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Design and synthesis of rutaecarpine analogs as potential cytotoxic agents for cancer chemotherapy treatment

(Diploma thesis)

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I declare that this Diploma Thesis is an original author's copy, written down by myself (under the supervision of PharmDr. Marta Kučerová, Ph.D.). All literature and other sources used in this diploma thesis are listed in the literature section. This diploma thesis was not used to gain another or the same academic degree. This study was supported by the specific academic research (SVV 260 183).

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ABSTRACT

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Cancer is a progressive multifactorial collection of diseases that causes disorders and decreases quality of life of patients and in some cases results in death. Cancer can affect people of all ages, sexes and races and can be diagnosed in tissues of whole body. It is the second leading cause of death in the world after cardiovascular disorders. There are many ways how to treat cancer and they variy with each type of cancer.

Nowadays treatment of cancer is based on surgery, chemotherapy, radiotherapy, biological treatment, immunotherapy and hormonal therapy. Chemotherapy represents the basis of cancer cure and is often used in combination with other approaches for tumour treatment and maximal effect of therapy and to prevent and cure metastases. Chemotherapy consists of cytotoxic agents that induce apoptosis by various pathways. These drugs interfere with cell cycle of all body cells but, in general, chemotherapy agents are aimed at fast proliferating cells more than cells with physiological cell cycle. Cytotoxic chemotherapy agents act by various mechanisms that lead to programmed cell death and are divided to five categories; these are alkylating agents, antimetabolites, anti-tumour antibiotics, topoisomerase inhibitors and tubulin binding drugs all aimed at modifications in DNA replication and transcription and cell division.

Rutaecarpine is an alkaloid found in fruits of *Evodia rutaecarpa* called Wu-Chu-Yu that have been widely used in traditional Chinese medicine for a very long time. Rutaecarpine has been proved to possess many pharmacological properties including cytotoxic activity. Its planar structure promises potential cytotoxicity through intercalation into DNA and derivatives of rutaecarpine have been proved to inhibit topoisomerase I and topoisomerase II. Topoisomerase

inhibitors are widely used agents in clinical practice either alone or in combination with other antineoplastic agents.

This work is focused on preparation of rutaecarpine derivatives that could show cytotoxic activity equal to rutaecarpine but would possess increased water solubility, because the natural alkaloid is very poorly soluble. Due to the position chosen for substitution also inhibitory activity against topoisomerases could be increased. Cytotoxic activity together with better solubility of prepared derivatives could bring new options for their potential use as anti-cancer treatment.

ABSTRAKT

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Rakovina je progresivní onemocnění multifaktoriálního charakteru s mnoha symptomy, které snižuje kvalitu života pacientů a v některých případech končí smrtí. Rakovina může postihovat lidi všech věkových skupin, pohlaví nebo ras a může být diagnostikována v tkáních po celém těle. Po kardiovaskulárních onemocněních je rakovina na světě druhým nejčastějším onemocněním způsobujícím smrt.

Dnešní léčba rakoviny spočívá v chirurgickém odstranění tumoru, chemoterapii, radioterapii, cílené terapii, imunoterapii a hormonální terapii. Chemoterapie představuje základní přístup protirakovinné léčby a je často aplikována v kombinaci s dalšími způsoby léčby pro maximalizaci účinku terapie a při léčbě a prevenci metastází. Chemoterapie v dnešním slova smyslu představuje léčbu cytotoxickými léčivy, která aktivují buněčné mechanismy vedoucí k apoptóze. Tyto látky narušují buněčný cyklus všech buněk v těle, ale obecně jsou zaměřeny především na rychle se dělící buňky. Cytotoxické látky působí různými mechanismy vedoucími k programované buněčné smrti a dělí se do pěti skupin podle jejich mechanismu účinku- alkylační látky, antimetabolity, cytostatická antibiotika, inhibitory topoizomeráz a látky vážící se na tubulin. Všechna cytostatika jsou zaměřena na změny v replikaci DNA a transkripci a dělení buněk.

Rutekarpin je alkaloid nacházející se v plodech rostliny *Evodia rutaecarpa* lidově nazývaných Wu-Chu-Yu, které se již dlouhou dobu hojně používají v tradiční čínské medicíně. Bylo prokázáno, že rutekarpin má mnoho farmakologických vlastností, mimo jiné byla prokázána i jeho cytotoxická aktivita. Pro jeho planární strukturu se nabízí jeho využití jako interkalační látky a jeho deriváty zase ukázali inhibiční aktivitu proti topoizomerasam I a II.

Inhibitory topoizomerasy jsou široce využívanou skupinou chemoterapeutik buď samostatně, nebo v kombinaci s dalšími látkami.

Tato práce je zaměřena na přípravu derivátů rutekarpinu, které zachovávají cytotoxickou aktivitu rutekarpinového pentacyklu, ale vykázaly by lepší rozpustnost ve vodě vzhledem k velmi špatné rozpustnosti přírodního alkaloidu. Zároveň zvolená pozice pro substituci slibuje možnou inhibiční aktivitu proti topoizomerázám. Cytotoxická aktivita společně se zvýšenou rozpustností derivátů by mohla přinést nové možnosti pro jejich potenciální využití v protirakovinné chemoterapii.

Content

1 LIST OF ABBREVIATIONS

5-FU	5-fluorouracil
AA	amino acid
ADP	adenosine diphosphate
AML	acute myelogenous leukaemia
Arg	arginine
AS	active site
BCNU	carmustine
bp	base pair
CCNU	lomustine
CGI	CpG islands
CINV	chemotherapy-induced nausea and vomiting
CPT	campthotecin
CT	clinical trials
DCM	dichlormethane
DMF	dimethylformamide
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DNAMT	DNA methyltransferase
dsDNA	double stranded DNA
ET_2O	diethyl ether
EtOAc	ethyl acetate
FDA	Food and Drug Administration
GARFT	glycinamide ribonucleotide formyltransferase
GHKL	gyrase, HSP90, histidine kinase, MutL
GI50	50% growth inhibition

GIT	gastrointestinal tract
His	histidine
Hsp	heat shock protein
Lys	lysine
MeOH	methanol
MP	mercaptopurine
MutL	DNA-mismatch repair enzyme L
NA	nucleic acid
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
PAH	polycyclic aromatic hydrocarbon
PI3K	phosphoinositol 3-kinase
PKB	protein kinase B
PTEN	phophatase and tensin homolog
RNA	ribonucleic acid
THF	tetrahydrofuran
TLC	thin layer chromatography
Topo I	topoisomerase I
Topo II	topoisomerase II
TopoIcc	topoisomerase I cleavage complex
TS	thymidylate synthase
TSG	tumour suppressor gene
Tyr	tyrosine
UTP	uridine triphosphate
VBL	vinblastin
VCR	vincristin
VDS	vindesine
VRL	vinorelbine

2 THEORETICAL PART

2.1 Cancer

Cancer is a generic term given to a collection of diseases. These diseases can affect any part of the body and they each have its own aetiology and clinical outcome. All diseases of this group lead to uncontrolled cell growth.[1]

Normal cells divide, grow and die in balance that depends on the organism's actual needs. Cancer cells break down these processes and keep on growing without being regulated. During cancer process some of many types of cells in human body start dividing uncontrolled forming a primary tumour and secondary spreading into other tissues. A group of growing cells forms a subset of neoplasms. When neoplasms form mass they grow to tumours that can be either solid or soft (as in case of leukaemias). Tumour is basically a mass of cells of unregulated growth that affect not only the tissue of its base but does affect also other surrounding tissues by pressure caused by its growth. These tumours caused by cancer are called malignant tumours and they can spread into other tissues or they can partially get to distant places by blood or lymphatic system and start new neoplasms there that lead to new tumours. This is called metastasis and it is the most life-threatening stage of cancer.

Also benign tumours can develop in human body but they are not as dangerous as malignant ones, they do not invade other tissues and they mostly do not grow back after removal.[2]

There are many types of cancer distinguished by the kind of tissue or organ affected by the unregulated growth of cells. Well known types based on names of organs that are affected are for example lung cancer, breast cancer, prostate cancer and according to the type of cells forming the tumour are for example sarcoma, carcinoma, melanoma or lymphoma.

Nowadays drug treatment comprises many cytotoxic chemotherapeutics divided into five main categories. We use alkylating agents, anti-metabolites, anti-tumour antibiotics, topoisomerase inhibitors and tubulin binding drugs.[3]

Other possibilities to cure are cancer surgery, radiotherapy or immunotherapy. The treatment of cancer is in most cases based on a combination of therapies, i.e. surgery and chemotherapy.

2.1.1 History

Cancer has been on this world even longer than humankind itself. Even dinosaurs have been found to suffer from cancer. There have been found tumours in dinosaur fossils and also in fossils of prehistoric people. First evidence of human cancer was found in 19th century in Egyptian papyri from 1600–1500 BC. The papyri were possibly based on much older material and consist of records concerning breast cancer and provide possibilities to cure it. Basic methods were surgery, magical and pharmacological treatment. A man's dead body of age approximately 2700 years that was found in Siberia provides the oldest scientific evidence of human disseminated cancer. Its skeletal lesions were confirmed by modern techniques to have prostatic origin.[4,5]

The scientific discipline studying cancer is called oncology based on antique Greece word *onkos* that was first used for tumours or masses of growing cells by Hippocrates. He was also the first to use words *karkinos* and *karkinomas* (nowadays' carcinomas) for neoplasms. Aulus Cornelius Celsus, Hippocrates successor, then distinguish between *cacoethes* that can be surgically removed from body and *carcinomas* that would grow back after removal and are practically untreatable. He also believed that the treatment would even worsen the disease and its progression.[4]

Galen described first described processes of fast cell growth in human body in a written document and divided them into three categories. He distinguished between physiologically enlarging organs and pathologically growing organs. While enlargement of uterus during pregnancy was understood as physiological, growths of lymph nodes caused by inflammation or tumours were taken as pathological. He saw the difference between these two categories, as well, and so he was the first man to write a document about tumours both benign and malignant. Galen also formulated a black bile theory of cancer origin.[4]

After the fall of Greek empire there was lack on new discoveries in medicinal care. Galen's and Hippocrates' knowledge was taken as standard for a very long time. During next centuries there was described nature of many kinds of cancer with various symptoms. It was also described that there can be seen swollen blood vessels that proliferate to provide tumours with necessary nutrients.[4]

After the release of post-mortem dissections during the 14th century new findings in cancer could be applied in medicine. In 1507 there was the first printed case report of stomach cancer which was found by Antonio Benivieni. He actually did not recognize it as cancer but according to evidence we know it was gastric carcinoma.[6]

New hypotheses of the origin of cancer were formulated for the first time in almost 1500 years history of belief in Galen's black bile theory. Paracelsus suggested that cancer is

caused by a series of *ens* (entities) such as *astrotum* (cosmic), *veneni* (toxic), *naturale et spirituale* (physical or mental) and *deale* (providential) which is a base for nowadays theory of cancer as a collection of diseases.[4]

Gabriele Fallopius described very precisely differences between benign and malignant tumours and many of them are applicable even today. He characterized malignant tumours as very firm cell masses of irregular shape often with surrounding swollen blood vessels and adhesion to neighbouring tissues. While benign tumours were identified as watery softer masses that are movable and not adherent to surrounding tissues.[4]

With the invention of microscope cells started to be studied and scientists wanted to find differences between physiological and cancerous cell. At first they believed there are specific essential cancerous cells in a body that end up with tumours but later this theory was disproved and scientists identified cancerous cells as secondarily defected. German scientist Theodor Boveri observed cancer development in sea urchins and suggested there is a role of somatic mutations as a starting point of neoplasms.[4]

In July 1896 there was first used radiation in form of X-rays to treat cancerous cell masses by Victor Despeignes. At the same time Pierre Curie observed skin burns on his body developed because of handling radioactive samples. He started to study radioactivity as treatment for cancer and later radium cure became known as Curie therapy that showed interesting results. Marie and Pierre Curie were later awarded Nobel Prize in physics for their research in radioactivity. Marie Curie carried on in research of Curie therapy after the death of her husband and founded the Radium Institute to use radium to cure tumours there. Marie Currie died in 1934 from exposure to X-ray and radioactivity.[4]

During 20th century cancer was addressed to the national and also international levels and a lot was done in research of treatment of this disease. Many hospitals and institutes aimed at cancer treatment were established all around the world and cancer became well-known and unfortunately also very expanded and today it has become the second most frequent cause of death in developed countries.

2.1.2 Prevalence and incidence

In most western countries the incidence and mortality rate of cancer decreases but in less developed countries the trend is inverse. Still more and more cancer cases are reported in less developed countries mainly those that have adopted western style of living i.e. eating unhealthy food and physical inactivity.[7] 32.6 million people lived with cancer diagnosis, 14.1 million patients were newly diagnosed cancer and 8.2 million people died from cancer in 2012 worldwide.[8] According to WHO data it is more than all strokes or all ischaemic heart disease.[9]

The highest number of new cases and deaths is connected with lung cancer, 12.9% of cases within incidence and 19.4% in case of mortality rate respectively. The second most common cancer is breast cancer with 11.9% of new cases per year and the third most common is colorectal cancer that affects 9.9% of people diagnosed with cancer. There is not a big difference between men and women; cancer affects more men but only of 6% which means 53% for men and 47% for women. The most common cancer for men is lung cancer with 16.7% out of all men cases and in case of women it is breast cancer with 25.2%. The overall cumulative risk of being diagnosed any kind of cancer by the age of 75 for both sexes is 18.5%. In case of deaths the difference between both sexes is similar to incidence but the overall risk of death from cancer by 75 is only 10.5%.[9]

The cancer site also varies among continents or between more developed and less developed countries. While in more developed countries the leading cancer site of new cases of diagnosed cancer is prostate for men in less developed countries it is lung. In case of women the leading site is breast for both groups of countries but the second most common site is colorectum for more developed countries and cervix uteri for less developed ones.[9] It is believed that colorectum points at bad eating habits and it is associated with unhealthy food while in case of less developed countries there are more cases of cancers associated with viral or bacterial infections. The overall prevalence ratio of cancer in less developed countries was 56% in 2008. It is believed to increase to over 60% in 2030 due to their faster acceptance of western lifestyle than western healthcare systems.[7]

The situation in the Czech Republic is very similar to the world situation. The most common cancer site in 2007 was colorectum in both sexes together. While in men the leading cancer site was lung cancer and prostate cancer, women are affected mostly by breast cancer. The 5-year prevalence of lung and prostate cancer in men was in years 2003–2007 17.4% that represents approximately 23300 patients diagnosed with this cancer site. The same prevalence for women's breast cancer was in these years 24.3% which means about 29300 cases of breast cancer diagnosis. Cumulative risk of being diagnosed any kind of cancer was 41.21% for men and 28.40% for women respectively in 2007. The increasing numbers of both prevalence and incidence are believed to be associated with more factors than only health. The most important factor is development in cancer healthcare and diagnostic methods that are very important instruments in fighting cancer. The other factors can be demographic such as ageing of the population of the Czech Republic or different kinds of supportive programmes for earlier detection of cancer.[10]

All data used in these paragraphs do not include non-melanoma skin cancer.

2.1.3 Causes

As cancer is not only one disease but a collection of them there are very lot of factors playing role in causing malignant neoplasms. Cell mutation that could start unregulated growth of group of cells that would end up with a malignant life-threatening tumour can be either genetic based or gained due to our behaviour during our lives. It is commonly said that cancer is mainly genetic but it is believed that only 5–10% of cancer cases could be associated with genetic predisposition. The rest 90–95% is attributed to our lifestyle and environmental causes.[11] Studies have shown that genetic related groups of people are at higher risk than others. Dizygotic and monozygotic twins have been proved to get cancer more likely than other relatives when one of the pair already got it. But actually the presumption is not as high as we would expect it. The concordance of breast cancer between dizygotic and monozygotic twins has been shown to be only 12% and 20%, respectively.[12,13] It shows that the association between cancer and genetic predisposition is not the main factor. The real problem is how we live.

The main risk factors within environment and human lifestyle are tobacco, alcohol use, diet, obesity and physical inactivity, infectious agents – either viral or bacterial – then environmental and occupational carcinogens and exposure to radiation.[11,14]

2.1.3.1 Tobacco

Tobacco is associated not only with lung cancer but it is also connected with at least 13 other kinds of cancer, as well. It contains more than fifty carcinogens and it affects all organs and all parts of body. Tobacco causes cancer in smokers but also second-hand smokers are at risk. Most deaths are associated with tobacco use (around 30%) and it causes death in 87% of cases of lung cancer.[11] Primary prevention in form of non-smoking can save most lives worldwide and it should be aimed at this field primarily. Smoking has decreasing trend in more developed countries while in less developed countries it stagnates or the number of people smoking is raising and they are at higher risk of developing cancer.[14] For example the decrease of tobacco use in USA from 1950s can be associated with almost 40% drop in deaths from lung cancer from 1991 to 2003.[15]

2.1.3.2 Alcohol use

Alcohol is associated with cancer of aero-digestive tract i.e. liver, oropharynx, larynx and oesophagus cancer in both sexes. In addition it is also linked to breast cancer in women and it is identified as a cause in 3% of new cases of breast cancer. Every day use of alcohol over 60 g also increases risk of colorectal cancer. Alcohol also affects liver and increases risk for potential hepatitis infection that can lead to hepatocellular carcinoma by alcohol caused cirrhosis. Ethanol itself does not belong to list of carcinogens but it is metabolised in human

body to acetaldehyde and free radicals are released during the process. They can then bind to DNA and proteins and cause secondary mutation and hyperproliferation.[11]

2.1.3.3 Diet, obesity and physical inactivity

Diet, obesity and sedentary lifestyle are associated with each other and they can play a very important role in tumour formation. It is understood that physical inactivity and bad eating habits lead to obesity which is considered a risk factors of many diseases including cancer.

Diet itself plays a role and is associated significantly with colorectal cancer. It is connected with 70% of deaths caused by colorectal cancer. Also most carcinogens that are ingested come from food.[11] Risky food for colorectal cancer is represented mainly by red and processed meat as well as smoked or charcoal grilled meat and low fibre meals. A lot of carcinogens are produced by meal preparation, e.g. heterocyclic amines are produced by cooking meat. 2-Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine that is produced by cooking beef is a mutagen responsible for approximately 20% of total mutagenicity found in fried beef that is largely consumed in fast food restaurants.[11,14] Diet can be related to as many as fourteen types of cancer and not only with GIT cancer types.[11]

Obesity or overweight is linked to 14% in men and 20% in women of all deaths from cancer in the United States.[16] Obesity is a huge problem of developed countries and is becoming a problem even in less developed countries due to their acceptance of western lifestyle and bad eating habits. This also causes increasing incidence of cancer in less developed countries. Among compounds associated with obesity and cancer we can find insulin-like growth factor-1 (IGF-1), insulin or sex steroids. Also factors like insulin resistance and inflammation is connected with obesity and cancer.[17]

Physical inactivity leads to increase of BMI that can secondarily become a cause of colorectal, breast and endometrial cancer.[14] Physical activity can decrease the risk of colorectal cancer by 17–24%, breast cancer by 20–40% and endometrial cancer by approximately 27%. It lowers the risk through the decrease of BMI and also through other not yet well understood mechanisms. Recreational physical activity has been shown to be more effective than professional physical activity.[18]

2.1.3.4 Infectious agents

An estimated 16% of malignant neoplasms were associated with infectious agents in 2008 worldwide. The percentage was higher in less developed countries than in more developed ones by 15%. The most often infectious agents associated with cancer are *Helicobacter pylori*, human papillomaviruses and hepatitis viruses B and C. Cervical cancer in women caused by infectious agents represents around a half of cases and liver and gastric cancers in men caused

by infectious agents represent approximately 80% of these cancers diagnosed.[14] Hepatitis B virus infection generates reactive oxygen through chronic inflammation and indirectly causes cancer this way while human papillomavirus is acting directly through activation of viral genes E6 and E7 that are mutagenic and cause cervical cancer. The major risk factor causing cancer during infection is the inflammation. There is evidence that almost all mutagenic – either directly or indirectly – infectious agents activate an inflammatory marker known as NF-κB, also Helicobacter pylori's components activate this marker. Due to this anti-inflammatory drugs can play a role in prevention.[11]

2.1.3.5 Occupational and environmental carcinogens

Combating occupational carcinogens dates back to 1921 when the first report concerning aromatic amines was published by International Labour Organization. By now the reduction of exposure to carcinogens at work has saved many lives and protected many people from being diagnosed cancer. The main occupational carcinogens are aromatic amines, asbestos, benzene or benzidine. Strict rules were first used to measure asbestos exposure at workplace in the USA and Sweden and thirty years later there was recorded decrease in mesothelioma risk. These factors once the carcinogens are known are the most easily preventable in population.[14]

More than 15 types of cancer can be associated with environmental carcinogens. Environmental pollution includes arsenic, inside and outside air pollution, metallic pollution and food pollutants and additives for instance nitrates or organochlorines. Outside air pollution includes pesticides, petrol and diesel exhaust or carbon particles linked to polycyclic aromatic carbons (PAHs). Inside air pollution comprise tobacco smoke and volatile organic compounds e.g. benzene or formaldehyde. PAHs and nitric oxide have been proved to be associated with increased occurrence of lung cancer. Nitric oxide has also shown to be an inductor of metastases of lung cancer. Another environmental pollutant from incinerators – dioxane – has been found to be the cause of risk increase of sarcoma or lymphoma. Also something as common as water can be increasing the risk of cancer development when it is chlorinated and the exposure is long term. As well sunlight is dangerous in term of skin cancer and it is definitely easily preventable.[11,14]

2.1.3.6 Radiation

Radiation can be the cause of cancer in more than 10% of overall cancer cases. Both ionizing and nonionizing radiation induces cancers such as sarcoma, lymphoma and leukaemia or breast cancer. Even X-ray examination gives raise to cancer when restrictions and special handling are not applied. Women are at increased risk of breast cancer after X-ray chest irradiation during puberty when breasts are developing. The most dangerous carcinogens from this group are radon, radium and uranium either at home or surroundings or at work, e.g. mine

workers are at risk. Also low-frequency electromagnetic field can be a source of mutagenic effect to cells. High-voltage power lines, transformers, electric engines can increase the risk of carcinogenic mutation. Actually all electronic equipment can raise the potential of cancer development which is now a commonly discussed question, i.e. cell phones and their prolonged daily use have shown that they can increase the risk of brain tumour development.[11]

2.1.4 Prevention

Prevention is basically to avoid all of the above mentioned causes of cancer and to get examined early and regularly to increase the chances of cancer survivor when diagnosed. A study from the United Kingdom from 2010 says that 45% of cancer in men and 40% in women could have been prevented by reduction of risk factor to optimum level or by their elimination.[19] Obviously the proportion would be definitely various in different parts of the World and there will be a difference between less and more developed countries. Diet contributes most to overall cancer development – between 30–35% – and it is followed by tobacco and infectious agents. Among patients with lung cancer almost 90% of them are cigarette smokers. Even smokeless tobacco can cause cancer; it is responsible for over 4% of all cancers worldwide.

The prevention includes eating of various kinds of fruits and vegetables, teas and spices, wholegrain foods, regular vitamins intake and regular physical activity. Molecules known to play a role in cancer prevention are for example carotenoids, quercetin, silymarin, catechins, curcumin, diallyldisulfide, capsaicin, piperine, ferulic acid, phenolic acids, vitamins such as vitamin C, D and E especially. Lack of exercise leads to higher serum insulin level, larger fat masses and lower concentrations of hormone binding globulin that could end up for instance with hormone dependent breast cancer.[11] In case of infectious agents based cancers, vaccination is prevention and early and regular screening. Also some medication can be categorized as preventive in case of cancer, for example NSAIDs can reduce the risk of colorectal cancer but they obviously cannot be used only to prevent cancer due to their effect on cardiovascular system and their adverse effects on GIT.[20]

2.1.5 Signs and symptoms

Cancer does not cause any signs or symptoms at the beginning, they get produced after growth of tumours or after their ulceration. Neoplasms alone do not hurt; it is the pressure of tumours to surrounding tissues or the ulceration of cell masses. The ulceration leads to bleeding from various parts of body, e. g. lungs, colon etc., that can be detected by sight or by painful feeling in the place. In case tumours are close to body surface can be detected by touch. Basic symptoms for all cancers can be divided to several groups – skin changes, breast changes, thickening of neoplasms under or on the skin, long-term hoarseness or cough, changes in bowel habits, difficult or painful urination, eating discomforts, unexpected weight gain or loss,

abdominal pain, unexplained night sweats, unusual bleeding or discharge or feeling weak and unsteady.[21] People shall be also worried of unilateral pain in leg or arm. Because of low specificity of cancer symptoms and its signs the disease is often thought to be some other illness, even by doctors, and cancer can be diagnosed with delay or even never. [22]

2.1.6 Diagnosis

Diagnosis differs with every different type of cancer. It starts with auto-examination by patients themselves at home which should be the first preventive steps. The next step is getting examined by lab tests that can point to cancer but also to various less serious homeostasis changes or illnesses. When neoplasms and tumours already occur, they can be observed by screening or imaging procedures. They include X-ray, ultrasound, CT scan, magnetic resonance imaging or PET (positron emission tomography) scan and nuclear scan when patients receive a radioactive tracer that is consequently scanned. To make sure that a patient is affected by cancer, biopsy is required.[23] Biopsy is a screening of a sample of tissue, cells or liquid that is removed from patient's body and then it is put under analysis of nucleic acids.[24] The sample of tissue can be removed with a needle, with an endoscope or by surgery that can be either excisional when whole tumour is removed or incisional when only a part of tumour is removed and used for biopsy.[23] The outcome of biopsy tissue sample is called pathology report.

2.2 Pathophysiology of cancer

Cancer from the microscopic point of view is caused by so-called mutations in DNA as results of DNA damage.[25] These mutations lead to change in life cycle of cells so that they start to behave differently and whole process ends up with neoplasm and tumour formation. These so-called mutations are alterations in oncogenes, tumour-suppressor genes and micro-RNA genes of a cell's DNA or their expression and they enhance transcription and other mechanisms that lead to malignant neoplasms. Cancer is ordinarily not caused by a single mutation in DNA but by a series of mutations, that could not be inhibited before tumour growth is started.[26] In fact mutations are routine business in human body cells but normally they are suppressed and repaired by physiological mechanisms. Every day a cell undergoes tens of thousands of mutations in its DNA.[25] Only when pathological mechanisms overbalance the physiological ones cancer is considered to be at place.

Mutations can be either genetic or epigenetic and they occur as large-scale and small-scale events. Genetic mutations involve changes in DNA sequence and alterations of transcription and protein synthesis. Large-scale mutations are losses or gains of whole parts of chromosomes. They are distinguished into two groups – genomic amplifications and translocations. Genomic amplification is characterised by a big number of copies of the same small chromosomal region. It often contains one or more oncogenes and neighbouring genetic

material. Translocation arises when two neighbouring chromosomes exchange some genetic regions, usually in specific locations. Small-scale mutations include deletion, insertion or point mutations. All of the above mentioned mechanisms could, without repairing mechanisms, lead to overexpression of an oncogene and to tumour formation or it may change the function or stability of the protein that is encoded in a specific gene sequence.

Epigenetic changes refer to mechanisms that include alterations of gene expressions without changing the DNA sequence. These alterations include DNA methylation, histone modification and posttranscriptional gene regulation by a noncoding RNA strand called micro-RNA. These processes are responsible for repression or expression of genes.[27]

2.2.1 Oncogenes

Oncogenes encode synthesis of proteins that are responsible for apoptosis and cell proliferation, or both together. They can be activated by large or small-scale mutations, gene fusion and juxtaposition to enhancers and by amplification. While translocations and mutations usually appear as initiative events of tumour formation, amplification often occurs later during the progression of the neoplasm.

The fact that cancer is caused by genetic mutations was first described in studies of Burkitt's lymphoma where the oncogene MYC from chromosome 8q24 is juxtaposed to an enhancer element from different chromosomes 14q, 22q, 2p by translocation. The enhancer element is a locus for immunoglobulin heavy chain coding gene. The oncogene MYC gets then induced by the enhancer element from the immunoglobulin heavy chain gene locus and initiates proliferation and malignant neoplasm.[26,28]

Other studies showed that transgenic mice with activated human oncogenes suffer from developing malignant neoplasms that resemble the human tumours. There have to be possibly more different alteration in genes for the tumour to progress because there is a latent period during the mice's cells reproduce and die ordinarily with no tumour formations. An activated oncogene is believed to be necessary but not the only cause of developing cancer.[26,29]

Oncogene products can be divided into six main groups; these are transcription factors, growth factors and their receptors, chromatin remodelers and apoptosis regulators.[26]

2.2.2 Proto-oncogenes

Proto-oncogenes are genes normally occurring genes in DNA strand and they can undergo a mutation to form oncogenes. One of the first known families of oncogenes called *ras* oncogenes has been proved to have proto-oncogenic origin. There are three different *ras* genes, H-*ras*, K-*ras*, N-*ras* that can be converted to *ras* oncogenes by mutations in codons 12, 13 and

61, respectively. These mutations can be observed in a lot of various tumours with different incidence depending on the tumour type.[30]

2.2.3 Tumour suppressor genes

Tumour suppressor genes (TSGs) encode inhibiting effects on proliferation and cell division. These genes are in fact transcription factors that regulate transcription of DNA to RNA with RNA polymerase. TSGs function as a repressor of the transcription process. They get activated by DNA damage or by some kind of cellular stress and their activation leads to suppression of cells' growth and mitosis. While oncogenes can lead to cancer by activation of proto-oncogenes, tumour suppressor genes lead to malignant neoplasm by their inactivation. This inactivation is a result of mutation of TSGs or adjacent genes.

There is a big difference between the domination of oncogenes and TSGs. Oncogenes have to get activated to cause proliferation and cell growth and they are dominant. It means that for their activation only one allele needs to get mutated while the other can stay inactivated and the neoplasm progresses anyway. There is only one mutation necessary to activate oncogene. TSGs have to get inactivated to result in the same result and they require a "two-hit" inactivation and so they are recessive. There is a need to get mutated both alleles for TSGs to lose their anti-proliferative and cell growth stopping effect. When there is a mutation in one allele of a TSG there is a big chance that neoplasm never develops because a second mutation in the other allele coding the TSG is necessary for its inactivation.[31]

The best described TSG is a p53 transcription factor and it is associated with more than 50% of all cancers. Its deletion findings in human colorectal cancer led to analysis of the second allele that showed mutations in this allele and it implicated p53 as a driving force of cancer.[32]

2.2.4 Micro-RNA genes

Micro-RNA (miRNA) genes are non-coding RNAs of approximately twenty-two nucleotides and are responsible for gene silencing after translation of mRNA into proteins and for regulation of gene expression. The alterations in miRNA genes are epigenetic and the DNA sequence does not change. Malignant neoplasm is caused either by their up-regulation or down-regulation. Their expression leads to increased or decreased expression of a number of genes that could lead to cancer. Their effect depends on the targets in specific tissues and can play a role of either oncogene or tumour suppressor gene. MiRNA are transcribed by RNA polymerases II and III when RNA polymerase II transcription is more usual.

MiRNA genes have been proved to appear in chromosomal segments involved in cancerous mutations, specifically amplifications and deletions. They have also been found in chromosomal alterations in cancers where oncogenes and tumour suppressor genes were not

involved. MiRNA genes are predicted to be involved in regulation of more than 60% of protein translation and in many other cell's regulation processes. It has also been predicted that there are approximately 1000 types of miRNA in the human genome.

One of the first described families of miRNAs, so-called LET-7, has an important role in regulation of mRNA translation and cell division. Members of the family target *ras* oncogenes. When LET-7 genes are down-regulated *ras* oncogenes get overexpressed and it leads to unregulated cell division. LET-7 family members have been described to be down-regulated or totally deleted in lung cancer. Another example of association of miRNA with TSGs is miR21 that inhibits expression of the tumour suppressor gene PTEN. PTEN gene encodes phosphatase that is a part of PI3K signalling pathway. Due to overexpression of miR21 PTEN is mutated or silenced that plays a role in advanced breast, prostate, lung or gastric cancer.[26,27]

2.2.5 Other epigenetic mutations

DNA methylation is a common epigenetic mutations and it plays a role in maintenance in cellular development. Cytosine's ring position 5' usually gets methylated within CpG dinucleotides and the result of methylation of these CpG dinucleotides is silencing of genes that starts with this segment. The overall methylated cytosines account for 1% of the total number of nucleotides but approximately for 75% of all CpG pairs in human genome. The total number of these segments is around 29000 that are distributed in whole genome but of higher concentration occurs in so-called CpG islands. Promoters of transcription are situated within CGIs in 50–60% of cases. The methylation is catalysed by DNA methyltransferase enzymes (DNAMTs). In group of DNAMTs are main three enzymes DNAMT1-3 and lack of some members of the group leads to death in mice. For example DNAMT3A and B are *de novo* enzymes that are highly expressed during embryogenesis while there is lack of them in adult tissues. Methylation of CpG segments of genome can down-regulate gene expression by inhibition of promoter and the transcription of particular genes by RNA polymerase cannot be started. When the down-regulated genes participate in cell development and its growth and division regulation malignant neoplasm can occur.[27]

Histone modification is another possible epigenetic mutation that can lead to tumour formation. Histones are proteins that form nucleosomes connected with linker DNA and nucleosomes concentrate to an ordered structure called chromatin. Nucleosomes consist of octamers of histone proteins with approximately 146 base pairs and are formed by two subunits of each of histone proteins H2A, H2B, H3 and H4. Histones contain a globular domain and a so-called histone tail that is a flexible charged NH_2 end. These histone tails undergo posttranslational modifications in form of methylation, acetylation, phosphorylation or ADP

ribosylation. The modifications have an important role in gene expression. Chromatin is divided to two parts; heterochromatin and euchromatin. Heterochromatin has got dense organised structure given by the formation of nucleosomes and it is not transcriptionally active while euchromatin is lightly organised with big spaces between histones connected with linker DNA. Euchromatin is transcriptionally active and rich in histone modifications like acetylation and trimethylated histone proteins. Heterochromatin lacks histone modifications. The modifications are catalysed by enzymes, for instance histone deacetylases, histone acetyltransferases or histone methyltransferases. Their overexpression or mutation can lead to down-regulation or up-regulation of their function and gene expression via formulation of either open structure of euchromatin or closed structure of heterochromatin.[25,27]

2.3 Cancer chemotherapy treatment

Chemotherapy treatment from the historical point of view means any kind of chemical used to treat diseases. Nowadays meaning of chemotherapy is reserved for cancer and antimicrobial treatment with chemical agents and the previous sense of the word as any kind of chemical drug used to treat other diseases is now being expressed in pharmacotherapy.[33]

Cytotoxic chemotherapy treatment remains the mainstream systemic cure for cancer. These drugs interfere with cell cycle and processes involved in cell proliferation. Cytotoxic agents kill cells by various mechanisms that lead to DNA damage or damage on key proteins for cell division and the death is caused by apoptosis. They are aimed on fast growing and dividing cells but they can also affect normal cells and cause adverse effects in other parts of the body, especially bone marrow and mucous membranes. Therapy of cancer is usually combining various ways of therapy, such as radiotherapy, surgery and systemic therapy. Systemic therapy is required especially in cases of disseminated cancer or in patients with high risk of cancer metastases. Cytotoxic chemotherapy has systemic effect as well as immune therapy, hormonal therapy or targeted therapy.[3,34]

Cell cycle is divided into four stages; G1 phase, S phase, G2 phase and M phase. All stages vary in length from each other and can vary according to cell type. G1 or gap1 phase is initial growth stage and it is the first phase of cell cycle after division. During this phase the cell is growing and number of proteins and organelles is being increased. The G1 checkpoint mechanisms take control of the stage and check if the cell is ready to get to S phase and that DNA strand could get replicated. S phase or synthetic phase is a stage when synthesis of DNA takes place. DNA is being replicated and the number of chromosomes gets doubled but the ploidy of the cell remains the same. G2 phase or gap2 phase is another stage of cell growth when the cell is getting ready for mitosis and its size is getting almost double the original size. The G2 checkpoint control mechanisms confirm everything is prepared for the cell to get to

mitosis. M phase or mitosis phase is the last stage when cell growth is stopped and chromosomes, nucleus, cytoplasm, organelles and the cell membrane get divided into two new cells from one mother cell. Chromosomes get fixed to fibres that pull sister chromatids to opposite sides of nucleus and cytokinesis takes place during which nucleus gets divided followed by membrane folding inward and dividing the rest of cell's material roughly in halves and forming two identical new cells. M phase consists of four other phases called prophase, metaphase, anaphase and telophase and they are followed by the cytokinesis.

Cytotoxic agents in chemotherapy used for cancer treatment can be divided to five main categories of derivatives according to their mechanism of action. These groups are alkylating agents, anti-metabolites, anti-tumour antibiotics, topoisomerase inhibitors and tubulin binding drugs.[3]

2.3.1 Alkylating agents

Alkylating agents are drugs that affect DNA by forming covalent bonds in its strands and causing single-strand or double-strand DNA breaks while the cell is trying to replicate or repair the alkylated strands. These breaks lead to cross-linking of DNA between both strands of DNA (interstrand crosslink) and within one strand of DNA (intrastrand crosslink). They are also able to form covalent bonds in proteins and strands of RNA. The covalent bond formations and following breaks in DNA strands lead to apoptosis – programmed cell death. Alkylating agents are independent on cell cycle, they can affect cell in any stage and cause the apoptosis. Certain percentage of cells is killed by certain dose of alkylating cytotoxic drugs and so their effect is dose-dependent and cell cycle-independent. These drugs are used extensively. Alkylating agents can be categorised by their chemical structure to eight classes; nitrogen mustards, oxazaphosphorines, platinum based drugs, alkyl alkane sulphonates, nitrosoureas, tetrazines, aziridines and non-classical alkylating agents. [34,35]

Nitrogen mustards most commonly alkylate DNA strands on N7 nitrogen of guanine and form covalent bonds between these bases. Other molecules can be also alkylated but in less cases. Chlorambucil and melphalan are the most common nitrogen mustard derivatives used in clinical practice. Chlormethamine (chlormethine) is rather a sulphur mustard agent and it is rarely used for cancer chemotherapy treatment.

Platinum compounds get activated inside cells and form reactive derivatives that alkylate nucleotides and lead to inter- and intrastrand crosslink of DNA. Nowadays treatment comprises three of these agents; cisplatin, carboplatin and oxaliplatin. Platinum based derivatives are commonly used in chemotherapy following surgical removal of tumours but strong adverse events are observed often when used especially severe nausea and vomiting after chemotherapy (CINV). Patients treated with this platinum compounds are at highest risk of

CINV and medical treatment combating these adverse effects should be used as prophylaxis right from the beginning of the chemotherapy.[36]

Cyclophosphamide was developed in 1960's to be selectively activated by over-expressed enzyme phosphoramidase but later it was discovered that cyclophosphamide and its derivatives are activated by liver and not by the tumour produced phosphoramidase. Nevertheless **oxazaphosphorines** act as alkylating agents but with strong side effects.

The only member of **alkyl alkane sulphonates** is busulfan and its main use is in chronic myeloid leukaemia.

Nitrosoureas gets decomposed quickly in aqueous solution into chloroethyl diazonium ion and an isocyanate derivative. While the isocyanate derivative can carbamoylate proteins, the chloroethyl diazonium ion can alkylate DNA and lead the cell to apoptosis. Nitrosoureas when decomposed usually alkylate O6 position of guanine. Members of this group of cytotoxic agents are carmustine (BCNU), lomustine (CCNU), semustine, fotemustine and streptozotocin. While BCNU and CCNU are mostly used in brain tumours due to their lipophilic structure, streptozotocin as a natural sugar based nitrosourea is used for treatment of pancreatic islet cell tumours.

Tetrazines acts the same way like nitrosoureas by releasing a reactive diazonium ion to alkylate DNA strands. Two members of this group are used in clinical practice, dacarbazine and temozolomide, both used for malignant melanomas.

Thiotepa and mitomycin C are two prodrug members of **aziridines** and procarbazine and hexamethylmelamine (altretamine) are also prodrugs from a nonspecific group of non-classical alkylating agents.[34]

2.3.2 Antimetabolites

Antimetabolites are cytotoxic chemotherapeutic agents that interfere with DNA and RNA synthesis. These derivatives resemble naturally occurring compounds in the body by their structure. They have two main mechanisms of action – they either inhibit key enzymes involved in synthesis of NAs or they get incorporated to the strands of NAs and they cause strand breaks and prevent mitosis. In general their effect is cell cycle-dependent and they act in S-phase of cell cycle. Their effect is maximal after continuous use for a period of time. Antimetabolites are not dose-dependent. NAs strands are constructed from two groups of bases; pyrimidines and purines and antimetabolites resembling both these groups are called antipurines and antipyrimidines. There is another group of antimetabolites called antifolates and it resembles folic acid and inhibits enzymes required for purine and thymidylate production that are essential for DNA synthesis. [3,34]

Antifolates are involved in processes of folic acid that is resembled by them. Folic acid plays a role of a coenzyme to methyltransferases involved in biosynthesis of thymidylate and purine and certain amino acids. Folic acid is reduced to dihydrofolic acid (DHF) and it is then reduced by dihydrofolate reductase (DHRF) to tetrahydrofolate (THF). THF is required for purine and thymidylate synthesis as a cofactor. THF is oxidized during these processes to DHF and then it can be recovered by DHFR again to THF and the whole process can start once again. Methotrexate and pemetrexed as two members of antifolates clinically used inhibit function of DHFR and the reduction from DHF to active THF. Due to the lack of THF, synthesis of thymidylate and purines is reduced and it leads to reduced synthesis of NAs. In addition, pemetrexed inhibits also thymidylate synthase (TS) and glycinamide ribonucleotide formyltransferase (GARFT) enzymes essential for synthesis of thymidylate and purines. A second generation antifolate drug is raltitrexed that is a selective TS inhibitor.[34]

Antipyrimidines interfere with pyrimidine synthesis *via* various paths. They resemble pyrimidines and they either inhibit key enzymes involved in pyrimidine or DNA synthesis or they get incorporated into NAs. 5-Fluorouracil (5-FU) is a prodrug used for chemotherapy treatment. It gets metabolised intracellularly to a derivative called fluorouridine monophosphate (FUMP) that gets metabolised to fluorouridine triphosphate (FUTP) and fluorodeoxyuridine monophosphate (f-dUMP). Both metabolites interfere with NA synthesis but both by different mechanism of action. While FUTP gets incorporated to RNA strands analogically to UTP and disturbs its function and cause strand breakage, f-dUMP acts as substrate for TS instead of deoxyuridine monophosphate (dUMP) and binds covalently to the enzyme and its cofactor 5,10-methylene THF, which results in inhibition of *de novo* thymidine synthesis and synthesis of DNA strand. Capecitabine and ftorafur are other drugs from this category and they get metabolised to 5-FU. Arabinosides are nucleosides with altered sugar moiety of the molecule. Arabinose as a non-physiological sugar is used with a pyrimidine base cytosine in case of cytarabine. It gets converted to the form of nucleotide and then incorporated to DNA strands. It also acts as an inhibitor of DNA polymerase that is one of key enzymes for DNA synthesis. Gemcitabine is another member of arabinosides; it is a difluoro- derivative of cytarabine and it gets also converted to a triphosphate form and acts similarly to cytarabine. In addition the nucleotide of gemcitabine inhibits enzyme responsible for gemcitabine deactivation called deoxycytidine reductase and it prolongs the effect of gemcitabine nucleotide.[34]

Two drugs of the group of **antipurines** are in clinical practice use nowadays. 6-Mercaptopurine (MP) is a sulphurated derivative of purine and it gets converted by ribonucleotide reductase to 6-sulfanyl-2'-deoxyguanosine-5'-triphosphate and this metabolite gets incorporated to DNA strand and causes lethal strand breaks. Patients do not react on treatment with MP immediately but cells continue dividing for a period of time before dying.

Thioguanine (TG) is a sulphurated analogue of guanine and it gets incorporated to DNA strand and recognized by mismatch reparation enzymes causes a lethal DNA damage that results in apoptosis. These drugs are very well absorbed from GIT and they are usually used orally.[34,37]

2.3.3 Anti-tumour antibiotics

Anti-tumour antibiotics have nonspecific effect on cells when various mechanisms play roles in their cytotoxicity. Anthracyclines are produced by fungus *Streptomycetes* spp. They intercalate between DNA base pairs and lead to DNA strand breaks and they form oxygen free radicals that cause strand breakage, too. Anthracyclines also act as inhibitors of DNA topoisomerase I and DNA topoisomerase II. The members of anti-tumour antibiotics are bleomycin, mitoxantrone (pixantrone in Czech Republic) and anthracyclines e.g. doxorubicin, epirubicin or idarubicin.[3,34,35]

2.3.4 Tubulin binding drugs

Microtubules are responsible for many important actions in cell cycle. They maintain the cell shape, control intracellular transport, secretion, meiosis and mitosis. Microtubules are of hollow rod shape and they consist of α-tubulin and β-tubulin. Their structure is dynamic and they get assembled and disassembled permanently. Tubulin binding drugs, according to their name, bind to tubulins and microtubules and prevent them from their assembly or disassembly, respectively. There are two groups of tubulin binding drugs; vinca alkaloids and taxanes and their mechanisms of action are totally opposite.[34,35]

Vinca alkaloids bind to tubulins and inhibit their concentration and formation of microtubules. Microtubules cannot be polymerized and their function gets lost so mitosis cannot be completed and apoptosis takes place. Vinca alkaloids are cell cycle-dependent and they bind to tubulins during S-phase and inhibit the formation of microtubules that is necessary for M-phase. Unfortunately their effect is not aimed only on cancer cells but on normal cells, too, and they do act more or less during whole cell cycle because microtubules are involved in a lot of processes in cell. Due to this they have many side effects but they remain the second most used cancer chemotherapy drugs in clinical practice. Clinically used vinca alkaloids are vinblastine (VBL), vincristine (VCR), vinorelbine (VRL), vindesine (VDS). While VBL and VCR are completely natural alkaloids, VRL and VDS are semisynthetic.[38]

Taxanes' mechanism of action is totally opposite to vinca alkaloids and they prevent microtubules from disassembly. They bind to β-tubulin and induce the tubulin polymerization and formation of microtubules. Microtubules get stabilized and the dynamic state of microtubules is disturbed. Microtubules' stabilization and disassembly inhibition lead to apoptosis. Taxanes act during S and G2 to M-phase and due to their inhibiting effect on

microtubules lead the cell to apoptosis. Paclitaxel and docetaxel and kabazitaxel are three members of taxane group while both act a little differently. Paclitaxel induces apoptosis most on the boundary of G2/M-phase while docetaxel's maximum cytotoxic effect is during S-phase. All members are widely used in clinical practice for treatment of breast cancer, lung cancer and ovarian cancer.[39]

2.3.5 Topoisomerase inhibitors

Topoisomerase inhibitors are cytotoxic agents that inhibit one group of key enzymes in DNA replication and transcription and interfere with cell's normal function during its cycle. There are two main DNA topoisomerases types Topo I and Topo II with slightly different activities and the drugs of this category can be divided into two groups according to which Topo type they inhibit. Topo I inhibitors comprise clinically used drugs based on natural alkaloid campthotecin irinotecan and topotecan. Topo II inhibitors used in clinical practice are etoposide, teniposide, doxorubicin, idarubicin, epirubicin and mitoxantrone.

2.3.5.1 Topoisomerases

DNA topoisomerases are enzymes catalysing structural changes of DNA molecule and forming its isomer. There are two main groups of enzymes in human body encoded as EC 5.99.1.2 for DNA topoisomerase I and EC 5.99.1.3 for DNA topoisomerase II.

Topoisomerases are enzymes that break strands of DNA. They are responsible for DNA topology in cells. These enzymes can relax topological strains in DNA strands. They play a role in catenation or decatenation of circular DNA, e.g. mitochondrial DNA, and in positive and negative supercoiling of DNA. While group of Topo I breaks single strand DNA, type Topo II enzymes breaks double strand DNA. Topo I category includes subcategories Topo IA, IB and IC and submember groups the other Topo II are Topo IIA and Topo IIB. Their function is required during life-important processes of DNA replication and transcription and also other various processes in cell cycle. The enzyme temporarily binds to the strand(s) via a phosphodiester covalent bond and the energy of the bond is kept. Topo I breaks one strand while the other from the duplex is passed through the gap and the number of supercoils get changed by one. Topo II changes the number of supercoils by two. The enzyme gets bound to 5' phosphate ends of both strands of the duplex and the other segment of the DNA double strand is passed through the break.[40]

DNA Topo IA is typically approximately 67 kDa in molecular weight and has a characteristic toroidal fold. It is composed of four domains named domain I to IV. Domain I plays a role in ion interactions with Mg ions non-covalently bound to it and it consists of a Toprim domain. Toprim domain is found in DNA gyrase-primases and topoisomerases. Domain

III and IV contains helix-turn-helix (HTH) domains and the tyrosine catalytic residue can be found in HTH of domain III. Domain II forms a flexible bridge between domains III and IV. The active site (AS) is formed by conformational changes and important rearrangements in domain IV are also necessary for binding the DNA single strand. Topo IA binds to one strand of separated duplex of the DNA molecule and relaxes negative supercoiling of the strand by one. The enzyme binds to a 5' phosphate end of the DNA strand with the tyrosine moiety of the enzyme's AS via a phosphodiester bond. The cavity of the Topo IA gets open, pulls the strand and breaks it. The complementary strand of the DNA then gets passed through the cleft and the broken strand gets connected to the 3' end with the enzyme again. The 3' end of the broken strand also interacts with the Topo IA but non-covalently. Magnesium ions are required for the enzyme to catalyse the cleavage and they also keep the 3' end of the broken strand in the catalytic site of the enzyme in the right position for ligating the strand back. The cavity gets closed afterwards and the enzyme gets separated. Topo can perform many acts quickly even without disconnection of the DNA strand but the supercoiling always changes by one by an act of Topo IA. Whole process is ATP- independent and none of the energy of the previous bond of the DNA molecule gets lost. Different type of Topo IA is prokaryotic DNA gyrase (or reverse gyrase) that works exactly opposite way and is found in thermophilic prokaryotes. DNA gyrase catalyses positive supercoiling of one strand of DNA and it prevents the DNA molecule from melting at higher temperature. Energy in form of ATP is required for this process.[40,41]

Topo IB are typically larger than IA group, their molecular weight is approximately 90 kDa in eukaryotes. They share the common fold around the AS with Topo IA. These enzymes consist of core domain or central domain and then C- and N-terminal regions while the N-region is dispensable for topoisomerase activity. The N-terminal domain is involved in DNA-enzyme interactions but the enzyme's activity is fully operated by the larger core domain with the C-terminal region. The AS is formed by a pentad of amino acids (AAs) - catalytic Tyr and Arg, Arg, Lys and His and the catalytic Tyr can be found in C-terminal region of the enzyme while the rest of the pentad is situated in the core domain. Topo IB group of enzymes comprise eukaryotic DNA Topo I, vaccinia Topo I and DNA Topo V of *Methanopyrus kandleri* that is often categorised as a type Topo IC. Topo IB not only relaxes negative supercoiling of DNA but it also relaxes positive supercoiling of the double-stranded molecule. The mechanism of action of Topo IB differs from Topo IA. Whole enzyme binds to the DNA over 22 bp. Topo IB's Tyr residue of the AS of the enzyme binds to the 3' OH-group of a DNA base and the 5' end of the broken strand rotates so the supercoiling gets relaxed. The 5' end is bound to the enzyme only via non-specific ion interactions and so it can rotate freely. Every time the strand rotates the number of supercoils changes by one but it can rotate more than once per one act of the enzyme from binding to the DNA and its breakage to the ligation of the strand. Unlike

Topo IA, Topo IB binds to dsDNA and breaks one strand of it. It is thought that the enzyme binds to the dsDNA then gets in the closed conformation and the rotation of the domains occurs at this moment in closed conformation but the strand breakage appears upon enzyme regaining open structure. Then the torsion tension gets released and the enzyme gets to closed conformation by which the strand is ligated again. During the rotation of the complementary strand the enzyme does not open to any other substantial extent. The mechanism has not been still fully understood.[40,41]

Topoisomerases II are enzymes responsible for breaking dsDNA and passing the intact helix through the nick to change DNA topology. They are categorized to two groups of Topo IIA and Topo IIB. Topo IIA comprises DNA gyrase, eukaryotic Topo II and bacterial Topo IV while the other category includes only one member Topo VI. Topo IIB-type Topos are found only in *Archaea* and some higher plants. Topo II molecular weight is approximately 170 kDa to 180 kDa. They consist of four main domains; N-terminal GHKL (gyrase, HSP90, histidine kinase, MutL) ATPase unit, Toprim domain, central DNA- binding core and C-terminal domain. GHKL ATPase represents an ATP binding superfamily of enzymes found in DNA gyrase B, Hsp90, His kinase and DNA-mismatch repair enzyme MutL.[42] Topo IIs form dimers or tetramers. While prokaryotic Topo II forms heterodimers- tetramers, eukaryotic type Topo II forms a homodimer. The dimer is connected in both C- and N-termini and it forms a hole in the middle of the dimer approximately 20Å in diameter. The catalytic Tyr of AS is situated in winged helix domain of the central DNA-binding core. The Topo IIA appear in two isoforms Topo IIα and Topo IIβ while Topo IIα concentration increases during G2/M 2–3 fold and it is found in higher levels in fast proliferating cells like cancer cells. The Topo IIβ does not change much during the cell cycle. Unlike Topo I type, the act of Topo II is ATP dependent and energy is spent to catalyse the DNA topology modification. Topo II binds DNA via phophodiester bond at the 5' end of the double strand. Each subunit of the dimer is bound to this end. Two segments of DNA double strand are involved in the process, they are known as G segment or gate segment and T segment or transported segment. While the G segment is a subject of cleavage the T segment is passed through the new gap. The G segment gets bound to N-terminal gate of the enzyme and consecutively ATP is captured and DNA T segment enters the enzyme through the N-domain that gets closed and the T segment is passed inside. Capture of the G segment leads to dimerization of ATPase domain that binds ATP and starts the hydrolysis. The hydrolysis and closure of N-terminal induces conformational changes of the enzyme and it stimulates the breakage of the G segment of DNA. Then T segment is passed through while this act is regulated by the catalytic Tyr of the AS. After T segment passes through the G segment gets ligated again and T segment is released via C-terminal of the enzyme. ATP hydrolysis brings the AS's Tyr closer to the phosphodiester bonds to position sufficient for catalysis. The

enzyme's reaction requires ATP and Mg^{2+} ions and one act modifies the number of supercoiling by two.[40,43,44]

2.3.5.2 Topoisomerase I anticancer drugs

Topotecan and irinotecan are water soluble intravenous drugs approved for cancer chemotherapy treatment. These derivatives are based on natural alkaloid campthotecin (CPT) that was proved to inhibit Topo I activity. CPT derivatives act by trapping Topo I cleavage complex and getting stuck between bp of the DNA because of their planar aromatic structure. CPTs bind at the interface of Topo I-DNA complex and so inhibit the enzyme's activity. They intercalate at the cleavage site of the complex by π-π interactions and form a network of H-bonding in Topo I residues. When the TopoIcc is trapped by CPTs it prevents the complex from religation by displacing the 5' OH end relative to the 3' phosphotyrosyl and results in lot of negatively supercoiled region of the strand. These negatively supercoiled sections can form circles in DNA strand and they get converted to DNA damage by collision between the complex and replication fork or by transcription block. Both pathways end up with DNA damage response and lead to apoptosis. The CPTs tend to trap preferentially positively supercoiled DNA than negatively supercoiled molecule. In fact CPTs act and their cytotoxicity is caused by trapping the TopoIcc rather than by inhibiting the Topo I catalytic activity.

Topotecan is approved by FDA for treatment of small-cell lung cancer and ovarian cancer. It was the first drug based on CPT to undergo clinical trials (CT). It acts mainly in fast reproducing cells and so in cancer cells as well as in bone marrow cells, for instance. Due to this haematological toxicity is a common side effect of topotecan therapy. Bone marrow cells destruction can lead to infections through loss of white blood cells, anaemia with fatigue via low levels of red blood cells or bleeding due to low level of platelets. In general, patients feel tired and very sick and CINV can be induced by topotecan chemotherapy; it can be controlled with anti-emetic drugs. Topotecan has also teratogenic effect.

Irinotecan is approved by FDA for treatment of colorectal tumours. Unlike topotecan it is a prodrug and it gets converted by liver to 7-ethyl-10-hydroxy-CPT (SN-38). This pharmacologically active metabolite is responsible for majority of anti-tumour activity of irinotecan. Common side effects include the same as above in case of topotecan and severe diarrhoea and liver dysfunction which is temporary and mostly asymptomatic.[45,46,47]

2.3.5.3 Topoisomerase II anticancer drugs

There are two classes of Topo II anticancer drugs. They are distinguished by their action on Topo II. First class is called Topo II poisons because it leads to increased levels of Topo II-DNA covalently bound complexes like the CPT derivatives in case of Topo I. The

second class is called Topo II inhibitors and includes molecules that act by inhibition of Topo II catalytic activity while they do not raise the level of enzyme-DNA covalent complexes. It is believed that their cytotoxic activity is caused by elimination of the essential enzymatic activity of Topo II. Elimination of Topo II activity by either mechanism results in DNA replication and transcription block and DNA strand breaks. Topoisomerase poisons' mechanism of action is not fully understood yet and it is believed that they act similarly like CPTs on Topo I.[43,47]

Etoposide has been used since 1983 for treatment for various types of cancer including leukaemia, lung cancer, non-Hodgkin's lymphoma, soft tissue sarcomas, neuroblastoma and other. It is a Topo II poison binding covalently to Topo II-DNA complex and prevents the broken double strand from religating. It is believed that etoposide targets almost specifically Topo IIα of which levels are increased in rapidly proliferating cells like in cancer. Topo II binds to dsDNA with two scissile bonds. Etoposide is required to act on both these bonds as a two-site inhibitor to induce enzyme mediated breaks in dsDNA. Etoposide-Topo II induced DNA damage results in apoptosis. Common side effects of etoposide are bone marrow suppression, CINV and hair loss, also liver toxicity and fever and chills may occur when high-dose therapy is used. Etoposide has been identified as carcinogenic to humans by WHO. It is associated with development of acute myelogenous leukaemia (AML). Other Topo II inhibitors can be related with development of AML, as well. Teniposide is an analogue of etoposide and it was approved by FDA in 1993. It is used for treatment of acute lymphocytic leukaemia in paediatric patients with poor prognosis and for treatment of CNS malignancies. Its mechanism of action is also based on forming TopoIIcc that prevent religation of dsDNA as in case of etoposide. It has been proposed that the covalent complexes arrest transcription and start 26S proteasome-mediated Topo IIβ degradation. The toxicity of teniposide is similar to etoposide but hypersensitivity reactions are more frequent with teniposide infusions.[43,47,48]

Anthracyclines are a group of antibiotics used as antineoplastic agents. They are used for treatment of breast cancer, leukaemia, lymphoma, and sarcoma. They act as Topo II poisons as well as they intercalate into DNA molecule. Currently approved anthracyclines are doxorubicin, daunorubicin, idarubicin and epirubicin. Anthracyclines act as Topo II poisons in a similar manner to etoposide so they bind covalently to TopoIIcc and inhibit the double-strand religation. They also intercalate between base pairs of DNA molecule because of their planar aglycone moiety and they can modify the action of nuclear helicases that dissociate DNA duplex into two single DNA strands. Anthracyclines also undergo one- and two-electron reduction due to their quinone-based structure and produce reactive analogues that can cause damage in lipid membranes and macromolecules. Common adverse reactions of anthracyclines include CINV, alopecia, mucositis, dose-limiting myelosuppression that can be severe and cardiotoxicity. Cardiotoxicity is the most serious side effect of anthracyclines and it can be

acute, chronic or subclinical. Subclinical cardiotoxicity may not occur in paediatric patients until they are adolescent or adult. Cardiotoxicity is thought to be caused by the free radicals formed by reduction of anthracyclines. AML can also appear in association with anthracycline based cancer chemotherapy treatment.[43]

Mitoxantrone is another Topo II poison used in clinical practice to treat primarily breast cancer, prostate cancer, leukaemias and lymphomas. It has got similar therapeutic range like anthracyclines but it lacks the ability of forming quinone type free radicals and so it causes less cardiotoxicity at the same cytotoxic doses. Mitoxantrone acts also by binding covalently to Topo II and results in formation of TopoIIcc and prevents the enzyme from religation of the DNA duplex. Mitoxantrone caused DNA damage leads to NF-κ B activation and induction of apoptosis. Mitoxantrone's dose limiting side effect is myelosuppression like in case of other Topo II poisons. Other adverse reactions include cardiotoxicity, CINV and alopecia. Mitoxantrone has also been used for multiple sclerosis treatment.[43,48]

2.3.6 Rutaecarpine and its derivatives

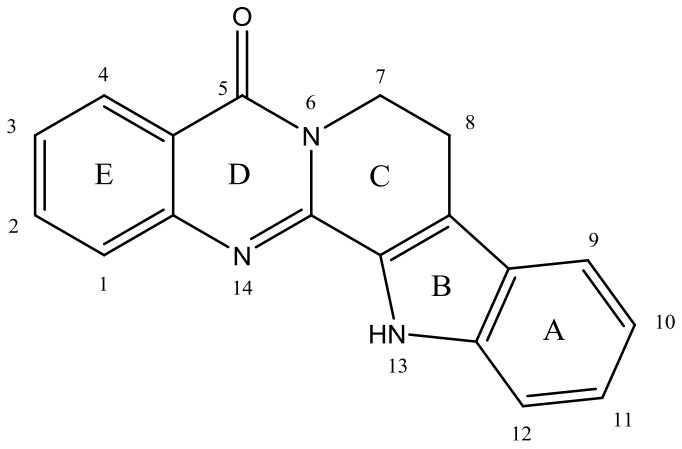


Fig. 1– Structure of rutaecarpine

Rutaecarpine is an alkaloid naturally occurring in herbs of *Rutaceae* family especially in *Evodia rutaecarpa* and its fruits. The fruits of *Evodia rutaecarpa* are originally called Wu-Chu-Yu and rutaecarpine is the major component of them. The herb and fruits has been widely and long used in traditional Chinese folk medicine to treat gastrointestinal disorders, amenorrhoea, headache or postpartum haemorrhage.[49] Rutaecarpine has been proved to have various medical properties such as anti-thrombotic, anti-inflammatory and analgesic, anti-tumour, thermoregulatory, vasorelaxing or anti-obesity activity. Also positive effects on cardiovascular and endocrine systems have been proved.[50] Rutaecarpine was first isolated in 1915 by Asahina and Kashiwaki from *Evodia rutaecarpa* acetone extracts after treatment with base and later from Wu-Chu-Yu fruits. Rutaecarpine structurally belongs to a group of quinazolinones fused with piperidine condensed with indole ring system.[51]

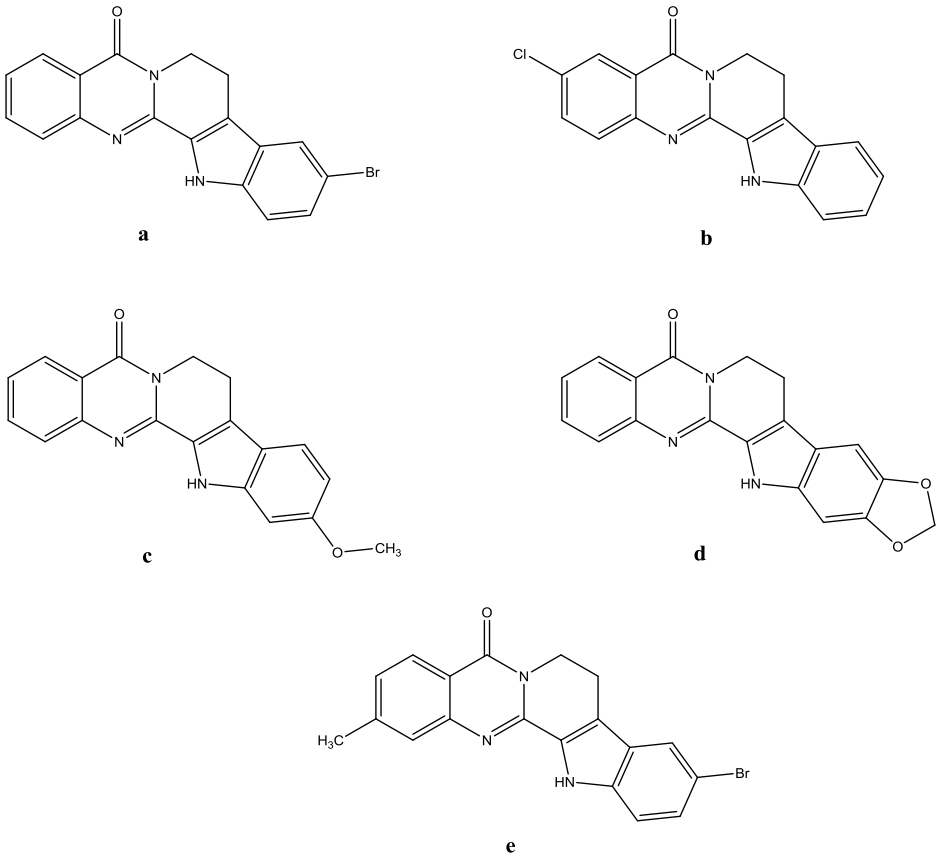


Fig. 2– rutaecarpine derivatives

Rutaecarpine's structure can be found in two isoforms. The crystallographic structure of rutaecarpine is planar where rings A and B and rings D and E are coplanar while ring C is in half-chair conformation. The half-chair conformation of ring C can undergo a ring flip and form two isoforms. Rutaecarpine has shown cytotoxic activity against various cell lines. Basically substitution on its ring core results in selectivity for a specific cell line. Substitutions on ring E increase the cytotoxicity more than substitutions on other rings of rutaecarpine. Rutaecarpine itself did not show significant inhibiting activity against Topo I and II but its derivatives did. Also the inhibitory activity against Topo I and Topo II seems to be raised by substitution on ring E while substitutions on other rings do not affect it. 10-Bromorutaecarpine (**a**) showed very good inhibitory activity as the only member of non-E-ring substituted derivatives at levels comparable to CPT and etoposide as well as 3-chlororutaecarpine (**b**) with substituted E-ring.[52,53] 11-Methoxyrutaecarpine (**c**) showed selectivity in cytotoxicity to renal cancer cell line (786-0) at 0.31 μM GI_{50} or against lung cancer cell lines (A549/ATCC and NCI-11460) at 0.75 μM and 1.38 μM GI_{50} respectively. 10, 11-Methylenedioxy derivative (**d**) has shown selectivity against ovarian cancer cell line panel (OVCAR-4) at 1.08 μM GI_{50} while 10-methylsulafanylrutaecarpine has shown specific cytotoxicity against renal cancer cell line (ACHN) at level 0.08 μM GI_{50}. Also 10-bromo-2-methylrutaecarpine (**e**) has shown good

cytotoxicity value against the same line of cancer cells at 0.3 μM GI_{50} as well as against CNS cancer cell line (U251) at equivalent level.[50]

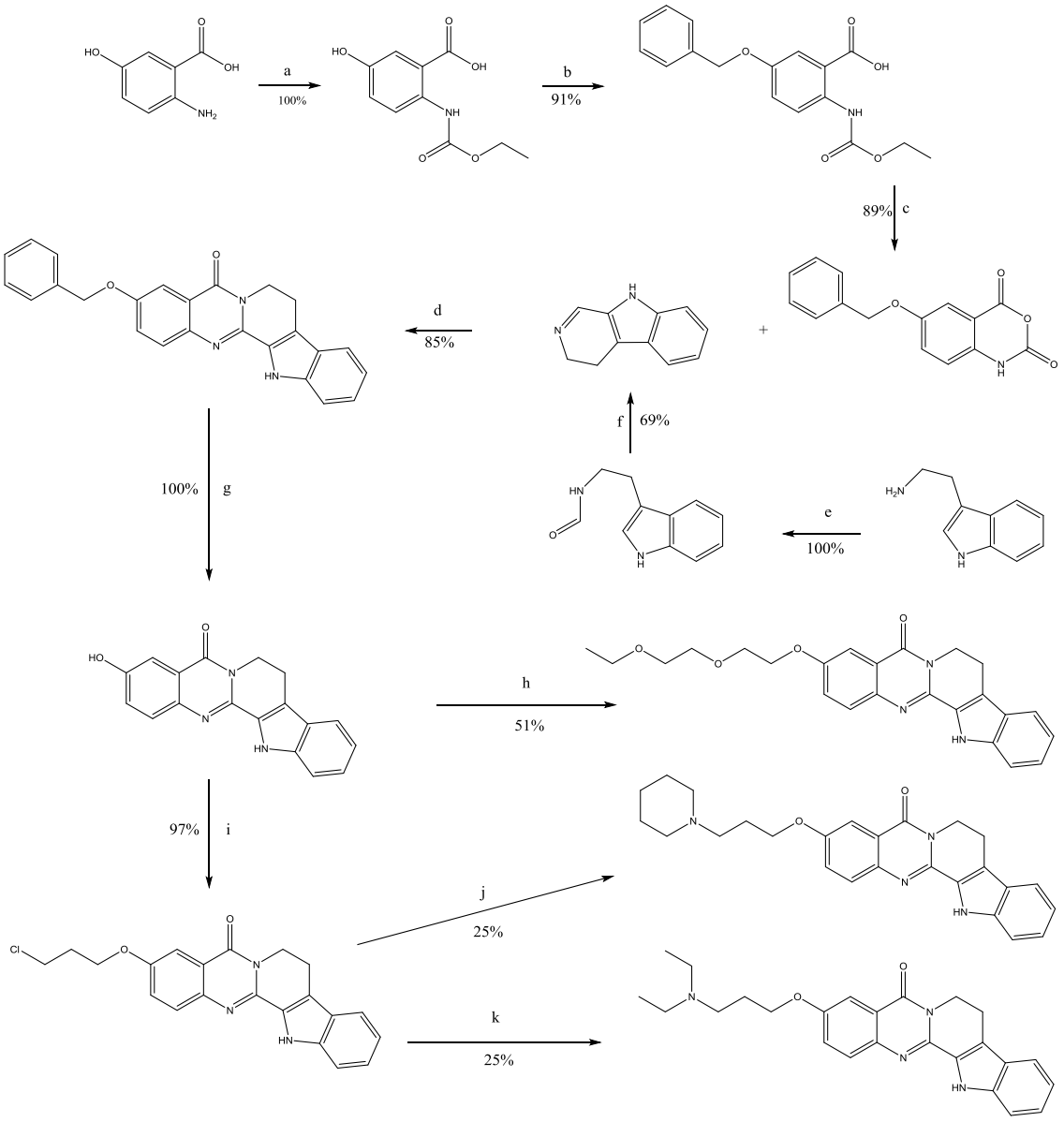
Rutaecarpine's metabolism has been studied only *in vitro* on rat and human liver microsomes and *in vivo* in rats. There are two phases of rutaecarpine's metabolism. It is in majority metabolised to 6 various monohydroxylated metabolites on rings A and E. Rutaecarpine gets metabolised with CYP 3A4 mainly and metabolites are excreted in urine, faeces and bile. C-ring hydroxylated metabolite accounts for 47% of all metabolites *in vitro* while *in vivo* it has not found at all. Minor amount of rutaecarpine is metabolised to dihydroxylated derivatives and they are excreted in urine and faeces. The hydroxy groups are introduced to rings A and C in majority and to rings C and E. In phase II rutaecarpine gets glucuronided and sulfated to form monosulfate and monoglucuronide metabolites and they get excreted in urine and faeces.[50]

3 EXPERIMENTAL PART

3.1 Design of 3-alkyloxyrutaecarpine derivatives

Rutaecarpine is an alkaloid with very good cytotoxic activity but poor water solubility. The aim of our work was preparation of rutaecarpine derivatives with equivalent cytotoxic activity but better solubility. We focused on two approaches. The first one represented the synthesis of a better soluble rutaecarpine derivative itself; the other approach was preparation of a well soluble salt. We chose three different moieties to get connected to rutaecarpine via ether bond and short polymethylene chain. Two of the moieties were supposed to provide tertiary amine derivatives to form hydrochloride salts and so to increase water solubility. The last moiety chosen was very hydrophilic aliphatic polyethoxy chain. CPT derivatives used in clinical practice topotecan and irinotecan were prepared also by introduction of tertiary amine moieties and poor water solubility of CPT was increased by preparation of HCl salts of both molecules. The planar structure of rutaecarpine is believed to be responsible for its cytotoxic activity, so that it could intercalate between base pairs of DNA molecule like CPTs. The introduced moiety and the linker should not disturb the pharmacophore in the molecule inducing cytotoxic effect. The moieties were introduced to rutaecarpine´s E-ring in position 3. The reason for choise of E-ring was supported by the provement, that substitution on this ring can positively affect the inhibitory activity to Topo I and Topo II unlike substitution on the other rings.[50]

3-Hydroxyrutaecarpine was prepared by a simple heterocyclic fusion reaction of isatoic anhydride with tryptamine 3-ring derivative that was developed by Huang et al.[54] in the Department of Pharmaceutical Chemistry of University of Wuerzburg. This synthesis provides 3-hydroxyrutaecarpine in six steps with no purification necessity and in very good yield. During the fusion reaction, the 3-hydroxy group was protected in order to obtain pure product and to avoid by-products. After removing the protective group, particular ether derivatives were provided by Williamson ether synthesis.



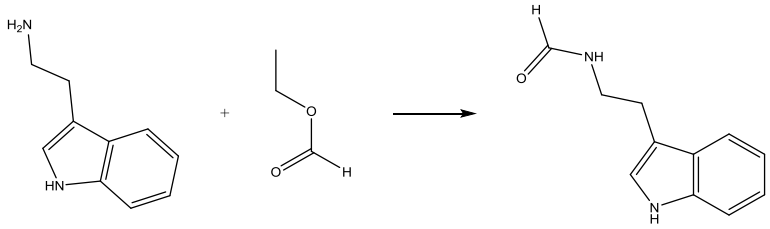
(a) Ethylchloroformate in dry dioxane, 108°C, 4h; **(b)** benzyl bromide, K_2CO_3 in dry DMF, r.t.18 hrs; KOH (water solution), 95°C, 2hrs; **(c)** oxalyl chloride in THF, reflux, 2 hrs; Et_2O, reflux, 10 mins; **(d)** ethylformate, 60°C, 24 hrs; **(e)**DMF, 95°C, 24 hrs; **(f)** $POCl_3$, DCM, r.t., 2hrs; **(g)** H_2, Pd/C in MeOH/EtOAc, r.t., 24 hrs; **(h)** PEG, K_2CO_3 in dry DMF, r.t., 24 hrs; **(i)** 1-chloro-3-iodopropane, K_2CO_3 in dry DMF, r.t. 24 hrs; **(j)** piperidine, K_2CO_3, KI in dry DMF, 95°C, 72 hrs; **(k)** diethylamine, K_2CO_3, KI in dry DMF, 50°C, 72 hrs

Fig. 3– Synthesis scheme of rutaecarpine derivatives

3.2 Synthesis

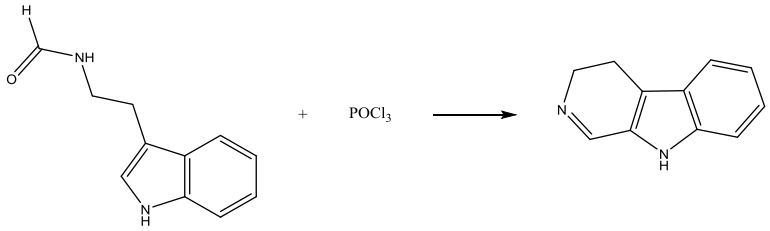
All used solvents and reagents were supplied by Merck, Sigma-Aldrich and Alfa-Aesar and were provided by the University supplier. Reaction progress and detection of by-products were monitored by TLC. Flash and column chromatography with silica gel (Kieselgel 40, 0.040–0.063 mm; Merck, Germany) was used for chromatographic separations and purification. Chemical structures of reaction products were checked by NMR spectroscopy with a Bruker AV-400 NMR instrument (Bruker, Karlsruhe, Germany) in DMSO-d6. Analytic HPLC was performed on a system from Shimadzu Products equipped with a DGU-20A3R controller, LC20AB liquid chromatograph, and a SPD-20A UV/Vis detector. Stationary phase was a Synergi 4U fusion-RP (1504.mm) column. As mobile phase, a gradient MeOH-TFA (0.01%)/water-TFA (0.01%) (phase A/phase B) were used. Gradient mode: 0-8 min (30–90% phase A), 8–13 min (90% phase A), 15–18 min (90–30% phase A).

***N*-[2-(1*H*-indol-3-yl)ethyl]formamide (1)**



1 g (6.22 mmol) of tryptamine was dissolved in ethyl formate (8 mL, 99 mmol, 16 eq) and heated under reflux (60°C) for 24 hours. The solvent was then evaporated under reduced pressure to obtain the title product as brown oil (1.17 g, 100%). No further purification was needed.[55]

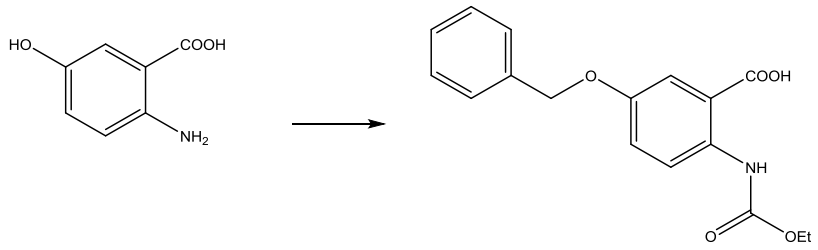
4,9-dihydro-3*H*-pyrido[3,4-b]indole (2)



To the solution of *N*-[2-(1*H*-indol-3-yl)ethyl]formamide (1.17 g, 6.22 mmol) in dichlormethane (5 mL) was added phosphorus oxychloride (1.5 mL, 16.35 mmol, 2.6 eq) drop wise while cooled down to 0–5°C. Then the reaction mixture was stirred at room temperature for 2 hours. The solvent and the excess of phosphorus oxychloride were evaporated under reduced pressure and the dark solid was suspended in ethyl acetate (100 mL) and extracted with 10% aqueous solution of acetic acid (4×40 mL). Combined water layers were basified with ammonia up to pH 9. Yellow solid precipitated and it was extracted with dichlormethane (3×40 mL) and dried with sodium sulphate to give the title product as yellow foam (732 mg, 69%).[55]

^{1}H NMR (400 MHz, DMSO-d_6) δ 11.30 (s, 1H), 8.37 (t, *J* = 2.3 Hz, 1H), 7.60 – 7.52 (m, 1H), 7.41 (dt, *J* = 8.3, 1.0 Hz, 1H), 7.20 (ddd, *J* = 8.3, 7.0, 1.2 Hz, 1H), 7.05 (ddd, *J* = 8.0, 6.9, 1.0 Hz, 1H), 3.84 – 3.75 (m, 2H), 2.84 – 2.76 (m, 2H). Spectral data are in accordance with literature.[55]

5-(benzyloxy)-2-[(ethoxycarbonyl)amino]benzoic acid (3)

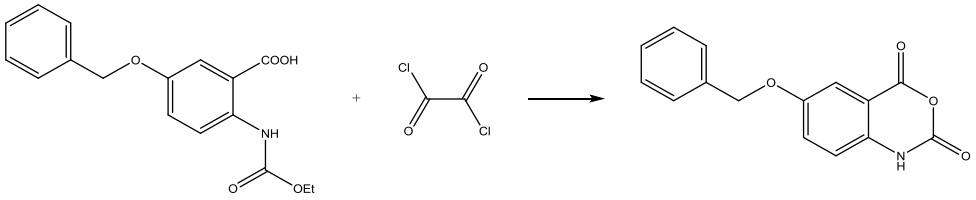


To the solution of 2-amino-5-hydroxy-benzoic acid (1 g, 6.53 mmol) in dry dioxane (20 mL) under argon atmosphere was added drop wise ethyl chloroformate (1.42 g, 1.25 mL, 13.06 mmol, 2 eq). The reaction mixture was heated at 108°C for 4 hours and the mixture became clear solution. The solvent was evaporated under reduced pressure and the intermediate was checked on TLC and used without further purification as grey to silver prisms (1.47 g, 6.53 mmol, 100%)

To the solution of 2-[(ethoxycarbonyl)amino]-5-hydroxybenzoic acid (1.47 g, 6.53 mmol) in dry dimethylformamide (10 mL) with potassium carbonate (5.4 g, 39.18 mmol), benzyl bromide (2.33 mL, 19.59 mmol) was added drop wise and the reaction mixture was stirred under nitrogen atmosphere for 24 hours. The reaction mixture was then poured into crushed ice (150 g) and stirred until the ice melted. Precipitated brown solid was collected by filtration and there was given the first batch of benzyl-5-(benzyloxy)-2-[(ethoxycarbonyl)amino]benzoate. The filtrate was extracted with diethyl ether (3×50 mL), then washed with water (3×30 mL) and brine (1×30 mL) and dried over sodium sulfate. Then the solvent was evaporated under reduced pressure to give the second batch of benzyl-5-(benzyloxy)-2-[(ethoxycarbonyl)amino]benzoate. The two batches together were mixed in methanol (10 mL), some white solid precipitated and brown oil occurred. After addition of methanol (20 mL) and dioxane (10 mL), the oil was completely dissolved. To the methanolic solution, solution of potassium hydroxide (733 mg, 13.06 mmol) in water (10 mL) was added and the mixture was heated at 95°C for 2 hours. The solvent was evaporated and the aqueous solution diluted with water and acidified with 2M hydrochloric acid (1.5 mL) in ice-water bath. The precipitate was collected by filtration and the solid was put under vacuum overnight to obtain the title product as yellow solid (1.86 g, 91%).[55]

^{1}H NMR (400 MHz, DMSO-d_6) δ 10.33 (s, 1H), 8.15 (d, J = 9.2 Hz, 1H), 7.53 (m, 1H), 7.47 – 7.45 (m, 2H), 7.42 – 7.38 (m, 2H), 7.36 – 7.27 (m, 2H), 5.11 (s, 2H), 4.14 (q, J = 7.1 Hz, 2H), 1.24 (t, J = 7.1 Hz, 3H). Spectral data are in accordance with literature data.[55]

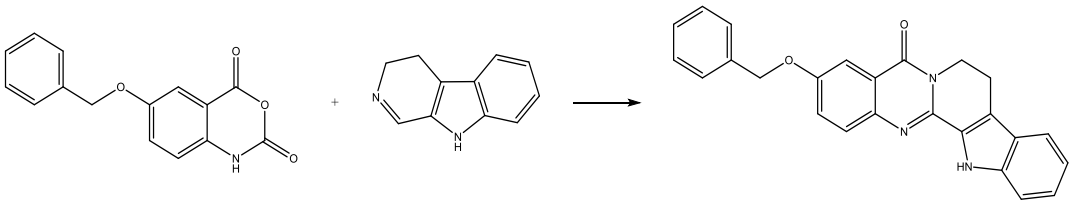
6-(benzyloxy)-2*H*-benzo[d][1,3]oxazine-2,4(1*H*)-dione (4)



To the solution of 5-(benzyloxy)-2-[(ethoxycarbonyl)amino]benzoic acid (3 g, 9.51 mmol) in dry tetrahydrofuran (50 mL), oxalyl chloride (8.74 g, 5.8 mL, 41.36 mmol, 68.9 eq) was added drop wise. The mixture was then heated at 60°C under argon atmosphere for 2 hours. The solvent and excess of oxalyl chloride were evaporated and white solid appeared. It was suspended in diethylether and the mixture was heated at 40°C for 10 min. The solid was collected by filtration and washed with diethylether and the title product was obtained as white prisms (2.27 g, 89%).[55]

^{1}H NMR (400 MHz, DMSO-d_6) δ 11.61 (s, 1H), 7.49 – 7.30 (m, 7H), 7.12 (dd, J = 8.7, 0.6 Hz, 1H), 5.18 (s, 2H). Spectral data are in accordance with literature data.[55]

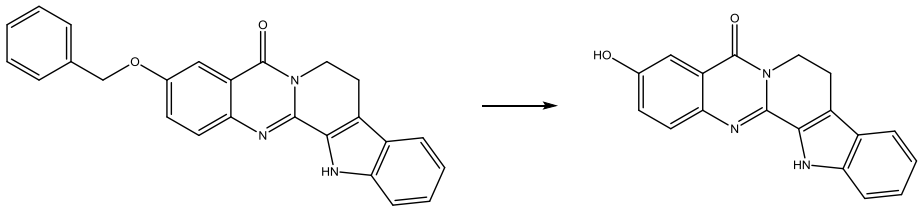
3-(benzyloxy)-8,13-dihydroindolo[2',3':3,4]pyrido[2,1-b]quinazolin-5(7*H*)-one (5)



6-(Benzyloxy)-2*H*-benzo[d][1,3]oxazine-2,4(1*H*)-dione (269 mg, 1 mmol) was dissolved in dimethylformamide (5 mL) and 4,9-dihydro-3*H*-pyrido[3,4-b]indole (221 mg, 1.3 mmol, 1.3 eq) was added. The reaction mixture was heated at 95°C for 24 hours without protective atmosphere. Then water (20 mL) was poured into the solution and light solid precipitated. It was collected by filtration, washed with water and put under vacuum overnight. The title product was obtained as off-white solid (324 mg, 85%).[54]

^{1}H NMR (400 MHz, DMSO-d_6) δ 11.81 (s, 1H), 7.70 – 7.61 (m, 3H), 7.55 – 7.46 (m, 4H), 7.45 – 7.39 (m, 2H), 7.39 – 7.33 (m, 1H), 7.29 – 7.23 (m, 1H), 7.09 (t, *J* = 7.5 Hz, 1H), 5.26 (s, 2H), 4.46 (t, *J* = 6.8 Hz, 2H), 3.17 (t, *J* = 6.8 Hz, 2H). Spectral data are in accordance with literature data.[54]

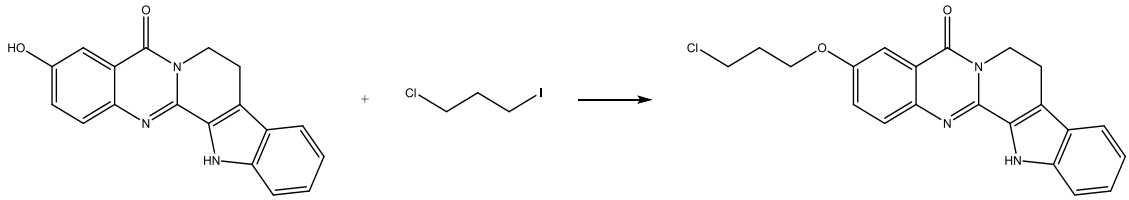
3-hydroxy-8,13-dihydroindolo[2',3':3,4]pyrido[2,1-b]quinazolin-5(7*H*)-one (6)



To the solution of 3-(benzyloxy)-8,13-dihydroindolo[2',3':3,4]pyrido[2,1-b]quinazolin-5(7*H*)-one (1.25 g, 3.35 mmol) in ethyl acetate/methanol (250mL/200mL), palladium on carbon (125 mg, 10% of weight) was added and the mixture was treated with hydrogen for 22 hours. Then it was filtered and the solvent was evaporated under reduced pressure and the title product precipitated as white solid (1.01 g, 100%).[54]

^{1}H NMR (400 MHz, DMSO-d_6) δ 11.82 (s, 1H), 10.12 (s, 1H), 7.69 (dd, *J* = 7.9, 1.0 Hz, 1H), 7.63 (d, *J* = 8.8 Hz, 1H), 7.55 – 7.50 (m, 2H), 7.34 (dd, *J* = 8.8, 2.9 Hz, 1H), 7.30 (ddd, *J* = 8.3, 6.9, 1.2 Hz, 1H), 7.14 (ddd, *J* = 7.9, 6.9, 1.0 Hz, 1H), 4.49 (t, *J* = 6.8 Hz, 2H), 3.22 (t, *J* = 6.8 Hz, 2H). Spectral data is in accordance with literature data.[54]

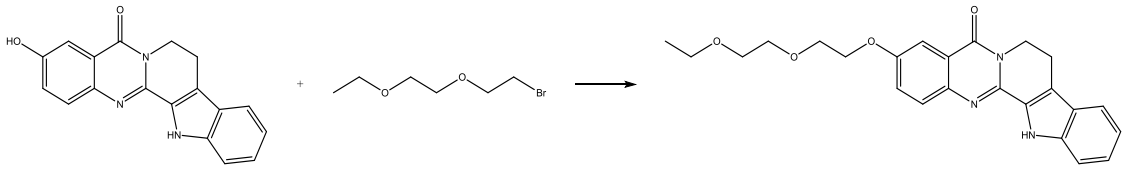
3-(3-chloropropoxy)-8,13-dihydroindolo[2',3':3,4]pyrido[2,1-b]quinazolin-5(7*H*)-one (7)



To the solution of 3-hydroxy-8,13-dihydroindolo[2',3':3,4]pyrido[2,1-b]quinazolin-5(7*H*)-one (1.18 g, 3.88 mmol) in dry dimethylformamide (10 mL), potassium carbonate (1.61 g, 11.64 mmol, 3 eq) and 1-chloro-3-iodopropane (1.19 g, 625 µl, 5.82 mmol, 1.5 eq) were added and the mixture was stirred under nitrogen atmosphere at room temperature for 24 hours. Light solid precipitated and the solution became unclear. Then water (40 mL) was poured into the mixture and a lot of white solid precipitated. It was collected by filtration, washed with water and the product was obtained as white solid (1.42 g, 97%).

^{1}H NMR (400 MHz, DMSO-d_6) δ 11.80 (s, 1H), 7.67 – 7.62 (m, 2H), 7.58 (d, *J* = 3.0 Hz, 1H), 7.51 – 7.46 (m, 1H), 7.45 (dd, *J* = 8.9, 3.0 Hz, 1H), 7.26 (ddd, *J* = 8.2, 7.0, 1.2 Hz, 1H), 7.09 (ddd, *J* = 8.0, 6.9, 1.0 Hz, 1H), 4.46 (t, *J* = 6.8 Hz, 2H), 4.23 (t, *J* = 6.1 Hz, 2H), 3.84 (t, *J* = 6.5 Hz, 2H), 3.18 (t, *J* = 6.9 Hz, 2H), 2.24 (m, *J* = 6.3 Hz, 2H).

3-[2-(2-ethoxyethoxy)ethoxy]-8,13-dihydroindolo[2',3':3,4]pyrido[2,1-b]quinazolin-5(7*H*)-one (8)



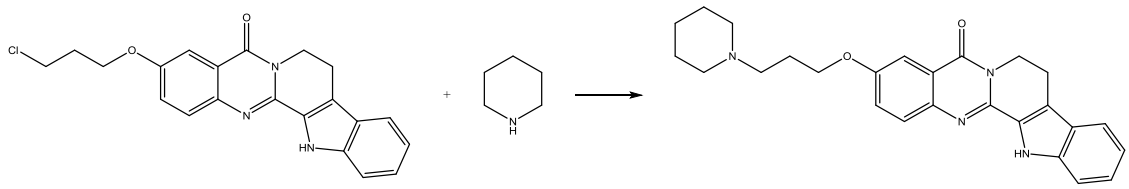
To the solution of 3-hydroxy-8,13-dihydroindolo[2',3':3,4]pyrido[2,1-b]quinazolin-5(7*H*)-one (100 mg, 0.33 mmol) in dry dimethylformamide (3 mL), potassium carbonate (137 mg, 0.99 mmol, 3 eq) and 1-bromo-2-(2-ethoxyethoxy)ethane (133 mg, 103 μl, 0.66 mmol, 2 eq) was added and the mixture was stirred under nitrogen atmosphere at room temperature for 24 hours. Water (10 mL) was poured into the solution and white solid precipitated. It was filtered and purified by column chromatography using dichlormethane:methanol (20:1) to obtain the title product as white solid (70 mg, 51%).

^{1}H NMR (400 MHz, DMSO-d_6) δ 11.81 (s, 1H), 7.64 (d, *J* = 8.8 Hz, 2H), 7.58 (d, *J* = 2.9 Hz, 1H), 7.51 – 7.47 (m, 1H), 7.45 (dd, *J* = 8.9, 3.0 Hz, 1H), 7.26 (ddd, *J* = 8.3, 7.0, 1.2 Hz, 1H), 7.09 (ddd, *J* = 7.9, 6.9, 1.0 Hz, 1H), 4.46 (t, *J* = 6.8 Hz, 2H), 4.27 – 4.20 (m, 2H), 3.83 – 3.78 (m, 2H), 3.64 – 3.60 (m, 2H), 3.54 – 3.50 (m, 2H), 3.49 – 3.41 (m, 2H), 3.21 – 3.15 (m, 2H), 1.11 (t, *J* = 7.0 Hz, 3H).

^{13}C NMR (101 MHz, DMSO) δ 160.77, 157.06, 143.88, 142.25, 138.99, 128.66, 127.71, 125.44, 124.91, 124.63, 121.94, 120.25, 120.12, 117.46, 112.94, 108.06, 70.48, 69.70, 69.31, 68.24, 66.04, 41.42, 19.44, 15.58.

HPLC purity: 98%

3-[3-(piperidin-1-yl)propoxy]-8,13-dihydroindolo[2',3':3,4]pyrido[2,1-b]quinazolin-5(7*H*)-one (9)

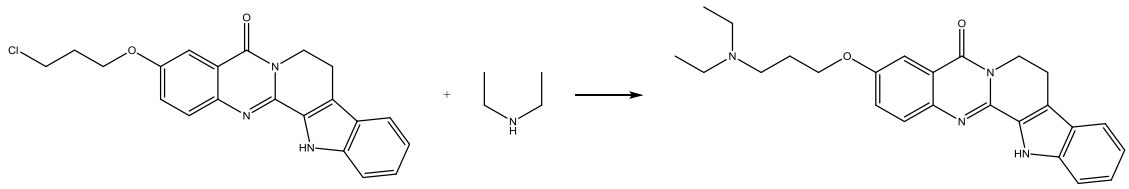


To the solution of 3-(3-chloropropoxy)-8,13-dihydroindolo[2',3':3,4]pyrido[2,1-b]quinazolin-5(7H)-one (185 mg, 0.49 mmol) in dimethylformamide (7 mL), potassium carbonate (337 mg, 2.44 mmol, 5 eq), potassium iodide (40 mg, 0.24 mmol, 0.5 eq) and piperidine (62 mg, 72 µl, 0.73 mmol 1.5 eq) were added and the mixture was heated under argon atmosphere at 95°C for 24 hours. TLC showed a big spot of starting material, that's why 6.5 eq of piperidine was added and the reaction mixture was heated for further 48 hours. Then water (20 mL) was poured into the solution, and tiny particles precipitated. The mixture was acidified with 2M hydrochloric acid and it became clear again. The acidified solution was washed with dichlormethane (4×10 mL), then it was basified with 2M sodium hydroxide and yellow solid precipitated and first batch of the title product was collected by filtration. Structure and purity was confirmed by NMR. The organic layers were washed with water (3×10 mL) subsequently with brine (1×10 mL), dried with sodium sulphate and purified by column chromatography by using dichlormethane:methanol:ammonia (20:1:0.1) to obtain the second batch of the tile product. The two batches together gave the product as yellow solid (51 mg, 25%).

^{1}H NMR (400 MHz, DMSO-d_6) δ 11.80 (s, 1H), 7.69 – 7.60 (m, 2H), 7.55 (d, *J* = 2.9 Hz, 1H), 7.48 (d, *J* = 8.3 Hz, 1H), 7.42 (dd, *J* = 8.9, 2.9 Hz, 1H), 7.26 (t, *J* = 7.7 Hz, 1H), 7.09 (t, *J* = 7.5 Hz, 1H), 4.46 (t, *J* = 6.8 Hz, 2H), 4.13 (t, *J* = 6.5 Hz, 2H), 3.18 (t, *J* = 6.8 Hz, 2H), 2.41 (t, *J* = 7.1 Hz, 2H), 2.39 – 2.30 (m, 4H), 1.92 (p, *J* = 6.8 Hz, 2H), 1.56 – 1.46 (m, 4H), 1.44 – 1.34 (m, 2H).

^{13}C NMR (101 MHz, DMSO) δ 160.80, 157.24, 143.81, 142.15, 138.99, 128.65, 127.72, 125.44, 124.91, 124.66, 121.94, 120.26, 120.12, 117.42, 112.94, 107.90, 67.10, 55.53, 54.59, 41.43, 26.65, 26.09, 24.64, 19.44.

3-[3-(diethylamino)propoxy]-8,13-dihydroindolo[2',3':3,4]pyrido[2,1-b]quinazolin-5(7*H*)-one (10)



To the solution of 3-(3-chloropropoxy)-8,13-dihydroindolo[2',3':3,4]pyrido[2,1-b]quinazolin-5(7*H*)-one (200 mg, 0.53 mmol) in dimethylformamide (7 mL), potassium carbonate (364 mg, 2.63 mmol, 5 eq), potassium iodide (44 mg, 0.26 mmol, 0.5 eq) and diethylamine (385 mg, 545 µl, 5.27 mmol, 10 eq) were added and the mixture was heated under argon atmosphere at 50°C for 24 hours. TLC showed a lot of starting material, that's why the reaction time was prolonged and it was heated for further 48 hours. Then water (15 mL) was added to the solution and light solid precipitated and it was collected by filtration. The product was purified by column chromatography by using dichlormethane:methanol:ammonia (20:1:0.1) to obtain the title product as off-white solid (55 mg, 25%).

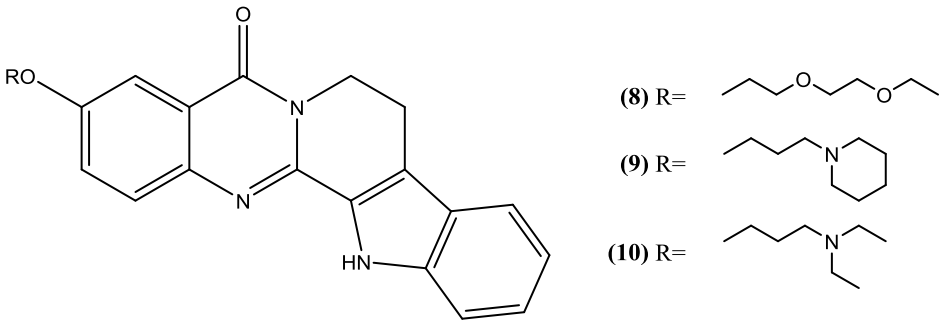
^{1}H NMR (400 MHz, $CDCl_3$) δ 9.39 (s, 1H), 7.63 (d, *J* = 2.9 Hz, 1H), 7.54 (dd, *J* = 8.0, 1.0 Hz, 1H), 7.50 (d, *J* = 8.9 Hz, 1H), 7.28 – 7.25 (m, 1H), 7.24 – 7.20 (m, 2H), 7.09 (ddd, *J* = 7.9, 6.8, 1.1 Hz, 1H), 4.52 (t, *J* = 6.8 Hz, 2H), 4.06 (t, *J* = 6.3 Hz, 2H), 3.15 (t, *J* = 6.9 Hz, 2H), 2.56 (t, *J* = 7.4 Hz, 2H), 2.48 (q, *J* = 7.1 Hz, 4H), 1.96 – 1.83 (m, 2H), 0.96 (t, *J* = 7.2 Hz, 6H).

^{13}C NMR (101 MHz, $CDCl_3$) δ 161.41, 157.60, 143.03, 141.88, 138.15, 128.13, 127.42, 125.72, 125.23, 124.74, 121.91, 120.50, 119.91, 117.40, 111.99, 107.71, 67.00, 49.36, 47.02, 41.27, 26.99, 19.70, 11.83.

3.1 Results and discussion

Rutaecarpine is a privileged natural structure with biological activity. The aim of our work was preparation of 3-substituted ether derivatives of rutaecarpine, that would possess better water solubility than the parent molecule.

Starting molecule were commercialy available tryptamin and 3-hydroxylated anthranilic acid. Tryptamine afforded in two step reaction partially hydrogenated β-carbolin skeleton in overall yield 69% (product **2**). The other part of 5-ring structure of ruteacarpine started from 2-amino-5-hydroxybenzoic acid. First, it was acylated with ethyl chloro formate and after protection of hydroxyl group with benzyl, the intermediate was submitted to cyclization reaction with oxalyl chloride. The resulting 6-substituted 2*H*-benzo[d][1,3]oxazine-2,4(1*H*)-dione is actually derivatized isatoic anhydride (product **4**) and it was obtained in overall yield 81%. This synthon was fused with intermediate **2** in a simple heterocyclic fusion reaction developed in group of prof. Decker[54] as an alternative to Bergman's reaction under acidic condition. Neutral conditions and no tedious chromatographic purification are advantages of this method.



Product **5** resulting from the fusion reaction served after deprotection to product **6** as starting compound for derivatization of hydroxyl group in position 3 with different alkyls. In total three derivatives with hydrophilic chain linked with etheric oxygen to rutaecarpine were obtained *via* Williamson ether synthesis in good yields (products **8**–**10**). Product **8** contains hydrophilic aliphatic polyethoxy chain. Products **9** and **10** with tertiary amino group in the side chain were gained from 3-(3-chloropropoxy)rutaecarpine (**7**). The new three semisyntethic products of rutaecarpine have been purificated by column chromatography and characterized by NMR spectra. While product **8** prepared with Williamson synthesis in was obtained in good yield and with little amount of by-products in 24 hours, the other two products prepared by substitution on aliphatic chain needed to be treated with more reactants and for three times longer. Even after use of ten equivalents of diethyl amine in synthesis of **10** and after 72 hours a little spot of starting material remained on TLC and it did not disappear even after addition of reactants. The purification of **9** had to be done in two different ways to obtain higher yield of the final product.

Solubility of these new derivatives of rutaecarpine is supposed to be better than one of the origin molecule. It is to be tested. Piperidinylpropoxy and diethylaminopropoxy derivative (**9** and **10**) enable conversion to hydrochlorides.

Cytotoxic tests will also be accomplished on these rutaecarpine derivatives. The planar structure is supposed to be responsible for their intecalating mechanism and substitution in position 3 of E-ring should not influence the biological activity of these better soluble derivatives.

The aim of this work was to prepare 3- substituted ether derivatives of rutaecarpine. Three different molecules were obtained and their synthesis was established. Experimental part also focused on optimization of the synthesis of 3- ethers of rutaecarpine and the established synthesis provided good overall yields with purification necessary only in the last step of whole synthesis. Two of the products (**9, 10**) were provided with tertiary amino- group that can form salts with HCl to increase its water solubility and one product (**8)** was provided with highly hydrophilic group and showed raised solubility itself.

As rutaecarpine is a strong cytotoxic agent due to its core structure we can propose similar effects of our final compounds. They could also show raised Topo I and Topo II inhibitory activity comparing to rutaecarpine because of the substitution of E-ring of the core. Cytotoxicity tests are going to be performed and they will show how potent our 3- substituted rutaecarpine ethers are.

4 CONCLUSION

Cancer as the second most deadly disease has been in the centre of medicinal research for many years but there are still not all mechanisms elucidated. It is complicated by the fact, that cancer is a collection of diseases and not a model illness, i. e. one cause – one disease. In the last decades cancer treatment and curative processes have gone a long path and the chances to get cured are much higher than they used to be. There are new drug discoveries e.g. in the field of biological treatment and even whole new drug groups but cytotoxic chemotherapy treatment remains the mainstream therapy of cancer of various types. Searching natural compounds is one of the main ways of new drug discoveries and also Chinese traditional medicine has already shown its potential in this way. Also some cytotoxic agents are based on natural alkaloids and there are still many others waiting.

Rutaecarpine was first isolated 100 years ago but there is so little known about this alkaloid. Only few papers a year are published about rutaecarpine and its cytotoxic acting in human body is still not fully understood. Derivatives of rutaecarpine are being prepared aiming to better solubility and increased cytotoxicity to use its natural potential in the fight of humankind with cancer.

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