EFFECT OF CYCLINE-DEPENDENT KINASE AND MATRIX METALLOPROTEINASE INHIBITORS ON HEMATOPOIETIC AND LEUKEMIC CELLS

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Cover: The Chinese national painting “Horse” is from the famous painter Bei Hong Xu.
月亮以不常满为心，
大海有容乃能容之度。
ABSTRACT
Rapid advances in molecular and cellular biology have improved the understanding of the mechanisms involved in leukemia development. Cyclin-dependent kinases (CDKs) and matrix metalloproteinases (MMPs) have been suggested as potential therapeutic targets and a number of pharmacologic inhibitors of CDKs and MMPs have been developed.

This thesis aimed to increase knowledge about pharmacokinetics and cytotoxic effects of the CDK inhibitor roscovitine and MMP inhibitors from tetracycline analogues.

The effect of roscovitine on hematopoietic progenitors was studied in vitro and in vivo in mouse model. In vitro, bone marrow (BM) was incubated with roscovitine and the clonogeneic capacity of BM cells was assessed using semisolid methylcellulose-based media. Roscovitine decreased colony formation in concentration- and cell type-dependent manner, CFU-GEMM were the most sensitive, followed by BFU-E and last CFU-GM. In vivo, only transient decrease in BFU-E was observed after the treatment which probably is due to the low distribution of roscovitine to BM (1.5%) compared to that detected in plasma.

Pharmacokinetics and distribution of roscovitine was studied in adult and in 14-days old rats. Pharmacokinetics of roscovitine in plasma and brain fitted a two-compartment model in both adult and pups. Pharmacokinetics was age-dependent with a terminal elimination half-life of 7h in brain and plasma in pups compared to <0.5 h in adult rats. Brain exposure to roscovitine expressed as AUC brain/ AUC plasma was 100% in rat pups and 20% in adult rats. Moreover, significant but transient CDK5 inhibition and Erk1/2 activation were observed in hippocampus, frontal cortex and cerebellum.

Cytotoxic effect of roscovitine was studied in three cell lines representing different types of leukemia: the myeloid HL-60, the lymphoblastic Jurkat and the CML K 562. Roscovitine decreased viability and proliferation in a concentration- and time-dependent manner in all cell lines. Cell morphology induced by roscovitine was consistent with apoptosis. Loss of mitochondrial membrane potential, release of cytochrome c, active fragment of caspase-3 and cleaved PARP were observed in all three cell lines, however, the pattern of activation of caspase-2 and -8 showed different pattern among the cell lines.

The cytotoxic effects of tetracycline analogues (TCNAs) doxycycline, minocycline and COL-3 (chemical modified tetracycline-3) were studied in the human leukemia HL-60 cells. TCNAs decreased proliferation and induced apoptosis in concentration- and time-dependent manner. A apoptosis induced by TCNAs was mitochondria-mediated and caspase-dependent. COL-3 exerted the strongest anti-proliferative and proapoptotic effect.

In conclusion, both roscovitine and TCNAs exerted cytotoxic effect through mitochondria-mediated and caspase-dependent apoptotic cell death in leukemic cells. Roscovitine did not affect hematopoietic progenitors in vivo that might be explained by the low distribution of roscovitine to bone marrow in combination with rapid elimination of the drug. However, age-dependent pharmacokinetics and high distribution of roscovitine to brain in rat pups was observed. This may be implicated in the treatment of pediatric CNS leukemia and brain tumors.


IV. **Hairong Song**, Kim R. Maguire, Åke Siden, Zuzana Hassan. Cytotoxic effect of tetracyclines (doxycycline, minocycline and COL-3) on acute myeloid leukemia HL-60 cells. Submitted to Leukemia and lymphoma.
LIST OF ABBREVIATIONS

AIF  Apoptosis-inducing factor
ALL  Acute lymphoblastic leukemia
AML  Acute myeloid leukemia
AUC  Area under concentration-time curve
BFU-E Burst-forming unit-erythroid
Caspases Cysteine aspartate-specific proteases
CDK  Cyclin-dependent kinase
CDKI Cyclin-dependent kinase inhibitor
CFU-GM Colony-forming unit-granulocyte-macrophage
CFU-GEMM Colony-forming unit-granulocyte-erythrocyte-monocyte-macrophage
Cmax Maximum concentration
CL  Clearance
CML Chronic myeloid leukemia
CNS Central nervous system
COL-3 Chemically modified tetracycline-3
DISC Death-inducing signaling complex
DOXY Doxycycline
FADD Fas-associated protein with death domain
MDR Multidrug resistance
MINO Minocycline
MMP Matrix metalloproteinase
MMPI Matrix metalloproteinase inhibitor
PARP Poly-(ADP-ribose)-polymerase
$\tau_{1/2}$ Elimination half-life
TCNA Tetracycline analogue
TIMP Tissue inhibitor of metalloproteinase
TRIAL Tumor necrosis factor-related apoptosis-inducing ligand
Vd Volume of distribution
1 INTRODUCTION

1.1 LEUKEMIA
Leukemia is a heterogeneous group of hematopoietic malignancies that occurs worldwide and includes acute/chronic and myeloid/lymphocytic leukemias (Chen et al., 2007).

1.1.1 Epidemiology of leukemia

1.1.1.1 Acute myeloid leukemia
Acute myeloid leukemia (AML) is the most common myeloid leukemia, with a prevalence of 3.8 cases per 100 000 rising to 17.9 cases per 100 000 adults aged 65 years and older. The median age at presentation is about 70 years. The gender ratio (M/F) is 3/2. There are several risk factors for acquiring AML, such as exposure to ionizing radiation, benzene, and cytotoxic chemotherapy (Chen et al., 2007).

1.1.1.2 Acute lymphoblastic leukemia
Acute lymphoblastic leukemia (ALL) is a malignant disorder that originates in a single B- or T-lymphocyte progenitor. The disease is most common in children, but can occur in any age group. The incidence among infants, children and adolescents is 31 per 10^6 per year (Gaynon and Siegel, 2002).

1.1.1.3 Chronic myeloid leukemia
Chronic myeloid leukemia (CML) accounts for approximately 15% of all leukemias and has an annual incidence in the 1 to 1.5 cases per 100 000 population. The disease is essentially one of adult life and is uncommon in childhood. Final confirmation of CML is done by detection of the Philadelphia Chromosome (Ph) or BCR-ABL transcripts (Barnett and Eaves, 2002).

1.1.1.4 CNS leukemia
The first leukemic infiltration of the central nervous system (CNS) has been described in 1967 (Hardisty and Norman, 1967). CNS relapse confers a dismal prognosis (Wellwood and Taylor, 2002). Since CNS relapse developed in more than 80% of children who remained in bone marrow remission (Evans et al., 1970), CNS-directed treatment or CNS prophylaxis became an essential component of the therapy of ALL in the early 1970s. The most widely used approach was combination of cranial irradiation and MTX administered intrathecally (Chessells, 1994). Later the cytarabine or steroids have been added, as well as high dose dexamethasone i.v. as systemic chemotherapy (Pullen et al., 1993). However, the CNS prophylaxis resulted in the neuropsychological problems, early puberty and impaired growth. Moreover, CNS relapse was frequently followed by bone marrow relapse (George et al., 1985). Therefore, prevention of CNS relapse is currently a standard approach in the treatment of ALL, and the focus is on minimizing early and late toxicity (Wellwood and Taylor, 2002).

1.1.2 The molecular genetics of leukemia
Different types of leukemia usually have specific chromosome translocations that cause the activation of oncogenes (Chen et al., 2007; Gao et al., 2007). AML blasts
develop from normal cells that have been affected by genetic events encompassing two major groups, I. mutations that activate signal transduction pathways, II mutations that affect transcription factors or components of the transcriptional co-activation complex (Frohling et al., 2005; Kelly and Gilliland, 2002). Group I of genetic events results in activation of cell surface receptors, such as RAS, or tyrosine kinases (FLT3 and c-KIT); group II exemplified by overexpression of HOX genes or formation of fusion genes (Downing, 2003). Group I mutations accounts for approximately 50% in AML patients, thus, inhibitors of these pathway are attractive as potential therapeutic agents (Tallman et al., 2005). The formation of abnormal fusion genes, such as AML-ETO \([t(8;21)]\) occurs in approximately 40% of M2-type AML (Li et al., 2006; Zhang et al., 2006). In M3-type AML (acute promyelocytic leukemia, APL), the fusion gene PM L-RAR\(_\alpha\) involving a translocation of chromosomes 15 and 17 \([t(15;17)]\) was found. This fusion gene blocks myeloid differentiation by antagonizing the effects of the normal RAR\(_\alpha\) or inhibits endogenous PML pathway (Cheng et al., 1999; Nichols and Nimer, 1992). The destruction of PM L-RAR\(_\alpha\) by ATRA and AS2O3 is a good example of targeted cancer therapy (Chen et al., 2007). BCR-ABL is reciprocal translocation between chromosomes 9 and 22, and is found in the leukemic cells of >95% of patients with CML (Rowley, 1973). STI-571 (imatinib) targets and binds to the tyrosine kinase domain of BCR-ABL and thus, is another example of targeted therapy based on molecular genetics (Schindler et al., 2000).

1.2 STANDARD CHEMOTHERAPY AGENTS USED IN LEUKEMIA

1.2.1 Mechanism of cytotoxicity

During the past four decades, many studies have investigated a wide variety of cytotoxic antileukemic agents (Tallman et al., 2005). Contemporary acute leukemia treatment programs include induction, intensified consolidation, maintenance phase and CNS prophylaxis (Apostolidou et al., 2007; Tallman et al., 2005). Conventional chemotherapeutic agents either interfere with DNA, inhibit nucleic acid synthesis, block protein synthesis, or target the mitotic spindle apparatus (Pui and Evans, 2006).

1.2.2 Adverse effect

Adverse effects of cytostatics may be classified according the target organs, severity, onset or reversibility. According to severity, adverse effects are classified as mild, severe or life-threatening; according to onset, as early, delayed and latent (Gale, 1985). The organ toxicity of cytostatic agents may be expressed as different clinical symptoms, such as nausea and vomiting caused by gastro-intestinal toxicity and stimulation of chemoreceptor trigger zone in brain; hepatitis and cholestasis caused by hepatic toxicity; veno-occlusive disease caused by endothelial damage, adults respiratory distress syndrome caused by toxic or allergic lung injury, nephrotoxicity and hemorrhagic cystitis caused by toxicity to urinary tract. The major dose-limiting toxicity of vast majority of cytostatic agents is myelosuppression (leucopenia, neutropenia, thrombocytopenia, and anemia). It is also one of the most frequent and serious adverse effects. The mechanisms of myelosuppression is divided into two major groups, direct and indirect (Table 1).
Table 1. Mechanisms of myelosuppression (Gale, 1985)

Assessment of myelosuppression is applicable to both human being and animal. Different methods such as peripheral blood cell counts, bone marrow cellularity measurements, assays of CFU-S, CFU-E, BFU-E, CFU-GEMM and long term cultures are used (Gale, 1985). Figure 1 shows the differentiation of hematopoietic progenitors in mice.

Figure 1. Hierarchical model of normal hematopoietic cell differentiation in mice.

1.2.3 Mechanism of multidrug resistance

Over the past 20 decades, information generated from cell biology has strengthened our understanding of how malignant cell survive toxic insults and become resistant to antineoplastic (Chen et al., 2007; Mahadevan and List, 2004). Cells exposed to toxic agents can develop resistance by a number of mechanisms including pre-target, target and post-target. However, the mechanisms of BCR-ABL maintain the proliferation of cells while resisting drugs is unique.
1.2.3.1 Pre-target

ATP-binding cassette (ABC) transporters contribute to multidrug resistance. This super-family of active transporters comprises 49 functionally distinct transmembrane proteins (de Jonge-Peeters et al., 2007; Li et al., 2006). Two members of ABC super-family of transporter proteins, P-glycoprotein (P-gp, also referred to as MDR1) and breast cancer resistance protein (BCRP, encoded by the ABCG2 gene) has been described to be correlated with multidrug resistance in leukemia (Raaijmakers et al., 2002; Scharenberg et al., 2002; Zhou et al., 2001). MDR1 is able to cause a degree of resistance to amphipatic drugs such as anthracyclines and vinca-alkaloids (Fisher and Sikic, 1995). The efflux pump, flippase model and vacuum cleaner model have been considered as three mechanisms of pumping (Bolhuis et al., 1997; Borst and Elferink, 2002; van der Kolk et al., 2002). High expression of BCRP mRNA has been observed in 30% of high risk AML cases. A potentially independent role of this transporter in drug resistance in leukemia has been suggested (Ross et al., 2000).

1.2.3.2 Target

Topoisomerase II plays a role in DNA replication, chromosome scaffold formation and segregation, and possibly gene transcription (Bradbury and Middleton, 2004; Davies et al., 1988; Wang, 1985). The mechanisms of resistance to anti-topoisomerase II drugs may include: point mutation and abnormally functioning topoisomerases, methylation, upregulation of topoisomerase I and form of topoisomerase II(β) (Hochhauser and Harris, 1991). DNA repair was enhanced through the enzyme O^6^-alkylguanine alkyltransferase (AGT), base excision repair, nucleotide excision repair, recombination repair and mismatch repair (Bradbury and Middleton, 2004; Hochhauser and Harris, 1991). The level of repair proteins has been reported to increase as tumors become resistance to chemotherapy (Chu, 1994). The p53 protein is an important regulator of the cell cycle and is sensitive to DNA damage. Mutation or loose of p53 function will allow cells with DNA damage to continue replicating, which means resistance to the drugs (Petty et al., 1998).

1.2.3.3 Post-target

Many tumors intrinsically resistant to chemotherapy are defective in the ability to undergo apoptosis (Burger et al., 1999; Gao et al., 2007; O'Gorman and Cotter, 2001). The antiapoptotic proteins Bcl-2 is expressed in over 87% of AML cases (Bensi et al., 1995). Up-regulation of Bcl-2 or down-regulation of BAX expression and increased resistance to treatment-induce apoptosis was observed (Burger et al., 1999; Russell et al., 1995). Transfection of Bcl2 or Bcl-X_L into cell lines greatly increased resistance to numerous insults, including cytotoxic drug-induced apoptosis (Minn et al., 1995; Miyashita and Reed, 1992). Ras transformed cells have enhanced resistance to Fas-induced death (Fenton et al., 1998).

Tyrosine kinase inhibitor

Imatinib is a small molecule binding to BCR-ABL, and blocking BCR-ABL function by blocking its ability to use ATP. Imatinib-resistant clones are appearing and allow the disease to progress (Frame, 2007). Five main mechanisms are currently known that may result in the resistance to imatinib, including plasma protein binding, drug efflux, mutation of the BCR-ABL kinase, independent of BCR-ABL and gene amplification (Kantarjian et al., 2006; Larson et al., 2008; Picard et al., 2007).
1.3 NEW THERAPEUTIC STRATEGIES FOR THE TREATMENT OF LEUKEMIA

The efficacy of most antineoplastic drugs is dose-dependent, higher doses result in increased response rates and a higher proportion of cures. The cure rate of childhood ALL is more than 80%, but the survival rate for adult ALL remains below 40%. The survival rate of AML patients who are younger than 60 years is 20-75%, but less than 10% of elderly patients (Estey and Dohner, 2006; Nakanishi et al., 1999; Pui and Evans, 2006). Moreover, antineoplastic drugs are nonspecific, have narrow therapeutic window, often produce adverse cytotoxic effects in various normal tissue and their effect may be reduced due to multidrug resistance of the cancer cells (Pui and Jeha, 2007). Thus new treatment strategies are required aiming to improve the treatment efficacy and reduce side effects. The new agents are expected to be specific against the molecular targets in cancer cells, to overcome multidrug-resistance and to have minimal toxicity against normal tissues, which may improve cure rate and quality of life. Currently, scientists investigate the new therapeutic strategies for the cancer treatment including the new formulations of the old drugs and new agents based on discoveries of molecular biology of cancer cells:

1) Encapsulate old drugs into liposome or PEG: VCR, anamycin, daunorubicin and asparagines have been encapsulated in liposome or PEG (Cortes et al., 1999; Guthlein et al., 2002; Leonetti et al., 2004; Pui and Jeha, 2007);

2) New nucleoside analogues: clofarabine, nelarabine, forodesine (Miles et al., 1998; Parker et al., 1991; Rodríguez and Gandhi, 1999);

3) Monoclonal antibodies: rituximab, anti-CD19, alemtuzumab (Gilleece and Dexter, 1993; Goulet et al., 1997; Schulz et al., 2004);

4) Molecular therapeutics:
   A. Tyrosine kinase inhibitors: imatinib, dasatinib, nilotinib (Fausel, 2006);
   B. FLT3 inhibitors: CEP-701, SU5416, PK C412 (Stirewalt and Radich, 2003);
   C. Proteasome inhibitors: bortezomib (Cortes et al., 2004; Yu et al., 2003a);
   D. Histone-deacetylase inhibitors: LAQ824, AN9 (Cutts et al., 2001; Kosugi et al., 1999)
   E. Cyclin-dependent kinases inhibitors (CDKI): roscovitine, flavopiridol, olomoucine (Arguello et al., 1998);

5) Angiogenesis inhibitors: bevacizumab, VEGF inhibitor, matrix metalloproteinase inhibitor (M MPI), CDK1 (Karp et al., 2004; Lin et al., 2007; Nyormoi et al., 2003) (Figure 2).
1.3.1 Cyclin-dependent kinase inhibitors

1.3.1.1 Cyclin-dependent kinases

Cyclin-dependent kinases (CDKs) are small serine/threonine protein kinases that require association with a cyclin subunit for their activation. Eleven CDKs and sixteen cyclins have been identified. They are critical regulators of cell cycle progression and RNA transcription (Shapiro, 2006). CDK 1/CyclinA, CDK 2/CyclineA, CDK 4/6/CyclinD, CDK 7/cyclinH and CDK 11/cyclinL regulate the cell cycle; CDK 7/CyclinH, CDK 8/CyclinC, CDK 9/CyclinT and CDK 11/cyclinL connect with the transcription machinery (M einhart et al., 2005; P relich, 2002)(Figure 3, Figure 4); CDK 5/p35 or p39 is indispensable for normal neural development and function (Dhariwala and Rajadhyaksha, 2008). CDKs are regulated by positive phosphorylation by kinases such as CDK-activating kinase (CAK), as well as negative phosphorylation by INK 4 family (p16<sup>INK4a</sup>, p15<sup>INK4b</sup>, p18<sup>INK4c</sup> and p19<sup>INK4d</sup>), or Cip/Kip family (p21<sup>Cip1</sup>, p27<sup>Kip1</sup>, p57<sup>Kip2</sup>) (Hall and Peters, 1996; Sherr and Roberts, 1995).
Figure 3. Cell cycle interactions. CDK/cyclin complexes are kept inactive though association with CDKIs or Weel. Phosphorylation of CDK by a CDK-activating kinase (CAK, CDK7/cyclinH/MAT1), and removal of the inhibitory phosphates by a Cdc25 phosphatase.

Figure 4. Transcriptional activity of CDKs on RNA polymerase II and elongation of RNA. CDT: carboxy-terminal domain

**CDK5**

CDK 5 was found in 1992 as an unusual molecule and most abundant in the nervous system. It appears to be indispensable for normal neural development and function (Hellmich et al., 1992). It is activated by non-cyclin proteins p35 and p39. p25 is a proteolytic fragment of p35, generated by a calcium-activated protease, calpain. p25 has a longer half-life than p35, thus, binding of p25 to CDK5, maintains CDK5 in hyperactive state, which is often associated with neurotoxicity (Lee et al., 2000; Patrick et al., 1999). In normal cells, the physical function of CDK5 is obligatory for migration and differentiation of neurons in developing brain. The young rat model has shown its peak expression and activity in brain at day 14 postnatal. Deregulation of CDK5 has been implicated in Alzheimer’s disease, amyotrophic lateral sclerosis, Parkinson’s disease, Huntington’s disease and acute neuronal injury (Brion and Couck, 1995; Paudel et al., 1993; Wang et al., 2003; Wu et al., 2000). Regulators of CDK5 activity are considered as potential therapeutic molecules for degenerative disease. Currently, some studies have documented that CDK5 controlled process of survival and metastasis in several tumor cell lines (Goodyear and Sharma, 2007; Strock et al., 2006).
Animal development from a single-cell zygote to fertile adult requires many rounds of cell division. During each division, cells complete ordered series of events collectively called cell cycle, which includes S, M, G1 and G2 phases. The cell cycle is controlled by numerous mechanisms such as CDKs/cyclin complex, CDK inhibitory protein, p53 and the Rb/2F pathway ensuring correct cell division (van den Heuvel, 2005). But in cancer, there are fundamental alterations in the genetic control of cell division, resulting in an unrestrained cell proliferation (McDonald and El-Deiry, 2000; Sherr, 1996). Hematopoietic malignancies show clear evidence of aberrations in the Rb pathway, also cyclin D1 translocation, or loss in endogenous CDK inhibitors in leukemia and hyperactivation of CDK 4/6 in human T cell leukemia (Dreyling et al., 1997; Neuveut et al., 1998; Siebert et al., 1996).

**Cyclin-dependent kinase inhibitors**

Abnormal regulation of kinases and phosphatases appears to contribute directly to the onset of some diseases. Theses observations have encouraged the active search for pharmacological inhibitors of kinases and phosphatases. Treatment with these inhibitors may induce both cell cycle arrest and apoptosis. and inhibition of CDKs important for RNA transcription, including those encoding anti-apoptosis family members, cell cycle regulators, as well as p53 and nuclear factor-kappa B-responsive gene targets. Based on the critical role, the CDKs pathways comprise an attractive set of targets for novel anticancer drug development (Shapiro, 2006). Different strategies for therapeutic intervention can modulate CDK activity by different ways: direct (inhibiting CDK kinases) and indirect (targeting the regulators of CDK activity). More than 50 inhibitors have been described such as purine analogues, plant cytokinin analogues and pyrimidine analogues (Vermeulen et al., 2003).

1.3.1.2 Roscovitine

Fundamental research on cell division from starfish oocytes model in 1970s has lead to discovery of the first inhibitory purines in 1980s as non-specific kinase inhibitors. Roscovitine was synthesized as the third generation of purine inhibitors in 1997 by Dr L Meijer Roscoff, France (Meijer and Raymond, 2003).

Its molecular formula is \(C_{19}H_{26}N_6O\) and molecular weight is 354.5. It has a purine ring (Figure 5). The crystal structure of roscovitine build complex with CDK 2 has been determined. In addition, the R-isomer has about twice the inhibitory capacity than the L-isomer. Roscovitine is soluble in chloroform and in DM SO.

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**Figure 5. Chemical structure of roscovitine**
Molecular mechanism
Roscovitine is a 2,6,9-trisubstituted aminopurine analogue that competes with ATP for binding to the active site on CDKs. Roscovitine selectively inhibited CDK2/E (0.13 ± 0.07µmol/L), CDK2/A (2.2 ± 0.7µmol/L), CDK1/B (14.1 ± 2.7µmol/L), CDK4/D1 (14.7 ± 3.6µmol/L), CDK6/D3 (50 ± 6µmol/L), CDK7/H (0.46 ± 0.09µmol/L), CDK9/T1 (0.78 ± 0.15µmol/L) and Erk2 (36 ± 4µmol/L). Its inhibitory potential was higher than other analogue such as olomoucine, bohemine (Raynaud et al., 2005). It also inhibited rRNA processing, MDM2 expression, Rb phosphorylation and caused DNA damage. Roscovitine can induce apoptosis in p53-dependent or p53-independent manner (Alvi et al., 2005; Hahntow et al., 2004; Ljungman and Paulsen, 2001; Lu et al., 2001; Maggiorella et al., 2003; Whittaker et al., 2004; Vitali et al., 2002).

Preclinical trails
A. Dose or concentration
Roscovitine was well tolerated up to 2000 mg/kg with good oral bioavailability (86%) in mice (Raynaud et al., 2005). IC50 of cancer cells lines in vitro ranged from 7.9 to 30.2 µM, and Roscovitine in concentration of 20µM induced high levels of apoptosis (about 80%) in a variety of cultured cancer cells (McClue et al., 2002).

B. Metabolism
Major mouse urinary metabolites of roscovitine were found as the carboxylic acid analogue (M1: MW =313, M 2: M W =353, M 3: M W =369, M 4: M W =371, M 5: M W =371, M 6: M W =371), as well as minor metabolite like C8-oxo-R-roscovitine and N9-desisopropyl-R-roscovitine were identified (Nutley et al., 2005). Major rat metabolite M 1 (MW =368) in plasma, M 2 (MW =370) in liver and kidney, and M 3 (MW =312) in several organs were identified (Vita et al., 2005). The other major metabolites of roscovitine determined in vivo are conjugates, with glucuronidation being the principal route of metabolism (McClue and Stuart, 2008).

In human, single oral dose was given to healthy male after breakfast and roscovitine and its carboxylate metabolite were studied in plasma and urine (de la Motte and Gianella-Borradori, 2004).

C. Cellular effect
Several studies have shown that roscovitine has cytotoxic effect on a broad range of tumor cell lines in vitro, and have the ability to suppress human colon and uterine cancer xenografts in vivo (Tirado et al., 2005). Synergistic effect of roscovitine with conventional cytostatic drugs such as gemcitabine, doxorubicine and cisplatin have been found (Crescenzi et al., 2005; MacCallum et al., 2005; Raje et al., 2005).

Summarizing the data, roscovitine has four major actions.
1. It clearly inhibits proliferation through cell cycle arrest in either G1 or G2. It inhibited proliferation of rabbit retinal pigment epithelial cells in G2/M phase (Wu et al., 2008), and reinforced the G1 arrest in cancer cells treated by doxorubicine (Crescenzi et al., 2005). It decreased cell proliferation and matrix production and improved renal function in experimental glomerulonephritis (Pippin et al., 1997). These effects are due to inhibition of CDK2, CDK1 or Erk1/2 (Knochkaert et al., 2002).
2. It induces apoptosis of human cancer cell lines. Roscovitine has been found to induce apoptosis in chronic lymphocytic leukemia cells, Hodgkin’s lymphoma cells and multiple myeloma cells by different mechanism. Roscovitine and its analogue olomoucine dramatically enhanced the farnesyltransferase-induced apoptosis of human cancer cell lines (Edamatsu et al., 2000; Foell et al., 2008; Hahntow et al., 2004; MacCallum et al., 2005; Raje et al., 2005).
3. It shows antiviral activity. Roscovitine inhibits HSV replication, transcription of E or IE gene, and viral DNA synthesis. It inhibits replication of HIV-1, V S. and H1M by inhibition of CDKs rather than viral proteins (Agboatb et al., 2005; Schang, 2002).

4. It shows a neuroprotective action. Apoptosis plays a key role in the progression of neurodegenerative disorders such as Alzheimer’s and Parkinson’s diseases.

A ntia apoptotic effects of roscovitine on CGN (cerebellar granule neurons) are due to its anti-proliferative efficacy and effect on the mitochondrial apoptotic mechanism. Roscovitine’s analogue flavopiridol inhibited both the release of cytochrome c and the activation of caspase-3 by targeting CDK 5 on CGN (Jorda et al., 2003; Verdaguer et al., 2004).

According to summary above, the potential of clinical application would be as either anticancer or antivirus agents, as well as treatment of glomerulonephritis and neurodegenerative disorders.

Clinical trials
Roscovitine has entered the clinical phase II trials in patients with cancer as presented in Table 2 (Senderowicz, 2003).

<table>
<thead>
<tr>
<th>Dose</th>
<th>MTD</th>
<th>DLT</th>
<th>t(\frac{1}{2}) (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phase I</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100, 200, 800 mg</td>
<td>1600 mg/day</td>
<td>skin rash</td>
<td>4</td>
</tr>
<tr>
<td>Bid x 7d</td>
<td></td>
<td>nausea, vomiting</td>
<td></td>
</tr>
<tr>
<td>w 3w</td>
<td></td>
<td>non-hematological toxicity</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>increasing creatinine</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>hypokalemia</td>
<td></td>
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<tr>
<td>Phase II A</td>
<td>2500 mg/day</td>
<td>hypokalemia</td>
<td>3</td>
</tr>
<tr>
<td>5d x 3w</td>
<td>3200 mg/day</td>
<td>vomiting</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>increasing creatinine</td>
<td></td>
</tr>
<tr>
<td>Phase II B</td>
<td>2000 mg/day</td>
<td>skin rash</td>
<td></td>
</tr>
<tr>
<td>10d</td>
<td>2000 mg/day</td>
<td>vomiting</td>
<td></td>
</tr>
</tbody>
</table>

Table 2. Clinical trial of roscovitine. MTD = maximum tolerated dose; DLT = dose limiting toxicity; \(t\frac{1}{2}\) = elimination half-life

1.3.2 Matrix Metalloproteinase Inhibitors

1.3.2.1 Matrix metalloproteinases

The matrix metalloproteinases (MMPs) are a family of zinc-dependent proteinases capable of degrading components of the basement membrane and the components of extracellular matrix (ECM) (Chambers and Matriasian, 1997; Kahari and Saarialho-Kere, 1999; Kleiner and Stetler-Stevenson, 1999; Ray and Stetler-Stevenson, 1994). The human MMP gene family consists of at least 18 structurally related members that fall into five classes according to their primary structure and substrate specificity: collagenases (MMP-1, MMP-8, and MMP-13), gelatinases (MMP-2 and MMP-9), stromelysins (MMP-3, MMP-7, MMP-10, MMP-11, and MMP-12), membrane type (MT)-MMPs (MT1-MMP, MT2-MMP, MT3-MMP and MT4-MMP), and non-classified MMPs (RASS1-1 and Enamelysin) (Kleiner and Stetler-Stevenson, 1999). Most MMPs are secreted as latent precursors (zymogens) that are proteolytically activated in the extracellular space to execute physiologic processes, such as uterine involution, bone resorption and wound healing (Murphy et al., 1999). MMPs also
modulate the proliferation, differentiation and migration of hematopoietic stem or progenitor cells of different lineages through extracellular matrix (ECM) proteolysis and modulate release of growth factors/cytokines (Turpeenniemi-Hujanen, 2005; Yu and Han, 2006).

1.3.2.1.1 Role of MMPs in tumor development

MMPs play an important role in cancer cell progression, invasion and metastasis. Tumor progression is associated with an angiogenic switch (Liotta, 1986; Liotta et al., 1980). Tumor-induced angiogenesis is regarded to be important to sustain growth of solid tumor to the size when they become invasive and capable of generating metastasis (Bergers and Benjamin, 2003; Stetler-Stevenson, 1999). MMP-9 as a major contributor and a crucial factor triggering activation of quiescent vasculature (Bergers et al., 2000). It mediated release of VEGF from the ECM and recruitment of pericytes to the angiogenic vasculature (Bergers et al., 2000; Giraudo et al., 2004). A few researches strongly suggest a functional role for the localization of proteolytically active MMPs to the tumor stroma border and leading edge of invasive tumors. MMP-2, -9, -7 and -14 at the invasive protrusions of tumor cells has been predictable (Hofmann et al., 2003). The mechanisms of metastasis include loosening of cell-to-cell adhesion and extensive remodeling of the ECM, but until now, no clearcut evidence has been proved for the direct role of MMPs in this process (Condeelis and Segall, 2003; Condeelis et al., 2005). MMP-2 (gelatinase A) and MMP-9 (gelatinase B) are found abundantly in cancer tissues and have been also shown to be involved in the growth and invasion of myeloid and lymphoid neoplasmas (Stefanidakis and Koivunen, 2006).

1.3.2.2 Matrix Metalloproteinase Inhibitors

Matrix Metalloproteinase Inhibitors (MMPI) can be classified as natural and pharmacological.

A. Natural inhibitors: MMPs are inhibited by nonspecific protease inhibitors such as alfa-2-macroglobulin and alfa-1-antiproteinases, and specific tissue inhibitors of metalloproteinases (TIMPs: TIMP-1, -2, -3 and -4) (Kahari and Saarialho-Kere, 1999).

B. Pharmacologic inhibitors: MMPs play pivotal role in the process of malignant progression, thus a number of pharmacologic inhibitors of MMPs have been developed for the treatment of cancer. Inhibitors of MMPs fall into three pharmacologic categories: 1) collagen peptidomimetics and non-peptidomimetics, 2) tetracycline derivatives, and 3) bisphosphonates (Hidalgo and Eckhardt, 2001). Among these inhibitors, tetracycline analogues (TCNAs) have shown the relatively low toxicity (Deryugina and Quigley, 2006; Hidalgo and Eckhardt, 2001; Onoda et al., 2006; Seftor et al., 1998). The potential therapeutic interventions inhibiting MMPs are shown in Figure 6.
1.3.3 Tetracycline and its analogues

1.3.3.1 Chemistry

The tetracyclines (TCNs) were discovered in 1948 as broad-spectrum antibiotics. Currently TCN family is composed of three groups: natural products, semisynthetic compounds and chemically modified tetracyclines (Sapadin and Fleischmajer, 2006; van den Bogert et al., 1986). Tetracycline and its analogues (TCNAs) show a basic chemical structure consisting of a tetracyclic naphthacene carboxamide ring system that is surrounded by upper and lower peripheral zones (Figure 7). Tetracyclines with antibiotic property have a dimethylamine group at carbon 4 (C4) in ring A. Removing C4 can reduce antibiotic ability, but enhance non-antibiotic actions. Modification of the lower peripheral region reduces both antibiotic and non-antibiotic properties, while activity against the biological targets may be enhanced by modifying the upper zone particularly in C7 through C9 of D ring (Golub et al., 1992; Nelson, 1998).
1.3.3.2 Molecular mechanism

1.3.3.2.1 Antibiotic effects

The bacteriostatic effect of the TCNAs is due to their inhibition of bacterial protein synthesis by preventing the binding of the aminocycl1 t-RNA to the ribosome in the bacterial cell (Schnappinger and Hillen, 1996).

1.3.3.2.2 Non-antibiotic effect

**Metal chelation:** TCNAs are also ionophores and bind to divalent metal cations such as \( \text{Ca}^{2+} \) and \( \text{Mg}^{2+} \) to form lipid-soluble complexes, and transport them across hydrophobic membranes (Nelson, 1998).

**Inhibition of inflammation:** During inflammation process, TCNAs inhibit lymphocytic proliferation or production of \( \text{MMP-9} \), suppress neutrophilic migration and chemotaxis, up-regulate anti-inflammatory cytokine IL-10, or accelerate degradation of nitric oxide synthetase (Sapadin and Fleischmajer, 2006).

**Proteolysis:** TCNAs inhibit matrix metalloproteinases (MMPs) that play important role in the remodeling of the connective tissue and are involved in physiologic processes, tumor invasion, growth and metastasis.

**Angiogenesis:** Angiogenesis is a prominent feature of numerous diseases, including cancer and arthritis, and appears to play an important role in kidney disease and hypertension (Jackson, 2002). MMPs are involved in the process. For instance, activation of \( \text{MMP-2} \) and \( \text{MT1-MMP} \) directly degrades numerous matrix components, possibly activates the extracellular signal-regulated protein kinase cascade during angiogenesis (Gingras et al., 2001). TCNAs may inhibit the process from protein and mRNA levels by affecting migration of endothelial cells (Hanemaaijer et al., 1998).

**Apoptosis:** TCNAs have been shown to induce apoptosis in several cancer cell lines in vitro, such as melanoma, osteosarcoma, prostate, breast, colorectal, cervix carcinoma and leukemia (Bettany et al., 2000; Liu et al., 1999; Ouratidis et al., 2007; Onoda et al., 2006; van den Bogert et al., 1986; Wu et al., 2006). Their proapoptotic effect on mast cells offer a novel means to treat disorders with inappropriate expansion such as rheumatoid arthritis and systemic mast cell disease (Sandler et al., 2005). However, TCNAs may have antiapoptotic properties. Minocycline prevented neuronal cell death in mice after traumatic brain injury (Sanchez Mejia et al., 2001). Minocycline also protects cardiac necrotic and apoptotic cell death from ischemia/reperfusion (I/R) injury by inhibiting caspases and reducing leakage of mitochondrial proteins (Scarabelli et al., 2004).

**Bone metabolism:** Osteoclast-mediated osteolysis is dependent on MMPs during unmineralized matrix degradation. MMPs are also important mediators of metastasis formation in bone, especially patients with tumors of the prostate or breast (Duivenvoorden et al., 2002). Minocycline was found to stimulate the colony-forming efficiency of marrow stromal cells to increase bone formation in rat model (Williams et al., 1998). In mouse model of bone metastasis, TCNAs decease tumor burden probably through inhibition of \( \text{MMP} \), induction of apoptosis in mature osteoclasts and inhibition of osteoclastogenesis (Saikali and Singh, 2003).
1.3.3.3 Clinical applications
Tetracyclines have been suggested to have therapeutic potential in different
diseases, as summarized in Figure 8.

![Diagram showing clinical applications of TCNA](image)

Figure 8. Clinical applications of TCNAS.

1.4 CELL DEATH
Five categories of dynamic cellular activities that lead to cell death have been
described: apoptosis (type I), autophagy (type II), necrosis (type III), mitotic
catastrophe and senescence (Figure 9) (Dimri, 2005; Okada and Mak, 2004).
A apoptosis and autophagy have been considered to be “programmed” (Danial and
Korsmeyer, 2004; Lum et al., 2005).

![Diagram showing types of cell death](image)

Figure 9. Schematic illustration of the different types of cell death.

1.4.1 Programmed cell death
Programmed cell death (PCD) occurs during organ development, and plays an
important role in cellular homeostasis. A apoptosis was described in 1972 by Kerr and
Wyllie. Morphologic characteristics of apoptosis include cell membrane blebbing, cell
shrinkage, chromatin condensation, and nucleosomal fragmentation (Kerr et al., 1972),
finally ending with the engulfment by macrophages or neighboring cells, thus avoiding an inflammatory response in surrounding tissues (Savill and Fadok, 2000). Apoptosis has been considered a major mechanism of chemotheraphy-induced cell death. There are two distinct molecular signaling pathways that lead to apoptotic cell death: the intrinsic and the extrinsic (Ricci and Zong, 2006).

1.4.1.1 The extrinsic pathway

The extrinsic pathway is activated by ligand-bound death receptors mainly including TNF-TNFR1, FasL-Fas and TRAIL-DR4 or -DR5 (Baud and Karin, 2001). The complexes recruited to the death domain-containing adaptor proteins such as TRADD, RAIDD, or FADD, which allows binding of pro-caspase-8 or -10 to form DISC. DISC leads to activation of caspase-8 and -10 (Jin and El-Deiry, 2005). According to their requirement for mitochondrial pathway in Fas-induced apoptosis, cells can be divided into two groups: type I and II. In type I cells, processed caspase-8 is sufficient to activate caspase-3 that executes apoptosis. In type II cells, caspase-8 mediates cleavage of Bid and subsequently release of mitochondrial proapoptotic factors that activate caspase-3 (Scaffidi et al., 1998). Radical oxygen species (ROS) is mediated in part by the JNK pathway as mediators of TNF induced cell death (Sakon et al., 2003).

1.4.1.2 The intrinsic pathway

The intrinsic apoptotic pathways are initiated inside cells. The apoptotic signals is trigged, regulated and amplified by mitochondria, endoplasmic reticulum stress, lysosomes and nucleus (Green and Kroemer, 2004; Jin and El-Deiry, 2005).

Mitochondria: Mitochondria are vital for cellular bioenergetics and play a central role in determining the point-of-no-return of apoptosis (Galuzzi et al., 2006). Activation of the mitochondrial pathway results in biochemical changes, such as a loss of mitochondrial membrane potential, permeability transition pore forming and release of mitochondrial proteins into cytosol (Del Poeta et al., 2003).

Mitochondrial membrane potential (Δψm): Mitochondria undergo major changes in membrane integrity. These changes concern both the inner and the outer mitochondrial membranes, leading to the dissipation of the inner transmembrane potential which is a constant feature of early apoptosis, and/or to cause the release of proteins through the outer membrane (Hirsch et al., 1997; Loeffler and Kroemer, 2000).

Permeability transition pore (PTP): PTP is now thought to be due to the formation of dynamic multiprotein ensembles at inner or outer membrane contact sites (Zoratti and Szabo, 1995). The PTP is composed by outer membrane protein ( peripheral benzodiazepin receptor) (PBR), VDAC, intermembrane protein ANT, and at least one matrix protein ( cyclophilin D ) (Bernardi and Petronilli, 1996; Brustovetsky and Klingenberg, 1996). PTP is formed in the inner mitochondrial membrane in response to radical oxygen species overproduction and overload Ca2+. Besides PTP, another pores have been hypothesized, such as mitochondrial apoptosis-inducing channel (MAC), a proteolipid pore formed in response to outer membrane caused by oligomerized forms of BAX and BAK after their activation by tBid; a lipid channel formed by the lipid ceramide (Belizario et al., 2007).

Bcl-2 family: Bcl-2 family plays important role in apoptosis via intrinsic pathway. The family consists of proapoptotic and antiapoptotic members inducing or inhibiting apoptosis. Most Bcl-2 family members posses a hydrophobic C-terminal transmembrane domain, which allow them to localize into membranes of mitochondria,
ER or nucleus (Krajewski et al., 1993). The proapoptotic group includes BAX, BOK, and BAK, and the BH3-only group such as BID, BAD, BIK, BIM, PUMA, and NOXA. The antiapoptotic members include Bcl-2, Bcl-X_L, Bcl-w, Mcl-1 and A1 (Youle and Strasser, 2008). BAX and BAK are crucial for inducing permeabilization of the outer mitochondria membrane and the subsequent release of apoptogenic molecules, which leads to caspase activation. Both of them were inhibited by Bcl-2 and Bcl-X_L. BH3-only proteins sense signals to induce apoptosis and transduce the information to core Bcl-2 family members to initiate cell death. Moreover, their expression can be induced by transcription factors, for example, NOXA and PUMA, that are induced by the tumor suppressor p53 in response to DNA damage (Nakano and Vousden, 2001; Oda et al., 2000; Yu et al., 2001). The final fate of a cell may be determined by the balance or ratio of anti- and proapoptotic proteins (Fadde et al., 1999).

**Endoplasmic reticulum stress:** Besides the mitochondria, the ER is a second compartment in intrinsic apoptosis. If stress (oxidative, chemical toxicity, Ca^{2+} ionophores) to the ER cannot be solved, induction of necrosis or apoptosis becomes inevitable (Breckenridge et al., 2003). Both of BAX and BAK localize in ER to discharge Ca^{2+} ions and activate caspase-12 (Nutt et al., 2002). Other molecules such as GADD153/CHOP, ALG-2, VCP and BAP31, may also be involved in ER stress-induced apoptosis (Rao et al., 2004).

1.4.1.3 Crosstalk between extrinsic and intrinsic pathways

Despite the difference in the initiation manner, the two pathways converge on the activation of effector caspases (Danial and Korsmeyer, 2004). Death receptor-mediated apoptotic signaling can activate the mitochondrial pathway through the BH-3 only protein, BID (Li et al., 1998). Another crosstalk is that the Smac/Diablo are released from the mitochondria during death receptor-mediated apoptosis (Deng et al., 2002). The crosstalk even exists between the upstream components of the two pathways. For example, in response to DNA damage, p53 targets the BAX and PUMA to activate the intrinsic pathway, as well as upregulates the genes of FasL and TNF/DR5 in the extrinsic pathway (Kasibhatla et al., 1998; Wu et al., 1997).

1.4.1.4 Caspase-dependent pathway

Caspases are synthesized in normal cells as inactive proenzymes (Thornberry and Lazebnik, 1998). Fourteen members of the caspases family have been identified. They are either involved in apoptosis or control of inflammation (caspase-1, -4, -5, -11, -12, -13, -14) (Thornberry et al., 1992). During apoptosis, initiator caspases as upstream signal transducers activate the effector caspases. The initiator caspase-8 and -10 contain a death effector domain (DED) to interact with adaptor proteins. A caspase recruitment domain is found in caspase-2 and -9, which bind and activate effector caspases (Picard et al., 2007). Effector caspases-3, -6, and -7 act on a variety of substrates resulting in proteolysis of cellular proteins and death by apoptosis. The best characterized substrates are poly-(ADP-ribose) polymerase (PARP), ICAD (inhibitor of caspase-activated DNA se), lamins, fodrin and actin. Caspase-dependent cleavage of DNA-protein kinase (DNA-PK), cell cycle regulators (Rb), NF-kB and PKB (protein kinase B) has been reported (Barkett et al., 1997; Duriez and Shah, 1997; Earnshaw et al., 1999; Song et al., 1996; Tan et al., 1997; Widmann et al., 1998).
1.4.1.5 Caspase-independent pathway

In apoptosis, the mitochondria can play a key role, but also other organelles such as lysosomes and the endoplasmic reticulum have an important function in release and activation of death factors such as mitochondrial proteins, cathepsins, calpains, and other proteases (Broker et al., 2005).

1.4.1.5.1 Mitochondria

Release of toxic proteins (AIF, Endo G, Omi/HtrA2) from intermembrane space of the mitochondria was involved in caspase-independent apoptosis. Mature AIF translocates to the nucleus in response to PARP activation due to DNA damage (Yu et al., 2002). Overexpression of AIF induces peripheral chromatin condensation and high molecular weight (50kbp) DNA fragmentation (Ravagnan et al., 2001). Endo G digests nuclear DNA in the absence of caspase activity or the caspase-activated deoxyribonuclease CAD/DFF. Endo G-dependent DNA laddering was found in isolated nuclei (Li et al., 2001; van Loo et al., 2001). Omi/HtrA2 contributes to both caspase-dependent and caspase-independent programmed cell death (PCD). Overexpression of mature Omi/HtrA2 induces cell death independent of caspase action relying on the catalytic activity (Suzuki et al., 2001; Verhagen et al., 2002).

1.4.1.5.2 Lysosomes

Lysosomal proteases rather promote cell death more indirectly by triggering mitochondrial dysfunction and subsequent release of mitochondrial proteins (Boya et al., 2003; Guicciardi et al., 2000). tBID is cleaved and translocated to the mitochondria after lysosomal disruption (Cirman et al., 2004). Cathepsin D can trigger activation of BAX leading to selective release of AIF from mitochondria and PCD (Bidere et al., 2003).

1.4.1.5.3 Endoplasmic reticulum

Intracellular calcium influx caused by ER stress induces activation of a family of cytosolic proteases. BAX is involved in the cross-talk between the calpain and caspase proteolytic system (Liu et al., 2004; Neumar et al., 2003). It has been reported that calpain was independent of caspase activation during apoptosis (Mathiasen et al., 2002; Narvaez and Welsh, 2001), indicating that the ER may be important in caspase-independent cell death.

1.4.2 Apoptosis and Leukemia

Blockage of apoptosis or senescence of hematopoietic progenitor cells may allow the cell to acquire further mutations, hyperproliferate and eventually become malignant (Brady, 2003). CD95 plays a critical role in immune system homeostasis. Patients with autoimmune lymphoproliferation syndrome (ALPS) exhibit a significant increase in the incidence of lymphomas, since mature T lymphocytes fail to undergo apoptosis following stimulation via CD95 or T-cell receptor triggering (Straus et al., 2001). Overexpression of Bcl-2 blocks apoptosis activated via the mitochondria (Seto et al., 1988). Many studies have shown that overexpression of the Bcl-2 in leukemic cell lines confers increased resistance to cytotoxic drugs (Miyashita and Reed, 1993). In relapse of acute lymphoid leukemia, the Bcl-2 expression increases and the level of mutant p53, BAX, FAS, and active caspase-3 declines (Prokop et al., 2000). High levels of Mcl-1 were found to correlate with a failure to achieve complete remission in CLL.
patients (Kitada et al., 1998). PML-RARα fusion protein is found in most cases of acute promyelocytic leukemia (APL) (Dyck et al., 1994). This fusion protein inhibits apoptosis in leukemic cell lines, possibly because of binding endogenous PML and interfering with its localization to promyelocytic oncogenic domains (PODs) (Grignani et al., 1993). The BCR-ABL oncogene in chronic myelogenous leukemia (CML) inhibits apoptosis, in part, by constitutively activating STATs, which directly binds the Bcl-XL gene promoter and activate Bcl-XL gene transcription (Nieborowska-Skorska et al., 1999). Thus, it is important to study the regulation of apoptosis in leukemia and to understand how to the leukemic cells die in response to chemotherapy.

1.4.3 Apoptosis-based therapies for leukemia

1.4.3.1 Intrinsic pathway

Bcl-2 family proteins are the most prominent targets of the intrinsic pathway for cancer drug discovery. Drugs may target at different levels, DNA, mRNA or protein (Reed and Pellecchia, 2005). Some synthetic retinoids reduce levels of Bcl-2 or Bcl-XL mRNA in leukemic cells, suggesting a potential proapoptotic effect of these agents. Some of them have already been approved for clinical use (Reed, 1999). Antisense oligodeoxynucleotides (ODNs) targets the Bcl-2 mRNA are undergoing clinical phase III trial in relapsed CLL, AML and myeloma (Buchele, 2003). Small-molecule inhibitors such as gossypol directly binding Bcl-2 or related antiapoptotic proteins (Bcl-XL and Mcl-1) have also entered clinical trials for cancer (Kitada et al., 2003).

1.4.3.2 Extrinsic pathway

TRAIL and agonistic antibodies directed against TRAIL receptor exhibit potent antitumor activity and often synergize with chemotherapy (Ashkenazi et al., 1999). NF-kB and FLICE are modulators of sensitivity to TNF family (Irmler et al., 1997). The importance of NF-kB for suppression of TNF-induced apoptosis is well established, the chemical inhibitors of NF-kB kinase (IKKs) display proapoptotic activity against cultured malignant cells (Burke et al., 2003). FLIP has role in producing Fas- and TRAIL-resistant states in tumor cells, since destruction of FLIPs induce apoptosis in vitro of cultured leukemia cells (Ikeda et al., 2004; Pedersen et al., 2002). Certain effector caspases are targets of suppression by an inhibitor of apoptosis proteins (IAPs) and overexpression of IAPs has been documented in cancer and leukemia (Altieri and Marchisio, 1999; Deveraux and Reed, 1999). SURVIVIN is a member of the IAP family and downregulation of SURVIVIN by antisense oligonucleotides has induced apoptosis. Thus, further evaluation of SURVIVIN as a treatment target in cancer therapy is warranted (Chen et al., 2000).
2 AIMS OF THE THESIS

The overall aim of my thesis was to increase the knowledge on the effects of CDK inhibitor roscovitine and matrix metalloproteinases inhibitors (tetracycline analogues) on leukemic and hematopoietic progenitor cells; and to illustrate the effect and distribution of roscovitine in the brain of the rat. This knowledge may help in designing new treatment strategies for leukemia in order to improve therapeutic efficacy and to decrease the adverse effects.

Specific aims of the study

I. To study the pharmacodynamic effect of roscovitine on hematopoietic progenitors in vitro and in vivo in mice.

II. To investigate mechanisms of cytotoxic effects of roscovitine on human leukemic cell lines in vitro.

III. To study pharmacokinetics of roscovitine in rat pups and the effect of roscovitine on CDK5 and Erk in the brain.

IV. To investigate mechanisms of cytotoxic effects of matrix metalloproteinase inhibitors from tetracycline analogue group on a leukemic cell line in vitro.
3 MATERIALS AND METHODS

3.1 CHEMICALS

The following materials were used: RPMI 1640 medium, Dulbecco’s phosphate-buffer saline (PBS), fetal bovine serum (FBS), Iscove’s modified Dulbecco’s medium (IMDM) from Invitrogen AB, Stockholm, Sweden; 3H-Thymidine from Amersham Pharmacia Biotech AB, Uppsala, Sweden; doxycycline, minocycline, dimethylsulfoxide (DMSO), propidium iodide (PI) and ribonuclease A (RNase A) from Sigma-Aldrich Sweden AB, Stockholm, Sweden; resazurin from R&D System, Stockholm, Sweden; Complete mini protease inhibitor cocktail from Roche AB, Stockholm, Sweden; Z-VAD-FMK from Bachem AG, Bubendorf, Switzerland; MethoCult GF M 3434, MethoCult M 3534 and MethoCult M 3334 from Stem Cell Technologies Inc., Vancouver, Canada; roscovitine from LC laboratories, Boston, USA; etoposide from Bristol- Myers Squibb, Bromma, Sweden. COL-3 was generously provided by Collagenex Pharmaceuticals, Newtown, PA, USA. All other chemicals were of analytical grade and purchased from Merck, Darmstadt, Germany or Sigma-Aldrich Sweden AB, Stockholm, Sweden, unless otherwise stated.

Stock solutions of roscovitine and COL-3 were prepared in DMSO and further diluted with sodium chloride or culture media immediately before use. Stock solutions of DOXY and MINO were prepared in sterile water and further diluted in media immediately before use.

3.2 ANIMALS (STUDY I AND III)

All animal experiments were approved by the regional ethics committee for animal research in accordance with the Animal Protection Act, the Animal Protection Regulation and the Regulation of the Swedish National Board for Laboratory Animals. All animal experiments were designed according to the guidelines established by the Committee on the Care and Use of Laboratory Animals.

Female Balb/c mice at age 8-12 weeks used in paper I and Sprague Dawley rat pups at the age of 14 days and adult male rats used in study III were obtained from B&K Universal (Sweden). The animals were allowed to adapt to their surroundings for one week before starting the experiments. Animals were kept in fully acclimatized room at constant temperature and humidity on a 12 h light/dark cycle and were fed standard pellet and water ad libitum.

3.3 HUMAN LEUKEMIC CELL LINES (STUDY II, IV)

The myeloid HL-60 cell line was purchased from DSMZ (Braunschweig, Germany). The lymphoblastic Jurkat and the CML K 562 cell lines were purchased from ATCC (LGC Promochem AB, Boras, Sweden). Cells were seeded at a concentration of 2 x 10^5 cells/ml, and grown in RPMI 1640 medium supplemented with 10% heat-inactivated FBS at 37°C in 95% humidified 5% CO2 atmosphere. All experiments were run in exponentially growing cells.

3.4 EXPERIMENTAL METHODS

3.4.1 Animal treatment and sample collection

In study I, Balb/c mice were treated with different doses of roscovitine and sampled depending on the purpose. A Pharmacokinetic and distribution of roscovitine: roscovitine was administered intraperitoneally (i.p.) in a dose of 50 mg/kg. Mice were
killed at 10, 20, 30 min and 1, 2, 3, 4, 6, 8 h and blood and bone marrow were collected.

B. Effect of single dose of roscovitine on hematopoietic progenitors in vivo: The mice were treated with a single dose of 50, 100 or 250 mg/kg. Mice were killed on days 1, 3, 6, 9 and 12 after the treatment and bone marrow was sampled.

C. Effect of repeated doses of roscovitine on hematopoietic progenitors in vivo: The mice were treated with roscovitine in a dose of 350 mg/kg/day for four consecutive days and bone marrow was examined on day 1 and 5 after the last dose of roscovitine.

In study III, Sprague Dawley rat pups were injected i.p. with a single dose of roscovitine 25mg/kg. Blood for PK profile was collected at 3, 10, 20, 30 and 60 min, and 2, 3, 4, 6, 15 and 24 h. Brains for assessment of activity of CDK5 and Erk1/2 were sampled at 1, 2, 6, 15, 24, 38, and 48 h.

Untreated animals served as controls, animals treated with DMSO were used for assessment of the effect of the solvent. The final concentration of DMSO was 10%.

3.4.2 Sample preparation
Blood was collected by heart puncture into tubes containing heparin and centrifuged. Plasma was transferred to new tubes and either stored at -70°C, or proteins were precipitated with acetonitrile. After centrifugation clear supernatant was transferred to new tubes and stored at -20°C until assay.

Femurs were removed and cleaned; bone marrow was flushed out with sodium chloride 9 mg/ml and single-cell suspension was prepared by gentle flushing through needle and syringe. Nucleated cells were counted using Türk solution.

Brain was divided into two hemispheres and then after removal of olfactory bulb dissected into frontal cortex (FC), hippocampus (HC) and cerebellum (Cr). The samples were immediately frozen to -70°C until assay.

3.4.3 Roscovitine analysis
Roscovitine concentration in plasma, bone marrow and brain were analyzed using high performance liquid chromatography (HPLC) methods. The HPLC system consisted of a Gilson 234 auto-injector with a 200-µl loop, a LKB 2150 pump, a Zorbax SB-C18 column (3.5 µm; 4.6 x 75 mm), a UV-detector with variable wavelength "LDC Analytical Spectro- Monitor 3200", and a CSW chromatography Station Integrator. The mobile phase consisted of tetrahydrofurane: 0.1% phosphoric acid in water. The flow rate was 1 ml/min and the injection volume was 50 µl. The data were collected after running for 6 minutes, and processed using the integration system.

For analysis, 100 µl of plasma or bone marrow samples were mixed with 100 µl acetonitrile and vortexed for 30 seconds, then centrifuged at 7000 rpm for 10 min. 50 µl of the supernatant was injected into the system. Brain samples were homogenized by sonication in 0.9% NaCl in water (1:2 w:v), 60 – 100 µl of homogenate was added to equal amount of acetonitrile, vortexed for 1 min and centrifuged at 3000 g for 10 min. 50µl of supernatant was injected into the system.

3.4.4 Pharmacokinetic analysis
Pharmacokinetic analysis of roscovitine in plasma, bone marrow and different brain was performed using WinNonlin (Pharsight, Mountain View, CA, USA). Bone marrow data were derived after adjustment of concentrations to 10⁹ cells (approx. 1 g of tissue). Area under the concentration–time curve (AUC) was estimated using both trapezoidal
rule and WinNonlin program. All other parameters such as distribution volume, terminal half-life, the maximum concentration and clearance were calculated using WinNonlin.

3.4.5 Clonogenic assay

2 x 10^4 nucleated marrow cells in 1.1 ml of MethoCult M 3534 and 2 x 10^5 nucleated cells in 1.1 ml of MethoCult M 3334 were plated and scored with invert microscope on day 7 for CFU-GM and BFU-E, respectively. 2 x 10^4 nucleated cells were plated in 1.1 ml of MethoCult M 3434 and scored for CFU-GM, BFU-E and CFU-GEMM on day 12. The plates were incubated at 37 °C with 5% CO2 and 100% humidity. CFU-GM were defined as consisting of 50 or more cells, BFU-E as consisting of 30 or more cells.

3.4.6 Treatment of cells in vitro (Study I, II, IV)

Bone marrow cells from untreated mice were incubated with roscovitine in vitro in three different schedules.

A. The bone marrow cells were incubated with roscovitine in concentration of 25, 50 or 100 for 1, 3, 6, 12 and 24 h. Cells were washed before plating in MethoCult M 3534 and MethoCult M 3334.

B. The bone marrow cells were incubated with roscovitine in concentration of 25, 50, 100 or 250 for 4 h. Cells were washed before plating in MethoCult M 3434.

C. The bone marrow cells were plated in MethoCult M 3434 containing roscovitine a final concentration of 1, 10, 25 or 100 µmol/L. Colony formation was examined after 12 days.

In all three schedules, DMSO in a final concentration of 0.3% was used as a control for solvent toxicity. Untreated cells served as controls.

HL-60, Jurkat and K562 exponentially growing cells were incubated with roscovitine in final concentrations of 5, 25, 50, 100 and 200µmol/L up to 72 h. Etoposide (VP16) in a final concentration of 6 µg/ml for 24 h served as a positive control for apoptosis. The cells incubated with DMSO in a final concentration of 0.2% served as control solvent effects. The cells grown in complete medium served as controls.

The HL-60 cell line was incubated with DOXY, MINO and COL-3 in final concentrations of 0.5, 1, 2.5, 5, 10, 25, 50 and 100 µg/ml for 6 h and 24 h. The general caspase-inhibitor Z-VAD-FMK in a final concentration of 100µmol/L was used for 1 h prior to TCNAs. The controls were incubated with complete medium. In experiments with COL-3, DMSO in a final concentration of 0.2% was used as a control for solvent toxicity.

3.4.7 Viability and Proliferation (Study I, II, IV)

Cell viability was assessed using resazurin assay. Ten thousands of leukemic cells or 2 x 10^5 crude bone marrow cells were seeded in triplicates on 96 wells black microplates and incubated with roscovitine or TCNAs. Resazurin was added to each well in final concentration of 10% for 2 h at 37°C. Fluorescence was read by Fluostar Optima (BMG L Labtech, Offenburg, Germany) at a wavelength 590nm.

Proliferation was studied using 3H-thymidine incorporation assay. After the appropriate treatment, aliquots of 0.2 ml of cell suspension were incubated in triplicates in 96 wells microplates with 1µCi 3H-labelled thymidine for 4 h at 37°C. Labeled cells
were harvested by aspiration upon membrane filters, and dissolved in scintillation fluid (Optiscint Hisafe, Amersham Pharmacia Biotech AB, Uppsala Sweden), then counted by scintillation counter (WALLAC, EG&G Comp, Turku, Finland).

3.4.8 Assessment of apoptosis (Study II, IV)

Cells were cytopspined on slides and stained in May-Grünwald-Giemsa. Apoptotic cells were identified with morphological changes such as apoptotic body, condensed chromatin and fragmented nuclei. Percentage of apoptotic cells were estimated in a minimum of 400 cells per slide.

3.4.9 Cell cycle analysis (Study II)

Following appropriate treatment, the cells were washed with PBS, fixed in 70% ethanol in PBS, and then stored at -20°C until analysis. The cells were stained with hypotonic propidium iodide (PI) solution (20µg/ml) containing RNase A (100 µg/ml) at 37°C for 30 minutes. The DNA profile was analyzed using FACScan flow cytometer and CELL Quest software (Becton Dickinson, San Jose, CA, USA).

3.4.10 Assessment of mitochondrial membrane potential (Study II, IV)

Tetramethylrhodamine methyl ester (TMRM, Molecular Probes, Carlsbad, California, USA) specifically accumulates into mitochondria depending on ∆Ψm. Thus, TMRM was used to assess the mitochondrial membrane potential. Cells treated with roscovitine, TCNA or DMSO and controls were incubated with TMRM in a final concentration of 25 nmol/L at 37°C for 30 min. The cells were washed and resuspended in PBS, and analyzed using FACScan flow cytometer and CELL Quest software.

3.4.11 Western Blotting (Study II, III, IV)

Immunoblotting was performed in cell lines and in brain tissues using slightly different modifications (for details see study II, III and IV). Total cellular protein or subcellular fractions (cytosol and pellet) were prepared from cell lines. Brain tissues were homogenized by sonication in ice-cold isolation buffer and supernatant was prepared by centrifugation. A fter determination of protein content, the equal amounts of protein were separated by gel electrophoresis (SDS-PAGE). Then the proteins were transferred to nitrocellulose or PDF membrane. A fter blocking, the membrane was incubated with primary antibodies. The following primary antibodies were used: rabbit antibodies against caspase-2, -3, -8, -9, Bid, Bcl-XL (Becton Dickinson, Stockholm, Sweden), caspase-3, pErk1/2 (Cell Signaling, Stockholm, Sweden), CDK 5-p35 antibodies (C19)(Santa Cruz Biotech Inc., Santa Cruz, CA, USA), and actin (Sigma, St. Louis, MO, USA), and mouse antibodies against caspase-7, cytochrome c (Becton Dickinson, Stockholm, Sweden), Bcl-2 (Dako Sweden AB, Stockholm, Sweden), actin (Sigma, St. Louis, MO, USA), and PARP (Oncogene Research Products, Boston, MA, USA).

A fter washing, the membrane was incubated with horse-radish peroxidase-conjugated secondary antibodies (Amersham Pharmacia Biotech AB, Uppsala, Sweden). The protein bands were visualized using either ECL Plus Western Blotting Detection Reagents (Amersham Pharmacia Biotech AB, Uppsala, Sweden) or Supersignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL, USA). Optical density of the bands was quantified with scion image (Scion Corporation,
USA). The membrane were stripped in stripping buffer and re-blotted whenever appropriate.
4 RESULTS
4.1 PHARMACOKINETICS AND DYNAMICS OF ROSCOVITINE IN ANIMALS (STUDY I, III)

4.1.1 Effect of roscovitine on bone marrow in vivo and in vitro in mouse model

In study I, we investigated the effect of roscovitine on clonogenic capacity of bone marrow in vivo and in vitro, and the exposure of bone marrow cells to roscovitine after i.p. administration of roscovitine in mouse model.

4.1.1.1 Effect on hematopoietic progenitors in bone marrow in vivo and in vitro

The viability of bone marrow cells incubated with roscovitine for 4 h decreased in a concentration-dependent manner. The highest studied concentration of roscovitine 250 µM decreased the viability of bone marrow cells to 70% compared to the controls ($P = 0.015$) (Figure 10).

![Figure 10. Viability of bone marrow cell after exposure to roscovitine. Bone marrow cells were incubated with roscovitine for 4 hours. Viability was assessed using resazurin assay and expressed as % of the control. Results are presented as mean ± SD of three animals. * indicates significant level of $p<0.05$.](image)

Colonies formation decreased in a concentration- and time-dependent manner, when bone marrow cells were exposed to roscovitine until 24 h. However, a decrease in colony formation was observed in the controls at 12 and 24 h of incubation. No effect of DMSO on the colony formation was observed compared to the controls. Therefore, long time exposure was studied after addition of roscovitine to MethoCult media that optimally support the colony formation. CFU-GM growth was blocked at 100 µmol/L roscovitine, BFU-E formation was decreased by roscovitine at 25 µmol/L and completely inhibited at 100 µmol/L. CFU-GEMM growth was stopped at concentrations of 25 and 100 µmol/L. Thus, the suppression of colony formation was dependent on cell type and concentration (Figure 11).
Figure 11. Effect of roscovitine on colony formation in vitro. Roscovitine at final concentration of 1, 10, 25 or 100 µM or DMSO was added to methylcellulose-based media. Twenty thousand of nucleated bone marrow cells were added. CFU-GM (a), BFU-E (b) and CFU-GEMM (c) were counted at day 12 using inverted microscope. Results are expressed as mean ± SD from 5 mice. * indicates significant level of p<0.05, ** indicates significant level of p<0.01.

Then, we studied the effect of roscovitine on clonogenic capacity of bone marrow in vivo. Cellularity of bone marrow was expressed as number of nucleated cells per femur. Bone marrow cellularity was not affected by roscovitine administered as either single or multiple doses. Clonogenic capacity of bone marrow was not affect by single dose of roscovitine up to 250 mg/kg at any time point examined. Multiple doses of roscovitine decreased transiently the BFU-E growth, but did not have any significant effect on CFU-GM and CFU-GEMM growth (Figure 12).
Figure 12. Effect of roscovitine on hematopoietic progenitors in vivo. Mice were treated with roscovitine in dose 350 mg/kg divided into two daily doses for 4 days. Mice treated with DMSO served as controls for assessment of the effect of the solvent. Untreated animals were used as controls. Bone marrow was examined 1 and 5 days (numerals 1 or 5) after the last dose of roscovitine. CFU-GM (a), BFU-E (b) and CFU-GEMM (c) were counted on day 12 using inverted microscope. Each group consisted of 5 mice, control group of 8 mice. Results are expressed mean ± SD. * indicates significant level of p<0.05.

4.1.1.2 Pharmacokinetics of roscovitine in plasma and bone marrow

Pharmacokinetic parameters in plasma and bone marrow of mice are presented in Table 3. The AUC of Roscovitine in bone marrow was 1.5% of AUC of roscovitine reached in plasma.

<table>
<thead>
<tr>
<th></th>
<th>AUC trapez nmol/L . hr</th>
<th>AUC inf nmol/L . hr</th>
<th>Cmax nmol/L</th>
<th>CL L/hr</th>
<th>V d L</th>
<th>t½ hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma</td>
<td>282518</td>
<td>275781</td>
<td>202014</td>
<td>0.05</td>
<td>0.015</td>
<td>0.82</td>
</tr>
<tr>
<td>BM</td>
<td>4096</td>
<td>4588</td>
<td>4946</td>
<td>0.62</td>
<td>0.54</td>
<td>0.61</td>
</tr>
</tbody>
</table>

Table 3. Plasma and bone marrow pharmacokinetic parameters following i.p. administration of roscovitine 50 mg/kg in Balb/c mice. BM = bone marrow; AUC = area under the concentration-time curve; AUC trapez = AUC estimated using trapezoidal rule; AUC inf = AUC derived using WinNonlin analysis; Cmax = estimated maximum concentrations; CL = clearance; V d = apparent volume of distribution; t½ = half-life

4.1.2 Pharmacokinetics and effect of roscovitine in rat pups

In study III, we investigated pharmacokinetics of roscovitine in brain of rat pups and adult rats. Moreover, we studied the effect of roscovitine on activity of CDK 5 and Erk1/2 in different brain regions of rat pups.

4.1.2.1 Age-dependent pharmacokinetics of roscovitine in rat model

Pharmacokinetic parameters in plasma and brain regions after single i.p. injection of roscovitine in dose of 25 mg/kg are presented in Table 4. Both plasma and brain
concentrations of roscovitine were fitted a two-compartment model. The distribution half-life of approximately 0.6 h was found in rat pups compared to 0.06 h found in adult rats. Brain exposure to roscovitine expressed as AUC was similar to plasma exposure in rat pups. However, the exposure of adult rat brain reached only 25% of that found in plasma. No significant differences in exposure of different brain regions to roscovitine were found within the same age category. The most striking difference between rat pups and adult rats was found in elimination half-lives, 7 h in both brain and plasma of rat pups compared to 20 and 30 min in brain and plasma, respectively, of adult rats. No significant difference in clearance between brain and plasma was observed in rat pups. The clearance found in the adult brain was 5-fold higher compared to that found in plasma. The Cmax was significantly higher (p < 0.05) in pup’s brain compared to that found in plasma. In adults rats, plasma Cmax was 4-fold higher compared to that observed the brain. No roscovitine metabolites were found in the brain of both adult and young rats.

<table>
<thead>
<tr>
<th></th>
<th>Pup</th>
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<th>Frontal cortex</th>
<th>Hippocampus</th>
<th>Cerebellum</th>
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</thead>
<tbody>
<tr>
<td>AUC</td>
<td>(h.μg/g)</td>
<td>66.8 ± 7.2</td>
<td>69.57 ± 14.9</td>
<td>74.9 ± 11.9</td>
<td>78.7 ± 11.2</td>
</tr>
<tr>
<td>Tα</td>
<td>(h)</td>
<td>0.59 ± 0.14</td>
<td>0.43 ± 0.11</td>
<td>0.48 ± 0.19</td>
<td>0.50 ± 0.09</td>
</tr>
<tr>
<td>Tβ</td>
<td>(h)</td>
<td>7.2 ± 1.4</td>
<td>6.8 ± 1.3</td>
<td>8.0 ± 1.7</td>
<td>7.7 ± 2.2</td>
</tr>
<tr>
<td>Cmax</td>
<td>(μg/ml)</td>
<td>15.8 ± 0.4</td>
<td>24.9 ± 1.8</td>
<td>24.8 ± 1.9</td>
<td>23.7 ± 1.4</td>
</tr>
<tr>
<td>Vd</td>
<td>(ml)</td>
<td>102 ± 13</td>
<td>86 ± 20</td>
<td>90 ± 21</td>
<td>88 ± 15</td>
</tr>
<tr>
<td>CL</td>
<td>(ml/h)</td>
<td>11.3 ± 1.2</td>
<td>1.1 ± 2.1</td>
<td>10.2 ± 1.5</td>
<td>9.7 ± 1.2</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Adult</th>
<th>Plasma</th>
<th>Frontal cortex</th>
<th>Hippocampus</th>
<th>Cerebellum</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUC</td>
<td>(h.μg/g)</td>
<td>3.01 ± 0.21</td>
<td>0.71 ± 0.14</td>
<td>0.58 ± 0.03</td>
<td>0.62 ± 0.06</td>
</tr>
<tr>
<td>Tα</td>
<td>(h)</td>
<td>0.081 ± 0.05</td>
<td>0.045 ± 0.02</td>
<td>0.062 ± 0.012</td>
<td>0.062 ± 0.018</td>
</tr>
<tr>
<td>Tβ</td>
<td>(h)</td>
<td>0.54 ± 0.26</td>
<td>0.35 ± 0.13</td>
<td>0.36 ± 0.15</td>
<td>0.42 ± 0.18</td>
</tr>
<tr>
<td>Cmax</td>
<td>(μg/ml)</td>
<td>17.71 ± 4.42</td>
<td>4.47 ± 0.70</td>
<td>4.64 ± 0.81</td>
<td>3.81 ± 1.22</td>
</tr>
<tr>
<td>Vd</td>
<td>(ml)</td>
<td>650 ± 223</td>
<td>1095 ± 167</td>
<td>2056 ± 219</td>
<td>1909 ± 484</td>
</tr>
<tr>
<td>CL</td>
<td>(ml/h)</td>
<td>1637 ± 118</td>
<td>7262 ± 1612</td>
<td>8737 ± 452</td>
<td>8139 ± 727</td>
</tr>
</tbody>
</table>

Table 4. Pharmacokinetic parameters of roscovitine in plasma and different brain parts of rats after single i.p. administration of 25 mg/kg. Results are presented as mean ± SD from 5 rats for each time point. Results are presented as mean ± SD of three to five animals for each time point. AUC = area under the concentration–time curve; Tα = absorption half-life; Tβ = elimination half-life; Cmax = maximum reached concentration; Vd = volume of distribution at steady state; CL = clearance.

4.1.2.2 Effect of roscovitine on activation of CDK5 and pErk1/2 in brain regions in vivo

Roscovitine induced a significant inhibition of CDK5 activity expressed as an increase in p35 levels in all studied brain regions in rat pups. A significant accumulation of p35 was observed in frontal cortex (140% of controls) at 1–2 h, and in hippocampus and cerebellum (150% and 200%, respectively) at 2 h after administration of roscovitine. However, these effects were transient and the levels of p35 normalized in all brain regions at 6–15 h after treatment. No change in p35 levels was observed in the adult brain regions.
In rat pups, a transient activation of pErk1/2 was observed at 1 and 2 h after injection in frontal cortex and hippocampus, respectively. In cerebellum, roscovitine induced a significant increase of pErk1/2 levels at 2 h, followed by a significant decrease at 6 h after administration. At later time points, pErk1/2 levels in all the brain regions were similar in treated and control rat pups (Figure 13).

Figure 13. Densitometric analysis of immunoblot bands for both p35 and pErk1/2 in the frontal cortex, hippocampus and cerebellum until 48 h after single i.p. injection of roscovitine. Data are mean ± SD of values expressed as percentage of control animals (*, p < 0.05 for analysis of p35 data; #, p < 0.05 for analysis of p-Erk data; ANOVA followed by Fisher’s PLSD post-hoc test).

4.2 CYTOTOXIC EFFECT OF ROSCOVITINE IN LEUKEMIC CELL LINES (STUDY II)

Cytotoxic effects of roscovitine was studied in three leukemic cell lines presenting different types of leukemia, the myeloid HL-60, the lymphoblastic Jurkat and the CML K 562.

4.2.1 Effect of roscovitine on viability and cell proliferation

Roscovitine decreased viability in a concentration-dependent manner in all cell lines (Figure 14). The IC<sub>50</sub> was 17, 24 and 47 µmol/L in HL-60, Jurkat and K 562 cells, respectively. Maximum effect of roscovitine was reached within 24 h of incubation since prolonged exposure to roscovitine did not further reduced viability of any of the cell lines. The cytotoxic effect was irreversible, since no recovery in viability was observed when the cells were incubated with roscovitine for 24 h, then washed and incubated in roscovitine-free media up to 72 h. In similarity, proliferation assessed using <sup>3</sup>H-Thymidine incorporation assay was decreased in a concentration- and time-dependent manner in all cell lines, however, the effect was most pronounced in HL-60 cells. Incubation with DMSO did not affect viability or proliferation of the cells compared to controls.
Figure 14. The effect of roscovitine on viability of HL-60, Jurkat and K562 cell lines. Cells were seeded in a concentration of 10000 cells per well in triplicates and exposed to roscovitine in concentrations of 0.5 to 100 µmol/L for 24h. Viability was assessed using resazurin assay. Cells incubated with complete medium served as controls. The results were calculated as a percentage of the appropriate control. Experiments were run three times and results are expressed as mean ± SD.

4.2.2 Roscovitine-induced cell death

Changes in cell morphology induced by incubation with roscovitine were consistent with apoptosis. A apoptotic type of cell death was also confirmed by sub-G1 peak in cellular DNA staining and caspase activation and decrease in ΔΨm.

Roscovitine induced apoptosis in concentration and time-dependent manner in HL-60, Jurkat and K562 cells (Figure 15). Apoptosis appeared at 3 h and markedly increased at 6 h of incubation with roscovitine in concentrations 25 and 50 µmol/L in HL-60 and Jurkat cells, and 100 and 200 µmol/L in K562 cells.
Figure 15. Roscovitine-induced apoptosis. Cells were incubated with roscovitine in concentrations of 5 to 200 µmol/L up to 24 h. Controls consisted of cells incubated with complete medium and controls for solvent toxicity consisted of cells treated with 0.2% DMSO. Apoptosis was assessed using morphological criteria in minimum of 400 cells on cytospined slides in Giemsa staining. Results are expressed as means ± SD of three independent experiments. (a) HL-60 cell line; (b) Jurkat cell line; and (c) K562 cell line.

Staining of cellular DNA content was used for detection of sub-G1 peak and cell cycle analysis. The maximum of sub-G1 cells appeared at 24 h, together with morphological apoptotic changes. In HL-60 and Jurkat cells, roscovitine (50 µmol/L) induced apoptosis without any phase arrest at 6 h and 24 h. In K562 cells, G2/M arrest was observed during the incubation with roscovitine in concentration of 50 µmol/L, while increase in sub-G1 peak was observed with higher concentration of roscovitine (200 µmol/L) (Figure 16).

Figure 16. Effect of roscovitine on cell cycle. Cells were incubated with roscovitine in concentrations of 5 to 200 µmol/L for 24 h, control cells were incubated with complete medium. Fixed cells were stained with propidium iodide and DNA profile was analyzed using flow cytometry. All experiments were run three times. Results are expressed as means ± SD of three experiments.

Loss of ΔΨm was detected in all three cell lines and was concentration- and time-dependent (Figure 17). In Jurkat and HL-60 cell lines, a marked loss of ΔΨm was observed at 2 h exposure to 25 and 50 µmol/L of roscovitine, complete loss of ΔΨm was observed at 24 h of incubation with roscovitine 50 µmol/L. Roscovitine in a concentration of 5 µmol/L did not induce a loss of ΔΨm in Jurkat or HL-60 cells. In K562 cells, a marked loss of ΔΨm was found at 24 h of incubation with 100 and 200 µmol/L of roscovitine. Lower concentrations of roscovitine induced only slight loss (50 µmol/L) or no loss (5 and 25 µmol/L) of ΔΨm. DMSO exposure did not affect ΔΨm compared to control.
Figure 17. The effect of roscovitine on mitochondrial membrane potential ($\Delta \Psi_m$). Cells were incubated with roscovitine (25 to 200 µmol/L) up to 24 h. Cells incubated with complete medium served as controls, while cells treated with DMSO (0.2%) served as a control for solvent toxicity. Loss of $\Delta \Psi_m$ was assessed using TMRM assay and flow cytometry. Results are expressed as means ± SD of three experiments.

4.2.3 Pathways of apoptosis

Caspase activation was observed in all three cell lines (Figure 18). Initiator caspase-2 was cleaved to 15 kDa fragment and caspase-8 to 23 kDa fragment already at 6 h in HL-60. In Jurkat cells, the cleavage of caspase-8 preceded cleavage of caspase-2, while in K562 cells, caspase-8 and caspase-2 were not cleaved. The 17 kDa fragment of effector caspase-3 was observed at 6 h from the start of incubation with roscovitine in HL-60 and Jurkat cells and at 24 h in K562 cells. Poly-ADP-ribose polymerase (PARP) was cleaved to 85 kDa, thus confirming caspase-3 activation. Antiapoptotic protein Bcl-2 was cleaved to 23 kDa fragment in HL-60 cells, but remained intact in Jurkat cells. Full-length Bcl-XL disappeared in Jurkat and K562 cells. Cytochrome-c release was observed in all three cells lines. Translocation of tBid to mitochondrial compartment was found in HL-60 and Jurkat cell lines. Actin remained intact in all three cell lines treated with roscovitine.
Figure 18. Protein analysis in roscovitine-induced apoptosis. Cells were incubated with roscovitine in concentrations of 5 to 200 µmol/L for 6 and 24 h. Controls (C) were treated with complete medium, controls for solvent toxicity were treated with DMSO (0.2%) (D). Cells incubated with etoposide (V) (6 µg/ml for 24 h) served as a positive control for apoptosis. Level of expression and/or integrity of proteins were investigated by immunoblotting. Blots were subsequently stripped and probed with antibody directed against actin to ensure equivalent loading and transfer. (a) HL-60 cell line; (b) Jurkat cell line; and (c) K562 cell line.

4.3 CYTOTOXIC EFFECT OF TCNAS ON THE MYELOID HL-60 CELL LINE (STUDY IV)

The cytotoxic effects of TCNAs doxycycline, minocycline and COL-3 were studied in the myeloid HL-60 cell line. A concentration-dependent decrease in the viability of HL-60 cells was observed following 24 h incubation with all three TCNAs. The greatest effect was observed after incubation with COL-3 compared to DOXY and MINO when expressed as IC50 (0.9 µg/ml for COL-3 and 9.6 µg/ml for both DOXY and MINO, respectively) (Figure 19).
Figure 19. HL-60 cells were incubated with doxycycline, minocycline or COL-3 in concentrations of 0.5, 1, 2.5, 5, 10, 25, 50, 100 µg/ml for 24 h. Viability was assessed using resazurin fluorescence assay and expressed as percent of the control. Results are presented as a mean ± SD of three separate experiments.

4.3.1 TCNA-induced apoptosis
A apoptosis was induced by DOXY, MINO and COL-3 in a concentration and time-dependent manner in HL-60 cells (Figure 20). A apoptotic morphology was observed at 6 h after the incubation start. In similarity to viability assay, the effective concentration of COL-3 was 1/10 of DOXY and MINO. DMSO did not induce apoptosis compared to controls.
Figure 20. TCNA-induced apoptosis in HL-60 cells. May-Grünwald-Giemsa staining was used for assessment of apoptotic morphology. Apoptosis is expressed as a percentage of 400 cells counted per slide. Results are presented as a mean ± SD of three separate experiments.

4.3.1.1 Mitochondrial membrane potential

Loss of $\Delta \Psi_m$ was observed at 2 h of incubation with COL-3 in concentrations of 2.5 and 5 µg/ml and DOXY 50 µg/ml and at 4 h of incubation with MINO in a concentration of 50 µg/ml. The decrease in $\Delta \Psi_m$ progressed in a concentration- and time-dependent manner. Complete loss of $\Delta \Psi_m$ was observed at 24 h incubation with the COL-3 in concentration of 5 µg/ml and DOXY and MINO in concentrations of 50 µg/ml, respectively (Figure 21). DMSO did not affect $\Delta \Psi_m$ compared to controls incubated with complete medium.

Figure 21. The effect of TCNAs on mitochondrial membrane potential ($\Delta \Psi_m$). HL-60 cells were incubated with doxycycline (D) or minocycline (M) in final concentrations of 25 and 50 µg/ml or COL-3 (C) in final concentrations of 2.5 and 5 µg/ml for 2, 4, 6 and 24 h. Controls were incubated with complete medium and cells incubated with DMSO in a final concentration of 0.2% served as a control for solvent toxicity. Mitochondrial membrane potential was assessed using tetramethylrhodamine methyl ester and flow cytometry. Results are expressed as mean ± SD of three independent experiments.

4.3.2 Pathways of apoptosis

Activation of caspase-2, -3, -7, -8 and -9 was observed after treatment with all three drugs (Figure 22). However, the pattern of appearance of caspase fragments differed among the drugs. A 17 kDa fragment of caspase-3 was observed at 6 h incubation with DOXY, MINO and COL-3, but in DOXY and COL-3 an intermediate fragment was detected that was not observed after incubation with MINO. 32 and 20 kDa fragments of caspase-7 were detected, similarly, at 6 h incubation with DOXY and MINO, however, only a 20 kDa fragment was observed after the treatment with COL-3. The caspase-2 antibody detected long (48 kDa) and short (35 kDa) forms, and 25 kDa fragments were detected following treatment with all three drugs, but no 15 kDa fragment was observed. Cleaved 85 kDa PARP and 23 kDa Bcl-2 were observed, but only intact actin was detected in all three treatments.
Figure 22. Proteolytic events during TCNAs-induced apoptosis. HL-60 cells were incubated with doxycycline or minocycline in final concentrations of 2.5, 10 and 25 μg/ml or COL-3 in final concentrations of 0.75, 1 and 5 μg/ml for 6 and 24 h. Cells incubated with complete medium and cells incubated with DMSO in a final concentration of 0.2% served as controls. The appropriate protein bands are indicated with arrows. (a) Doxycycline; (b) Minocycline; and (c) COL-3.

4.3.3 Effect of Z-VAD-FMK on TCNAs-induced apoptosis

Viability of cells pretreated with Z-VAD-FMK was higher compared to cells treated with TCNAs alone. Pretreatment with Z-VAD-FMK also reduced TCNA-induced apoptosis by 76% in DOXY, 69% in MINO, and 50% in COL-3 at 24 h of incubation (Figure 23). No cytotoxic effect of 0.2% DMSO and Z-VAD-FMK in a concentration of 100 μmol/L compared to controls was observed.
Figure 23. The effect of Z-VAD-FMK on TCNAs-induced apoptosis. HL-60 cells were treated with Z-VAD-FMK in a final concentration of 100 μmol/L for 1 h prior to treatment with TCNAs for 6 h and 24 h. Viability (A) and apoptosis (B) were assessed using resazurin assay and morphological criteria in Giemsa staining, respectively. Toxicity of solvent DMSO in final concentration of 0.2% and pancaspase inhibitor Z-VAD-FMK was assessed in both assays. DMSO and Z-VAD-FMK exerted no cytotoxic effect on HL-60 cells compared to control incubated with complete medium (data not shown on the graph). Results are presented as a mean ± SD of three separate experiments. D: DOXY 25μg/ml; M25: MINO 25μg/ml; Z: Z-VAD-FMK 100μmol/L; C: COL-3 5μg/ml in viability experiments (a) and 2.5μg/ml apoptosis experiments (b).
5 DISCUSSION

Leukemia is a heterogeneous group of blood malignancies. The treatment and patients’ survival have improved during the last decades due to improved diagnostic methods, better knowledge on risk factors, development of new drugs such as monoclonal antibodies and tyrosine kinase and proteasome inhibitors and finally yet importantly improved supportive therapy. However, the treatment results are still not satisfactory due to resistant disease, relapse and/or side effects of the treatment schedules. Advances in molecular and cellular biology improved the understanding of the mechanisms involved in leukemia. Several molecules that play a critical role in cell cycle, signal transduction and apoptosis have been identified and recognized as potential therapeutic targets. Thus, new treatment strategies aiming on higher efficacy and decreased adverse effects are investigated.

In the present thesis, pharmacokinetics and cytotoxic effects of CDK-inhibitor roscovitine and metalloproteinase inhibitors from tetracycline analogue group have been investigated.

The cytotoxic effect of roscovitine was studied in HL-60, Jurkat and K562 cell lines. These cell lines represent leukemias of different origin, myeloid, lymphoblastic and CML, respectively. Cytotoxic effect of roscovitine was concentration-dependent with the IC50 of 17 µmol/L, 24 µmol/L and 47 µmol/L in HL-60, Jurkat and K562 cells, respectively. The average IC50 of 15 µmol/L (range 5-36 µmol/L) was reported in cancer cell lines (Lacrima et al., 2007; MacCallum et al., 2005; McClue et al., 2002; Mohapatra et al., 2005; Raje et al., 2005; Raynaud et al., 2005). Thus, the IC50 observed in HL-60 and Jurkat cells were similar to published data, but the IC50 of K562 cells was higher. In primary B-CLL cells, roscovitine in concentrations of 10 and 20 µmol/L decreased the cell viability at 24 h of incubation and almost all cells died at concentrations of 50 µmol/L. Only minor effect of roscovitine in concentrations of 20 µmol/L was observed in normal peripheral blood mononuclear cells, but profound induction of cell death was induced with the highest concentration of roscovitine (Hahtow et al., 2004). Thus, roscovitine has cytotoxic effect on both cancer and normal blood cells.

Since myelosuppression is frequent complication to chemotherapy, we studied the effect of roscovitine on crude bone marrow and on hematopoietic progenitors. We have found that incubation with roscovitine in concentration of 250 µmol/L for 4 h decreased the viability of crude bone marrow cells to 70%. Since majority of crude bone marrow cells consists of myeloid cells at different stage of maturation, these results are in agreement with study by Rossi et al who reported that roscovitine induced apoptosis in mature blood neutrophils (Rossi et al., 2006). Further, we addressed the effect of roscovitine on primary hematopoietic progenitors studied as the clonogenic capacity of bone marrow cells. Incubation with roscovitine up to 250 µmol/L for 4 hours did not affect the clonogenic ability of hematopoietic progenitors. This might be due to that 4 h incubation was too short to exert the effect of roscovitine. Since longer incubation decreased the clonogenic capacity of the controls probably due to lack of relevant growth factors in media supplemented with FBS, we could not exclude the additive effect of roscovitine and growth factors deprivation in this experiment setting. Therefore, we examined the colony formation after addition of roscovitine to semisolid MethoCult media containing recombinant growth factors. Decrease in colony growth was cell type- and concentration-dependent. BFU-E colonies were more sensitive than
CFU-GM, but the most sensitive were CFU-GEMM. The higher sensitivity of erythroid progenitors has been observed for other cytotoxic drugs and xenobiotics compared to granulocyte-macrophage progenitors (Corazza et al., 2004; M alerba et al., 2002). To be able to compare our results from the primary hematopoietic progenitors to our and published data in cell lines, we estimated AUCs. Indeed, when reported $IC_{50}$ and incubation times are recalculated to AUCs, the exposure to roscovitine that inhibits hematopoietic progenitors is within the same range as exposure that inhibits cancer cells. This is an important finding since effective cancer treatment with roscovitine may result in myelosuppression. An interesting observation in in vitro experiment was that the time of exposure to roscovitine was an important factor that affected roscovitine-induced inhibition of hematopoietic progenitors. Lower roscovitine concentration with longer incubation time resulted in higher inhibition of colony formation compared to higher concentration of roscovitine with shorter incubation time despite that both concentration and time combinations gave the same AUC. This differs from other cytostatics, in which the linear relationship between the AUC and cytotoxicity independent of incubation times has been observed (Hassan et al., 2002; Hassan et al., 2001).

To address the myelosuppressive effect of roscovitine in vivo, we investigated the effect of roscovitine on hematopoietic progenitors in mouse model. Single dose of roscovitine up to 250 mg/kg did no affect the colony formation of bone marrow cells. This was unexpected since the total dose of 250 mg/kg administered i.p. has been reported to reduce tumor size by 85% compared to controls in xenograft model of Ewing’s sarcoma (Tirado et al., 2005). Then we studied effect of repeated doses of roscovitine twice daily for four consecutive days. Roscovitine in a dose of 350 mg/kg/day divided in two daily doses and administered i.p. for 4 consecutive days induced only transient decrease in BFU-E at day 1, but no decrease was observed 5 days after the last dose of roscovitine. CFU-GM and CFU-GEMM were not affected by the treatment. This difference between in vitro and in vivo may be explained by low distribution of roscovitine to bone marrow. Only 1.5% of administered roscovitine reached bone marrow when AUCs for plasma and bone marrow were compared. A another possibility is short elimination half life of roscovitine (Benson et al., 2007; de la Motte and Gianella-Borradori, 2004; Raynaud et al., 2005; Vita et al., 2005).

The $IC_{50}$ reported in cancer cell lines in vitro may be reached in vivo in plasma in mouse model (Raynaud et al., 2005). Exposure of cancer cells to the drug is an important factor in cancer therapy. The drug has to reach the target organ to be able to carry out the effect. It may be difficult to reach and maintain the adequate exposure of cancer cells in some target organs such as CNS. Therefore, we decided to study the distribution of roscovitine to brain in 14 days old rat pups and adult rats. We have found that exposure of brain to roscovitine expressed as AUC was similar to plasma exposure in rat pups, but the exposure of adult rat brain was only 25% of that found in plasma. The high distribution in pups brain may be due to the age-dependent variation in the maturity and function of blood-brain barrier (Butt et al., 1990) or due to age-dependent differences in pharmacokinetics of roscovitine. The most striking difference between rat pups and adult rats was found in elimination half-lives. In rat pups, $t_{1/2}$ was 7 h in both brain and plasma compared to 20 and 30 min in brain and plasma, respectively, in adult rats. Elimination half-life of roscovitine was reported in several species <30 min in rat, about 1 h in mice and 2-9 h in man (Benson et al., 2007; de la Motte and Gianella-Borradori, 2004; Raynaud et al., 2005; Vita et al., 2005). While no
significant difference in clearance between brain and plasma was observed in rat pups, clearance found in the adult brain was 5-fold higher compared to that found in plasma. Roscovitine has been shown to be metabolized through cytochrome P450 (McCue and Stuart, 2008). Thus, differences in elimination half-life of roscovitine between the 14-day pups and adult rats may be explained by immature CY P450 enzymes at the age of 2 weeks in rats (Rich and Boobis, 1997).

Roscovitine concentrations achieved in the brain and plasma of pups were >9.9 µmol/L and sustained up to 8 h. The estimated AUC of roscovitine in plasma and brain of rat pups was about 200 µmol/L.h. This exposure is similar to exposure required for induction of cytotoxic effects in cancer cell lines in vitro. These results may be implicated in the treatment of brain tumors or CNS leukemia in pediatric patients. But what would the effect of roscovitine on developing brain be? We addressed this question by experiments on the effect of roscovitine on CDK 5 and Erk1/2 in rat pups. CDK 5 plays an important role in brain development in young rats with peak expression and activity at day 14 postnatally (Wu et al., 2000).

Roscovitine is a potent inhibitor of CDK 5 (Bach et al., 2005). Roscovitine-induced inhibition of CDK 5 has been shown to result in activation of Erk1/2 in neuronal cells (Zheng et al., 2007). Single dose of roscovitine administered in this study resulted in transient inhibition of CDK 5 at 1-2 h after administration. Despite the fact that the concentrations of roscovitine found in brain at 6 h after the administration of roscovitine should be enough to sustain the inhibitory effect on CDK 5 (extrapolated from in vitro studies), the activity of CDK 5 was recovered at this time point. Possible explanations include the higher intraneuronal concentrations of ATP in vivo compared to those in vitro, drug internalization and high turnover of CDK 5 resulting in newly synthesized kinase free from roscovitine. Roscovitine-induced inhibition of CDK 5 has been shown to result in activation of Erk1/2 in neuronal cells (Zheng et al., 2007). The activation of Erk1/2 observed in this study was transient and return to normal levels. The effect of repeated doses of roscovitine on the development of the brain in rat pups remains to be investigated.

Despite the promising results from preclinical studies on roscovitine, the pharmacokinetic data in man may point some difficulties in further implementation. In healthy volunteers, the Cmax reached after single oral dose of roscovitine (800 mg) was only 3.4 µmol/L (de la Motte and Gianella-Borradori, 2004). This is several fold lower than average IC_{50} in cancer cell lines. In another study, clinical phase I trial, roscovitine was administered twice daily for 7 consecutive days to patients suffering from malignant solid tumors (Benson et al., 2007). The dose of 800 mg twice daily resulted in the Cmax about 10 µmol/L, thus approaching the IC_{50} of cancer cell lines. However, these concentrations did not sustain over the period required for antitumor effect in vitro and a wide variation in pharmacokinetic parameters was observed among the patients. Benson et al also reported side effect in relation to increased doses of roscovitine (Benson et al., 2007). The pharmacokinetic data have shown that there is a risk for too low exposure to roscovitine in man. To overcome these problems, a new formulation of roscovitine may increase the elimination half-life or combination with other drugs may potentiate the effect as reported in in vitro studies (Yu et al., 2003b).

Cytotoxic effect of roscovitine on leukemic cell lines resulted in cell death which had the morphological changes typical for apoptosis. Roscovitine induced apoptosis in concentration- and time-dependent manner. In K 562, the apoptosis was preceded by G2/M arrest, but no phase arrest was observed HL -60 or Jurkat cells. The results in HL-
60 are contradictory to recently published study in which HL-60 cells were arrested at G1 (Komina and Wesierska-Gadek, 2008). In several other studies the cell cycle arrest was observed either at G1 or G2/M check points (Meijer et al., 1997; Wesierska-Gadek et al., 2007).

Pathway of roscovitine-induced apoptosis was caspase-dependent and mitochondria mediated, however, the pattern of activation of caspases differed among the studied cell lines. Active fragments of caspase-2 and caspase-8 were found concomitantly in HL-60 cells, while the cleavage of caspase-8 preceded the cleavage of caspase-2 in Jurkat cells and no active fragments of either of these caspases was detected in K 562 cells. Caspase-2 has been implicated as an inducer caspase in DNA damage, and high levels of pro-caspase-2 were associated with very poor survival in AML (Estrov et al., 1998). The mechanism of caspase-2-induced apoptosis remains unknown (Guo et al., 2002). In both HL-60 and Jurkat cells, tBid was detected by Western blot analysis after exposure to roscovitine. It has been suggested that caspase-2, -8, and granzyme-B cleave Bid at different site of Ap in DNA damage pathway (Abal et al., 2004). Cleaved caspase-3 has been found in all three cell lines. Active caspse-3 contributes, as an effector caspase, to the morphological and functional changes in apoptosis, and is also responsible for the inactivation of the PARP processed during the later stages of apoptosis (Rodriguez-Hernandez et al., 2006).

We investigated the effect of matrix metalloproteinases inhibitors TCNAs DOXY, MINO and COL-3 on leukemic cell line HL-60. Matrix metalloproteinases are involved in degradation of extracellular matrix and basement membrane and play an important role in development of solid tumors and hematological malignancies including acute myeloid leukemia (Janowska-Wieczorek et al., 1999; Lin et al., 2002; Turpeenniemi-Hujanen, 2005).

A concentration-dependent cytotoxic effect was observed after incubation with TCNAs. COL-3 displayed the strongest effect among them as expressed as IC_{50}. Compared to other cancer cell lines tested in vitro, HL-60 cells were more sensitive to COL-3 and in the low range of sensitivity to DOXY (IC_{50} of COL-3 3-10 µg/ml and DOXY 10-20 µg/ml, respectively) (Lokeshwar et al., 2002; Onoda et al., 2004). Our results for DOXY are in agreement with the study by Tolemeo et al, but the IC_{50} of COL-3 is lower compared to their study (Tolomeo et al., 2001). The difference might be explained by different assays used in the studies (viability of the cell mass versus the viability of individual cells).

TCNAs have been reported to induce apoptosis in various cancer cells, however, the reported biochemical events involved differ (Liu et al., 1999; Mouratidis et al., 2007; Onoda et al., 2006; van den Bogert et al., 1985). The mitochondrion generates energy for a cell and also controls cell death by releasing death promoting factors into the cytosol. The death decision is centered on two processes: the inner membrane is promoted by the permeability transition pore (PTP), while Bid, BAX, and BAK play active roles in outer membrane permeabilization (Belizario et al., 2007). In our study, COL-3 5 µg/ml and DOXY 50 µg/ml induced significant loss of ΔΨm at 2 h, but this change appeared in MINO 50 µg/ml treatment after 4 h. Bcl-2 was cleaved at 6 h in COL-3 treatment, but this event was delayed in DOXY and MINO. These results may indicate that COL-3 changed the mitochondrial PTP on the inner membrane, thereby induced the collapse of ΔΨm, while putative pores on the outer mitochondrial membrane were formed after cleavage of Bcl-2. DOXY and MINO mainly targeted the PTP. The caspase cascade was activated by all three drugs, however, some
differences in the pattern were observed. We have been using polyclonal antibodies which can detect both pro-form and cleaved fragments of caspases. We have observed an intermediate of caspase-3 in DOXY- and COL-3-induced apoptosis, but this intermediate was not observed in MINO-induced apoptosis. Caspase-7 fragment of 32 kDa was observed in both DOXY- and MINO-induced apoptosis, but not in COL-3 treated cells. Moreover, long and short fragments of caspase-2 were detected and fragments of 25 kDa, but a 15 kDa fragment was absent. The origin of 25 kDa fragment and its role in apoptosis remain unclear. No actin cleavage was observed in any of the treatments. Cytoxic effects of all three TCNA s were reduced after preincubation with pancaspase inhibitor Z-VAD-FMK confirming that apoptosis induced by TCNA s was caspase-dependent.
6 CONCLUSIONS

In the present thesis, we have studied pharmacokinetics and cytotoxic effects of CDK-inhibitor roscovitine and metalloproteinase inhibitors from tetracycline analogue group in animal models in vivo and in leukemic cell lines and hematopoietic progenitors in vitro.

I In mouse model, roscovitine affected the clonogenic capacity of normal bone marrow which may be due to low distribution to bone marrow and short half-life of roscovitine in vivo. In vitro, roscovitine decreased the clonogenic capacity of hematopoietic progenitors. Cytotoxicity of roscovitine found in hematopoietic progenitors was obtained after similar exposure required for the cytotoxic effect in cancer cell lines.

II Roscovitine induced apoptosis through mitochondrial injury and caspase activation in human leukemia cells. The kinetics of the events differs depending on the cell type.

III Age-dependent pharmacokinetics of roscovitine was found in rat. The brain exposure to roscovitine was age-dependent. Higher brain exposure to roscovitine was found in rat pups compared to adult rats. Transient inhibition of CDK5 and activation of Erk1/2 was found in three different brain regions. Thus, roscovitine may have treatment potential in pediatric CNS leukemia and brain tumors.

IV TCNAs induced apoptosis in a mitochondria-mediated and caspase-dependent way in myeloid HL-60 cells. COL-3 had the strongest anti-proliferative and proapoptotic effect among tested TCNAs. Thus, TCNAs may have potential in the treatment of acute myeloid leukemia.
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