Collaboration Research Proposal

Title:	Novel Therapies Targeting Epigenetics and Autophagy in Breast and Liver cancer cell lines using Pyrazolopyrimidine Derivatives: Pharmacophore Modelling, Docking, Biological and Toxicological Evaluation.
Short Title:	Novel Drugs targeting Epigenetics for the Treatment of Breast and Liver Cancer
Keywords:	Breast cancer, hepatocellular carcinoma, Growth Factor, Pharmacophore model, Docking, Pyrazolopyrimidine.
Total Cost:	499650
Duration:	12 months
Research Theme	Medical sciences (pharmaceutics, dentistry, and medicine)

Novel Therapies Targeting Epigenetics and Autophagy in Breast and Liver cancer cell lines using Pyrazolopyrimidine Derivatives: Pharmacophore Modelling, Docking, Biological and Toxicological Evaluation

Table of Contents

English Summary:

Cancer is the second leading cause of death globally, accounting for an estimated 9.6 million deaths. Breast cancer is the second most common cancer overall in Egypt. Hence, the Egyptian government launched in 2019-2020 “The National Survey for Early Investigation of Breast Cancer”. Another genus of cancer that has a most frequent incidence in Egypt is liver carcinoma. Hepatocellular carcinoma (HCC) accounts for 75%-85% of the world's primary liver cancers.

Breast and liver cancer cells express various growth factors such as vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF). Nuclear Factor Kappa B (NFκB) is considered to be a transcription factor controlling expression of many proteins including VEGF and PDGF. Agents capable of suppressing NFκB activation have therapeutic promise and potential to inhibit carcinogenesis. The critical incidence rates of breast and liver carcinoma besides, these crucial biomarkers motivated our research group to design and synthesize novel drug like candidates with potential antitumor activity against breast and liver carcinoma. One of these potential candidates is pyrazolopyrimidine scaffold. Pyrazolo pyrimidines are fused heterocyclic ring systems which known as bio isosteres of adenine, that are necessary for every aspect of cell life. Current treatment strategies for different types of cancer are effective only in a small sector of patients. Many factors influence the therapeutic effect, including genetic variations. This study aims to: Design and synthesis of novel pyrazolopyrimidine derivatives with potential antitumor activity. This study will include drug designing, cell culturing and molecular studies techniques to assists and prove our hypothesis. Cell lines culture (HepG2 and MCF-7 cells): to assess and investigate the potential gold role of these pathways targeting as a novel therapeutic strategies. Following drug treatment, cells lysates and nuclear extracts will be subjected to western blotting, qRT-PCR, Western blotting and/or ELISA to determine the different levels of the parameters for investigation of the drugs mechanisms of actions. The safety profile of the most promising and effective candidates will be performed in-vivo using experimental animals after acute and subchronic treatment and mortality rate will be identified. These prototypes of highest efficacy and lower toxicity are expected to be transferred to the following pre-clinical phase of drug development in collaboration with industrial partners. The application of this complementary inter-disciplinary research project and the experience gained during its implementation will be of great impact on proper design and usage of central labs at college of Pharmacy AASTMT and for the scientists for the team in the beginng of their carreer to gain immense experience and transfer of know-how.

This project is the first step towards development of an effective treatment in collaboration with Egyptian industrial companies which will have strong economic impact on Egypt and AASTMT and will improve health care management and wealth of the Egyptian population.

Arabic Summary:

السرطان هو السبب الرئيسي الثاني للوفاة على مستوى العالم ، حيث يتسبب في ما يقدر بنحو 9.6 مليون حالة وفاة. سرطان الثدي هو ثاني أكثر أنواع السرطانات شيوعًا في مصر بشكل عام. ومن هنا ، أطلقت الحكومة المصرية في 2019-2020 "المسح القومي للتحقيق المبكر لسرطان الثدي". سرطان الكبد هو أحد أنواع السرطان الأكثر انتشارًا في مصر. يمثل سرطان الخلايا الكبدية75 (HCC) ٪85- ٪ من سرطانات الكبد الأولية في العالم.
الجدير بالذكر أن خلايا الصدر والكبد السرطانيه لديها تكون خطير من عوامل نمو الأوعيه الدمويه المساعده على نمو الخلايا السرطانيه السريع. واحد من اهم هذه العوامل هو NFκB ومن خلال دراسات حديثه اثبتت أن تثبيطه يعتبر واحد من أهم طرق العلاج الحديثه والفعاله للأمراض السرطانيه من خلال تأثيره على وقوف نمو هذه الخلايا السرطانيه. هذه الدلائل والعوامل حفزت فريق عملنا للتفكير فى استغلال ودراسه أدويه ومواد كيميائيه حديثه يعتبر لها نفس التاثير pyrazolopyrimidine scaffold) وذلك ما سوف نقوم بإثباته بالتجارب العمليه. هذه المركبات من خلال تركيبها الكيميائى اثبتت انها مهمه لدوره الحياه. بالإضافه الى ذلك العوامل الوراثيه سوف يتم دراستها حيث أنه وجد لها دور فعال ايضا ولا يجب غفلانه. لذلك الهدف من تلك الدراسه هو أثبات فعاليه تلك المركبات الجديده من خلال إجراء عدد من تجارب نموالخلايا والبيولوجيا الجزيئيه بالإضافه الى تجارب إنشاء وتحديث مركبات جديده لضمان فاعلياتها. خلال الدراسه سوف يتم استخدام خلايا سرطان الكبد والثدى لحساب الجرعه القاتله للخلايا السرطانيه. أيضا بعد الإنتهاء من اجراء تجارب الدواء حيث يتم تحضير عينات بلازما خلايا المحلله لقياس مستوى العوامل المختله التى من خلالها سوف يتم دراسة تأثير وطريقه عمل الادويه ونسب ومستوى سميتها على الفئران. سيتم إجراء ملف الأمان الخاص بالمرشحين الواعدين والأكثر فاعلية في الجسم الحي باستخدام حيوانات التجارب بعد العلاج الحاد وتحت المزمن وسيتم تحديد معدل الوفيات. ومن المتوقع أن يتم نقل هذه النماذج ذات الفعالية الأعلى والسمية الأقل إلى مرحلة ما قبل السريرية التالية لتطوير الأدوية بالتعاون مع الشركاء من الصناعة.
سيكون لتطبيق هذا المشروع البحثي التكميلي متعدد التخصصات والخبرة المكتسبة أثناء تنفيذه تأثير كبير على التصميم المناسب والاستخدام للمختبرات المركزية في كلية الصيدلة بالأكاديمية وللعلماء أعضاء الفريق الذين هم مستهل حياتهم الاكاديمية سيكتسبون خبرة هائلة ونقلا للمعرفة. هذا المشروع هو الخطوة الأولى نحو تطوير علاج فعال بالتعاون مع الشركات الصناعية المصرية والذي سيكون له تأثير اقتصادي قوي على مصر والأكاديمية وسيحسن إدارة الرعاية الصحية وثروة الشعب المصري.

Introduction:

Cancer is the second leading cause of death globally, accounting for an estimated 9.6 million deaths. Its burden continues to exerting tremendous physical, emotional and financial strain on individuals, families and communities. Breast cancer is the second most common cancer overall in Egypt (Figure 1.) Moreover, it is the most commonly occurring cancer in women according to Globocan 2020. [1,2], There were over 128.892 new cases with almost 86 thousand deaths. [3] Hence, the Egyptian government launched in 2019-2020 “The National Survey for Early Investigation of Breast Cancer”. Such governmental care as well as a highly increased incidence and mortality of breast cancer urged our research group to focus on discovering novel anti-breast cancer drug like molecules.

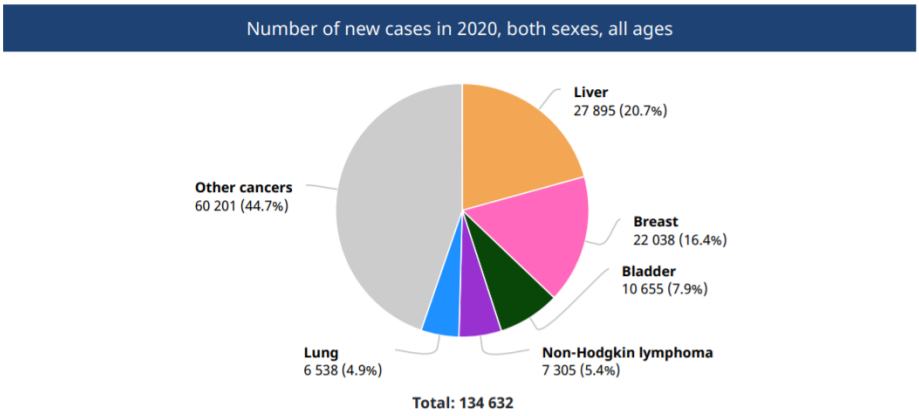


Figure 1: Number of new cases in 2020, both sexes, all ages, in Egypt. [2]

Another genus of cancer that has a most frequent incidence in Egypt is liver carcinoma. Hepatocellular carcinoma (HCC) accounts for 75%-85% of the world's primary liver cancers [4]. It is the sixth most prevalent cancer in the globe and the fourth most prevalent cause of death from cancer, accounting for 4.7% of all cancers in 2018, with approximately 841,000 new cases of liver cancer and 782,000 deaths annually [5]. In Egypt, HCC is the most prevalent malignancy in men, the 2nd most prevalent in women and the most prevalent malignancy in both sexes combined (Figure 2) [2].

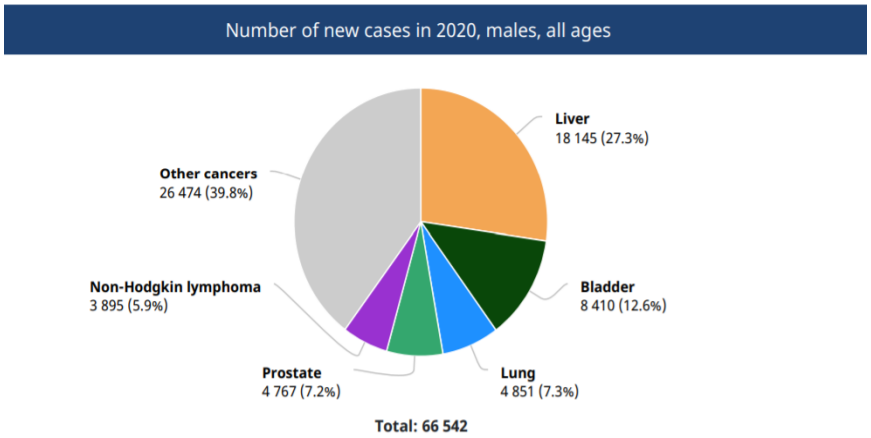


Figure 2: Number of new cases in 2020, males, all ages, in Egypt. [2]

If hepatocellular carcinoma could be diagnosed at an early stage, it can be treated by liver transplantation or surgical resection [6,7]. However, despite effective therapies for early stage and efforts at early diagnosis through screening of patients at risk for this cancer, most cases in Egypt are diagnosed at an advanced stage [8].

Breast and liver cancer cells express various growth factors such as vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), epidermal growth factor (EGF), fibroblast growth factor (FGF), and insulin-like growth factor (IGF), which induce cell proliferation in an autocrine fashion [9]. The receptors of these growth factors activate intracellular signals such as the RAF/MEK/ERK pathway and the PI3K/AKT/mTOR pathway, which induce proliferation of both cancer and endothelial

cells [10,11]. These growth factors, including their intracellular molecules, are considered to be a specific target for different cancer treatment [12].

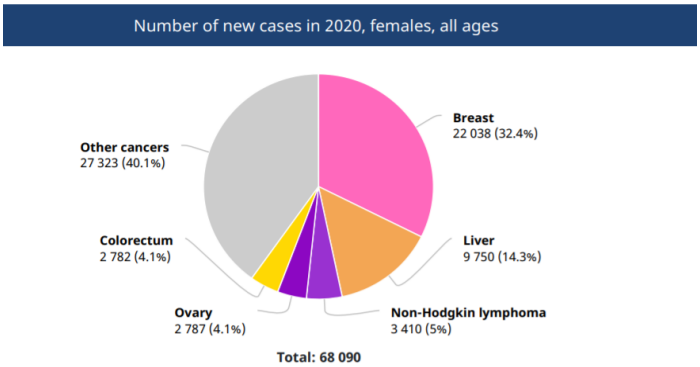


Figure 3: Number of new cases in 2020, females, all ages, in Egypt. [2]

Moreover the aberrant regulation of nuclear factor-κB (NFκB) and the signaling pathways that control its activity, are involved in breast and liver cancer development and progression, as well as in drug resistance, especially during chemotherapy and radiotherapy [13]. Blocking NFκB can cause tumor cells to cease proliferation or become more sensitive to the action of antitumor agents.

Thus, NFκB is the subject of intense study. Agents capable of suppressing NFκB activation have therapeutic promise and potential to inhibit carcinogenesis [14,15]. In different breast and liver cell types, downstream signalling of HGF/MET has been reported to be mediated through tyrosine phosphatase SHP-2, phosphatidylinositol 3-kinase (PI3K)/AKT, GTPases of the Rho family, glycogen synthase kinase 3 (GSK3), nuclear factor-κB (NF-κB), extracellular signal-regulated kinases (ERK), and p38 mitogen-activated protein kinase [16, 17].

The critical incidence rates of breast and liver carcinoma besides, these crucial biomarkers motivated our research group to design and synthesize novel drug like candidates with potential antitumor activity against breast and liver carcinoma. One of these potential candidates is pyrazolopyrimidine scaffold. Pyrazolopyrimidines are fused heterocyclic ring systems which known as bio isosteres of adenine, that are necessary for every aspect of cell life. Pyrazolopyrimidine derivatives have been explored for their inhibitory activity towards a variety of protein kinase enzymes and their function as anticancer agents [18-23]

Questions and Objectives

Problems Definition:

Despite the national and international efforts in the field of breast and liver cancer prevention and control, the prevalence of these two diseases remains the highest among all cancers in Egypt. In addition, gold standard treatment for breast and/or liver cancers showing high efficacy and low toxicity is far from being achieved. Furthermore, current treatment strategies for different types of cancer are effective only in a small section of patients, being influenced by many factors including genetic variations. For these reasons, the aim of our proposal is to:

- Design and synthetize novel pyrazolopyrimidine derivatives with potential antitumor activity.
- Determine the efficacy of these candidates on different types of cancer cells, particularly breast cancer and hepatocellular carcinoma, on which previous and known therapies failed to produce promising and stable effects
- Investigate the potential mechanisms of action for these new drugs especially on some signalling pathways recently proved of importance in the pathogenesis of these two cancer types.

Specific objectives:

1. Design of novel VEGFR inhibitors that may possess potential antitumor activity against breast cancer and hepatocellular carcinoma, through introducing pyrazolopyrimidine ring, bioisosteres of adenine. pyrazolopyrimidine Scaffold along with essential pharmacophoric features may compete with adenosine triphosphate (ATP) for the ATP-binding site of the VEGFR-2 intracellular kinase domain, thereby preventing the intracellular signalling. This leads to inhibition of tumor growth and metastasis.
2. 3D-ligand based pharmacophore model will be constructed to define the essential pharmacophore featured that are crucial to maintain high binding affinity to VEGFR-2. Consequently, novel VEGFR-2 inhibitors hits will be designed.
3. Chemical synthesis of the newly designed pyrazolopyrimidine derivatives through different synthetic pathways (average 15 compounds).
4. Screening of the newly synthesized pyrazolopyrimidine derivatives for other potential targets that may possess potential impact on Breast cancer and liver carcinoma using cytotoxicity test (MTT assay)
5. The tested new medicinal drug's potential mechanisms of action and their biochemical and molecular pathways and targets will be investigated on both liver and breast carcinoma.
6. The aim also is to minimize cytotoxic doses with the goal of reducing side effects, reducing toxicity and improving therapeutic outcomes. This would be achieved by testing combination between the most active candidates and reference drugs.
7. Perform Molecular docking studies on the most active compounds (1 up to 5 compounds) to explain their affinity to the binding site. Furthermore, the molecular modeling job will be enriched with an attempt to validate the stability of the of most active compounds – receptor complex through thermodynamics calculations.
8. Toxicological studies will finally be performed on the most promising compounds to evaluate their safety profile in experimental animals. Cardiovascular toxicity, liver functions and kidney functions in the presence of the proposed therapeutic doses of these newly developed compounds will be investigated using biochemical and histological methods. Mortality rate (LD_{50}) will also be studied.

<u>Preliminary data: Structure-based pharmacophore modelling and link to pathogenic pathways of cancer</u>

VEGFR-2 is one of the few thoroughly studied and well-validated targets in anticancer therapy. The main objective of the present work is to develop a model for designing of novel of VEGFR-2 kinase inhibitors. To achieve this goal within a reasonable time

frame, we need a fast and robust docking tool. Our initial studies were carried out with DS CDOCKER, since this is widely regarded as one of the best docking programs. The model was further used to identify new leads for VEGFR-2 kinase inhibitors.
Consequently, we established pharmacophore map (Figure 4) using the DS software. The pharmacophoric model was based on the crystal structure of 1YWN; the backbone amide-NH of Cys917 and Asp1044 used hydrogen bond donors; and the backbone carbonyl oxygen of Glu883 was the hydrogen bond acceptor. The results suggest the importance of the fine features of the pharmacophores: the presence of two hydrophobic groups, one hydrogen bond acceptor, and two hydrogen bond donors. This model was consisted with that obtained by Lee et al. [24].

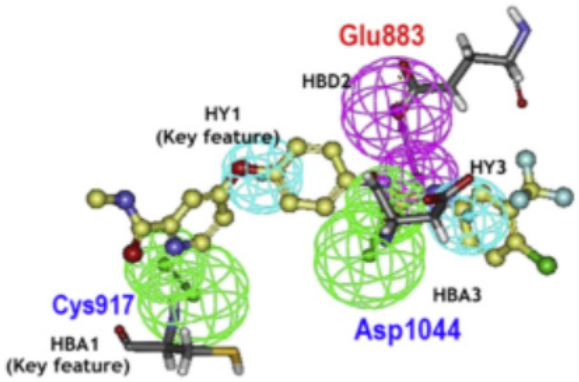


Figure 4. (binding with sorafenib) pharmacophoric model describing binding mode with sorafenib into the hinge region of VEGFR-2 kinase. Pharmacophoric features are color coded as follows: cyan, hydrophobic (HY); green, hydrogen bond acceptor (HBA); magenta, hydrogen bond donor (HBD).

The constructed model is used to design new pyrazolopyrimidine bearing sulfonamide moieties with the same essential pharmacophoric features of the reported and clinically used VEGFR-2 inhibitors, in addition, to being molecularly hybridized with pyrazolopyrimidine scaffold, bioiossteres of adenine in an attempt to get more potent inhibitors against liver and breast carcinoma. The main core of our molecular design rationale was carried out by bioisosteric modification strategies of VEGFR-2 inhibitors (sorafenib & pazopanib) at four different positions (Fig. 5).

Hepatocellular carcinoma (HCC) and breast cancer have a complex pathogenesis link with various risk factors. Different aspects of tumor biology, including development, progression, and response to therapy, can be affected by components of the tumor microenvironment. Suggested biomarkers which are considered to be part of the breast cancer and liver carcinoma microenvironment to be assessed are VEGF, p-Akt, m-TOR, HGF, Erk, EGF, PCL, PD-1 and NFκB, which are key regulators to their signaling pathways which are in turn main factors affecting and contributing to breast cancer and liver carcinoma. The activation of NF-κB, Akt/m-TOR, autophagic and

extracellular-regulated kinase (ERK) pathways promote HCC and breast cancer growth.

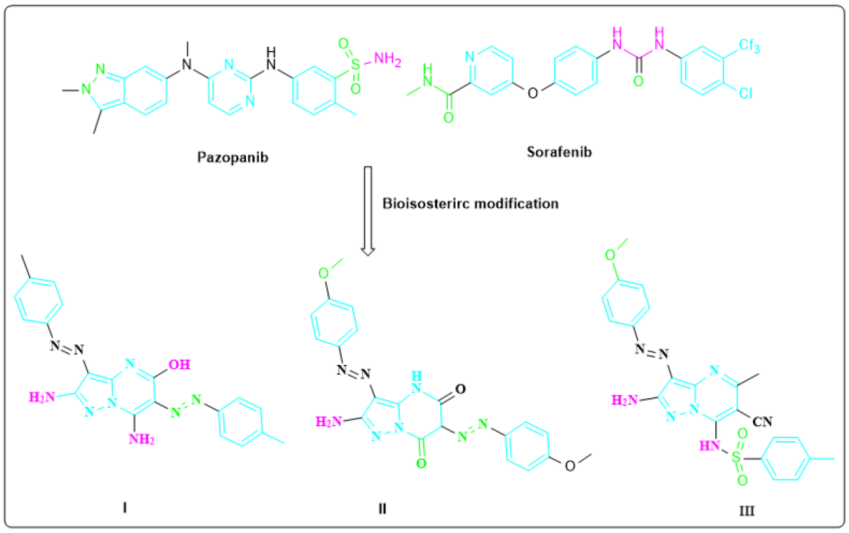


Fig. 5. Reported VEGFR-2 inhibitors and our derivatives.
Pharmacophoric features are color coded as follows: cyan, hydrophobic (HY); green, hydrogen bond acceptor (HBA); magenta, hydrogen bond donor (HBD).

Project Description

This is a one-year project applied for AASTMT by a team of scientists working in fields concerned with development and discovering of drug like molecules targeting Cancer. The project is based on design and synthesis of novel pyrazolopyrimidine derivatives with potential antitumor activity against breast cancer and hepatocellular. Furthermore, investigation of potential mechanisms of action for these new compounds will be established. This study will include drug designing, cell culturing and molecular studies techniques to assists and prove our hypothesis in a **complementary interdisciplinary manner.**

After computerized design and chemical synthesis, the project team will work to evaluate the potential activity of the newly-synthesized molecules against breast and liver carcinoma using cell lines. In addition, safety profile of the most effective candidates will be determined where toxicity on major organ functions in experimental animals will be studied.

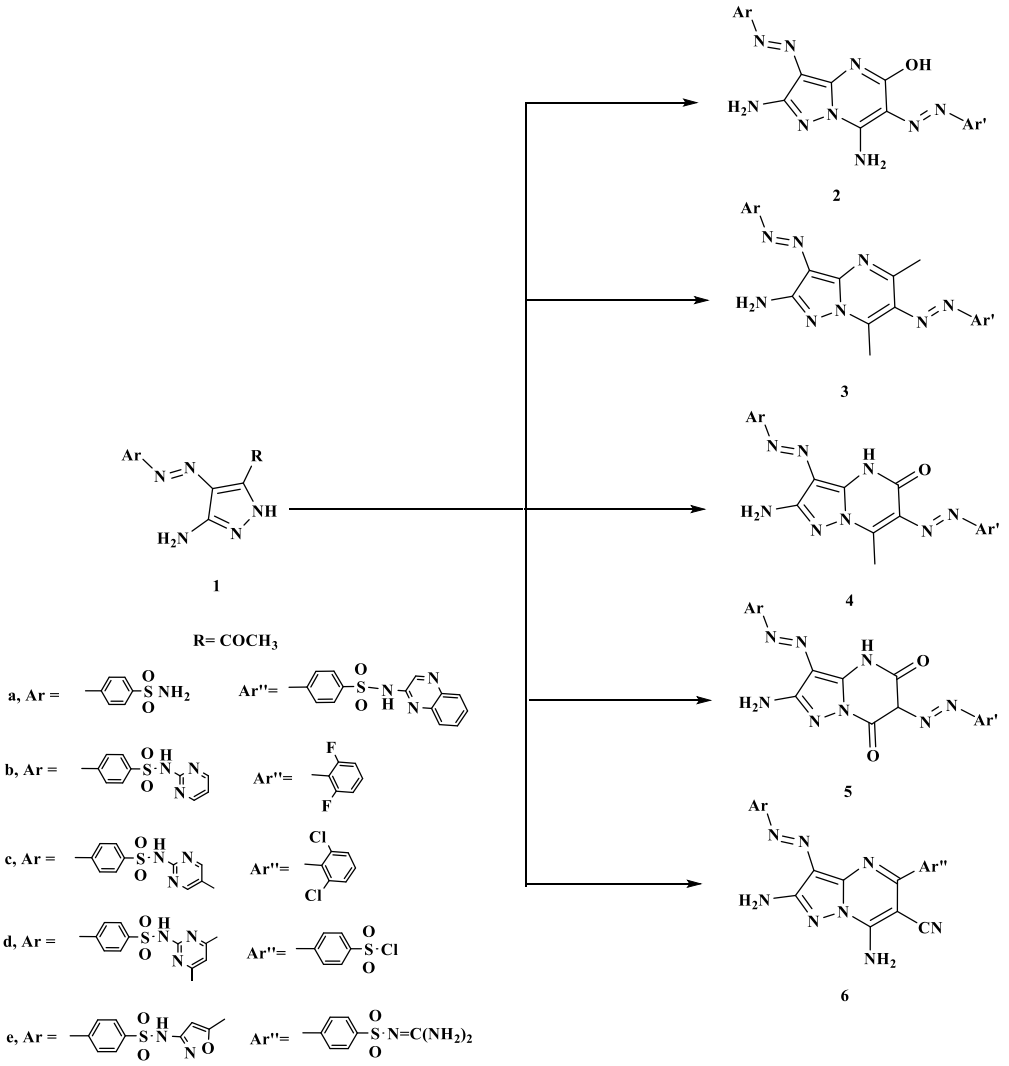
The research team is comprised of experts in Cancer research field, Molecular Biology and Toxicology from two institutes and three different disciplines. There is a great synergy between team members exhibited as complementation of the different work packages according to scientific interest and research experience, especially between the designing of the new molecules, synthesis of these candidate compounds, biological evaluation against liver and breast carcinoma and toxicological effects determination.

Work Package 1: Modelling and computerized design

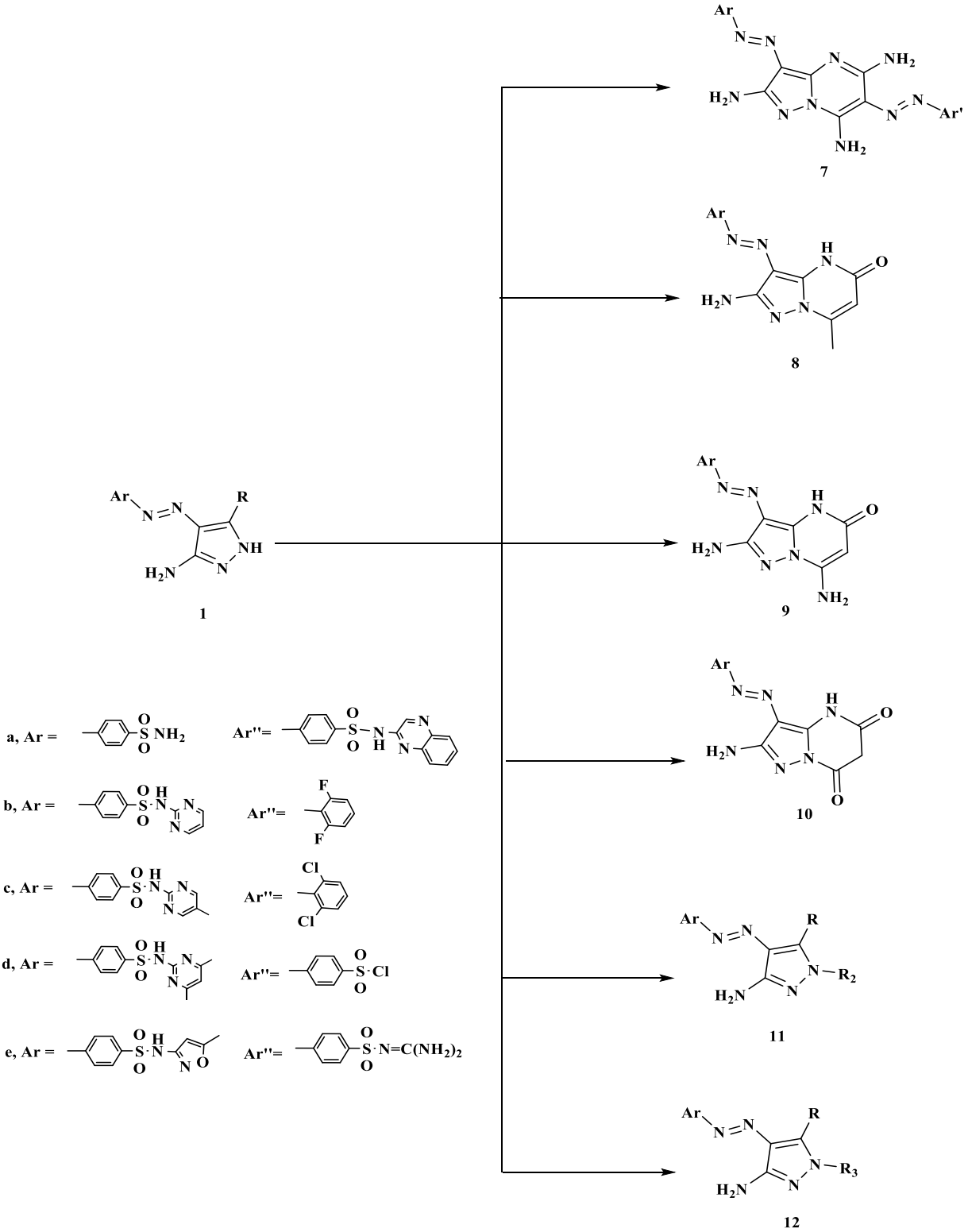
Discovery Studio (DS) 5.0 client (Accelrys) will be used to design the novel molecules. CDOCKER, docking algorithm within DS suite, will be used to perform the docking process. The detailed methodology is described in research design and method part of the project

Work Package 2: Laboratory chemical synthesis

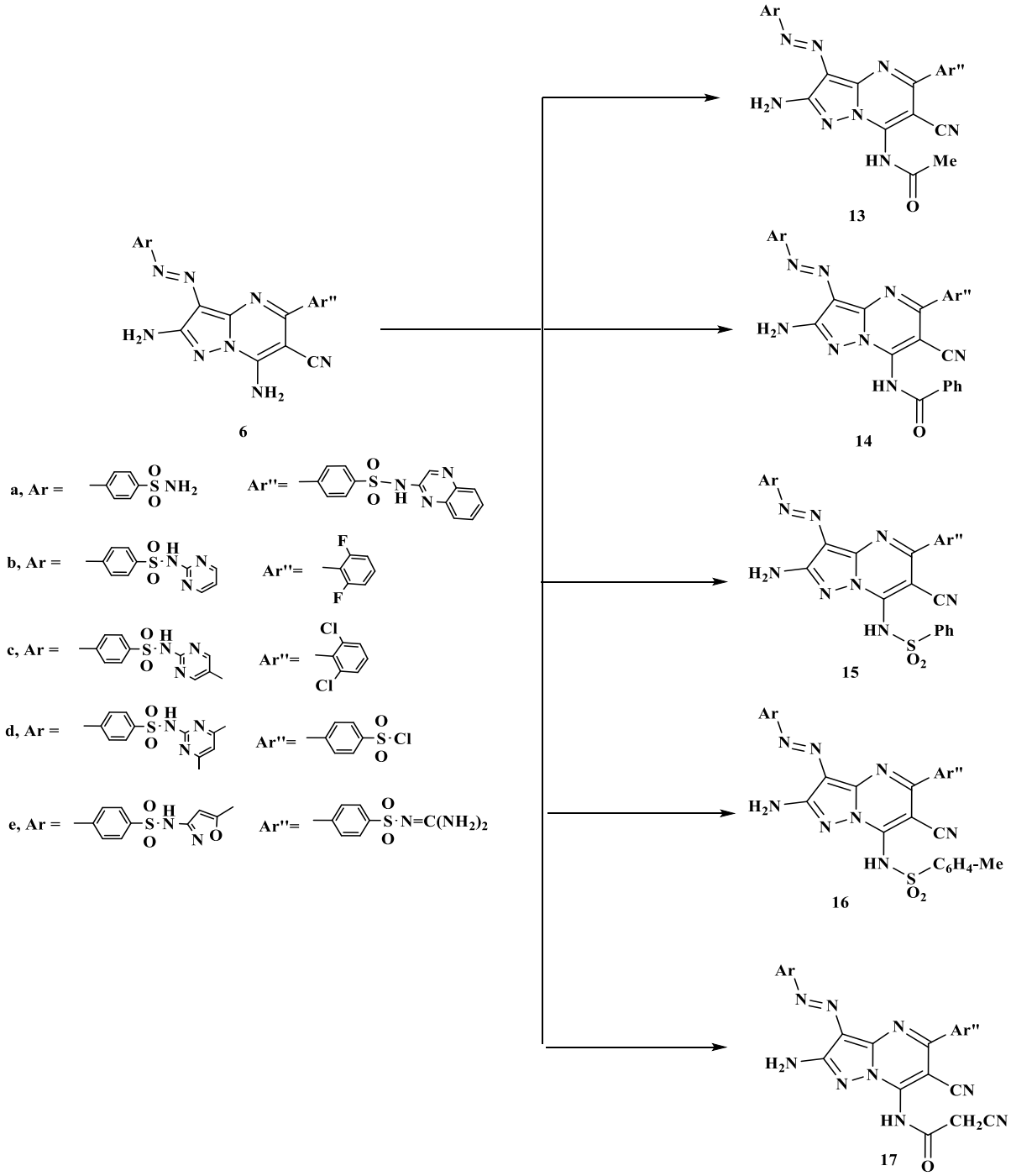
Using different 3-aminopyrazole derivatives as starting materials, the designed molecules will be synthesized according to the following synthesis pathways:



Synthetic pathway 1



Synthetic pathway 2



Synthetic pathway 3

Work Package 3: Biological evaluation of efficacy and understanding potential mechanism of action

Samples:

Cell lines culture (HepG2 and MCF-7 cells): to assess and investigate the potential role of these pathways targeting as a novel therapeutic strategy.

Screening of the new synthesized pyrazolopyrimidine derivatives for other potential targets that may possess potential impact on Breast cancer and liver carcinoma. This will be accomplished by determination of cytotoxic doses IC50 for each one of them using MTT assay.

The tested new medicinal drugs' potential mechanisms of action and their biochemical and molecular pathways and targets will be investigated by measuring and assessing different biochemical and molecular parameters using RT-PCR, ELISA and western blotting on cell lysate and nuclear extracts.

Then, reducing side effects, reducing toxicity and improving therapeutic outcomes testing will be accomplished by evaluating different targeted signaling pathways related proteins levels and making them therapy targets if possible by combining with reference drugs.

Furthermore, the mechanism of action of the potent candidates will be explained at molecular level using molecular modelling studies

Work Package 4: Toxicological studies

The safety profile of the most promising and effective candidates will be performed in-vivo using experimental animals after subchronic (21 days) treatment, using the proposed therapeutic dose and after acute treatment using three times the therapeutic dose. Liver, kidney and cardiac functions will be assessed using organ homogenates by laboratory analyses as well as histopathological examination. Complete LD_{50} study using dose-response curve method will be performed to determine mortality and rank the potential candidate drugs according to their safety.

Research Design and Methods

The main aim of the project is to Design and synthesize novel pyrazolopyrimidine derivatives with potential antitumor activity against breast cancer and hepatocellular. Furthermore, investigation of potential mechanisms of action and proving safety of these new compounds will be established. This main goal will be accomplished by the following objectives:

Objective 1*: Design of novel VEGFR inhibitors that may possess potential antitumor activity against breast cancer and hepatocellular carcinoma, through introducing pyrazolopyrimidine ring, bioisosteres of adenine.* This will be accomplished by bio-isosteric replacement of 1,2-pyrazolobenzene scaffold of Pazopanib inhibitor and pyridine ring of sorafenib with pyrazolo pyrimidine moiety that may compete with adenosine triphosphate (ATP) for the ATP-binding site of the VEGFR-2 intracellular kinase domain, thereby preventing the intracellular signalling.

Objective 2: *3D-ligand based pharmacophore model will be constructed to define the essential pharmacophore featured that are crucial to maintain high binding affinity to VEGFR-2.* Consequently, novel VEGFR-2 inhibitors hits will be designed. This pharmacophore model will be accomplished using DS software, where it is fast and robust molecular modelling software for constructing 3D-ligand based pharmacophore

model. The model was based on the crystal structure of VEGFR-2 with PDB ID: 1YWN [25].

Objective 3: *Chemical synthesis of the new designed of pyrazolopyrimidine derivatives.* This will be accomplished using the synthesis pathways 1-3 as shown in the previous section. Structure of the new synthetic molecules will be confirmed and elucidated using different spectroscopic instruments. Melting points will be determined in open-glass capillaries using a Graffin melting point apparatus. Following up of the reactions rates will be performed by thin-layer chromatography (TLC) on ready-made silica sheets from Merck and the spots were visualized by UV lamp at λ 254 nm. Infrared spectra (IR) will be recorded, using KBr discs, υ (cm-1), on Perkin-Elmer 1430 infrared spectrophotometer, Nuclear magnetic resonance spectra 1H-NMR (300 MHz) and 13C-NMR (75 MHz) will be performed in CDCl3 or DMSO-d6, and scanned on Brücker spectrophotometer. Microanalyses will be performed on Vario El Fab-Nr elemental analyzer.

Objective 4: *Screening of the new synthesized pyrazolopyrimidine derivatives for other potential targets that may possess potential impact on Breast cancer and liver carcinoma.* This will be accomplished by determination of cytotoxic doses IC50 for each one of them using MTT assay.

Objective 5: *The tested new medicinal drugs' potential mechanisms of action and their biochemical and molecular pathways and targets will be investigated.*

This will be accomplished by measuring and assessing different biochemical and molecular parameters using RT-PCR, ELISA and western blotting on cell lysate and nuclear extracts.

Objective 6: *The aim also is to minimize cytotoxic doses with the goal of reducing side effects, reducing toxicity and improving therapeutic outcomes.* This will be accomplished by evaluating different targeted signaling pathways related proteins levels and making them therapy targets if possible. Cell lines culture (HepG2, MCF-7): to assess and investigate the potential gold role of these pathways targeting as a novel therapeutic strategies.

Experimental design:

- **Cell culture** will be prformed based on method described by Polard, *et al.* [26] The replica of each of (HepG2 and MCF-7cells) cell lines will be purchased from the American type culture collection ((ATCC) (U.S. patent number: 4,393,133, USA). HEPG2 cells will be maintained as a monolayer culture in T-25 flasks at 37^{o}C and 5 % CO_2 in Dulbecco's Modified Eagle's Medium (DMEM) (Lonza Biowhittaker™, B-4800 Verviers, Belgium) supplemented with 10 % (v/v) fetal bovine serum (FBS) (Sigma-Aldrich Co., Germany). Penicillin/streptomycin (Lonza Biowhittaker™, B-4800 Verviers, Belgium) will be used at a concentration of 100 units/ml and 100 µg/ml, respectively. Phosphate buffered solution (PBS) pH 7.2 (Lonza Biowhittaker™, B-4800 Verviers, Belgium). 2.5% Trysin (Gibco™ life technologies Corporation, New York, USA).
- **Cell sub culturing:** HEPG2 cells will be passaged, when they will be 80 % confluent, about every third day. Media will be removed by aspiration and 5 ml of BPS will be added to wash the medium from the adherent cells. To detach the adherent cells, 2 ml

of 2.5 % (w/v) trypsin will be added to the T-25 flask and cells will be incubated for 5 minutes at 37°C. The cells will be observed under the inverted microscope (Micro master inverted digital microscope, Thermo fisher Scientific Inc., USA) every 2-3 minutes .

- After 5-10 minutes of incubation, cells will be detached and the trypsin cell suspension will be neutralized by adding an equal volume of complete growth medium to the T-25 flask. The cell suspension will be then transferred to a 15 ml falcon tube to which 5 ml of complete medium was added. Following resuspension, the cell suspension will be transferred into new pre-labeled T-25 flasks at a seeding density of about 4 x 104 viable cells/cm2. The flasks will be then incubated at 37oC in 5 % CO2 to allow cell attachment.
- **Cells counting:** Cell suspension (10 µl) will be mixed with equal volume of trypan blue and loaded in both chambers. Unstained cells (viable cells) will be counted under an inverted microscope at 10x magnification.
- **Cell Viability Assay:** Cell viability will be monitored using MTT assay. Generally, 5 × 103 cells will be allowed to grow in 96-well plates. After incubation with proposed drugs and their combination for 48 h, 10 μL MTT solution (0.5%) will be added to the medium for further incubation for 4 h. 100 μL DMSO will be added to every well to dissolve the insoluble formazan product after removing the medium. The absorbance of the colored solution will be measured at 570 nm with a spectrophotometer. All experiments were performed in triplicates.
- The tested new medicinal drugs' potential mechanisms of action and their biochemical and molecular pathways and targets will be investigated on HepG2 and MCF-7 cell lines on different cell lines control and treated groups with newly designed drugs and reference ones.

  Drug incubation period will be 3 days. Following drug treatment, cells lysates and nuclear extracts will be subjected to western blotting, qRT-PCR, Western blotting and/or ELISA to determine the different levels of the following parameters: VEGF, p-Akt, m-TOR, HGF, Erk, EGF, PCL, PD-1 and NFκB.

  qRT-PCR technique:

  Quantitative real time PCR (qRT-PCR) was applied to determine the relative expression of *tested genes* against *β-actin* as described by Nolan*, et al.*[27] qRT-PCR assay was carried out by using step one real time PCR system (Applied Biosystem, USA). The dye SYBR green was used, which absorbs light at 488 nm and emits light at 522 nm. SYBR green fluoresces when intercalated to double stranded DNA such that fluorescence intensity increases as the PCR amplicons increase. Reactions were performed using the SensiFast ^{TM} One Step RT-PCR kit with SYBR ® Green Hi ROX (Bioline Life science company, USA) which was designed for highly reproducible first-strand cDNA synthesis and subsequent real-time PCR in a single tube.

  A combination of the latest advances in buffer chemistry together with a reverse transcriptase and hot-start DNA polymerase system, ensures that SensiFAST SYBR Hi-ROX One-Step kit produces fast, highly-specific and ultra-sensitive one-step RT-qPCR. The SensiFAST SYBR Hi-ROX One-Step kit consists of a 2x SensiFAST SYBR

One-Step mix, as well as separate reverse transcriptase and RiboSafe RNase Inhibitor. Reactions were carried out in 48 wells PCR plate.
The cDNA synthesis step was performed at 45°C for 10 minutes then 95°C for 2 minutes for reverse transcriptase inactivation. The resulting cDNA was amplified by 40 cycles of PCR as follows: denaturation at 95°C for 15 seconds, annealing at 56°C for 1 minute, extension at 72°C for 15 seconds followed by final extension at 72°C for 10 minutes.
For each sample, Δ values were determined by subtracting the average of triplicate CT values of the target gene from that of the reference gene and relative expression was determined as $2^{-\Delta\Delta CT}$. ΔΔCT was determined from the equation:
$\Delta\Delta CT = (CT_{\text{gene}} - CT_{\text{reference gene}})_{\text{treated}} - (CT_{\text{gene}} - CT_{\text{reference gene}})_{\text{control}}$.

Enzyme-Linked Immunosorbent Assay

This particular immunoassay utilizes the quantitative technique of a “Sandwich” Enzyme-Linked Immunosorbent Assay (ELISA)) as described by Clark *etal*, [28] where the target protein (antigen) is bound in a “sandwich” format by the primary capture antibodies coated to each well-bottom and the secondary detection antibodies added subsequently by the investigator. The capture antibodies coated to the bottom of each well are specific for a particular epitope on the target protein, while the user-added detection antibodies bind to epitopes on the captured target protein.
After incubation and “sandwiching” of the target antigen, a peroxidase enzyme is conjugated to the constant heavy chain of the secondary antibody (either covalently or via Avidin/Streptavidin-Biotin interactions), allowing for a colorimetric reaction to ensue upon substrate addition. When the substrate 3, 3’, 5, 5’-Tetramethylbenzidine (TMB) is added, the reaction catalyzed by the peroxidase yields a blue color that is representative of the antigen concentration.
Upon sufficient color development, the reaction can be terminated through the addition of stop solution (2N sulfuric acid) where the color of the solution turns yellow. The absorbance of each well can then be read by a spectrophotometer.

Western blotting

The protein contents of each pooled group were assayed as described by Lowry et al., 1951.[29] Proteins [40 µg] from each group were added and mixed with the sample application buffer (SAB), and loaded on a 10% SDS-PAGE after boiling for 3 min. Proteins were transferred into nitrocellulose membranes. The membranes were washed with TBS buffer pH 7.3 (8 g NaCl, 0.2 g KCl and 3 g Tris-base/liter) for three times. The primary antibody of CYP2E1 and/or CYP3A4 was added after dilution of 1:1000. Then, each membrane was washed with Tween-TBS (0.2 ml Tween/1 L TBS) for four times. Anti-mouse horseradish peroxidase-conjugated secondary antibody [1:7000] was incubated with each membrane separately for 45 min. X-ray film was used to capture the signals of the protein expression of both isozymes. The band intensity of each isozyme was recorded using quantity one software program (version 4,6.9, Bio-Rad Co., California, USA).

Objective 7: *Perform Molecular docking studies on the most active compounds to explain their affinity to the binding site. Furthermore, the molecular modelling job will be enriched with an attempt to validate the stability of the of most active compounds –*

receptor complex through thermodynamics calculations. Modelling will be accomplished through ligand preparation, protein preparation and docking process.

Ligand preparation: Different 3-D conformations of the designed pyrazolopyrimidine derivatives were generated and energetically minimized using the "Generate Conformations" tool in Discovery Studio (DS) 5.0 client (Accelrys). The lowest energetic conformation thus obtained was subjected to the "Prepare Ligands" module to generate its isomers at physiological pH. The CHARMm force field was employed to develop the partial atomic charges on each atom of the isomer. The isomer with the lowest CHARMm energy was used for the docking study.

Protein preparation and docking process: The X-ray co-ordinates of VEGFR-2 PDB ID: 1YWN, resolution 1.71 Å) was retrieved from the protein data bank (www.rcsb.org). The "Prepare Protein" tool in DS was used to add missing atoms/chains and remove water molecules in the protein structure. The "Prepare Protein" algorithm was employed to protonate amino acid residues according to the physiological conditions. Determination of the binding site accomplished by choosing PDB site record. CDOCKER, a grid-based docking program, was used to dock the active compounds in the ATP binding domain, considering the default parameters. The most favourable pose of the docked compounds was identified based on the CDOCKER energy (-CDE).

Objective 8: *Evaluate the safety profile of the most promising candidates in experimental animals (mice).* Some side effects of selected compounds will be evaluated after single-dose administration (1 hr after administration of one acute high dose of the drugs equivalent to three times the therapeutic dose) and after subchronic administration (where drug or vehicle is administered intraperitoneally to groups of rats daily for 21 days in the therapeutic dose). The urine volume will be measured and urine sodium ions concentration (by flame photometry according to the method described by Harirforoosh and Jamali [30] and plasma urea (according to Fawcett and Scott [31] and plasma creatinine concentrations (according to Lustgarten and Wenk [32] using colorimetric kits, will be determined. After the 21st injection, blood samples will be collected for the determination of complete blood count, SGPT and SGOT. Liver, kidney and heart samples will be examined histologically. Mortality rate and complete LD_{50} study will be performed using dose-response curves with at least 4 doses by the method of Litchfield and Wilcoxon [33].

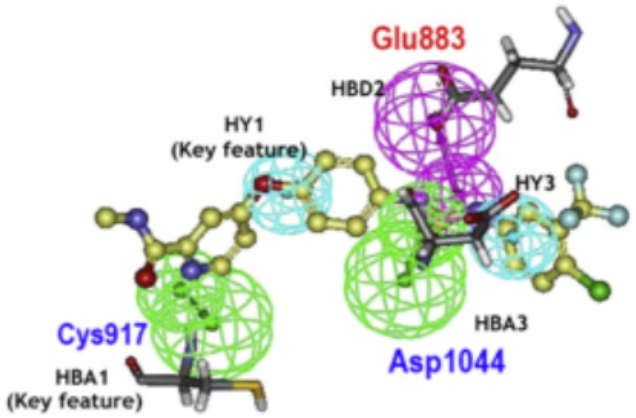
Anticipated Results and Evaluation Criteria

Results of the four work packages will be analysed periodically according to the GANTT chart below.

Representation of Novel structure designs will be performed. Chemical compounds synthesised in the lab will be available as prototypes and their chemical structure checked using different analytical techniques and represented as charts, tables and figures.

WP1: Molecular modelling evaluation will be based on the following criteria:

The constructed 3D model will be evaluated and compared with previously reported structure-based pharmacophore model on VEGFR-2, that has been obtained by the Lee et al [25].



*Pharmacophoric model describing binding mode with sorafenib into the hinge region of VEGFR-2 kinase.*Pharmacophoric features are color coded as follows: cyan, hydrophobic (HY); green, hydrogen bond acceptor (HBA); magenta, hydrogen bond donor (HBD).

The docked job will be validated through redocking of the most active VEGFR-2 inhibitors ((sorafenib & pazopanib) and subsequently, the CDOCKER binding energy of these potent inhibitors will be compared that obtained from our new designed candidates.

WP2: Charts obtained after the spectroscopic(IR, NMR and MS) analysis of the synthesized compounds will be used to confirm the structure of these compounds.

WP3: Biological testing will be evaluated through firstly; running MTT assay for IC50 doses determination followed by biochemical and molecular parameters will be measured for investigating potential drugs mechanism of actions on both breast cancer and liver carcinoma.

WP4: The battery of Toxicological studies is expected to yield as results:

Acute and Sub-Chronic studies: Tables and charts showing the change in laboratory parameters for liver, kidney, and cardiac function indices shown in methodology, in addition to a full report on body weight and vital parameters before, during and after the completion of drug-treatment. The results will be compared to that of the positive control anticancer drug.

LD_{50} determination: charts showing the number of dead animals versus dose to identify the dose that kills 50% of the mice, in addition to a table comparing the LD_{50} values for the most therapeutically effective prototypes. The compounds showing least LD_{50} is the highest in toxicity. These results are indispensable to choose the prototypes that will be transferred to the following pre-clinical phase in collaboration with industrial partners.

Statistical analysis: All data obtained will be presented as mean ± SE. Results will be analysed using one-way analysis of variance test (one-way ANOVA) followed by Tukey

post hoc test. Statistical analysis was performed using GraphPad Prizm software (version 3.0). For all the statistical tests, the level of significance is fixed at $p < 0.05$.

Expected Project Outcomes and Impact to AASTMT

I- Technical output and Impact:

After appropriate chemical and biological screening, the presented project will yield 1-5 prototype novel medicinal products which will be designed and formulated and potentially developed in the future into medications that may possess potential activity against breast and liver carcinoma. These prototypes of highest efficacy and lower toxicity are expected to be transferred to the following pre-clinical phase of drug development in collaboration with industrial partners.

As stated below, the results of this study will be published in at least one peer-reviewed journal specialized in drug discovery and development speciality, and one National conference. In all publications, the generous sponsorship of the AASTMT will be acknowledged with due respect.

College of Pharmacy is a newly-established institute in the AASTMT Abu-Kir campus. Generous budget is allocated to the construction and establishment of central laboratories (floors 3 and 4) specialized in green chemistry and molecular biology. The current collaboration between our team at AASTMT and Ain Shams University will enable the transfer of a valuable experience in these two fields concerning capacity building, proper use of specialized equipment and future sustainability plans for these newly-established research labs.

II- Financial feasibility & Socio-economic Impact:

Socioeconomic impacts

Liver and Breast cancer are the most prevalent cancer types in Egypt according to Globocan 2020 [1,2]. This disease is incapacitating to the patients and can progress to death which negatively affects the economic development of our Country and the wealth of its population. The contribution to the development of effective therapies will improve health care management and diminish the spreading of Breast and liver carcinoma in Egypt; providing good clinical solutions ensures better health, and better productivity.

The development of an effective treatment in collaboration with Egyptian industrial companies will have strong economic impact on Egypt and AASTMT. This proposal is the first step in achieving this impact. Markedly, liver and breast cancer research transforms and saves lives so our goal is to develop safe, economic and effective cure that is why we think AASTMT would accept to invest in our proposed project.

III – Publication:

Publication of from one to two research papers, in top peer reviewed specialized international journal (European Journal of medicinal chemistry, Q1, IF: 5.57; Bioorganic Chemistry, Q1, IF: 4.83; Bioorganic and medicinal chemistry, Q1, IF:3.07), for the new findings of this project.

Presentation of the project results in international specialized conference

Resources

Current resources that are used to carry out the proposed research project, as follows:

I- AASTMT Campus

- Personnel: Expert professors and lecturers (Professor. Amira Senbel, Dr. Botros Beshay and Dr. Mariam Shamaa) will be incorporated in this project. In addition to a number of professional technicians will be involved as well.
- Laboratory Space:

a. Chemistry lab equipped with hot plate magnetic stirrers, rotatory evaporator, UV lamp, melting point apparatus, glassware for carrying out the synthesis of the targeted designed molecules. The Chemistry lab will be used for carrying out the Chemical synthesis of targeted compounds

b. Computer aided drug design lab equipped with Discovery Studio software (DS) 5.0 client (Accelrys) for performing the modeling job.

c. Biological lab equipped with real-time PCR and cell cultures equipment and chemicals for running the biological experiments

d. Office and Computer Facilities: office space and computer facilities, together with all software deemed crucial to the research project are available.

II- Ain Shams University

- Personnel: Expert professors and lecturers (Ass.Professor. Nour El-Din Ahmed and Dr. Kurls Anwer) will be incorporated in this project.
- Laboratory Space:

Chemistry lab equipped with hot plate magnetic stirrers, rotatory evaporator, UV lamp, melting point apparatus, glassware for carrying out the synthesis of the targeted designed molecules. The lab is also equipped with laboratory Fume Cupboards to perform the chemical synthetic reactions that need high safety measures and could not be accomplished in AASTMT Campus.

III- Other Laboratory spaces

cell cultures labs are available for running the biological experiments at medical research institute, Smouha, Alexandria

Planned resources

The following equipment will be purchased: Microplate reader 2100-C for all immmounohistochemical analysis reading, Portable 3L liquid nitrogen storage tank container + storage tank for the proper transfer of samples between working sites, and Hot plate magnetic stirrer with thermometer for acurate chemical results.

Team Information

The team contributing to the implementation of this project belong to two institutions and three complementary scientific interests. The first and second WPs are assigned to the experts of medicinal chemistry (from AASTMT and Ain Shams University) to design, synthetize and confirm the structure of prototypes. WP3 is assigned to Dr. Marium Shamaa from AASTMT being specialized in the field of Molecular Biology, where the biological evaluation of effectiveness and relative potency of potential candidates will be proved. Using cell culture techniques and specific markers

determination, she will also aim to understand the pathological pathways modulated by these drugs. Docking techniques where the therapeutic effect is matched with receptor and enzymes molecular structure will be applied using specialized Computer program and this work will be done by Dr. Botros Beshay (Co-PI) from AASTMT. WP4 is dealing with final evaluation of side effects and toxicity of the most effective prototypes using experimental animals and the responsible personnel is Prof. Amira Senbel (PI).

1-Principle Investigator: Prof. Amira senbel

1. Basic Information		
Full Name in Arabic: اميرة مصطفي حلمي سنبل	**Full name in English**: Amira Mostafa Helmy Senbel	
Date of Birth	10/06/1976	
National ID	27606108800205	
Last University Degree Ph.D. 2005	**Faculty, University, Country** Faculty of Pharmacy, Alexandria University, Egypt	**Graduation info** Bachelor of Pharmaceutical Science, Faculty of Pharmacy, Alexandria University, June 1998
Title: Professor	**Field of specialization:** Pharmacology & Toxicology	
Affiliation:	College of Pharmacy, AASTMT, Abu-Kir Campus	
Current Position:	Vice-Dean for Training & Community Service	
Contact Information: **Mobile Phone:** 01006971669	**E-mail:** amira.senbel@aast.edu	

2. Scientific Achievements		
***h* index (SCOPUS only)** 10	**Citations (SCOPUS only)** 308	**Total no. of Int. publications in SCOPUS** 29

Last three recent publications	
1	Norel *et al.* International union of basic and clinical pharmacology. CIX. Differences and similarities between human and rodent prostaglandin E2 receptors (EP1-4) and prostacyclin receptor (IP): Specific roles in pathophysiologic conditions. Pharmacological Reviews, 2020, 72(4), pp. 910–968
2	Bassiouni, W., Senbel, A., Norel, X., Sildenafil corrects the increased contractility of rat detrusor muscle induced by alprostadil in vitro. Pharmacological Reports, 2019, 71(4), pp. 659–668.
3	Bassiouni, W., Daabees, T., Louedec, L., Norel, X., Senbel, A. Evaluation of some prostaglandins modulators on rat corpus cavernosum in-vitro: Is relaxation negatively affected by COX-inhibitors? Biomedicine and Pharmacotherapy, 2019, 111, pp. 1458–1466

Co- Principle Investigator: Dr. Botros Beshay

1. Basic Information		
Full Name in Arabic: بطرس يوسف جاب الله بشاي	**Full name in English**: Botros Youssef Gaballah Beshay	
Date of Birth.: 22-4-1986		
Last University Degree: PhD degree, September, 2019	**Faculty, University, Country** Faculty of Pharmacy, Alexandria University, Egypt	**Graduation info** Bachelor of Pharmaceutical Science, Faculty of Pharmacy, Assiut University, July 2008
Title: Lecturer	Field of specialization: 1-Drug Design, Molecular Modeling techniques applied to discover anticancer drug like molecules as well as antiviral candidates (HIV and COVID 19). 2- Antimicrobial drug like molecules and Serotonin receptor antagonists as anti- Alzheimer's agents, 3- Organic synthesis 4- Expert on Mastering of different computer aided drug Design software (Discover Studio, GOLD, SYBYL)	
Affiliation:	College of Pharmacy, Arab Academy for Science, Technology and Maritime Transport	
Current Position:	Lecturer of Medicinal and Organic chemistry	
Contact Information: **Mobile Phone:** +2 01007650839 **E-mail:** botros_beshay@aast.edu		

2. Scientific Achievements		
***h* index (SCOPUS only)** -	**Citations (SCOPUS only)** -	**Total no. of Int. publications in SCOPUS** -

Last three recent publications	
1	**Botros Y. Beshay**, Amira A. Abdellatef, Yasser M. Loksha, Salwa M. Fahmy, Nargues S. Habib, Alaa El-Din A. Bekhit, Paris E. Georghiou, Yoshihiro Hayakawa, Adnan A. Bekhit. Design and Synthesis of 2-Substituted-4-benzyl-5-methylimidazoles as New Potential

	Anti-breast Cancer Agents to Inhibit Oncogenic STAT3 Functions. *Bioorg. Chem.* ***(submitted Jan 22, 2021)***
2	**Botros Y. Beshay**, Sherief M. Abdel-Wahab, Zakaria K. Abdelsamii, Hanan A. Abdel-Fattah, Abdallah S. El-Etrawy, Louise N. Dawed and Paris E. Georghioua Synthesis and docking study structure-activity computation studies and a preliminary antibiotic evaluation of selected 2-aryl- and fluoroarylbenzimidazole-N1-acetamido conjugates. *Arch. Pharm*. ***(submitted December 15, 2020***
3	Botros Y. Beshay , Anwer, K , Marium M. Shamaa , Nour E.A. Abd El-Sattar, Design, green synthesis, molecular docking and anticancer evaluations of Pyrozolopyrimidine derivatives bearing sulfonamide moieties as VEGFR-2 inhibitors, bioorganic chemistry *(will be submitted April 1, 2021*)

AASTMT member: Dr. Mariam Shamaa

1. Basic Information		
Full Name in Arabic: مريم محمد حسن شمعه	**Full name in English**: Marium Muhamed Hassan Shamaa	
Date of Birth.: 1-1-1986		
Last University Degree: PhD degree, February, 2017	**Faculty, University, Country** Faculty of Pharmacy, Alexandria University, Egypt	**Graduation info** Bachelor of Pharmaceutical Science, Faculty of Pharmacy, Alexandria University, July 2007
Title: Lecturer	**Field of specialization:** ▪ Exploring the potential biological activity of recent biological compounds using in-vitro cytotoxicity assay and different mammalian cell lines. ▪ Extraction and isolation of DNA and RNA from whole blood and cell line. ▪ A good experience in the detection of genes using advanced techniques such as PCR, qRT-PCR, polyacrylamide and agarose gel electrophoresis. ▪ Dealing Excellency with cell culture techniques such as Thawing, Sub culturing and storage. ▪ Diagnostic biochemistry labs techniques (Kits and manual).	
Affiliation:	College of Pharmacy, Arab Academy for Science, Technology and Maritime Transport	
Current Position:	Lecturer of Biochemistry and Molecular Biology	

Contact Information: **Mobile Phone:** +2 01227537121 **E-mail:** marium.muhamed@aast.edu		
2. Scientific Achievements		
***h* index (SCOPUS only)** 3	**Citations (SCOPUS only)** 24	**Total no. of Int. publications in SCOPUS** 4
Last three recent publications		
1. Marium M. Shamaa (2020). **Sulfasalazine synergistically enhances the inhibitory effects of imatinib against hepatocellular carcinoma (HCC) cells by targeting NFκB, BCR/ABL, and PI3K/AKT signaling pathway-related proteins. *FEBS Open Bio*. doi: 10.1002/2211-5463.13052.** 2. Marium Shamaa; Mariam Arieby; Marie Mina; Monica Ossama; Heba Salamoony, **"Immunotherapy for different types of cancer: current status and future prospects"**, Dubai International Pharmaceutical & Technology Conference & Exhibition 2020, February, 2020. 3. Marium Shamaa, **"3D Cell Culture in Cancer Targeted Therapy Discovery"**, International Conference on Pharmaceutical & Healthcare Sciences-PHS, Alexandria University, 6-7 November 2019. 4. Botros Y. Beshay , Anwer, K , Marium M. Shamaa , Nour E.A. Abd El-Sattar, Design, green synthesis, molecular docking and anticancer evaluations of Pyrozolopyrimidine derivatives bearing sulfonamide moieties as VEGFR-2 inhibitors, bioorganic chemistry *(will be submitted April 1, 2021*)		

Member 1: Assoc Prof. Nour El-Din Ahmed

1. Basic Information		
Full Name in Arabic: نور الدين احمد عبد الستار	**Full name in English**: Nour El-Din Ahmed	
Date of Birth	3-6-1977	
National ID	27706030100559	
Last University Degree Ass Prof	**Faculty, University, Country** Ain Shams uni	**Graduation info** 2017
Title: Ass prof	**Field of specialization:** Chemistry	
Affiliation:	Ain Shams university	
Current Position:	Associate Professor	
Contact Information: **Mobile Phone:** 01012277219 **E-mail:** nourel-dinahmed@sci.asu.edu.eg		
2. Scientific Achievements		
***h* index (SCOPUS only)** 4	**Citations (SCOPUS only)** 4	**Total no. of Int. publications in SCOPUS** 19
Last three recent publications		

1	Nour E. A. Abd El-sattar, Eman H. K. Badawy, Eman Z. Elrazaz and Nasser S. M. Ismail, Discovery of pyrano[2,3-d]pyrimidine-2,4-dione derivatives as novel PARP-1 inhibitors: design, synthesis and antitumor activity, RSC Adv., 2021, 11, 4454–4464
2	Nashwa M. Saleh , Mohamed S.A. El-Gaby , Khaled El-Adl , Nour E.A. Abd El-Sattar, Design, green synthesis, molecular docking and anticancer evaluations of diazepam bearing sulfonamide moieties as VEGFR-2 inhibitors, bioorganic chemistry 104 (2020) 104350
3	Nour E. A. Abd El-Sattar, Eman H. K. Badawy, Wafaa H. AbdEl-Hady, Mohamed I. Abo-Alkasem, Asmaa A. Mandour,and Nasser S. M. Ismail, Design and Synthesis of New CDK2 Inhibitors Containing Thiazolone and Thiazolthione Scafold with Apoptotic Activity, Chem. Pharm. Bull. 69, 106–117 (2021)

Member 2: Dr. Kurls Ekram Anwar

1. Basic Information		
Full Name in Arabic: كيرلس اكرام انور	**Full name in English**: Kurls Ekram Anwer	
Date of Birth	24/4/1992	
National ID	29204240100413	
Last University Degree Ph.D.	**Faculty, University, Country** Science, Ain Shams university, Cairo, Egypt.	**Graduation info**
Title: Doctor	**Field of specialization:** Organic synthesis.	
Affiliation:	Chemistry department, faculty of Science, Ain shams University.	
Current Position:	Lecturer	
Contact Information: **Mobile Phone:** 01115478842 **E-mail:** Kurls_koko@yahoo.com kurlsEkram@sci.asu.edu.eg		

2. Scientific Achievements		
***h* index (SCOPUS only)** 3	**Citations (SCOPUS only)** 51	**Total no. of Int. publications in SCOPUS** 7

Last four recent publications	
1	Anwer, K. E., & Sayed, G. H. (2020). Conventional and microwave reactions of 1, 3-diaryl-5, 4-enaminonitrile-pyrazole derivative with expected antimicrobial and anticancer activities. Journal of Heterocyclic Chemistry, 57(6), 2339-2353
2	Anwer, K., Sayed, G., Hassan, H., & Azab, M. (2019). Conventional and Microwave Synthesis of Some New Pyridine Derivatives and Evaluation Their Antimicrobial and Cytotoxic Activities. Egyptian Journal of Chemistry, 62(4), 707-726.
3	Sayed, G. H., Azab, M. E., & Anwer, K. E. (2019). Conventional and Microwave-Assisted Synthesis and Biological Activity Study of Novel Heterocycles Containing Pyran Moiety. Journal of Heterocyclic Chemistry, 56(8), 2121-2133.

4	Sayed, G. H., Azab, M. E., Negm, N. A., & Anwer, K. E. (2018). Antimicrobial and cytotoxic activities of some novel heterocycles bearing pyrazole moiety. Journal of Heterocyclic Chemistry, 55(7), 1615-1625.

Research Team Information Table

Name of Res. Team Member in English	Name of Res. Team Member in Arabic	University / Institute In English	Position / Title	% of time spent on project	No. of months	Incentive per month (LE)	Number of other projects and their IDs	Total % of time spent on other projects	Contact No
Amira Senbel	اميرة سنبل	AASTMT(PI)	Professor	20%	12	1500	0	0	01006971669
Botros Beshay	بطرس بشاي	AASTMT (Co-PI)	Lecturer	40%	12	1500	0	0	01007650839
Marium Shamaa	مريم شمعة	AASTMT	Lecturer	40%	12	1500	0	0	01227537121
Nour El-Dina abdel Sattar	نور الدين عبد الستار	Ain Shams University	Associate Prof.	40%	6	1500	0	0	01012277219
Kurls Ekram Anwer	كيرلس اكرام انور	Ain Shams University	Lecturer	40%	6	1500	0	0	01115478842

Project Management

The proper management of the project implementation depends on:
- Division of tasks and expected outputs in the form of four work packages
- Each work package has a definite responsible personnel assigned among the team members according to his/her area and discipline of expertise
- Complementation of work packages chronologically
- Continuous self-assessment of each subtask of the GANTT chart taking the indices described under “Expected results” section as reference of proper achievement.
- Timely analysis of data and representation to ensure a smooth flow of work between the work packages and their responsible investigators
- Conduction of Bi-monthly Zoom meetings among the members of the team for revision of achievement and future planning.

- Exchange of live visits between the members of the team in their respective institutions in form of seminars to ensure an efficient transfer of scientific expertise.
- Assessment of risks (funding delays, purchasing delays, accidental absence of leave… etc) and preparing their substitution plan early ahead during execution.

Activity Name	M1	M2	M3	M4	M5	M6	M7	M8	M9	M10	M11	M12
1. Modelling and computerized design												
1.1. focus meetings and update review of literature	■											
1.2: Construct 3d pharmcophore model	■	■										
1.3: Validation of model	■	■										
1.4: Design of novel molecules	■	■										
2. Laboratory chemical synthesis of prototypes												
2.1. Purchasing Chemicals	■		■	■	■	■						
2.1: Synthetic pathway 1			■	■	■	■						
2.2: Synthetic pathway 2			■	■	■	■						
2.3: Synthetic pathway 3			■	■	■	■						
2.4: structure elucidation			■	■	■	■	■					
3. Biological evaluation of efficacy and understanding potential mechanism of action												
3.1. Purchasing equipment	■						■	■	■	■		
3.2 Purchasing Chemicals and Cell lines	■						■	■	■	■		
3.3: MTT cytotoxicity tests							■	■				
3.4: cells treatment								■	■			
3.5: biochemical and molecular parameters evaluation								■	■	■		
3.6: Docking studies											■	
4. Toxicological Studies												
4.1: Acute Studies										■	■	
4.2: Subchronic Studies										■	■	

4.3: Mortality test (LD_{50})determination												
4.4. Analysis of data and representation												
5. Publication												
5.1: Drafting manuscript												
5.2: Abstract presentation in conference												
5.3: Seminar												

Project Cost

Table of Eligible Cost

Eligible costs	Break downs		AASTMT support (L.E.)
(A) Staff Cost	Prof Amira Senbel		18000
	Dr. Marium Shamaa Dr. Botros Beshoy Dr. Dr.		18000 18000 9000 9000
	Technicians and/or Labor		----
	Consultation fees		---
	Total		72000
(B) Equipment	Equipment		109350
	Spare parts		----
	Total Equipment		109350
(C) Expendable Supplies & Materials	Stationary		1000
	Miscellaneous Laboratory, Field supplies, Materials Experimental animals		283050 5250
	Total expendable Supplies & Materials		289300
(D) Travel	Internal Transportation		1000
	Accommodation		2000
	Total travel		3000
€ Other Direct Costs	Services	Manufacture of specimens & prototypes	---
		Acquiring access to specialized reference sources databases or computer software	
		Computer services	
	Report preparation		
	Publications & patent Costs		25000
	Workshops organization or Training		1000
	Others (explain)		…….
	Total other direct costs		26000
(G) Total Costs			**499650**

Breakdown of Costs Other Grant(s)

Materials, Chemicals		
Size	Item	Price
1 L	DMSO	5000
2 bottles	MTT reagent	5000
4 flasks	Cell lines	8000
	Cell culturing reagents and glasswares	10000
	lab bench fees and analyses	30000
5 kits	Whole blood extraction kits	15000
	PCR Primers and master mix plus dyes (syber green)	10000
9 kits	ELISA kits	15000
0.1 mg	Rats Polyclonal AKT1 [p Ser473] Antibody	12670
0.1 mg	Rats Polyclonal TOR/mTOR [p Ser2448] Antibody	13320
0.05 ml	Rats Polyclonal ERK1/2 Antibody	11700
0.1 mg	Rats Monoclonal PD-1 Antibody (7A11B1)	11550
100 µg	Rat EGF Affinity Purified Polyclonal Ab	9100
100 µg	Rat VEGF MAb (Clone 123704)	8550
25 mg	4-aminobenzenesulfonamide (Sulfanilamide)	5850
10 mg	4-amino-N-(pyrimidin-2-yl)benzenesulfonamide	21000
10 mg	4-amino-N-(5-methylpyrimidin-2-yl)benzenesulfonamide	21000
10 mg	4-amino-N-(quinoxalin-2-yl)benzenesulfonamide	14500
5 g	2,6-difluoroaniline	1400
1 gm	2,4-dichloroaniline	2660
10 mg	4-amino-N-phenylbenzenesulfonamide	11400
10 mg	4-amino-N-cyclohexylbenzenesulfonamide	11400
1 g	4-aminobenzenesulfonyl chloride	3000
1kg	Malononitrile	6800
1kg	Ethyl acetoacetate	1750
1kg	Ethyl cyanoacetate	4000
250 g	Acetyl acetone	3200
250 gm	Di ethyl malonate	8700
500 gm	Sodium acetate	500
250 gm	Sodium nitrile	1000
	Total	**283050 LE**

Equipment name	description	cost
Microplate reader 2100-C	Micro Plate Reader, Model 2100-C, Comecta, Ivymen (EU)	75850
Portable 3L liquid nitrogen storage tank container + storage tank	Portable 3L liquid nitrogen storage tank container + storage tank	23000
Hot plate magnetic stirrer	With contact thermometer, made in koria by DAIHAN	10500
	Total	**109350 LE**

Plans for Disseminating Research Results / Sustainability of the action

Internal Dissemination of results

The tasks of the current project fall under four work packages which are sequential in order. Each team member is assigned the responsibility of one or more work packages. Dissemination of results among the team will depend on bi-monthly meetings between team members. Effective communication, self-evaluation and criticism is our proof of quality and our way to hand over reviewed results to the next responsible team members according to the order of work packages.

The transfer of know-how and experience between the two collaborating institutes will be achieved by physical visits to the labs and conduction of two seminars over the course of the project duration.

External dissemination of results

Dissemination of knowledge and novel data resulting from this project depend on:

- at least one participation in a specialized conference nationally
- Publication of from one to two research papers, in top peer reviewed specialized international journal (European Journal of medicinal chemistry, Q1, IF: 5.57; Bioorganic Chemistry, Q1, IF: 4.83; Bioorganic and medicinal chemistry, Q1, IF:3.07).

Sustainability

- The main output of this project is prototype chemical compounds proved effective and less toxic when compared to conventional therapies. These prototypes of highest efficacy and lower toxicity are expected to be transferred to the following pre-clinical phase of drug development in collaboration with industrial partners. Future collaboration with a leading Pharmaceutical company in Egypt will secure sponsoring the huge funding required to conduct preclinical studies (phase 2 and 3), application for patency, National Drug Authorities approval ..etc.
- The application of this complementary inter-disciplinary research project and the experience gained during its implementation with colleagues from Ain Shams

University will be of great impact on proper design and usage of central labs at college of Pharmacy AASTMT which are in their late phase of construction.
- Scientists in the beginning of their career contributing to this project will gain immense experience that will be of great value for themselves, as well as their post and undergraduate students in the near future.

LIST OF ABBREVIATIONS

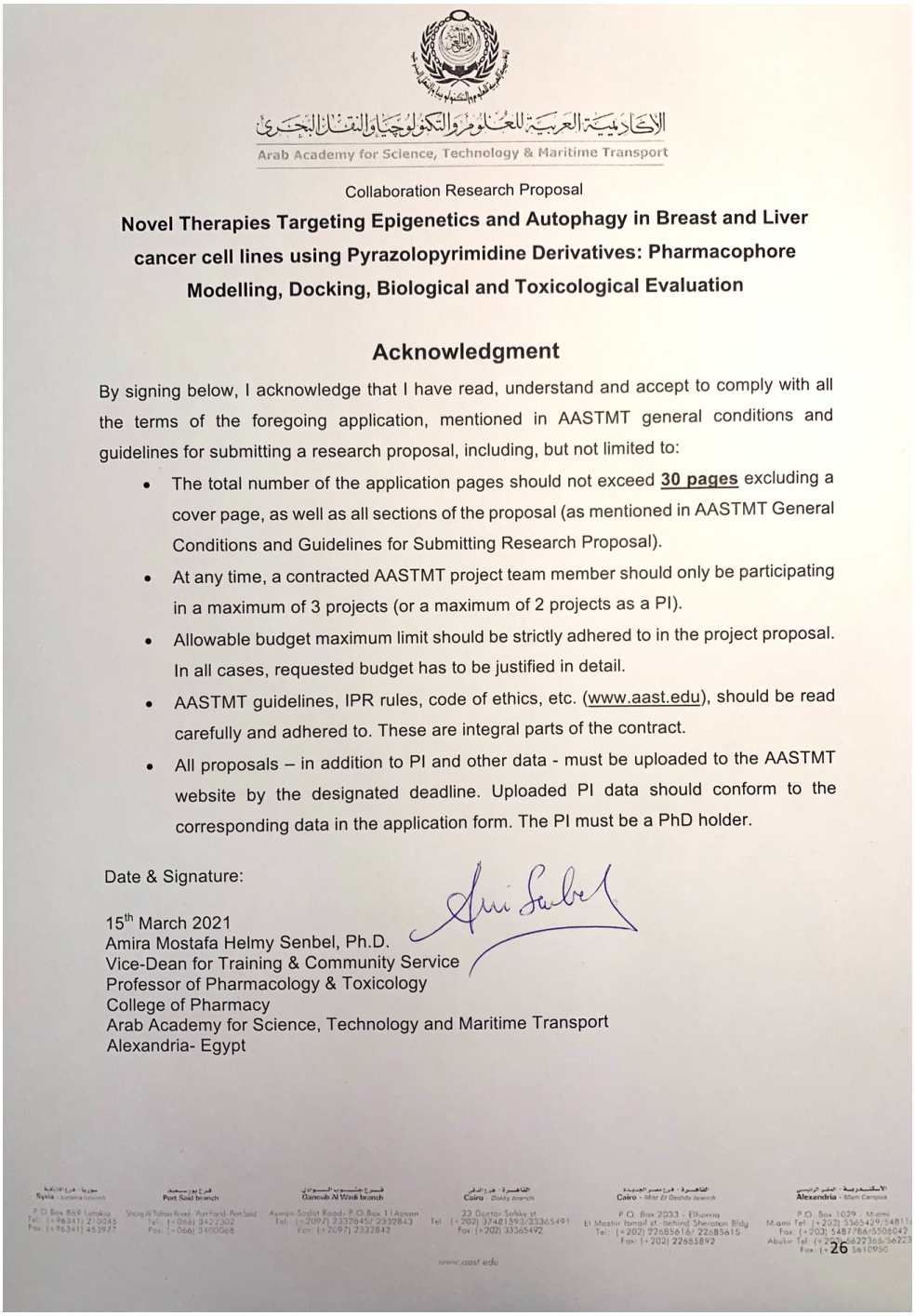
CT : Threshold cycle
DMEM: Dulbecco's modified eagle's medium
DMSO: Dimethyl sulfoxide
ELISA : Enzyme linked immunosorbent assay
ERK : Extracellular regulated kinase
HCC : Hepatocellular carcinoma
HGF : Hepatocyte growth factor
mTOR : Mechanistic target of rapamycin
MTT : Microculture tetrazolium test
NFκB : Nuclear Factor κB
PBS : Phosphate buffer solution
PD-1 : Programmed cell death protein 1
PDGF : Platelet derived growth factor
PI3K : Phosphatidylinositol 3-kinase
QRT-PCR Quantitative real time polymerase chain reaction
RAF : Serine-threonine protein kinase
VEGF : Vascular endothelial growth factor
VEGFR: Vascular endothelial growth factor receptor

Key Publications and references

1. https://www.who.int/health-topics/cance, accessed August 11, 2020.
2. GLOBOCAN 2020, https://gco.iarc.fr. accessed August 11, 2020.
3. A. S. Ibrahim, H. Baraka, H. M. Khaled, Hossam Kame, N. NH Mikhail. *Journal of Cancer Epidemiology,* Volume 2014, Article ID 437971.
4. *Jemal A, Bray F, Center MM, et al. Global cancer statistics. CA Cancer J Clin 2011; 61: 69–90.*
5. *Bray F, Ferlay J, Soerjomataram I, et al. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin 2018; 68: 394–424.*
6. Ghouri, Y.A., I. Mian, and J.H. Rowe, Review of hepatocellular carcinoma: epidemiology, etiology, and carcinogenesis. Journal of carcinogenesis, 2017. 16.
7. Shamaa, M.M., Sulfasalazine synergistically enhances the inhibitory effects of imatinib against hepatocellular carcinoma (HCC) cells by targeting NFκB, BCR/ABL, and PI3K/AKT signaling pathway-related proteins. FEBS Open Bio, 2020.
8. Yang, J.D., et al., A global view of hepatocellular carcinoma: trends, risk, prevention and management. Nature Reviews Gastroenterology & Hepatology, 2019: p. 1-16.
9. Shi, G.-J., et al., Involvement of growth factors in diabetes mellitus and its complications: a general review. Biomedicine & Pharmacotherapy, 2018. 101: p. 510-527.
10. Liu, L., et al., NO donor inhibits proliferation and induces apoptosis by targeting PI3K/AKT/mTOR and MEK/ERK pathways in hepatocellular carcinoma cells. Cancer Chemotherapy and Pharmacology, 2019. 84(6): p. 1303-1314.

11. Li, A., et al., BEZ235 increases sorafenib inhibition of hepatocellular carcinoma cells by suppressing the PI3K/AKT/mTOR pathway. American journal of translational research, 2019. 11(9): p. 5573.
12. Ji, B., et al., Hybrid Membrane Camouflaged Copper Sulfide Nanoparticles for Photothermal-chemotherapy of Hepatocellular Carcinoma: Consistent with the observations so far Acta Biomaterialia, 2020.
13. Duda, P., et al., Targeting GSK3 and associated signaling pathways involved in cancer. Cells, 2020. 9(5): p. 1110.
14. Patel, M., et al., NF-κB pathways in the development and progression of colorectal cancer. Translational Research, 2018. 197: p. 43-56.
15. Chen, P., et al., Epigenetic silencing of microRNA-204 by Helicobacter pylori augments the NF-κB signaling pathway in gastric cancer development and progression. Carcinogenesis, 2020. 41(4): p. 430-441.
16. Hervieu, A. and S. Kermorgant, The role of PI3K in met driven cancer: a recap. Frontiers in Molecular Biosciences, 2018. 5: p. 86.
17. Xiang, C., J. Chen, and P. Fu, HGF/Met signaling in cancer invasion: the impact on cytoskeleton remodeling. Cancers, 2017. 9(5): p. 44.
18. J. N., Philoppesa, M. A. Khedrb, M. H. A. Hassanc, G. Kameld, P. F. Lamiea, New pyrazolopyrimidine derivatives with anticancer activity: Design, synthesis, PIM-1 inhibition, molecular docking study and molecular dynamics. Bioorganic Chemistry 100 (2020) 103944.
19. A.Y. Hassan, N.M. Saleh, Mona. S. Kadh, E.S. Abou-Amra, New fused pyrazolopyrimidine derivatives; heterocyclic styling, synthesis, molecular docking and anticancer evaluation. J Heterocyclic Chem. 2020; 57:2704–2721.
20. F. Ran, Y. Liu, M. Liu, D. Zhang, P. Wang, J. Dong, W. Tang, G. Zhao, Discovery of pyrazolopyrimidine derivatives as potent BTK inhibitors with effective anticancer activity in MCL. Bioorg Chem, 2019 Aug;89:102943.
21. Ran F, Liu Y, Yu S, Guo K, Tang W, Chen X, Zhao G, Design and synthesis of novel 1-substituted 3-(6-phenoxypyridin-3-yl)-1H-pyrazolo[3,4-d]pyrimidin-4-amine analogs as selective BTK inhibitors for the treatment of mantle cell lymphoma. Bioorg Chem. 2020 Jan;94:103367.
22. A. Rahmouni, S. Souiei, M. A. Belkacem, A. Romdhane, J. Bouajila, H. B. Jannet, Synthesis and biological evaluation of novel pyrazolopyrimidines derivatives as anticancer and anti-5-lipoxygenase agents. Bioorg Chem 2016 Jun;66:160-8.
23. A. H. Shamroukh, A. E. Rashad, R. E. Abdel-Megeid, H. S. Ali, M. M Ali. Some pyrazole and pyrazolo[3,4-d]pyrimidine derivatives: synthesis and anticancer evaluation. Arch Pharm. 2014 Aug;347(8):559-65.
24. K. Lee ,Y. Kim ,K-W. Jeong ,Y. Lee, J. Y. Song ,M. S. Kim, G. S. Lee. European Journal of Medicinal Chemistry 45 (2010) 5420e5427.
25. Miyazaki, Y., Matsunaga, S., Tang, J., Maeda, Y., Nakano, M., Philippe, R.J., Shibahara, M., Liu, W., Sato, H., Wang, L., Nolte, R.T. Novel 4-amino-furo[2,3-d]pyrimidines as Tie-2 and VEGFR2 dual inhibitors. (2005) Bioorg Med Chem Lett 15: 2203-2207
26. Pollard, J. W. and J. M. Walker (1997). Basic cell culture protocols, Springer Science & Business Media.
27. Nolan, T., Hands, R. & Bustin, S. Quantification of mRNA using real-time RT-PCR. Nat Protoc 1, 1559–1582 (2006). https://doi.org/10.1038/nprot.2006.236
28. Michael F. Clark, Richard M. Lister, Moshe Bar-Joseph, ELISA techniques, Methods in Enzymology, Academic Press, Volume 118, 1986, Pages 742-766.
29. Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265–275.
30. Harirforoosh S, Jamali F. Effect of nonsteroidal anti-inflammatory drugs with varying extent of COX-2-COX-1 selectivity on urinary sodium and potassium excretion in the rat. Can J Physiol Pharmacol 2005;83(1):85–90.

31. Fawcett JK, Scott JE. A rapid and precise method for the determination of urea. J clin Pathol 1960;13:156–9.
32. Lustgarten JA, Wenk RE. Simple, rapid, kinetic method for serum creatinine measurement. Clin Chem 1972;18(11):1419–22.
33. Litchfield JT Jr, Wilcoxon FA (1949) Simplified method of evaluating dose-effect experiments. J Pharm Exp Ther 96:99–113.



الأكاديمية العربية للعلوم والتكنولوجيا والنقل البحري
Arab Academy for Science, Technology & Maritime Transport

Collaboration Research Proposal

Novel Therapies Targeting Epigenetics and Autophagy in Breast and Liver cancer cell lines using Pyrazolopyrimidine Derivatives: Pharmacophore Modelling, Docking, Biological and Toxicological Evaluation

Acknowledgment

By signing below, I acknowledge that I have read, understand and accept to comply with all the terms of the foregoing application, mentioned in AASTMT general conditions and guidelines for submitting a research proposal, including, but not limited to:

- The total number of the application pages should not exceed **30 pages** excluding a cover page, as well as all sections of the proposal (as mentioned in AASTMT General Conditions and Guidelines for Submitting Research Proposal).
- At any time, a contracted AASTMT project team member should only be participating in a maximum of 3 projects (or a maximum of 2 projects as a PI).
- Allowable budget maximum limit should be strictly adhered to in the project proposal. In all cases, requested budget has to be justified in detail.
- AASTMT guidelines, IPR rules, code of ethics, etc. (www.aast.edu), should be read carefully and adhered to. These are integral parts of the contract.
- All proposals – in addition to PI and other data - must be uploaded to the AASTMT website by the designated deadline. Uploaded PI data should conform to the corresponding data in the application form. The PI must be a PhD holder.

Date & Signature:

15th March 2021
Amira Mostafa Helmy Senbel, Ph.D.
Vice-Dean for Training & Community Service
Professor of Pharmacology & Toxicology
College of Pharmacy
Arab Academy for Science, Technology and Maritime Transport
Alexandria- Egypt

الأكاديمية العربية للعلوم والتكنولوجيا والنقل البحري
Arab Academy for Science, Technology & Maritime Transport

Collaboration Research Proposal

Novel Therapies Targeting Epigenetics and Autophagy in Breast and Liver cancer cell lines using Pyrazolopyrimidine Derivatives: Pharmacophore Modelling, Docking, Biological and Toxicological Evaluation

Declaration

I hereby declare as project Principle Inverstigator that the project entitled "*Novel Therapies Targeting Epigenetics and Autophagy in Breast and Liver cancer cell lines using Pyrazolopyrimidine Derivatives: Pharmacophore Modelling, Docking, Biological and Toxicological Evaluation*" is not submitted neither in whole or in part to any another funding programs.

As a PI, I equally declare that I was the recipient of a fund by STDF in collaboration with IFE (French Institute in Egypt) from March 2018- January 2019 for the project entitled "Potential role of AMPK activators and their interaction with prostacyclin and NO/cGMP pathways in the treatment of pulmonary hypertension". This funding has ended and final achievement report has been accepted and archived by STDF in May 2019.

Date & Signature:

15th March 2021
Amira Mostafa Helmy Senbel, Ph.D.
Vice-Dean for Training & Community Service
Professor of Pharmacology & Toxicology
College of Pharmacy
Arab Academy for Science, Technology and Maritime Transport
Alexandria- Egypt

سوريا - فرع اللاذقية
Syria - Latakia branch
P.O.Box 869 Latakia
Tel: (+96341) 210045
Fax: (+96341) 453977

فرع بورسعيد
Port Said branch
Sharq Al Tafraa Road - Port Fuad- Port Said
Tel: (+066) 3422302
Fax: (+066) 3400068

فرع جنوب الوادي
Ganoub Al Wadi branch
Aswan-Sadat Road- P.O.Box 11Aswan
Tel: (+2097) 2332845/ 2332843
Fax: (+2097) 2332842

القاهرة - فرع الدقي
Cairo - Dokky branch
23 Doctor Sobky st.
Tel: (+202) 37481593/33365491
Fax: (+202) 33365492

القاهرة - فرع مصر الجديدة
Cairo - Misr El Gedida branch
P.O. Box 2033 - Elhorria
El Moshir Ismail st.-behind Sheraton Bldg.
Tel: (+202) 22685616/ 22685615
Fax: (+202) 22685892

الاسكندرية - المقر الرئيسي
Alexandria - Main Campus
P.O. Box 1029 - Miami
Miami Tel: (+203) 5565429/5481163
Fax: (+203) 5487786/5506042
Abukir Tel: (+203) 5622366/5622388
Fax: (+203) 5610950

www.aast.edu

كلية العلوم
FACULTY OF SCIENCE

AIN SHAMS UNIVERSITY

Date: 13/03/2021

Dear Sirs

Arab Academy for Science, Technology and Maritime Transport (AASTMT)

I'd like to express my support of the project proposal entitled: "**Novel therapies targeting epigenetics and autophagy in liver and breast cancer cell lines using pyrazolopyrimidine derivatives: Pharmacophore modeling and docking, biological and toxicological studies** " being submitted to AASTMT Call for Collaboration Research and Innovation Project by **Dr. Kurls Ekram Anwer**.

I fully support the efforts of the research team from the Faculty of Science, Ain Shams University (ASU) as they seek external funding to implement their research work articulated in the submitted proposal.

Sincerely,

Prof. Dr. Mohamed Ragaa Mohamed ElSotohi

Dean of Faculty of Science

Ain Shams University

15/3/21

كلية العلوم
FACULTY OF SCIENCE

AIN SHAMS UNIVERSITY

Date: 13/03/2021

Dear Sirs

Arab Academy for Science, Technology and Maritime Transport (AASTMT)

I'd like to express my support of the project proposal entitled: "**Novel therapies targeting epigenetics and autophagy in liver and breast cancer cell lines using pyrazolopyrimidine derivatives: Pharmacophore modeling and docking, biological and toxicological studies** " being submitted to AASTMT Call for Collaboration Research and Innovation Project by **Dr. Nour El-din Ahmed Abd El-Sattar**.

I fully support the efforts of the research team from the Faculty of Science, Ain Shams University (ASU) as they seek external funding to implement their research work articulated in the submitted proposal.

Sincerely,

Prof. Dr. Mohamed Ragaa Mohamed ElSotohi

Dean of Faculty of Science

Ain Shams University