NATIONAL TECHNICAL UNIVERSITY OF ATHENS

SCHOOL OF CHEMICAL ENGINEERING

DEPARTMENT IV: SYNTHESIS AND DEVELOPMENT OF INDUSTRIAL PROCESSES

Evaluation of novel synthetic anillinoquinazolines as potential angiogenic modulators

DIPLOMA THESIS

of

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Acknowledgments

Some explanations may be useful to understand this diploma thesis. I first arrived from the National Institute of Applied Sciences of Toulouse (INSA) at NTUA in 2012 as an Erasmus student. After my integration in the national Greek fencing team in 2013, INSA and NTUA established a double degree in order to allow me to stay in Greece and continue my studies while fencing at the same time. This was extremely useful for me and allowed me among others to be ranked 21st in the Acropolis World Cup tournament and 26th in the European Championship in 2015, not to mention a silver medal in the Greek National Championship the same year. For this reason my first thought goes to Prof Boudouvis and Prof Maranges without whom these results and this thesis would not have occurred. Thank you very much. I would like to express my gratitude to Prof Kolisis for introducing me to the topic of this diploma thesis. Then I would like to thank Dr Heleni Loutrari for the useful comments, remarks and engagement through the learning process of this diploma thesis. Also, I like to thank the participants in my survey, who willingly shared their precious time during the process of interviewing. I would like to thank my family and my friends, who have supported me throughout entire process, both by keeping me harmonious and helping me putting pieces together.

Abstract

Angiogenesis is the sprouting of new blood vessels from pre-existing ones. It is an important process during the development of the embryo and the wound healing. The cells at the inner surface of the blood vessels, named endothelial cells (ECs), enter in division phase and create the new blood vessel. This process is induced by growth factors such as the vascular endothelial growth factor (VEGF) and the epidermal growth factor (EGF). They are realized in response to different types of stress (hypoxia, cut and other). These growth factors bind to tyrosine kinase receptor like vascular endothelial growth factor receptor (VEGFR) or epidermal growth factor receptor (EGFR). This bounding activate cascades of signals which then activate angiogenesis.

The angiogenesis plays also a key role in some diseases like cancer. In order to grow, tumors need oxygen and nutriments to feed the cancer cells. Such feeding can be done only by blood vessel. Consequently, in this, as well as in other diseases involving angiogenesis, the division of the endothelial cells is upregulated so as to create new blood vessel to feed the cancer cells. This is the driving force behind thousands of studies which took place up to now with the aim to find a solution to downregulate this mechanism of anarchic, uncontrolled and incessant cell proliferation.

From this perspective, the objective of this thesis was to study 22 novel anillinoquinazoline compounds. They were chosen because they are tyrosine kinase receptor inhibitors and it has already been proved that they present an inhibiting effect on EGFR. It will be interesting to verify this inhibition for VEGFR, as well.

The study was performed by using an in vitro model involving endothelial cells (EA.hy 926) and their proliferation was studied by using an MTT assay.

From the 22 compounds tested, it was found that four compounds present a promising impact to the inhibition of cell proliferation. Based on this result, it can also be expected that they present an inhibition action on angiogenesis.

KEY WORDS: Angiogenesis, Vascular Endothelial Growth Factor Receptor (VEGFR), Epidermal Growth Factor Receptor (EGFR), anillinoquinazoline, Cancer, MTT assay.

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1. Angiogenesis

1.1. GENERAL INFORMATION

The term of angiogenesis was first introduced by Hertig in 1935 to describe the formation of new blood vessels [1]. This definition has been refined over the years up to the most recent one: angiogenesis is the "sprouting of new blood vessels from pre-existing ones" [2]. It is a natural process which occurs in reproduction, development and wound repair. It takes place, physiologically, for a given period of time from few days, like ovulation, to months, as is the case of placentation [3]. This is one of the most important differences with the pathological angiogenesis which occurs for years. As presented in Figure 1, a large number of human diseases are angiogenesis-based. Angiogenesis is a complex mechanism involving endothelial cells, i.e. cells located on the inner membrane of the blood vessel. The angiogenesis is regulated by specific transduction pathways which are activated or inhibited by a great range of growth factors [4].

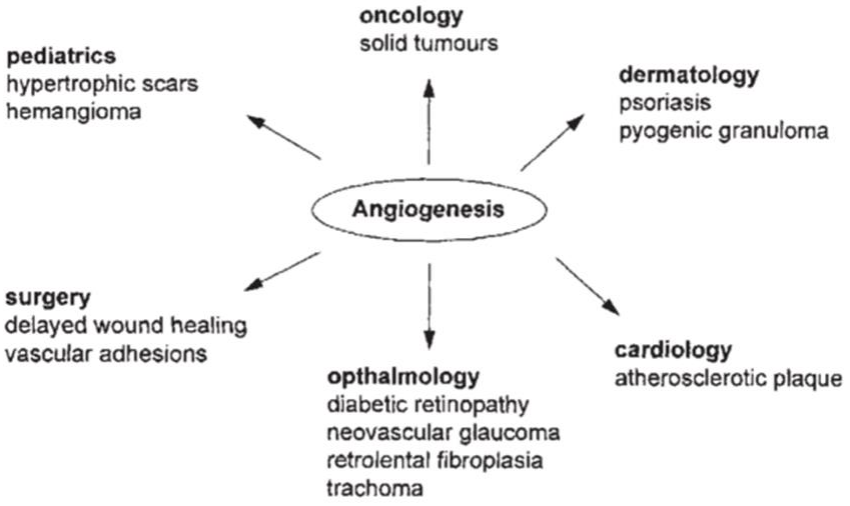


Figure 1: Principal angiogenesis-dependent diseases [4]**.**

1.2. STEPS OF ANGIOGENESIS

As previously mentioned, the angiogenesis is a process related to the endothelial cells. Figure 2 schematically depicts the structure and shows an electron microscopy micrograph of a blood capillary. Endothelial cells are positioned at the interface of blood capillary, between blood and tissues. Besides angiogenesis, (a) they have a barrier function, (b) they maintain the fluidity of the blood and (c) they are responsible for the development of inflammatory foci [5].

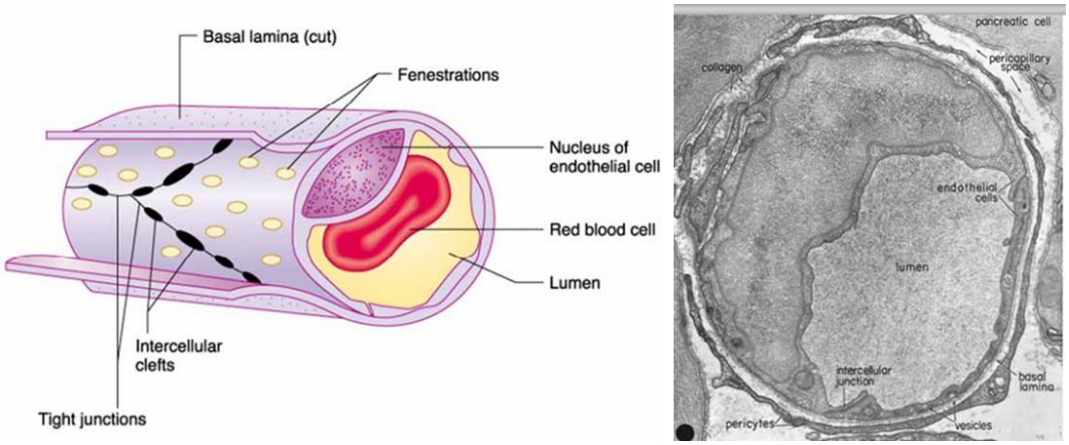


Figure 2: Structure of blood capillary [6]**.**

Angiogenesis takes place through several steps, illustrated in Figure 3:

- Endogenous signals (as hypoxia) activate cells that release signaling factors, known as angiogenic growth factors, which diffuse to the nearby tissues. Those angiogenic growth factors bind to specific receptors as the vascular endothelial growth factor receptor (VEGFR), the epidermal growth factor receptor (EGFR), the angiopoietin receptors (Tie-1, Tie-2) and the ephrin B2 located through the membrane of endothelial cells to stimulate them (steps 1, 2 and 3 in Figure 3).
- Stimulated Endothelial cells secrete protease (like plasminogen activator and collagenases) which locally degrades the basement membrane of the initial blood vessel and the extracellular matrix (step 4 in Figure 3).
- Endothelial cells proliferate and migrate through the formed gaps of the basal membrane in the direction of the angiogenic stimuli (known as chemotactic signal). Adhesion molecules (integrins), serve as grappling hooks in order to help the sprouting of new blood vessels (steps 5, 6 and 7 in Figure 3).
- The layers of endothelial cells can form a lumen. In this case, the edges of two neighborhood capillary sprouts connect and form a new capillary loop (steps 8 and 9 in Figure 3).
- The angiogenesis process is terminated with the formation of new basal membranes and the accumulation of pericyte cells (cells that inhibit the

proliferation of endothelial cells) and other perivascular cells near the newly formed capillaries (step 10 in Figure 3) [4].

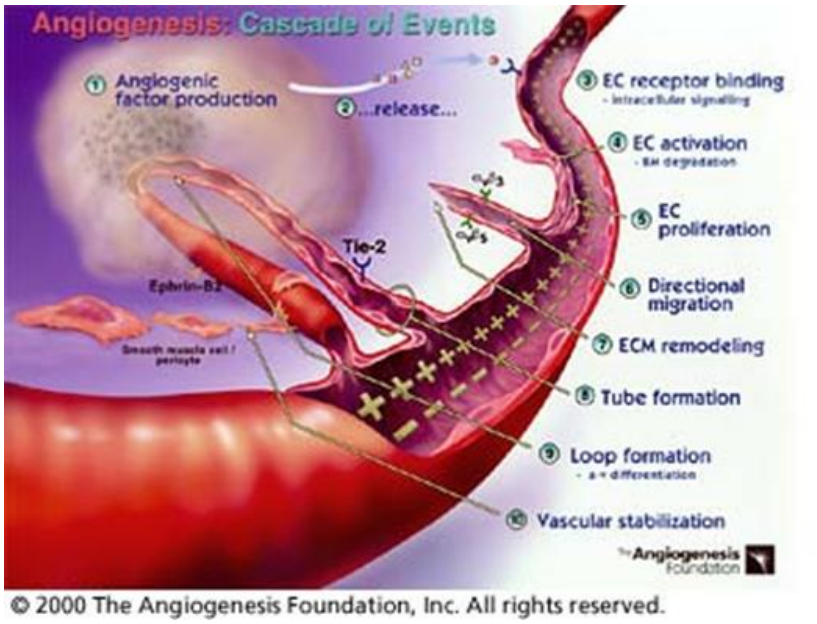


Figure 3: Angiogenesis: a cascade of events

After the presentation of the angiogenic steps, it will be interesting to understand its importance in an example of disease in which angiogenesis play an important role. Cancer was the disease chosen since angiogenesis is essential for the growth of the tumor. Indeed, the tumor can grow by its own until the size of 1-2mm. Over this size, the tumors must be supplied with nutriments and oxygen. In the organism, only the blood can bring those essential elements to the “organs”, that is why the tumor secretes signaling molecules to activate the angiogenesis. Those signals are over-produced and angiostatic factors i.e. proteins that inhibit the angiogenesis, lack.

1.3. VASCULAR ENDOTHELIAL GROWTH FACTOR (VEGF) AND ITS RECEPTORS (VEGFR)

The angiogenic growth factors are polypeptides that are synthesized when angiogenesis must be activated, during hypoxia for example.

The principal growth factor’s family of angiogenesis is the VEGF family. It is the predominant stimulator of angiogenesis and its receptor, VEGFR, is expressed specifically by endothelial cells. This growth factor acts as mitogenic stimulator of endothelial cells, as extracellular matrix remodeling, as regulator of embryonic stem cell development, as local generator of inflammatory cytokines and it is also a survival factor for the endothelial cell [7]. One inducer of VEGF transcription is hypoxia; when it occurs, hypoxia-inducible factor-1 (HIF-1) is released and it activates the transcription of the VEGF genes by binding to the binding site located on the VEGF promoter. Other proangiogenic factors such as EGF, fibroblast growth factor (FGF), transforming growth factors (TGF) can induce the VEGF mRNA expression and other can too but their action is not well known. The function of VEGFs is to act as ligand

which binds to a tyrosine kinase receptors, the VEGF monomer receptors, at the surface of endothelial cells. VEGFR and VEGF have a common pattern of 8 cysteine residues, which form the binding domain where bounding between the growth factor and its receptor takes place.

When VEGF binds to VEGFR monomer, a dimerization of VEGFR occurs which changes the 3D structure of tyrosine kinase domain. This change in spatial structure allows the autophosphorylation of the intracellular tyrosine kinase residue schematically depicted in Figure 4. The figure shows that, thanks to the ATP, tyrosine kinase phosphorylated serves as docking site for numerous signal transducers that initiate the cascade signaling pathway. VEGFR activated proteins form a linked complex and interactions among them activate the ras protein. It is a GTPase; i.e. a hydrolase which binds and hydrolyzes guanosine triphosphate. It regulates the cell proliferation, and activates the phosphorylation cascade which in turn activates mitogen protein kinase which initiates the cell cycle to cell division [8].

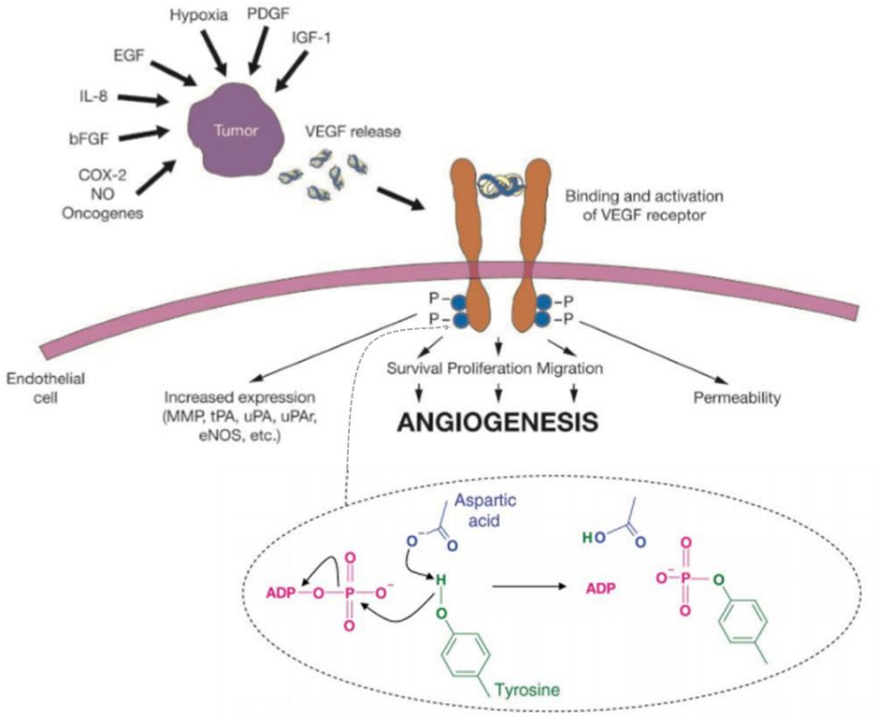


Figure 4: Process of phosphorylation of tyrosine kinase and activation of angiogenesis through VEGFR [9]**.**

Now it will be interesting to focus on the structure of VEGFR. VEGF is a gene family that includes placenta growth factor (PLGF), VEGFA, VEGFB, VEGFC and VEGFD. PLGF acts as an autocrine growth factor, VEGFA is a key regulator of blood vessel growth, VEGFB is responsible for heart vascularization and VEGFC and VEGFD regulate lymphatic angiogenesis. The endothelial cells express two VEGFRs receptor tyrosine kinases. The function of VEGFR1, also called Flt-1, is rather unclear. PLGF and VEGFB bind it but it seems to be a decoy receptor, which decreases the availability of the binding of the other growth factors, VEGFR2. VEGFR2, also named kinase-insert domain containing receptor (KDR) acts as cell surface receptor of VEGFA, VEGFC and VEGFD. It plays an essential role in the regulation of angiogenesis, vascular development, vascular permeability and embryonic hematopoiesis. Moreover, it promotes proliferation, survival, migration and differentiation of endothelial

cells [6]. As shown in Figure 5, the activation of angiogenesis, initiated by the proliferation, is one of the four consequences of the activation of VEGFR2 (survival, permeability, migration and proliferation).

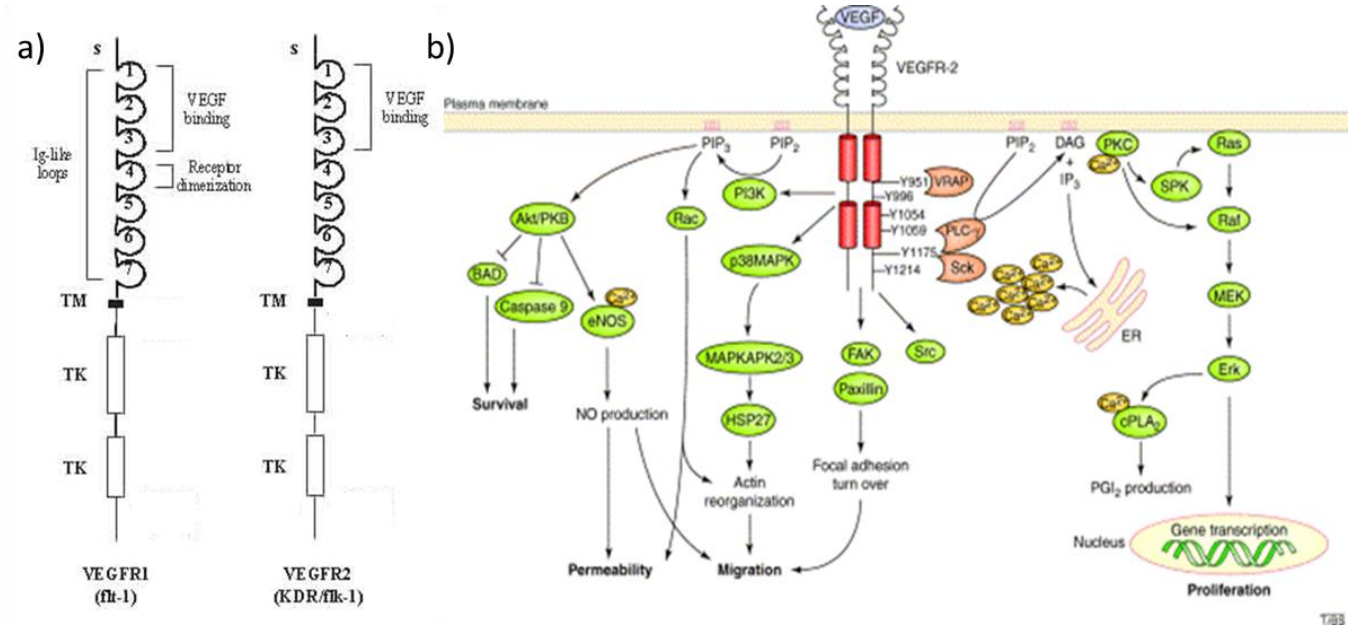


Figure 5 : a) Structures of VEGFR1 and VEGFR2. TM: transmembrane domain; TK: tyrosine kinase domain**. b) Signaling pathways activated by VEGFR2** [10][11]**.**

1.4. EPIDERMAL GROWTH FACTOR (EGF) AND ITS RECEPTORS (EGFR)

The EGFR is also a tyrosine kinase receptor and it must also be detailed because its pathway modulates, among other, angiogenesis by up-regulating VEGF. As shown in Figure 6, it consists in a ligand binding domain, a hydrophobic transmembrane region and a cytoplasmic domain which contains the tyrosine kinase domain. Like for VEGFR, EGFR dimerization is activated by the ligation of a growth factor. In this case the major growth factors are the EGF and the transforming growth factor β (TGF-β). Once the growth factor linked, a dimerization of EGFR occurs and the tyrosine kinase is phosphorylated which leads to activation of intracellular signaling pathways. The activation of EGFR pathway plays an important role in tumor progression (metastasis and cell survival), proliferation, adhesion, differentiation, migration, transformation and mobility (see Figure 6). When EGFR is activated by its growth factors (EGF and TGF-β) it induces the production of VEGF and it thus activates the VEGFR. Subsequently when EGFR is abnormally activated, VEGF is over-produced and VEGFR over activated [9].

It has been shown that the EGFR gene is overexpressed only in cancer cells like gastric, colorectal, pulmonary, bile duct, breast [12], glioblastoma [13][14] and T-lymphoblastic [15] carcinoma cells. It can be noticed that healthy endothelial cells do not express the EGFR [16].

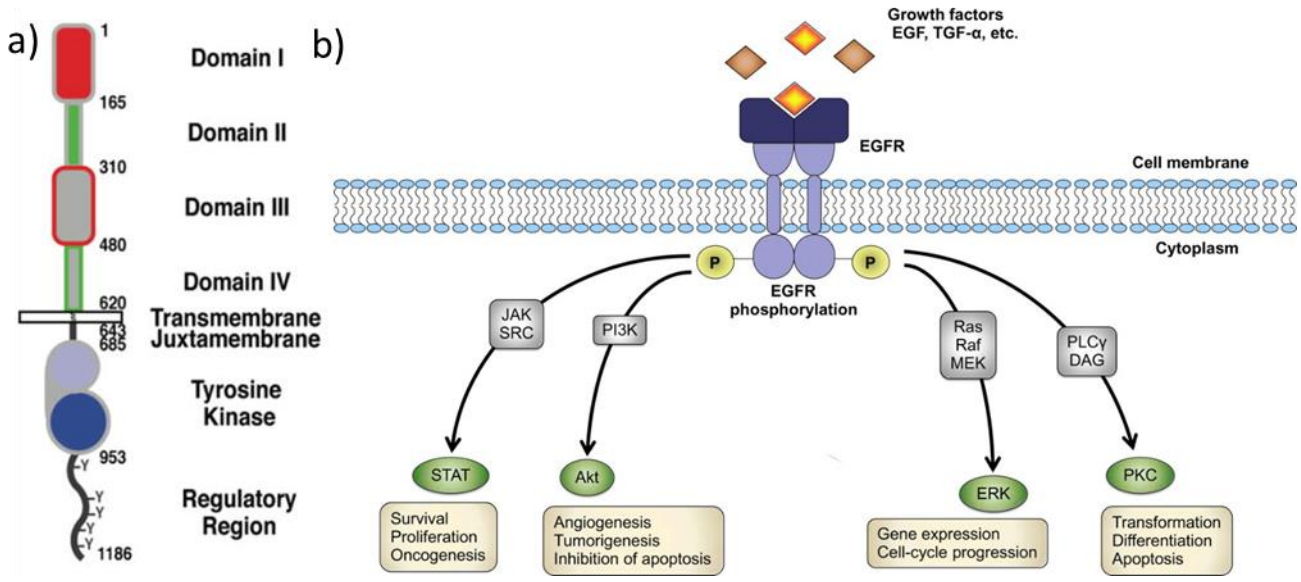


Figure 6 : a) Structure of EGFR. The extracellular domain is the bounding domain. Domain I and III adopt β-helix fold and the domain II and IV are responsible of the disulfide bonded module. The regulatory region is separated from the tyrosine kinase domain when thereof is phosphorylated. **b) EGFR signaling pathway** [17]**.**

1.5. MUTATIONS INDUCING ABNORMAL ANGIOGENESIS

The growth factor receptor mutation is the key for the abnormal activation of angiogenesis. The alterations that can occur are the mutation of the extracellular domain of growth factor receptors, the alteration of ATP binding motif and the modification of the catalytic portion of the kinase. Mutations on extracellular domain of growth factor receptors allow the ligand-independent kinase activation. Generally, mutation occurs at the breakpoints of chromosomal translocation. For example, in leukemia, there is a translocation between the chromosome 9 (break point cluster region: BCR) and 22 (Abelson tyrosine kinase gene: ABL). This fusion creates a new gene that produces a protein with several fold tyrosine kinase activity than normal ABL gene. Another example concern a substitution of leucine to arginine at the position 858 and a deletion in exon 19 of genes coding for the extracellular bonding domain of EGFR. Those mutation cause a "*constitutive activation of the tyrosine kinase of the EGFR by destabilizing its autoinhibited conformation, which is normally maintained in the absence of ligand stimulation*" [18]. This is the most common mutation.

Another mechanism which increases the angiogenic signal is the alteration in regulatory proteins or signaling pathways. An example is the increased of pro-oncogene tyrosine kinase (SRC) activity in colon cancer specimens. This pro-oncogene tyrosine kinase is down-regulated by phosphorylation of a critical carboxyl-terminal tyrosine. In 12% of colon cancer cases, this tyrosine on c-terminal is truncated which involves the remove of the down-regulation and activation of metastasis and consequently activation of the angiogenesis and progression of the cancer [19].

2. Inhibition of angiogenesis by synthetic compounds

2.1. A BRIEF HISTORY

Based on the theory explained in the previous paragraphs, it clearly appears that a way to fight against cancer is to stop the growth of the tumor. The mechanism of the growth of tumor is the angiogenesis, so research is focused on the way to inhibit angiogenesis. It was only when bioassay was developed, in the 1970's, that it was possible to begin research on angiogenic inhibitors. The first discovery was the antiproliferative activity of interferons. It was found that they inhibit the migration of endothelial cells. Some years later, a fumagillin (fungus antimicrobial molecule), TNP-470, was discovered to inhibit cell proliferation without causing endothelial cell apoptosis. It was the first synthetic angiogenesis inhibitor found. However it presented neurotoxicity. The next angiogenesis inhibitor discovered was the Thalidomine but it was related to inhibition of angiogenesis in developing fetal limb bud. Near 10 years later, a new molecule was created based on Thalidomine, the 3-amino Thalidomine, without the negative effects of Thalidomine, which is still used as a first-line therapy for the treatment of advance multiple myeloma. A last molecule discovered which presented angiogenetic inhibition was created on the basis of TNP-470 was Caplostatin which could be administrated in 10-fold dose range than the TNP-470 without causing neurotoxicity [20]. Figure 7 resumes the historical evolution of the discovery of angiogenesis inhibitors.

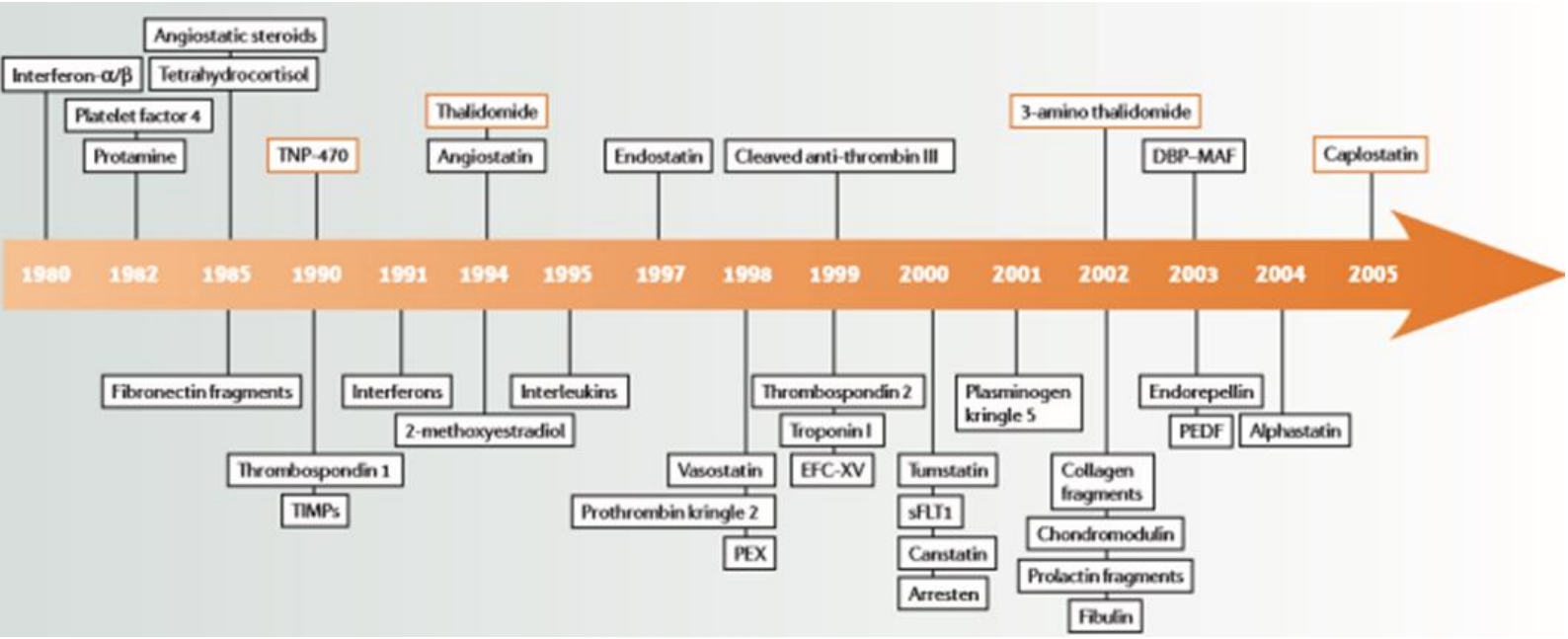


Figure 7 : History of angiogenesis inhibitors discovery. In orange keyline there are the synthetic angiogenesis inhibitors and in black keyline the endogenous angiogenesis inhibitors [3].

2.2. TWO MAIN APPROACHES OF TARGETED THERAPIES FOR ANGIOGENESIS: MONOCLONAL ANTIBODIES AND TYROSINE KINASE INHIBITORS

2.2.1. Monoclonal antibodies

The use of monoclonal antibodies in order to act on angiogenesis was first reported in 1992 when it was shown that monoclonal antibodies of VEGF slowed tumor in nude mice. By this date, great efforts have been done to develop anti-VEGF antibodies resulting in the formulation of the molecule Bevacizumab. It can inhibit all active forms of VEGF by preventing VEGF to bind to the VEGFR. The neutralization of VEGF prevent the formation of neovasculature by limiting the blood supply and hence limiting the development of immature and abnormal blood vessels. The consequence of these effects are the apoptosis of tumor endothelial cells and the decrease in interstitial fluid pressure on the tumor.

Another monoclonal antibody that can be cited is the Cetuximab. Contrary to bevacizumab, it is not bound to the growth factor but to the external part of the growth factor receptor, in this case EGFR. When Cetuximab is bound to EGFR, it prevents the dimerization of EGFR and the ligation of the ligands. Autophosphorilation doesn't occur and no signal to cell proliferation is emitted. Also, Cetuximab down-regulate the HIF-1, as it was mentioned before, it is a hypoxia inducer of angiogenesis. Moreover, Cetuximab binding to EGFR induces the internalization of this complex into the cytoplasm of the cell (endocytosis) for then being destroy by lysosomes [21][22].

However, this response to angiogenesis inhibition is reconsidered because angiogenesis is activated by much more than one angiogenic factors and tumor can turn away the blocking of one angiogenic factor by expressing in higher quantity other ones. Moreover, those monoclonal antibodies just inhibit the angiogenesis but to fight the tumor, they must be combined with chemotherapeutic drugs [23].

2.2.2. Tyrosine kinase receptor inhibitors

The tyrosine kinase receptors inhibitors are hydrophobic small molecules which can pass through the cell membrane. Instead of acting on growth factor or on the extracellular part of the growth factor receptor like antibodies, they act on the tyrosine kinase site of the intracellular part of the growth factor receptor, EGFR and VEGFR, and so, they block the activation of various downstream signaling pathways.

Those small molecules have a chemical structure which looks like the ATP chemical structure. More specifically, they must have the adenine ring that form hydrogen bonds with the ATP-bonding site of the growth factor receptor. Like this the kinase inhibitors can supersede the ATP on the ATP-bonding site. It is worth noting that the ATP-bonding site is the same for most of the protein kinase receptors so the tyrosine kinase receptor inhibitors

can be thought for more than one protein kinase receptor. Those inhibitors can be organized in three groups:

- The first are those who recognize the active conformation of the kinase, they bind to the ATP-binding site by creating one hydrogen bond instead of the three bonds that do normally the ATP. One example is the Sunitinib which targets the VEGFR.
- The second group recognizes the inactive form of the kinase. Like this, a hydrophobic pocket is created and the activation loop is inactivated. It is considered that those inhibitors "modulate kinase activity in an allosteric way". They indirectly compete with the ATP. Sorafenib is an example of such molecule. It acts on VEGFR, PDGFR and other.
- The third group is the "covalent inhibitors". They bind by covalence to cysteines at specific sites of the kinase. The cysteine residue possess a sulfur (electron rich atom) which reacts with an electrophilic group of the inhibitor. Like this the inhibitor and the cysteine residue form a covalent bound by sharing electrons. This prevents the bounding of the ATP to the ATP-binding site. Those inhibitors are principally quinazoline-based inhibitors. An example is Vandetanib which target both VEGFR and EGFR.

Those tyrosine kinase receptor inhibitors are multi-target kinase receptors. This is an advantage only if the inhibited tyrosine kinase receptors are known. Otherwise, this non-specific inhibition can act on other tyrosine kinase receptor resulting in toxicity effects (hypertension, diarrhea, nausea etc…). The first type on inhibition is the most toxic because the tyrosine kinase inhibitor must be as less specific as possible to fix any tyrosine kinase domain. The third is the most specific because of its irreversible mechanism of binding to a cytosine residue.

One of the problem of tyrosine kinase inhibitors is that mutations can occur on receptor and phosphorylation of the target kinase which allow the angiogenesis to take place. Another problem is the toxicity of those inhibitors because they can interfere on signaling pathways and decrease endothelial cell renewal capacity. To avoid this, genetic analyses must be done to predict treatment-related toxicity and, depending of results, adjust the dose or consider alternative inhibitors [24].

Quinazoline derived compounds :

The identification of a small molecule which can inhibit angiogenesis by inactivating many pathways without being toxic for human is a real challenge. Anillinoquinazoline or quinazoline derived compounds (shown in Figure 8) seem to be a good compromise. From a study done in the USA, it has been demonstrated that the quinazoline compounds act essentially on EGFR (Afatinib and Labatinib) and some of them on VEGFR too (Vandetanib) [25]. It seems that those compounds act even if the cell lines develop resistance to some inhibitors (Gefitinib and Erlotinib for example). Another advantage of using quinazoline compounds is that once the compound bonded to the ATP binding site, it is not released and so its action is permanent.

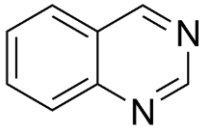


Figure 8 : Quinazoline structure

As said in the previous paragraph, quinazoline are essentially known to be covalent inhibitors thus it is this process that will be described hereafter. The process of this covalent inhibition is the redox reaction between the quinazoline ring of the inhibitor and a cysteine located near the ATP binding site. The goal is to find a molecule that can make covalent interaction with cysteines on VEGFR and EGFR either. The two important cysteines to do a covalent bond are the cysteine located at position 773 in EGFR and the cysteine located at the position 1045 in VEGFR. An example of a targeted molecule is presented in Figure 9 [26]. This example shows that from one molecule, two interesting pathways for this study of angiogenesis are inhibited. This is the aim of the novel research on quinazoline tyrosine kinase receptor inhibitors.

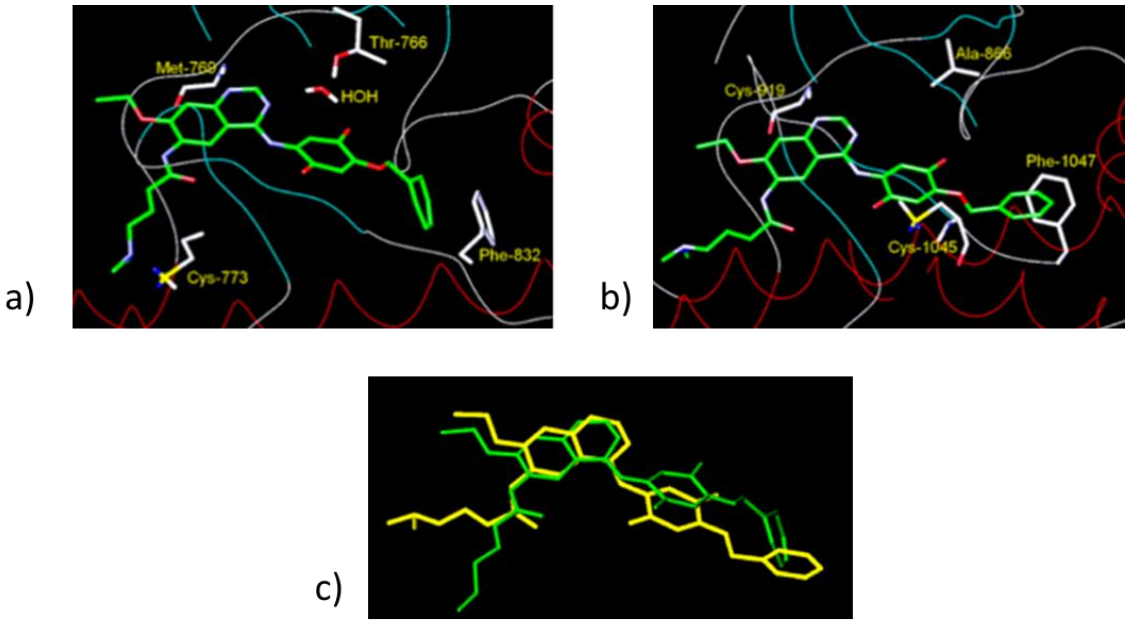


Figure 9 : : View of an overlap expected quinazoline derived molecule. a) quinazoline derived molecule bonded the kinase domain EGFR. b) quinazoline derived molecule bonded the VEGFR kinase domain. c) overlap of the same molecule in different 3D space. In green the bounding to the EGFR and in yellow the bounding to the VEGFR

The Figure 10 shows the action of the two types of angiogiogenic inhibitors.

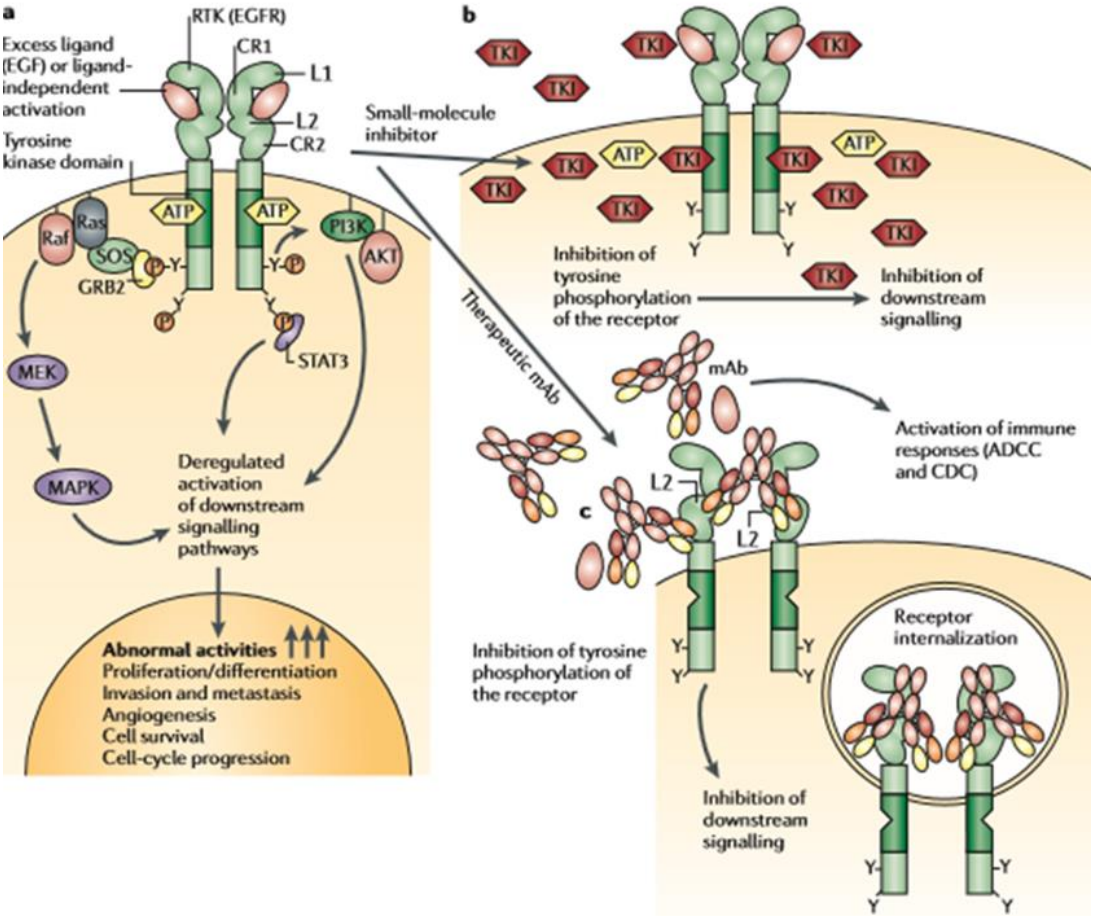


Figure 10 : Two principal approaches of inhibition by the EGFR pathway. A) EGFR normal pathway when there are an over production of growth factors. It can be notice that not only angiogenesis is amplified B) Inhibition of angiogenesis through tyrosine kinase inhibitor. C) Monoclonal antibody inhibitor for the angiogenesis inhibition [27].

3. Aim of the study

The goal of this study is to test 22 synthetized compounds if they have a negative effect on endothelial cell proliferation. By knowing the relation between cell proliferation and angiogenesis, it can be said that those which have an effect on endothelial cell proliferation, will also affect the angiogenesis.

4. Materials and methods

4.1. REAGENTS, INSTRUMENTS AND SUPPLIES

In this part, all the reagents, instruments and supplies which were used during this thesis will be presented in form of tables. For each of them, it will be also presented the company and the country of origin where they are purchased from.

Table 1: Reagents, companies and localizations

Reagents	Provider	Localization
Culture medium (DMEM 1X) *Dulbecco's Modified Eagle Medium*	**GIBCO**	**USA**
PBS Dulbecco 1X *Phosphate Buffer Saline w/o Ca^{2+} and Mg^{2+}*	**GIBCO**	**USA**
FBS *Fetal Bovine Serum*	**GIBCO**	**USA**
Antibiotics *Penicillin, Streptomycin*	**BIOCHROM A6**	**GERMANY**
Trypsin 0.05% - EDTA *EthyleneDiamineTetraacetic Acid*	**GIBCO**	**USA**
DMSO *Dimethyl sulfoxide*	**APPLICHEM**	**GERMANY**
MTT medium 10X	**SIGMA**	**USA/GERMANY**
HCL medium 1N	**SIGMA**	**USA/GERMANY**
HAT medium *Hypoxanthine-Aminopterin-Thymidine*	**GIBCO**	**USA**
BSA *Bovine Serum Albumine*	**SIGMA**	**USA/GRMANY**

Table 2: Instruments, companies and localizations

Instrument	Company production	Localization
Multimate *Laminar flow chamber*	**SAFEMATE 1.2** *Euroclone s.p.a*	**ITALY**
Centrifuge	**THERMO FISHER SCIENTIFIC**	**GERMANY**
Incubator *37°C – 5% CO_2*	**THERMO SCIENTIFIC**	**USA**
Microscope	**CARL ZEISS AG**	**GERMANY**
Water Bath	**BACACOS SCIENTIFIC**	**GREECE**
Photometer	**TECAN**	**AUSTRIA**
Special refrigeration container for cells -80°C *Liquid nitrogen*	**CRYOMED THERMO SCIENTIFIC**	**USA**
Hemocytometer	**REICHERT**	**USA**
Pipettes	**LABMATE**	**USA**
Pipette gun	**COSTAR**	**USA**

Table 3: Supplies, companies and localizations

Supplies	Company production	Localization
Sterile tubes *Falcon 10 mL, 50 mL*	**SARSTEDT**	**GERMANY**
Special cell culture plates *10cm*	**SARSTEDT**	**GERMANY**
Pipettes one use *5-10 mL*	**SARSTEDT**	**GERMANY**
Pipettes one use *25 mL*	**COSTAR**	**USA**
Eppendorf	**SARSTED**	**GERMANY**
Cryotubes	**GREINER BIO-ONE**	**GERMANY**
96 wells plate	**GREINER BIO-ONE**	**GERMANY**
Tips *Blue and white*	**GREINER BIO-ONE**	**GERMANY**
Tips *Yellow*	**CORNING INCORPORATED**	**USA**

4.2. SYNTHETIC COMPOUNDS

In this thesis, focus was put on 22 synthetic compounds, manufactured from urea and having a final shape based on quinazoline.

The compounds tested are synthetized by the Department of Pharmacy of the National and Kapodistrian University of Athens. They are synthetized based on anillinoquinazoline in order to create a core pyrimidopyrimidine (show in Figure 11). Some derivatives of pyrimidopyrimidines show an inhibition of proliferation of breast cancer cells [28] and may be worth investigating if they have an impact on angiogenesis and more specifically on VEGFR and EGFR.

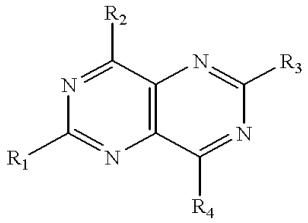
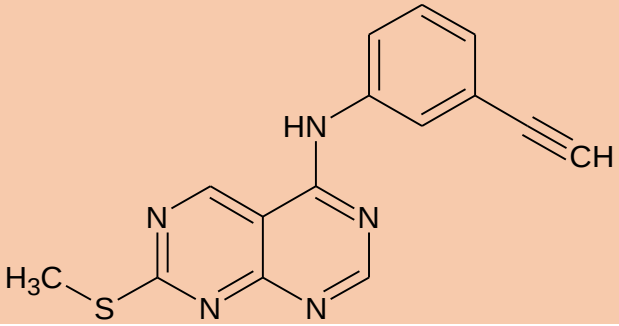
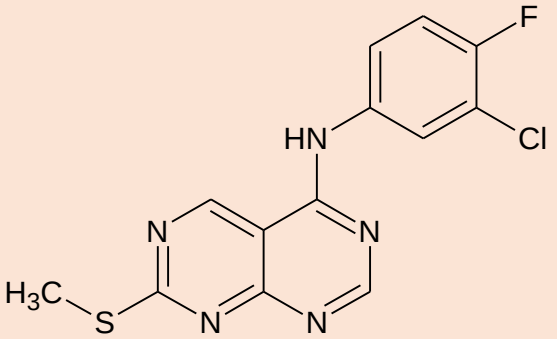
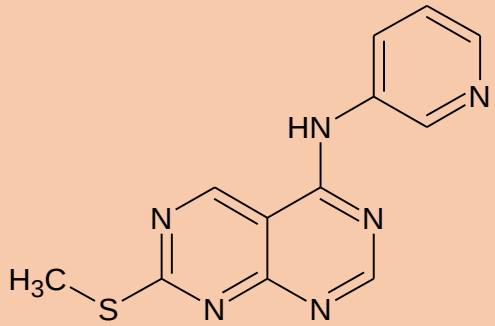
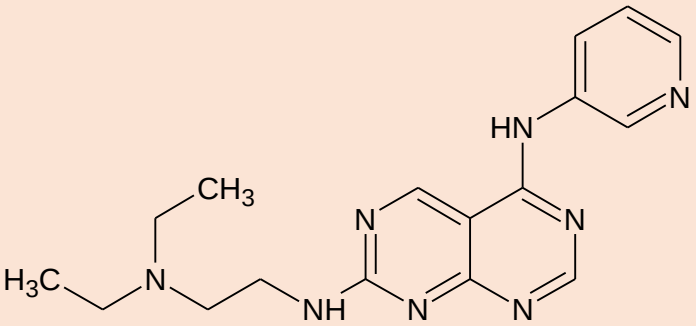
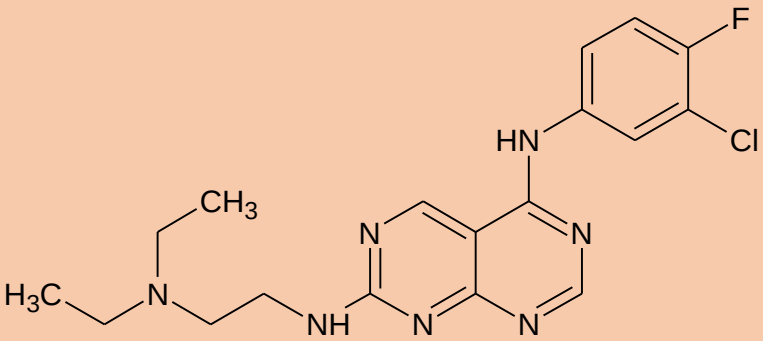
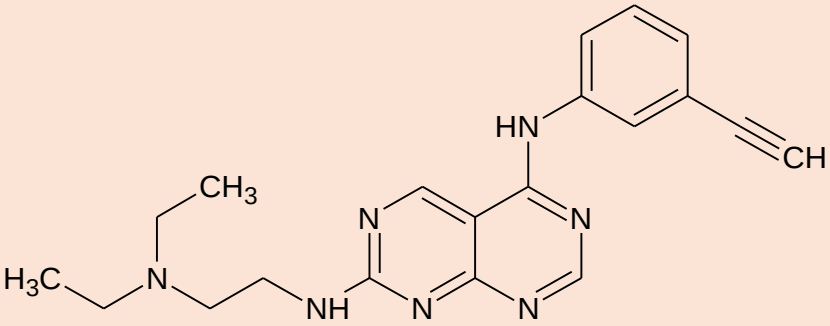
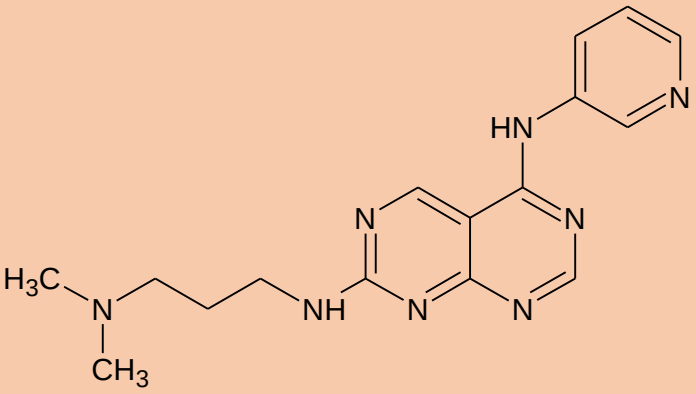


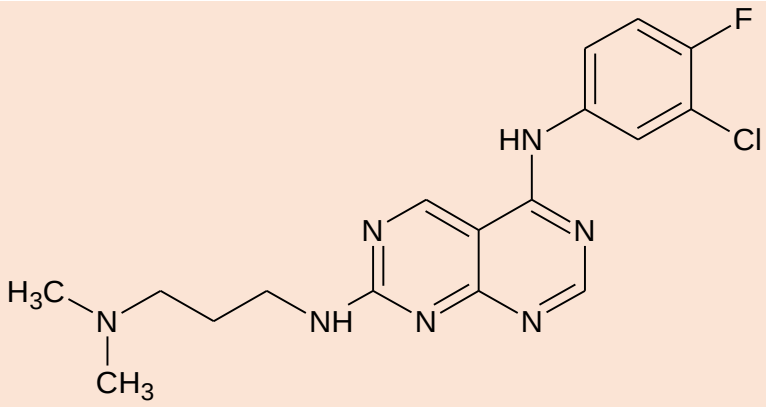
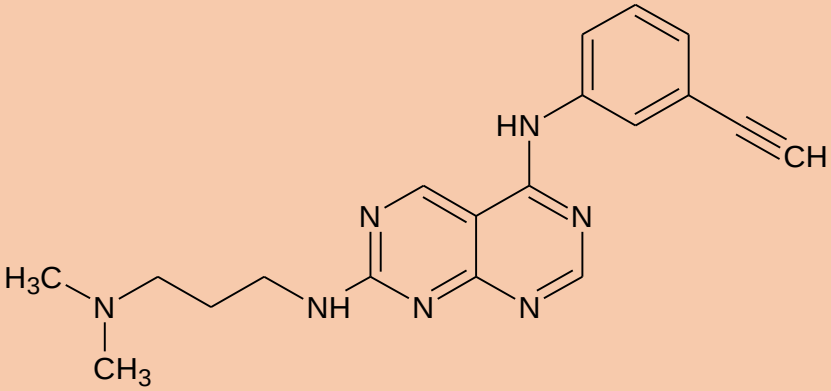
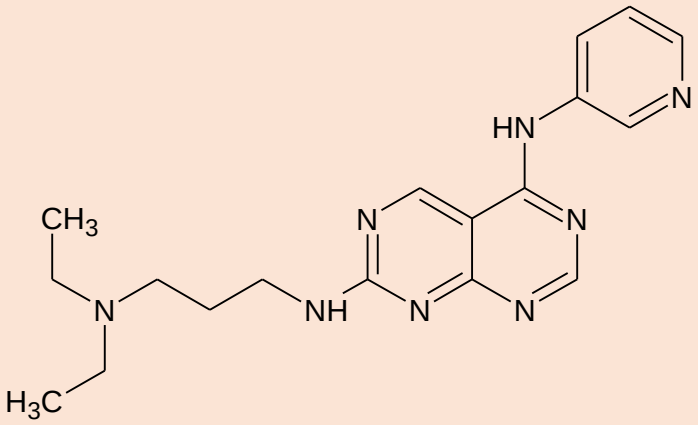
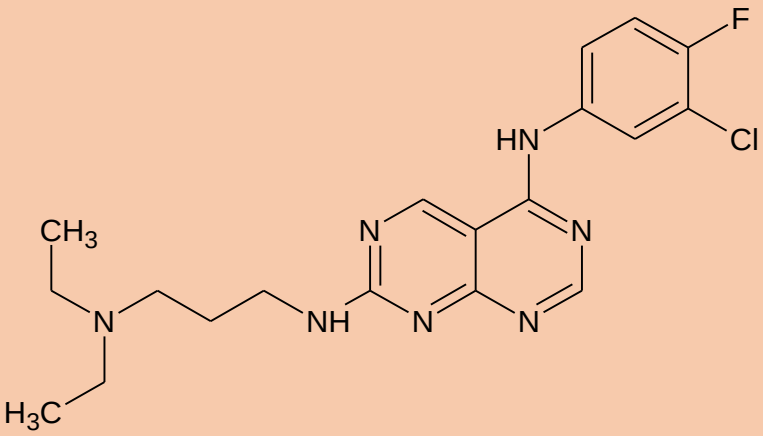
Figure 11: Structure of pyrimidopyrimidine

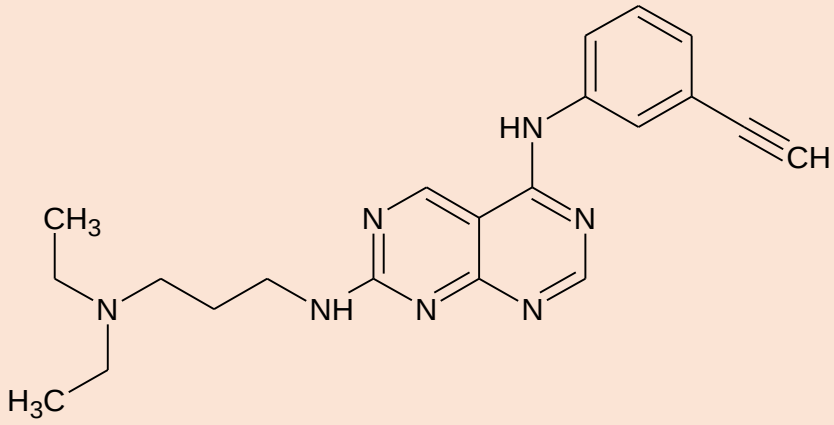
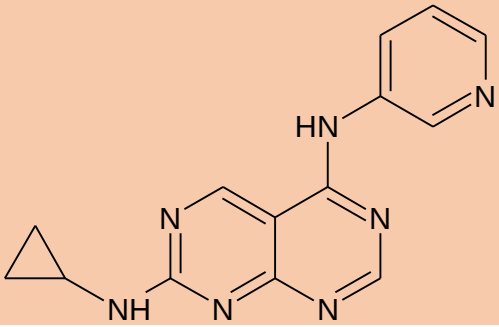
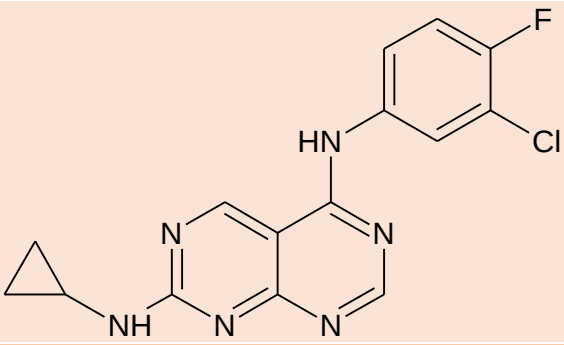
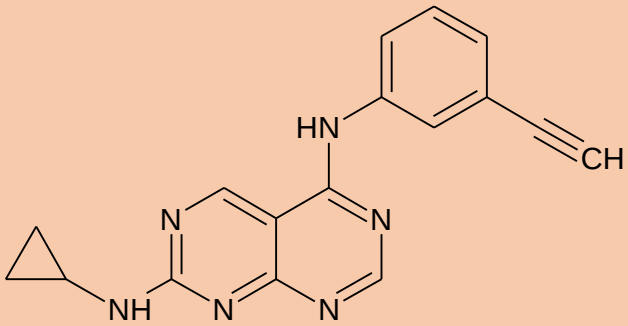
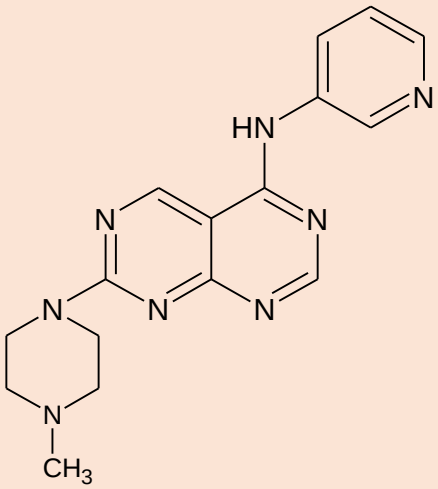
Table 4 resumes the chemical structures of the 22 tested compounds with their assigned number, their average mass and their molecular formula.

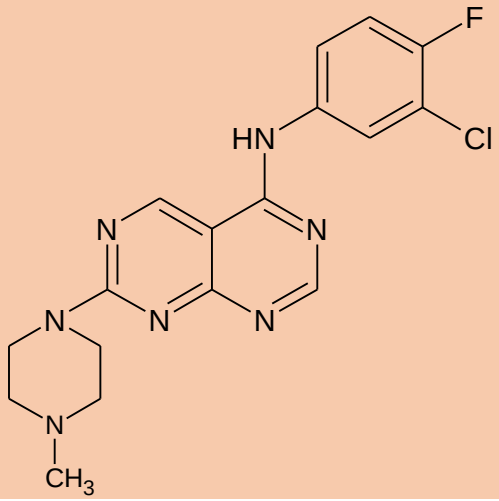
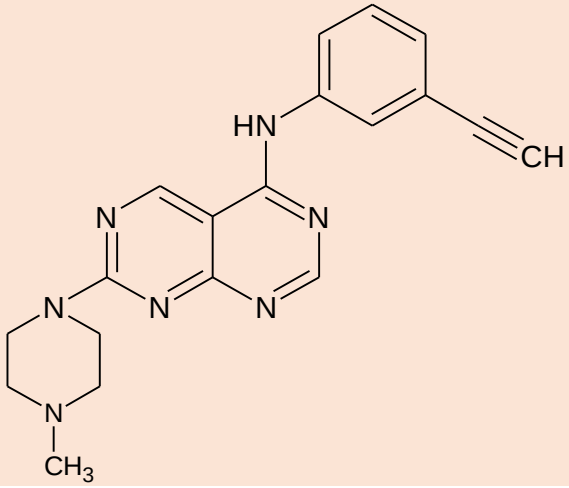
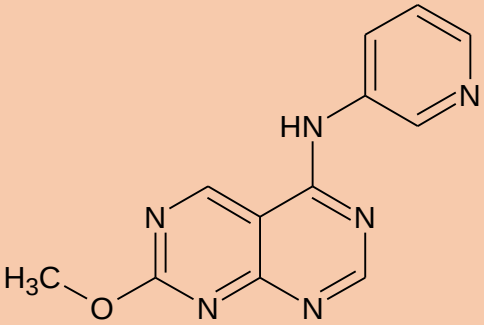
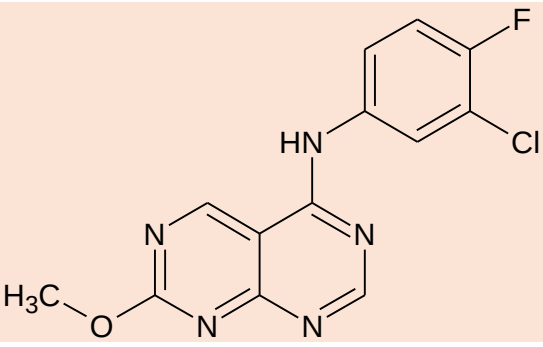
Table 4: Name, structure, average mass and molecular formula of tested compounds.

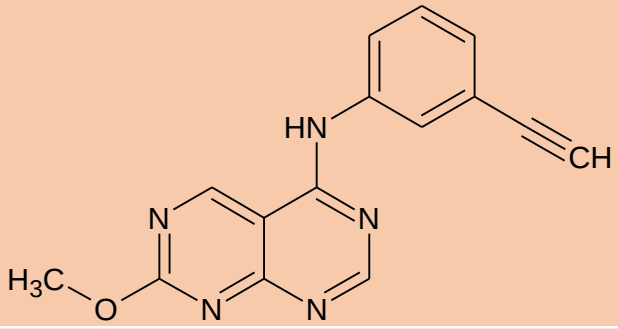
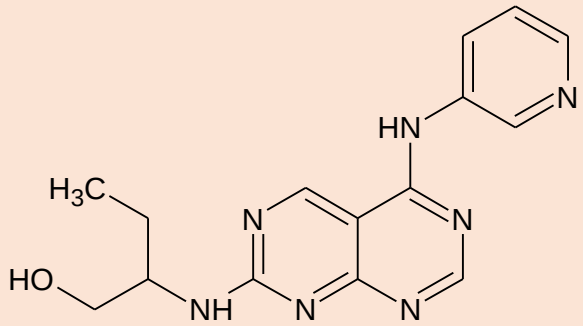
Name	Structure	Average Mass	Molecular Formula
CEM72		293.3463 Da	$C_{15}H_{11}N_5S$
CEM77		321.7605 Da	$C_{13}H_9ClFN_5S$

CEM80		270.313 Da	$C_{12}H_{10}N_6S$
CEM111		338.4102 Da	$C_{17}H_{22}N_8$
CEM78		389.8576 Da	$C_{18}H_{21}ClFN_7$
CEM149		361.4435 Da	$C_{20}H_{23}N_7$
CEM120		324.3836 Da	$C_{16}H_{20}N_8$

CEM121		375.8311 Da	$C_{17}H_{19}ClFN_7$
CEM122		347.4169 Da	$C_{19}H_{21}N_7$
CEM139		352.4368 Da	$C_{17}H_{23}N_8$
CEM140		403.8842 Da	$C_{19}H_{23}ClFN_7$

CEM141		375.4701 Da	$C_{21}H_{25}N_7$
CEM164		279.2999 Da	$C_{14}H_{13}N_7$
CEM162		330.7474 Da	$C_{15}H_{12}ClFN_6$
CEM163		302.3333 Da	$C_{17}H_{14}N_6$
CEM167		322.3677 Da	$C_{16}H_{18}N_8$

CEM168		373.8152 Da	$C_{17}H_{17}ClFN_7$
CEM169		345.4011 Da	$C_{19}H_{19}N_7$
CEM174		254.2474 Da	$C_{12}H_{10}N_6O$
CEM173		305.6949 Da	$C_{13}H_9ClFN_5O$

CEM175		277.2807 Da	$C_{15}H_{11}N_5O$
CEM97		311.3418 Da	$C_{15}H_{17}N_7O$

4.3. METHODOLOGY FOR THE TREATMENT OF ENDOTHELIAL CELLS BY CHEMICAL COMPOUNDS

4.3.1. Cell line and cell culture

Cell culture is used to study the behaviour of cells. It allows removing all reactions which take place *in vivo* in order to see the response at cell level only. By this method it is possible to control all the conditions of cell growth: culture medium, temperature and pH. The cell line used is EAhy 926. These are adherent endothelial cells from fusing human umbilical vein endothelial cells (HUVECs) with cells of cancerous lung tissue (A549) by exposure to polyethylene glycol (PEG) and then selected in HAT medium [29]. Some conditions are mandatory for the life of cells: operating in a vertical laminar flow chamber in order to ensure sterilized conditions, maintaining the incubator at 37°C and at a stable pH regulated by the injection of 5% $C0_2$. Finaly, the culture of the cells must be done with medium DMEM supplemented by 10% FBS, antibiotics penicillin and streptomycin and HAT to place the cells under their best environmental condition.

4.3.2. Passage of cells

The passage of cells is used to avoid the confluence and death of cells. It also ensures a specific amount of cells which is required for some experiments. In every case, the coverage of Petri dish by EAhy 926 cells mustn't exceed 90% of the plate surface. The situation according to which the cells cover all the Petri dish, is called confluence. When the cells become confluent, some proteins are produced which stop the cell division and create

contact inhibition. If this occurs, a dilution of the cells in a new Petri dish must be done, this is the passage of cells.

Methodology:

Passage of cells is used when the cells become confluent or they must be used for one experiment. All solutions used must be pre-warmed at 37°C in order to be at cell temperature.
The Petri dish with the cells is transferred from the incubator to the hood, in operation.
First, cell splitting is done. With this method, the adherent cells are detached from their support. The old medium of the Petri dish is aspirated with the Pasteur pipette under vacuum. Then the Petri dish is washed with PBS twice. The PBS is used to wash the cells from death cells and remaining old medium because it contains serum inhibiting the trypsin. Then, 3 mL of trypsin are added. Trypsin is a digestive enzyme found in pancreas. Its role is breaking down the cells bonds. The trypsin acts for 2-3 min and its activity is controlled by monitoring the detachment of cell and their rounded under microscope. Once the cells are detached, at least the same quantity of DMEM full (full culture medium) is added quickly to inactivate the trypsin. If the trypsin remains longer it kills the cells. Immediately after inactivating the trypsin, the mix (trypsin inactivated and DMEM full) is pipetted. This operation is realized many times all over the Petri dish to be sure that all the cells are taken. Then, all the solution, with the detached cells, is put into 15mL tubes for centrifugation. The centrifugation is carried out at room temperature and acceleration of 300 g for 5 min. Once the centrifugation is done, the supernatant (trypsin + medium) is discarded and the cells are re-suspended using 5-7 mL of DMEM full. Finally, the cells are re-suspended and put, with the new medium, on a new plate.

4.3.3. Cells counting

The cell counting by using a hemocytometer (or Neubauer chamber) is the simplest and cheapest method to access the number of many cells in a solution. The Neubauer chamber, shown in Figure 12, is a thick crystal slide with a thin glass slide. On its central area, there are two counting zones which can be independently filled. The counting area has a grid of 3 x 3 mm^2 and is subdivided into 9 squares of width 1 mm. Each of these 9 are divided into 16 squares to facilitate the counting [30].

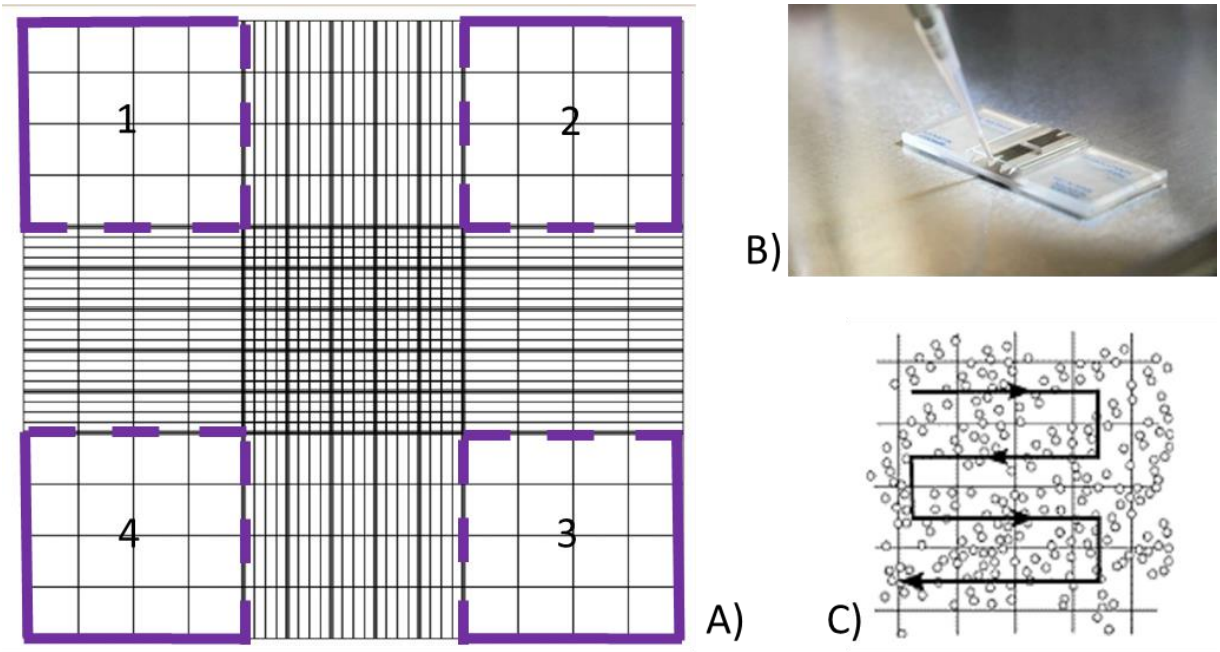


Figure 12 : Hemocytometer Neubauer. A) The 4 squares (1, 2, 3, 4) where counting takes place. Cells are counted on outside lines too (continuous violet line) but not on the inside lines (discontinuous violet lines). B) Method to fill the hemocytometer. C) Method of counting inside the square.

On the crystal slide, there are two ridges on which the coverslip can be placed and leaves a 1 mm space between those two compounds. It is in this space that the solution with the cells is placed and spreads. The volume of cell suspension of one square (1, 2, 3 or 4 in Figure 12) is 0.1 mm^3 (=10^{-4} mL), so the concentration (in cells/mL) of cells in the initial suspension is 10^4 higher than that on the square.

Methodology:

First, the hemocytometer is prepared. It is cleaned with 70% ethanol and the coverslip is bonded. Then, the cells are re-suspended with a pipet until not seeing any more pellets. 10 µL of the suspension are taken and put on the hemocytometer. After that, the Naubauer chamber is placed under the microscope at the 10X magnification and the cells are counted following the method explained in Figure 12. Finally, the cell number of the overall suspension is determined.

Example of counting the cell number:

Assume that there are 117 cells on the first square, on the second 101 cells, on the third 115 and on the fourth 108. So in total there are 441 cells on the 4 squares. This corresponds to:

$$\frac{441}{4} * 10^4 = 1{,}1025 * 10^6 \frac{cells}{mL}$$

This means that the concentration C1 of the suspension is 1,1025*10^6 cells/mL. If the overall suspension has a volume of 7mL, the number of cells in suspension is:

$$1{,}1025 * 10^6 * 7 = 7{,}7175 * 10^6\ cells$$

The next step is to evaluate the volume V1 that must be taken to have a final cells concentration C2 of $4x10^4$ cells/mL. Moreover, if the final volume of so as solution desired is 15 mL, it is needed:

$$C1 * V1 = C2 * V2$$

$$110.25 * 10^4 * V1 = 4 * 10^4 * 15$$

$$V1 = 0.544\ mL$$

So, 0.544 mL of the initial cell suspension must be taken and 14.456 mL of DMEM full (complete medium) must be added so as to have a final concentration of $4x10^4$ cells/mL.

4.3.4. Freezing and thawing cells

In order to preserve the cells after the complementation of an experiment, so as to avoid buying new ones, the freezing of cells was developed. With this method, the cells can be preserved for a long period of time in liquid nitrogen (LN2). Before freezing cells, it must be verified that the cells are in good shape, confluent and healthy.

Solutions:

- Freezing medium: Culture medium (DMEM 1X, *Gibco*) with 20% foetal bovine serum (FBS, *Gibco*), 1% antibiotics (Pen/Str, *Biochrom*), 1% HAT (*Gibco)* and 10% Dimethyl sulfoxide solution (DMSO, *Applichem*)
- Confluent cells in a 10cm Petri dish
- Thawing medium: It is the complete culture medium (Culture medium (DMEM 1X, *Gibco*) with 10% foetal bovine serum (FBS, *Gibco*), 1% antibiotics (Pen/Str, *Biochrom*), 1% HAT (*Gibco)*)

Methodology of freezing:

First of all, the cells must be detached from the Petri dish as explained above in paragraph 4.3.2. The solution is placed in a 15 mL tube and centrifuged. After having removed the supernatant, the cells are re-suspended into 1mL of freezing medium. The DMSO is used to protect the cells by partially solubilizing the membrane so that it is less prone to puncture and interrupting the formation or crystals. Moreover, in order to prevent the formation of crystals that can puncture the plasma membrane and lead to cell death the freezing must be very slow. The solution is then divided into two cryotubes of 0.5 mL each. Each cryotube is labeled, allowing identification of the nature of cells, of the date etc. The two cryotubes are transferred into a freezer of -80 °C for 12 h and then, they are transferred into LN2 where they can be stored for years if necessary.

Methodology of thawing:

The procedure must be as sterile and as fast as possible. The cells must remain as few as possible in DMSO which is toxic medium for them.

Firstly, the cryotube with the frozen cells is removed from LN2 and quickly transferred into the water bath at 37 °C. The cells remain there until half of the crystal is melt.
Then, all the cells are transferred into a 15 mL tube which contains 7 mL of complete culture medium. The aim of this step is to dilute the DMSO in order to limit its toxicity. It is followed by a centrifugation at 300 g during 5 min at room temperature. The supernatant is removed and 4 mL of complete culture medium is added. After homogenization of the cells, the cell solution is put on a 10cm Petri dish with additional 10mL of complete culture medium. The dish is put in the incubator at 37°C and 5% CO_2.

4.3.5. Starvation

The starvation is used for the synchronization of cell cycle to minimise the effect of growth factors present in serum that could mask the effects of tested compounds.

Solutions:

- Starvation medium: Culture medium with 0.125% BSA and antibiotics, HAT
- PBS Dulbecco 1X

Methodology:

Cells that are cultivated in 96 well plates are transferred into the hood. Each well is washed two times with 100µL PBS prior adding 100µL of starvation medium. The starvation medium consists in DMEM with 0.125% BSA. BSA is a major component of FBS. It provides protection from oxidative damage and stabilization of other media component [31].
Finally, the 96 well plates are placed for 3-4 h in the incubator.

4.3.6. Determination of cell proliferation by the MTT assay

The MTT assay is used for assessing cell viability. The NAD(P)H-dependent oxydoreductase enzymes produced in mitochondria of the cell reduce the MTT being on the culture medium and result in an insoluble product, the formazan. Only the viable cells can produce this enzyme so, by knowing the quantity of formazan, the number of living cells in suspension can be known. To break down the formazan, a solution of 0.1 N HCL is used. MTT is a colorimetric assay because the colour of the cell culture passes from yellow, colour of MTT, to orange dye with the formazan [32]. The mechanism of this reaction is schematically illustrated in Figure 13:

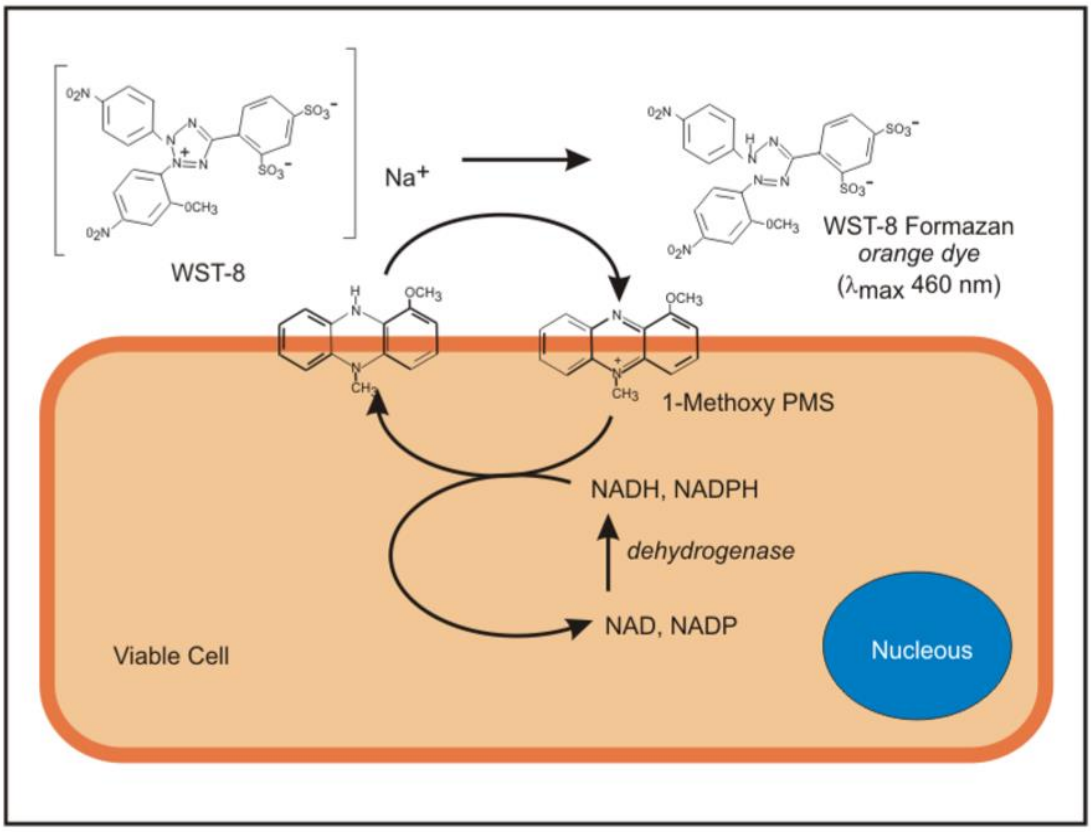


Figure 13 : MTT reaction. MTT reduction by mitochondrial reductase in live cells give insoluble formazan [32].

Solutions:

- Complete culture medium: Culture medium with 10% foetal bovine serum, antibiotics) and HAT
- PBS Dulbecco 1X
- MTT solution 10X
- HCL solution 0.1N
- BSA

Methodology:

Firstly, the cells are passaged in 96 well plates with the concentration of 4000 cells/0.1mL of complete culture medium in each well. After incubation overnight, the 96 well plates are washed twice with 100µL PBS and 100µL starvation medium added. During starvation time, the compounds are prepared.

The initial concentration of the compounds is 100mM or 75mM in 100% DMSO. It is achieved by a series of dilution with culture medium. DMSO is required to achieve the final concentrations of 10µM and 1µM. It is used because of its high ability to solubilize organic compounds. Its final concentration must always be 0.1% to not be toxic for cells.
Once the compounds are prepared and the starvation is done, the starvation medium is removed and 100µL of the solution with the compound is added. For the sake of reproducibility, 8 wells are completed for each concentration and compound. Moreover, 16 wells with cells are filled with complete culture medium with 0.1% DMSO and one well without cells is filled and plays the role of blank for the spectrophotometer. The obtained plate is schematically presented in Figure 14.

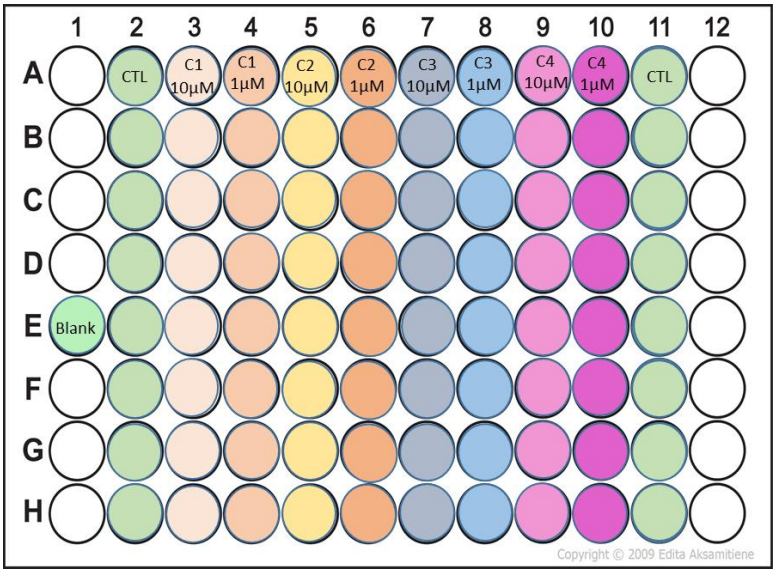


Figure 14 : Representation of a 96 well plate with 4 compounds (C1, C2, C3, C4) at 2 concentrations (10µM and 1µM) and with the controls (CTL) and the blank.

Then, the well plates are incubated for 48 h. During this incubation, it is essential to survey the cell proliferation in order to follow up the activity of the synthetic compounds.

4 h before the completion of the incubation, the 96 well plate is placed under the hood and 10µL of a solution containing 5mg/mL MTT is added on the top of each well. In this step, the use of MTT must be performed carefully and using aluminium foil because the MTT is photosensitive. The 96 well plate is replaced into the incubator. When the incubation is finished, 100 µL of isopropanol (with 0.1N HCL) is added in each well. As mentioned above, the isopropanol serves to break the crystals of formazan and by this way, allow the measure of absorbance of the suspension by a spectrophotometer.

After ensuring that all the crystals have been diluted (by pipetting with a multichannel pipette and seeing on microscope), the absorbance is read at 595 nm with a correction at 750 nm to remove the noise of the plate surface. Lastly, the results are analysed to assess the cell number by referring to the standard curve.

4.3.7. Standard Curve

The standard curve is used to establish the relation between cell number and absorption. So, for a known absorption, it will be possible to know the corresponding cell number. By knowing the latter, it is possible to evaluate the results of absorbance taken with the MTT assay.

Methodology:

The back plate which consists of a confluent 10cm Petri dish is taken from the incubator and put into the hood. Cell trypsinization and counting cells are done. Then, depending on the needed range of concentration of the standard curve, dilutions are prepared. In the following example, the initial concentration of cells is 2000 cells/µL and those values would

be taken to do the standard curve: 25000 cells, 20000 cells, 18000 cells, 15000 cells, 10000 cells and 8000 cells. 100µL of the solutions are put on wells of a 96 well plate.

The dilutions are done successively as explained in Figure 15.

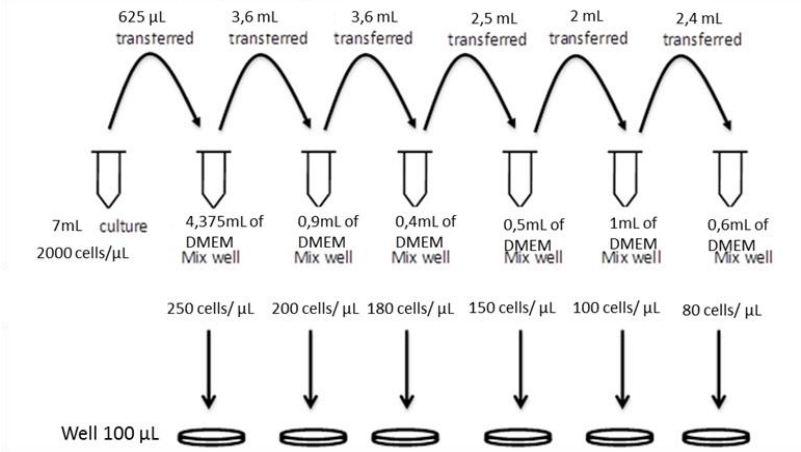


Figure 15 : Dilutions for standard curve

Next, the plate is put into the incubator overnight. The next day, by using MTT assay the number of viable cells is measured into each wells. Finally, the results feed an *Excel* sheetand the standard curve is done.

4.4. STATISTICAL ANALYSIS

The results taken from the MTT assay are transferred on an *Excel* sheet. The values obtained are expressed in optical density. Thanks to the standard curve the values are converted into cell number and then, in percentage of cell number of the control. Following, the results are transferred into the *Graphpad* software in order to proceed to a statistical analysis. The selected statistical model is the Mann-Whitney t-test analysis which provided estimates of how close are two series of measurements. In practice, this concept is expressed as p-value. When $p>0.05$, there is not significant difference (**ns**), when $0.01<p<0.05$, there is significant difference (*), when $0.001<p<0.01$ there is very significant difference (**) and when $p<0.001$ there is extremely significant difference (***). During this thesis, all obtained values are compared by t-test with the control. The final values, +/- SEM (standard error of the mean), are associated with a p-value.

5. Results

5.1. STANDARD CURVE

To facilitate understanding of the following results, those obtained with the standard curve will be presented first. In fact, the data taken from the MTT assay are expressed in optical density (OD) and must be first translated in cells number and then in percentage of control cells number. Control cells are those which are incubated with DMEM full and 0.1% of DMSO.

For that purpose, a standard curve must be created. This is achieved by creating a group of values of cells with known number and the corresponding OD, as explained in paragraph 4.3.7. The OD values are found thanks to photometry of the 96 well plate at 595nm and a correction at 750nm.

The Table 5 and Figure 16 hereafter summarize the results obtained from a representative standard curve.

Table 5: Optical density depending on cell number

Cell number	Optical density
2000	0.060
4000	0.114
10 000	0.225
18 000	0.286
25 000	0.302

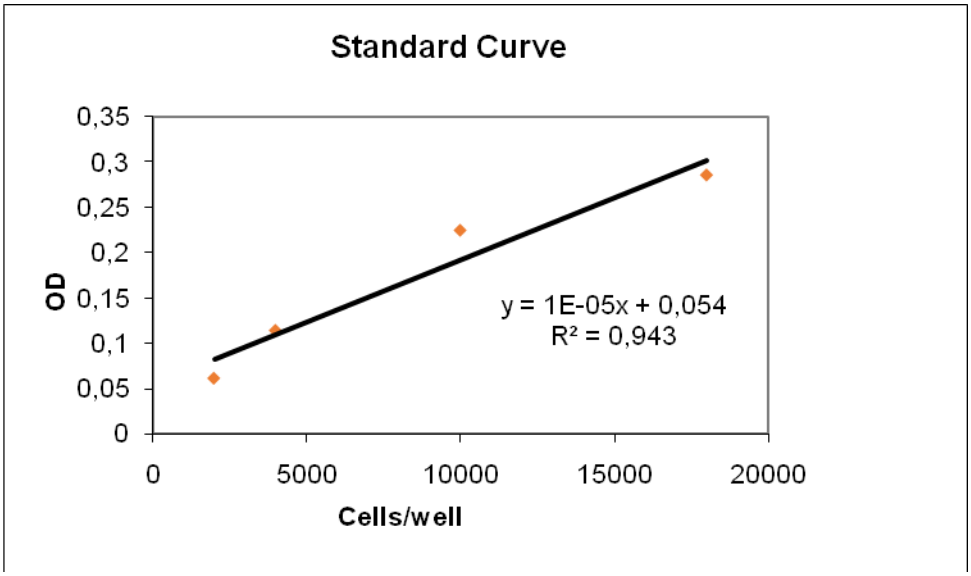


Figure 16 : Standard curve

In this example, the coefficient R^2 equals 0.943 which is a satisfactory value. Moreover, the corresponding equation found will be used to calculate the cell number if OD is known according to the formula hereafter:

$$OD = 1 * 10^{-5} * cell\ number + 0.0547$$

$$cell\ number = \frac{OD - 0.0547}{1 * 10^{-5}}$$

This formula is one of those used in the following calculations.

5.2. EFFECT OF TESTED COMPOUNDS ON CELL PROLIFERATION

Table 6 summarizes the results of the influence of all the compounds on cell proliferation.

Table 6: Summary table of the effect of compounds on cell proliferation.

Compound	Concentration	Cell number (% control)	% SEM	Mann-Whitney t-test
72	1 µM	107,34	2.52	*
	10 µM	42.58	3.79	***
77	1 µM	79.81	2.57	***
	10 µM	42.22	1.00	***
168	1 µM	94.21	2.93	ns
	10 µM	29.80	3.39	***
80	1 µM	99.70	3.58	Ns
	10 µM	76.52	2.57	***
78	1 µM	94,96	3,17	ns
	10 µM	89,25	3,40	**
97	1 µM	102,88	3,77	ns

	10 µM	88,47	4,98	**
111	1 µM	93,94	3,96	ns
	10 µM	83,18	3,66	***
122	1 µM	106,09	3,17	ns
	10 µM	81,58	3,91	***
149	1 µM	94,06	3,74	**
	10 µM	86,39	2,04	***
164	1 µM	85,34	3,07	***
	10 µM	83,63	1,53	***
173	1 µM	93.99	1.70	**
	10 µM	82.85	3.20	***
169	1 µM	92.92	1.24	***
	10 µM	80.42	2.81	***
162	1uM	95,59	2,59	ns
	10uM	93,24	3,24	ns
174	1uM	97,93	2,67	ns
	10uM	93,42	1,53	**
175	1uM	90,33	3,27	**
	10uM	100,02	2,58	ns
120	1uM	102,88	4,81	ns
	10uM	94,96	3,11	ns
121	1uM	95,98	2,40	ns
	10uM	98,37	3,34	ns
139	1uM	103,32	2,51	ns
	10uM	95,58	2,50	ns
140	1uM	100,42	3,69	ns
	10uM	98,61	3,52	ns
141	1uM	102,43	3,80	ns
	10uM	94,43	3,87	ns
163	1uM	102,71	3,78	ns
	10uM	99,59	3,59	ns
167	1uM	99,88	2,28	ns
	10uM	90,69	2,63	**

From the above table, it seems appropriate to separate the results taken from the MTT into three groups, depending on the effect of the compound on inhibition of cell proliferation at 10µM (the higher concentration of compound) express in % of control.

The first group concerns the compounds which lead to high cell proliferation inhibition. They are presented in Figure 17. In this group, there are three compounds: 72, 77 and 168. The corresponding cell numbers after growing with those compounds are 8975 cells/well, 7692 cells/well and 4936 cells/well (as reference there was 15744 cells/well in the control well). Those numbers seem to be very close to the number of the cells that there were put initially on the well (4000 cells/well); this is why it is said that those compounds mediate the cell proliferation inhibition. Also it is important to mention that at the concentration of 1 µM, the compound 77 shows an extreme significance at 1 µM, which means that there is a

concentration response (change in effect on an organism caused by differing levels of substance contact after a certain exposure time).

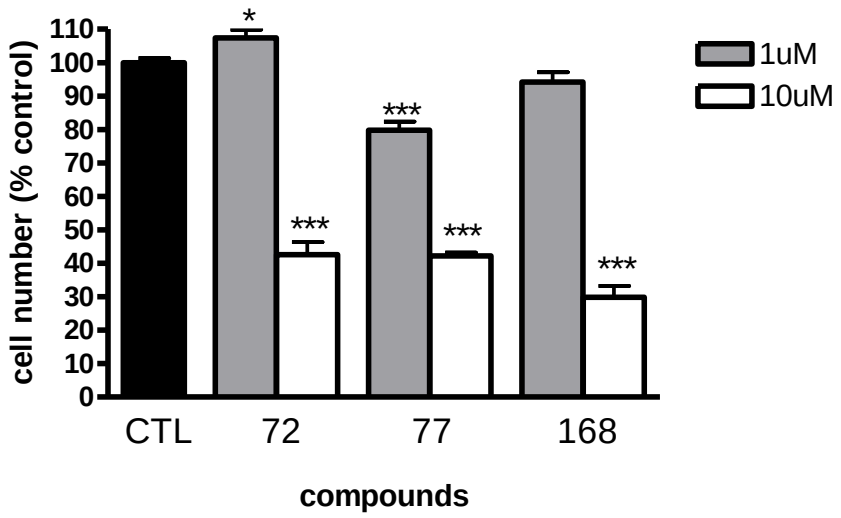


Figure 17 : Impact of the compounds 72, 77, 168 on cell proliferation as a percentage of control. The cells are incubated for 48 hours with the synthetised compounds before the determination of cell number. They are expressed as % of control +/- SEM.

The second group contains the compounds which have a relative impact on cell proliferation. The cell numbers vary from 70% to 90% in percentage of control sample. In this group, there are the compounds 80, 78, 97, 111, 122, 149, 164, 173 and 169. They are presented in Figure 18.

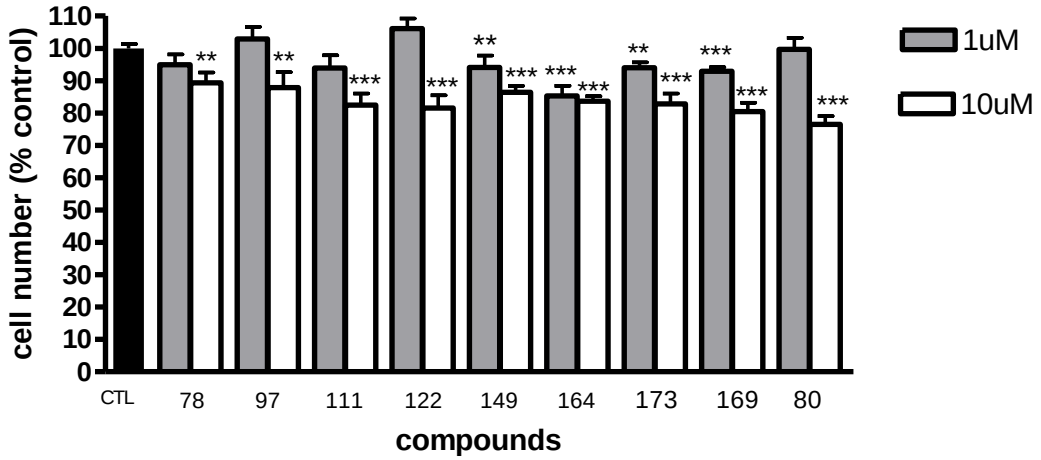


Figure 18: Impact of the compounds 78, 97, 111, 122, 149, 164, 173 and 169 on cell proliferation as a percentage of control. The cells are incubated for 48 hours with the synthetised compounds before the determination of cell number. They are expressed as % of control +/- SEM

It can be noticed that compounds 149, 169 and 173 at 1 µM seem to have an impact on cell proliferation (p-value shows a "very significant" relation between control and compound). This observation is particularly true for the compound 164. In this case, the concentration of the compound seems to have a concentration response.

The last group includes the compounds which don't have an impact on cell proliferation because the percentage of cell number is near 100% of control value. This category includes 10 compounds: 162, 174, 175, 120, 121, 139, 140, 141, 163 and 167. The results are plotted in Figure 19. All compounds in concentration 1 µM to 10 µM present a cell number between 90% and 104% of the control, so they can be removed from further investigations.

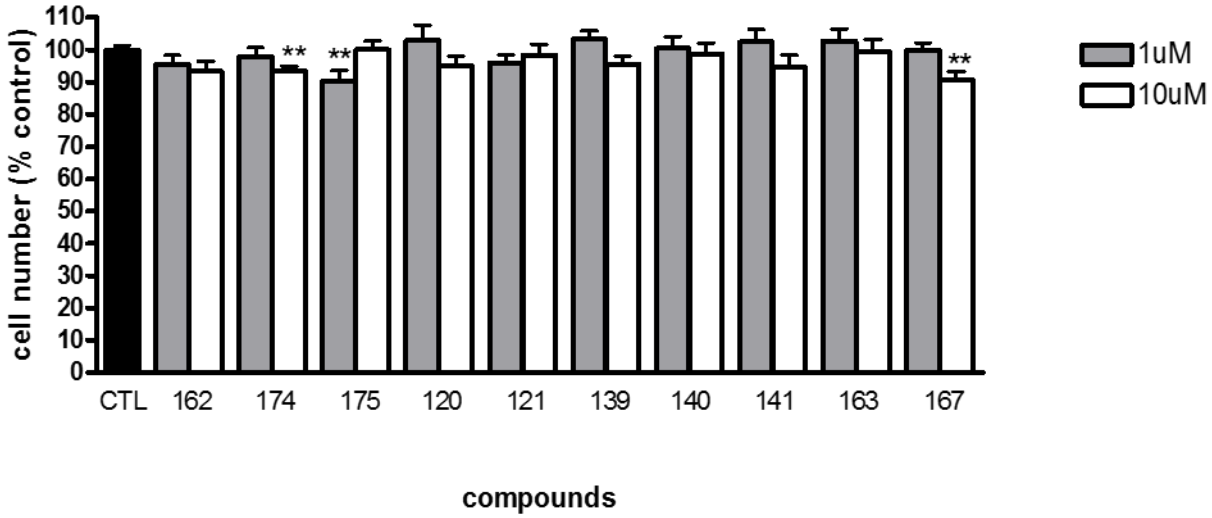


Figure 19: Compounds with no effect on cell proliferation. The cells are incubated for 48 hours with the synthetized compounds before the determination of cell number. They are expressed as % of control +/- SEM.

6. Discussion

From the 22 compounds tested, only the compounds 72, 77 and 168 induce a significant inhibition of endothelial cell proliferation at 10 µM. This might imply that they have a negative effect on angiogenesis. Moreover, compound 77 seems to have a dose response.

Also, compound 80 can be interesting because of its inhibition of cell proliferation at 76.5% in % of control.

Those four compounds seem to be very interesting for the study of the inhibition of angiogenesis. Especially compound 77 may be worth of further investigation because of its concentration response.

The other 18 compounds for which the cell number is around 80-100% of the control appear to be less interesting for the inhibition of cell proliferation. Therefore they may not present sufficient inhibition of angiogenesis.

In order to have a more general view of the action of those compounds, it will be interesting to compare the results of this project with the results taken from Kostakis's group unpublished data [33] on the action of those compounds on cancer cell lines to see if the compounds that have an activity on EGFR have also an activity on VEGFR.

The cell lines used to compare the above results are:

- **U87MG**: human glioblastoma cell lines
- **U87MGDeltaEGFR**: mutation of U87MG (in-frame deletion of 801 base pair that confer a high malignancy to tumor cells) [34]
- **MCF7**: breast cancer cell line [35]
- **CCRF**: T lymphoblastoid cell line with two DNA mutations on this cell line on p53 gene (p53 is a prominent tumor suppressor gene, when it is mutated it create a malignant phenotype and confers resistance to therapy) [36]
- **ADR**: ovarian and breast cell lines (high expression level of P-glycoprotein which make it very useful as a stable multidrug-resistant experimental model) [37]

Table 7 compares the impact of the 22 compounds on the cell proliferation for different cells types. These are expressed as percentage of cell number according to the control.

Table 7: Comparison of the effect of the 22 compounds with four other cell types CCRF, ADR, U87, U87MGDeltaEGFR and

MCF7.

Compound	EAhy		CCRF	ADR	U87	U87ΔEGFR	MCF7
	1uM	10uM	10uM	10uM	10uM	10uM	30uM
CEM 72	107,34 +/- 2,52	42,58 +/- 3,79	49,72 +/- 2,06	71,44 +/- 7,92	ND	ND	71,06 +/- 0,5
CEM 77	79,81 +/- 2,57	42,22 +/- 1,00	66,84 +/- 2,57	86,27 +/- 1,4	ND	ND	78,03 +/- 1,75
CEM 80	99,70 +/- 3,58	76,52 +/- 2,57	67,58 +/- 18,04	75,22 +/- 7,22	88,4 +/- 1,8	93,7 +/- 1,7	80,27 +/- 1,54
CEM111	93,94 +/- 3,96	83,18 +/- 3,66	87,54 +/- 2,72	93,84 +/- 6,96	ND	ND	ND
CEM 78	94,96 +/- 3,17	89,25 +/- 3,40	89,1 +/- 2,33	83,11 +/- 3,28	ND	ND	78,45 +/- 2,11
CEM 149	94,06 +/- 3,74	86,39 +/- 2,04	88,72 +/- 4,26	75,77 +/- 6,84	85 +/- 2,0	96,3 +/- 4,8	ND
CEM 120	102,88 +/- 4,81	94,96 +/- 3,11	94,82 +/- 3,12	80,82 +/- 4,36	ND	ND	ND
CEM 121	95,98 +/- 2,4	98,37 +/- 3,34	89,1 +/- 4,57	81,96 +/- 4,63	ND	ND	ND
CEM 122	106,09 +/- 3,17	81,58 +/- 3,91	78,95 +/- 2,83	83,19 +/- 2,11	ND	ND	ND
CEM 139	103,32 +/- 2,51	95,58 +/- 2,50	86,76 +/- 3,46	83,8 +/- 2,29	ND	ND	ND
CEM 140	100,42 +/- 3,69	98,61 +/- 3,52	81,57 +/- 2,23	88,9 +/- 1,97	80,7 +/- 2,0	88,4 +/- 2,9	ND
CEM 141	102,43 +/- 3,80	94,43 +/- 3,86	81,36 +/- 2,71	76,46 +/- 3,5	83 +/- 2,5	87,4 +/- 3,4	ND
CEM 164	85,34 +/- 3,07	83,63 +/- 1,26	73,04 +/- 4,85	88,53 +/- 4,75	89,8 +/- 2,4	96 +/- 3,4	ND
CEM 162	95,59 +/- 2,59	93,23 +/- 3,24	75,68 +/- 3,22	80,37 +/- 7,89	87,6 +/- 1,3	74,9 +/- 4,3	ND
CEM 163	102,71 +/- 3,78	99,59 +/- 3,59	79,76 +/- 3,54	75,52 +/- 8,28	ND	ND	ND
CEM 167	99,88 +/- 2,28	90,69 +/- 2,63	75,91 +/- 4,83	81,6 +/- 8,20	ND	ND	ND
CEM 168	94,21 +/- 2,93	29,80 +/- 3,39	78,24 +/- 1,22	68,4 +/- 6,28	84,6 +/- 1,6	77,2 +/- 2,3	ND
CEM 169	92,92 +/- 1,24	80,42 +/- 2,80	83,41 +/- 6,12	57,52 +/- 7,99	ND	ND	ND
CEM 174	97,93 +/- 2,67	93,42 +/- 6,12	80,23 +/- 2,08	71,91 +/- 6,51	ND	ND	ND
CEM 173	93,99 +/- 1,70	82,85 +/- 3,20	77,89 +/- 5,16	61,05 +/- 8,34	79,3 +/- 2,2	58,9 +/- 2,3	ND
CEM 175	90,33 +/- 3,27	100,02 +/- 2,58	90,88 +/- 4,62	73,35 +/- 4,56	78,2 +/- 1,8	73,4 +/-1,7	ND
CEM 97	102,88 +/- 3,77	88,47 +/- 4,98	82,84 +/- 3,37	68,82 +/- 2,88	ND	ND	85,58 +/- 0,78

The majority of the compounds have the same effect profile on cell number for EAhy and for CCRF, ADR and MCF7. The results vary for U87MGDeltaEGFR but this may be due to the deletion of the epithelial growth factor receptor.

The comparison will essentially concern the compounds which look promising for the inhibition of cell proliferation using the EAhy cell line.

- **Compound 72**: For the cell lines EAhy and CCRF, the obtained numbers of cell proliferation are very near and around 45% of control. This is very interesting because its means that compound 72 may act both on VEGFR and EGFR. However, for the cells lines ADR and MCF7, the values of cell proliferation are a little higher (72%).
- **Compound 77**: It shows a higher effect on EAhy than the other cells lines (42% against greater percentages: 65% to 86% for the other cell lines). This may indicate that compound 77 has a greater effect on VEGFR than EGFR.
- **Compound 168**: The same remark as the compound 77 can be made. Compound 168 seems to have a higher effect on EAhy than the other cells lines. This certainly means that this compound has a greater affinity on VEGFR than EGFR.
- **Compound 80**: Its action shows the same impact on cell proliferation for the cell lines EAhy, CCRF and ADR, around 70-75% of the control but this percentage is a little higher for the MCF7 and U87 (around 83%). Also it can be said that this compound has perhaps the same impact on VEGFR and EGFR but it is less specific between these receptors because the percentage is greater than the other compounds.

Finally, it will be interesting to continue the studies on compounds 72, 77, 168 and 80 because of their high inhibition to cell proliferation for the cell lines with a particular interest for compound 72 which seems to act on both VEGFR and EGFR with high affinity.

7. Conclusions and perspectives

The work performed in this thesis is the first step of a research which aims at identifying quinazoline based compounds which inhibit angiogenesis. It is premature to draw general conclusions from this work, because the behavior of cells *in vitro* is far from the reality and the complexity of the reactions that take place *in vivo.* Also, because signaling pathways in which each compound act are very complex. Finally, the best concentration at which the compound must be administrated should be known. For those reasons, some perspectives are given regarding the continuation of this work:

- The IC50 of the compounds 72, 77, 168 and 80 must be determined. The IC50 is the concentration of a drug that gives half-maximal response. By this way, it will be clear which concentration must be used for each of these compounds to have a specific cell proliferation.
- Then, it will be interesting to isolate the proteins of the cell cultured with the 4 compounds and to do a western blot in order to determine the influence of these compounds on proteins involved in angiogenesis (EGFR, VEGFR…).
- Finally, those compounds should be investigated in *in vivo* models like mice.

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