus: potential role of endogenous sialidase in antiviral immunity.* J Immunol. 178. (5): 2651-2654.

Moulton JE (1990). Tumors of the Mammary Gland. Animals TiD, University of California Press**:** 518-550.

Nakamori S., Ota D. M., Cleary K. R., Shirotani K. and Irimura T. (1994). *MUC1 mucin expression as a marker of progression and metastasis of human colorectal carcinoma.* Gastroenterology. 106. (2): 353-361.

Narita T., Funahashi H., Satoh Y., Watanabe T., Sakamoto J. and Takagi H. (1993). *Association of expression of blood group-related carbohydrate antigens with prognosis in breast cancer.* Cancer. 71. (10): 3044-3053.

Neufeld E. F. and d'Azzo A. (2001). *Biosynthesis of normal and mutant beta-hexosaminidases.* Adv Genet. 44. 165-171.

Opdenakker G., Rudd P. M., Ponting C. P. and Dwek R. A. (1993). *Concepts and principles of glycobiology.* FASEB J. 7. (14): 1330-1337.

Orr F. W. and Wang H. H. (2001). *Tumor cell interactions with the microvasculature: a rate-limiting step in metastasis.* Surg Oncol Clin N Am. 10. (2): 357-381, ix-x.

Paoloni M. and Khanna C. (2008). *Translation of new cancer treatments from pet dogs to humans.* Nat Rev Cancer. 8. (2): 147-156.

Pinho S. S., Osorio H., Nita-Lazar M., Gomes J., Lopes C., Gartner F. and Reis C. A. (2009). *Role of E-cadherin N-glycosylation profile in a mammary tumor model.* Biochem Biophys Res Commun. 379. (4): 1091-1096.

Powell L. D., Whiteheart S. W. and Hart G. W. (1987). *Cell surface sialic acid influences tumor cell recognition in the mixed lymphocyte reaction.* J Immunol. 139. (1): 262-270.

Ravn V. and Dabelsteen E. (2000). *Tissue distribution of histo-blood group antigens.* APMIS. 108. (1): 1-28.

Reis C. A., Osorio H., Silva L., Gomes C. and David L. (2010). *Alterations in glycosylation as biomarkers for cancer detection.* J Clin Pathol. 63. (4): 322-329.

Renkonen J., Paavonen T. and Renkonen R. (1997). *Endothelial and epithelial expression of sialyl Lewis(x) and sialyl Lewis(a) in lesions of breast carcinoma.* Int J Cancer. 74. (3): 296-300.

Roggentin P., Rothe B., Kaper J. B., Galen J., Lawrisuk L., Vimr E. R. and Schauer R. (1989). *Conserved sequences in bacterial and viral sialidases.* Glycoconj J. 6. (3): 349-353.

Rutteman SJ Withrow; EG MacEwen; GR (2001). Tumours of the Mammary Gland. Oncology SAC, W. B. Saunders Company**:** 455-477.

Saito M., Fronda C. L. and Yu R. K. (1996). *Sialidase activity in nuclear membranes of rat brain.* J Neurochem. 66. (5): 2205-2208.

Saito T., Taylor G. and Webster R. G. (1995). *Steps in maturation of influenza A virus neuraminidase.* J Virol. 69. (8): 5011-5017.

Saussez S., Lorfevre F., Lequeux T., Laurent G., Chantrain G., Vertongen F., . . . Kiss R. (2008). *The determination of the levels of circulating galectin-1 and -3 in HNSCC patients could be used to monitor tumor progression and/or responses to therapy.* Oral Oncol. 44. (1): 86-93.

Sawada M., Moriya S., Saito S., Shineha R., Satomi S., Yamori T., . . . Miyagi T. (2002). *Reduced sialidase expression in highly metastatic variants of mouse colon adenocarcinoma 26 and retardation of their metastatic ability by sialidase overexpression.* Int J Cancer. 97. (2): 180-185.

Sawada M., Moriya S., Shineha R., Satomi S. and Miyagi T. (1998). *Comparative study of sialidase activity and G(M3) content in B16 melanoma variants with different metastatic potential.* Acta Biochim Pol. 45. (2): 343-349.

Schauer R. (2000). *Achievements and challenges of sialic acid research.* Glycoconj J. 17. (7-9): 485-499.

Schengrund C. L., Rosenberg A. and Repman M. A. (1976). *Ecto-ganglioside-sialidase activity of herpes simplex virus-transformed hamster embryo fibroblasts.* J Cell Biol. 70. (3): 555-561.

Schneider R. (1970). *Comparison of age, sex, and incidence rates in human and canine breast cancer.* Cancer. 26. (2): 419-426.

Shiozaki K., Yamaguchi K., Takahashi K., Moriya S. and Miyagi T. (2011). *Regulation of sialyl Lewis antigen expression in colon cancer cells by sialidase NEU4.* J Biol Chem. 286. (24): 21052-21061.

Siegel R., Naishadham D. and Jemal A. (2012). *Cancer statistics, 2012.* CA Cancer J Clin. 62. (1): 10-29.

Soares R., Marinho A. and Schmitt F. (1996). *Expression of sialyl-Tn in breast cancer. Correlation with prognostic parameters.* Pathol Res Pract. 192. (12): 1181-1186.

Sorenmo K. (2003). *Canine mammary gland tumors.* Vet Clin North Am Small Anim Pract. 33. (3): 573-596.

Springer G. F. (1984). *T and Tn, general carcinoma autoantigens.* Science. 224. (4654): 1198-1206.

Springer G. F. (1997). *Immunoreactive T and Tn epitopes in cancer diagnosis, prognosis, and immunotherapy.* J Mol Med (Berl). 75. (8): 594-602.

Tokuyama S., Moriya S., Taniguchi S., Yasui A., Miyazaki J., Orikasa S. and Miyagi T. (1997). *Suppression of pulmonary metastasis in murine B16 melanoma cells by transfection of a sialidase cDNA.* Int J Cancer. 73. (3): 410-415.

Tringali C., Lupo B., Anastasia L., Papini N., Monti E., Bresciani R., . . . Venerando B. (2007). *Expression of sialidase Neu2 in leukemic K562 cells induces apoptosis by impairing Bcr-Abl/Src kinases signaling.* J Biol Chem. 282. (19): 14364-14372.

Uemura T., Shiozaki K., Yamaguchi K., Miyazaki S., Satomi S., Kato K., . . . Miyagi T. (2009). *Contribution of sialidase NEU1 to suppression of metastasis of human colon cancer cells through desialylation of integrin beta4.* Oncogene. 28. (9): 1218-1229.

Ueno S., Saito S., Wada T., Yamaguchi K., Satoh M., Arai Y. and Miyagi T. (2006). *Plasma membrane-associated sialidase is up-regulated in renal cell carcinoma and promotes interleukin-6-induced apoptosis suppression and cell motility.* J Biol Chem. 281. (12): 7756-7764.

van den Brule F. A., Berchuck A., Bast R. C., Liu F. T., Gillet C., Sobel M. E. and Castronovo V. (1994). *Differential expression of the 67-kD laminin receptor and 31-kD human laminin-binding protein in human ovarian carcinomas.* Eur J Cancer. 30A. (8): 1096-1099.

Varghese J. N., Smith P. W., Sollis S. L., Blick T. J., Sahasrabudhe A., McKimm-Breschkin J. L. and Colman P. M. (1998). *Drug design against a shifting target: a structural basis for resistance to inhibitors in a variant of influenza virus neuraminidase.* Structure. 6. (6): 735-746.

Varki A. (1993). *Biological roles of oligosaccharides: all of the theories are correct.* Glycobiology. 3. (2): 97-130.

Vereecken P., Zouaoui Boudjeltia K., Debray C., Awada A., Legssyer I., Sales F., . . . Heenen M. (2006). *High serum galectin-3 in advanced melanoma: preliminary results.* Clin Exp Dermatol. 31. (1): 105-109.

Victorzon M., Nordling S., Nilsson O., Roberts P. J. and Haglund C. (1996). *Sialyl Tn antigen is an independent predictor of outcome in patients with gastric cancer.* Int J Cancer. 65. (3): 295-300.

Wang B. L., Springer G. F. and Carlstedt S. C. (1997). *Quantitative computerized image analysis of Tn and T (Thomsen-Friedenreich) epitopes in prognostication of human breast carcinoma.* J Histochem Cytochem. 45. (10): 1393-1400.

Wells L., Vosseller K. and Hart G. W. (2001). *Glycosylation of nucleocytoplasmic proteins: signal transduction and O-GlcNAc.* Science. 291. (5512): 2376-2378.

Wilson J. C. and von Itzstein M. (2003). *Recent strategies in the search for new anti-influenza therapies.* Curr Drug Targets. 4. (5): 389-408.

Woronowicz A., Amith S. R., Davis V. W., Jayanth P., De Vusser K., Laroy W., . . . Szewczuk M. R. (2007). *Trypanosome trans-sialidase mediates neuroprotection against oxidative stress, serum/glucose deprivation, and hypoxia-induced neurite retraction in Trk-expressing PC12 cells.* Glycobiology. 17. (7): 725-734.

Yamaguchi K., Hata K., Wada T., Moriya S. and Miyagi T. (2006). *Epidermal growth factor-induced mobilization of a ganglioside-specific sialidase (NEU3) to membrane ruffles.* Biochem Biophys Res Commun. 346. (2): 484-490.

Yamanami H., Shiozaki K., Wada T., Yamaguchi K., Uemura T., Kakugawa Y., . . . Miyagi T. (2007). *Down-regulation of sialidase NEU4 may contribute to invasive properties of human colon cancers.* Cancer Sci. 98. (3): 299-307.

Yogeeswaran G. and Salk P. L. (1981). *Metastatic potential is positively correlated with cell surface sialylation of cultured murine tumor cell lines.* Science. 212. (4502): 1514-1516.

Yonezawa S., Tachikawa T., Shin S. and Sato E. (1992). *Sialosyl-Tn antigen. Its distribution in normal human tissues and expression in adenocarcinomas.* Am J Clin Pathol. 98. (2): 167-174.

Yu L. G., Andrews N., Zhao Q., McKean D., Williams J. F., Connor L. J., . . . Rhodes J. M. (2007). *Galectin-3 interaction with Thomsen-Friedenreich disaccharide on cancer-associated MUC1 causes increased cancer cell endothelial adhesion.* J Biol Chem. 282. (1): 773-781.

Zaccai N. R., Maenaka K., Maenaka T., Crocker P. R., Brossmer R., Kelm S. and Jones E. Y. (2003). *Structure-guided design of sialic acid-based Siglec inhibitors and crystallographic analysis in complex with sialoadhesin.* Structure. 11. (5): 557-567.

Zhao Q., Barclay M., Hilkens J., Guo X., Barrow H., Rhodes J. M. and Yu L. G. (2010). *Interaction between circulating galectin-3 and cancer-associated MUC1 enhances tumour cell homotypic aggregation and prevents anoikis.* Mol Cancer. 9. 154.