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REGULATION OF ONGOING DNA SYNTHESIS IN NORMAL AND NEOPLASTIC BRAIN TISSUE

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“Nothing shocks me. I'm a scientist”

(Indiana Jones)

To my parents

Summary

The treatment of human brain tumour is challenging in part due to the blood brain barrier and in part due to the specific biology of brain tumours that confer resistance to chemotherapy. For instance, the 5 years survival rate for patients carrying intracranial glioblastoma multiforme has remained at 4-5 % for the last 30 years. The knowledge of the brain tumour biology as well as the biology of the normal brain tissue would help to design new therapeutic strategies and to develop new and less toxic antineoplastic drugs for brain tumour treatment. Normal tissue must be studied in order to identify tumour-specific vulnerabilities and ways to inhibit toxicity in the host.

The present thesis describes a series of investigations of potential antineoplastic drugs performed in normal rat cerebral cortex, human brain tumour specimens and RG2 gliomas, performed "in vitro" in order to 1) better understand factors controlling the cell cycle and DNA replication in normal and neoplastic brain tissue, and thus, exploiting potential targets for new drugs 2) better apply the available antineoplastic drugs for the treatment of human brain tumours while producing no or low side effect on normal tissue.

A novel assay, which preserves the metabolic and proliferative properties of the tissue was developed and used to study ongoing DNA synthesis and its regulation by protein phosphorylation and proteolysis. The effect of low MW drugs (protein kinase and protease inhibitors) on these processes was evaluated. By analyzing the effects of different chemically unrelated inhibitors of protein kinases we found that many of these inhibitors might act through long term mechanism of action (e.g. inhibiting cell cycle transitions) rather than a direct effect on the DNA replication machinery, although some of these drugs are currently used as "DNA synthesis inhibitors". We suggest that, from the clinical point of view, it would be important to distinguish between these long and short-term mechanism of action. Our results also suggest that different sets of protein kinases and proteases yet not clearly identified regulate "ongoing DNA replication".

A more detailed study was carried out using roscovitine, a highly specific cyclin-dependent kinase inhibitor. The effect of roscovitine on DNA synthesis was evaluated in normal rat cerebral cortex, specimens obtained from human brain tumours and in a pilot experiment using a rat glioma model. We found that roscovitine is a potent inhibitor of ongoing DNA synthesis in the developing rat cerebral cortex as well as in human gliomas but showed little or no effect in adult normal tissue. Moreover, roscovitine inhibited preferentially DNA synthesis connected with replicative processes rather than DNA synthesis connected with DNA repair.

In addition, some *in vitro* studies of redox regulation of topoisomerases and the effects of thiol reacting drugs on this enzyme are presented.

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ORIGINAL PAPERS (I-VI)

This thesis is based in the following papers, which will be referred to in the text by their Roman numerals.

I. **Yakisich JS**, Sidén Å, Idoyaga Vargas V, Eneroth P, Cruz M. Fast and sensitive method for simultaneous measurement of cell proliferation rate and drug sensitivity in rat cerebral cortex. Exp Neurol. 1998 Jun;151(2):194-202

II. **Yakisich JS**, Sidén Å, Idoyaga Vargas V, Eneroth P, Cruz M. Early inhibition of DNA synthesis in the developing rat cerebral cortex by the purine analogues olomoucine and roscovitine. Biochem Biophys Res Commun. 1998 Feb 24;243(3):674-7.

III. **Yakisich JS**, Sidén Å, Vargas VI, Eneroth P, Cruz M. Early effects of protein kinase modulators on DNA synthesis in rat cerebral cortex. Exp Neurol. 1999 Sep;159(1):164-76

IV. **Yakisich JS**, Boethius J, Lindblom IO, Wallstedt L, Vargas VI, Sidén Å, Cruz MH. Inhibition of DNA synthesis in human gliomas by roscovitine. Neuroreport. 1999 Aug 20;10(12): 2563-7.

V. **Yakisich JS**, Sidén Å, Eneroth P, Cruz M. Disulfiram is a potent in vitro inhibitor of DNA topoisomerases. Biochem Biophys Res Commun. 2001 Nov 30;289(2):586-90.

VI. **Yakisich, JS**, Sidén, Å, Boethius, J.,Tasat, D.R., Hofer, A.,Idoyaga Vargas, V., Eneroth, P.,Cruz, M. Preferential inhibition of replicative DNA synthesis in the developing rat cerebral cortex by the purine analogue Roscovitine *(Manuscript)*

CONTENTS

ABBREVIATIONS

BBB	Blood-Brain Barrier
BSA	Bovine Sera Albumin
DNA	Deoxyribonucleic Acid
Ca I I	Calpain Inhibitor I (MG-101; ALLN; N-Ac-Leu-Leu-norleucinal)
Ca I II	Calpain Inhibitor II (ALLM; N-Ac-Leu-Leu-methioninal; LLM)
CDK	Cyclin-Dependent Kinase
CDKI	Cyclin-Dependent Kinase Inhibitor
CLC	Clasto-Lastacystin-β-Llactone
CNS	Central Nervous System
DMEM	Dulbecco's Modified Eagle´s Medium
EDTA	Ethylenediaminetetraacetic acid
LC	Lactacystin
MEM	Minimal Essential Media
MG-132	Cbz-leucynil-leucynil-leucinal (Cbz-LLn-Val; Z-Leu-Leu-Leu-CHO; N-carbobenzoxil-L-Leucynil-L-leucynil-L-Leucinal; LLL)
PBS	Phosphate-Buffered Saline
PK	Protein Kinase
PP	Protein Phosphatase
PMSF	Phenylmethylsulfonilfluoride
PSI	Proteasome Inhibitor I (Cbz-ile-Glu-(O-t-Bu)-Ala-Leucinal; Z-Ile-Glu-(OtBu)-Ala-Leu-CHO)
TCA	Trichloroacetic Acid
TLCK	Nα-p-Tosyl-L-Lysine Chloro-methyl Ketone
TPCK	N-Tosyl-L-Phenylalanine Chloromethyl Ketone

INTRODUCTION

Gliomas

Gliomas are the most common primary neoplasms of the central nervous system (CNS), comprising over 50% of all such tumours in adults (Greenle *et al*, 2000; Gupta and Sarin, 2002). Pathophysiologic and clinical properties of gliomas include infiltrative growth, induction of vasogenic edema, local compression and destruction of brain tissue, elevation of intracranial pressure, obstruction of cerebrospinal fluid flow, venous occlusion and hemorrhage. Damaged brain tissue often causes focal seizures by irritation or neurologic deficits by depression of CNS functions.

The more malignant forms (high grade) of these tumours have a very poor prognosis with a median survival of 12–24 months for patients classified as having a favorable prognosis (younger or good performance status) or 6–9 months for those having a poor prognosis (older or with poor performance status) (Gupta and Sarin, 2002). The treatment of this pathology is challenging in part due to the blood-brain barrier (BBB) and in part due to the specific biology of brain tumours that confer resistance to chemotherapy (Bredel, 2001; Bredel and Zentner 2002). The knowledge of the brain tumour biology as well as the biology of the normal brain tissue would help to design new therapeutic strategies and to develop new and less toxic antineoplastic drugs for brain tumour treatment.

Standard therapy usually consists of surgery followed by radiotherapy and possibly, adjuvant chemotherapy (Chamberlain and Kormanik, 1998; Fine, 1994; MacLendon and Halperin, 2003; Levin *et al*, 1999; Shapiro, 1986). After diagnosis, the median survival time of patients with highly malignant glioma is less than 6 months with surgery alone. Radiotherapy can increase this period by some months, and additional chemotherapy gives some effect (Stewart, 2002) but does not lead to a substantial improvement of this dismal prognosis. The median survival time is 3-5 months after surgery and supportive care. Postoperative conventional radiotherapy increases survival time to 9-12 months for glioblastoma multiforme (GBM) and to 36 months for anaplastic astrocytoma (Leibel *et al*, 1994; Walker *et al*, 1979; Walker *et al*, 1980). These figures have not substantially changed during the past two decades,

even when treatement with the most aggressive regimens of surgery, radiation, and chemotherapy were applied (Collins, 1995; Davis *et al*, 1998; Sasaki and Plate, 1998; Sheline, 1990). For instance, the 5 years survival rate for patients carrying intracranial glioblastoma multiforme has remained at 4-5 % for the last 30 year. Furthermore, this poor survival rate seems to be an overestimation since three studies, after careful histological review, determined that the survival rate was around 2 % (McLendon and Halperin, 2003).

Malignant gliomas do not usually metastasize outside the brain (Bellail *et al*, 2004). This feature may have crucial implications for some novel therapeutic strategies. On the other hand, gliomas are highly proliferative and diffusely infiltrative into regions of normal brain (Jung *et al*, 1998; Aboody *et al*, 2000; Bellail *et al*, 2004). Individual neoplastic cells migrate away from the primary brain tumor mass, along white matter pathways (Silbergeld and Chicoine, 1997). These cells are responsible for the recurrent tumor growth near the borders of the resection cavity (Aboody *et al*, 2000). Even if total surgical resection of these tumors is preformed, the tumor generally recurs within one year (Kanzawa *et al*, 2004). Approximately 80-90 % of gliomas recur within two cm of the original tumour indicating that improved local control is needed (Hochberg and Pruitt, 1980; Guerin *et al*, 2004). Failure of chemotherapy has been attributed to many factors including tumour-cell resistance, tumor heterogeneity and the existence of the BBB. Resistance to chemotherapy is complex and may involve multiple mechanisms such as genomic aberrations, deregulation of membrane transporting proteins and cellular enzymes, and an altered susceptibility to apoptosis (For review see, Bredel, 2001; Bredel and Zentner, 2002). Malignant gliomas show considerable heterogeneity even within a single tumor. Indeed, extensive karyotypic heterogeneity has been reported to exist within one tumor (Coons *et al*, 1995). Glioblastoma multiforme, the most malignant form of gliomas is thought to arise from astrocytes or astrocyte precursors but its heterogeneity in morphology and behavior makes difficult to clearly establish its origin (For review see Wechsler-Reya and Scott, 2001). Another of the major problems for drug treatment of brain tumors is the poor tissue penetration mainly due to the existence of the BBB. Although the role of the BBB as an obstacle to chemotherapy efficiency is controversial because it is commonly disrupted in malignant gliomas (Leggett *et al*, 1999; Risau *et al*, 1998; Stupp *et al*, 2001), most systemically delivered drugs do not adequately penetrate brain tumor tissue due to

residual BBB function. Those who do penetrate require high doses that result in systemic toxicity (Guerin *et al*, 2004). Therefore, novel therapies for malignant gliomas need to be actively investigated. Moreover, since these tumours are at present considered to be incurable, treatments that improve the neurological deficits and/or increase survival maintaining the best possible quality of life of the patient are important. As Rosemblum has pointed out, the normal tissue must be studied in order to identify tumour-specific vulnerabilities and ways to inhibit toxicity in the host (Rosemblum *et al*, 1989).

There are striking parallels between normal development and cancer: oncogenes and cancer suppressors play key roles in cell growth and differentiation during development and proteins originally described as regulators of pattern formation in invertebrates and vertebrates have been implicated in a variety of human cancers (For review see Wechsler-Reya and Scott, 2001). Insights into the cellular and molecular mechanisms that regulate normal brain development could lead to novel therapeutic strategies to inhibit malignant glial growth (Stevens and Fields, 2002). Genetic and epigenetic mechanisms underlying glioma neoplasia are continuously elucidated but this success has not been associated with parallel improvements in the treatment of malignant brain tumors. As stated by Noble and Dietrich, "One possible explanation for this failure is that the most important variables that support growth of malignancies are not yet identified. Another possible explanation is, however, that multiple variables important in neoplastic progression combine to create a level of disease complexity not taken into account by current therapeutic approaches. The study of development and neoplasia in the CNS provides some of the strongest support for the latter view, a view that if correct, would suggest that a radical rethinking of the biology of malignancy is required if we are to make progress in the treatment of this important medical condition" (Noble and Dietrich, 2004).

The cell cycle

In eukaryotes, the cell cycle is divided into two phases, interphase and mitosis. Interphase consists of G_1, S and G_2 phases. Mitosis can be sub-divided into prophase, metaphase, anaphase and telophase. Chromosomes condense during prophase, align during metaphase, separate during anaphase and decondense during telophase. In

mammalians, cell division is timely regulated by a family of protein kinase holoenzymes, the cyclin-dependent kinases (CDKs) and their heterodimeric cyclin partners. CDKs are serine/threonine kinases that become active only when associated with a regulatory partner (e.g. cyclins or other proteins). CDK/cyclin holoenzymes are activated by phosphorylation, which is catalyzed by CDK-activator kinase (CAK). The activity of CDKs is negatively regulated by direct interactions with proteins referred to as CDK inhibitors. Regulation of CDK activity occurs at multiple levels, including cyclin synthesis and degradation, phospho- and dephosphorylation, CDK inhibitor (CKI) protein synthesis, binding and degradation, and subcellular localisation (Pines, 1995; Harper, 1997; Cerutti and Simanis, 2000). Sequential turnover of certain cell cycle regulators, are mediated by the 20S proteasome, which promotes proteolytic degradation through the ubiquitin/proteasome pathway.

There are several control points during the cell cycle (Figure 1): in late G_1, called Start in yeast or the restriction point in mammals, in late G_2 and just prior to anaphase. To pass each point, cells have to fulfill several prerequisites. Before passing Start, cells can go through two developmental programs, i.e. entry into the mitotic cell cycle or undergo sexual development. Adequate nutritional conditions and a critical cell size are required to traverse Start. During G_2, cells have to check whether DNA replication is completed and ensure that DNA is not damaged (Ohi and Gould, 1999). Before chromosome separation cells also examine whether chromosomes are aligned and spindles are formed properly. These cell-cycle check-points are the mechanisms that govern the order of the cell-cycle events, because if the order of the events is incorrect then a full complement of genetic information is not transmitted at cell division, which may lead to cancer in higher eukaryotes (Murakami and Nurse, 2000). Thus, check-points are essential surveillance mechanisms that ensure proper cell-cycle progression (Nurse, 1997). A first checkpoint (the Restriction Point) at late G_1 integrates both positive and negative external and internal signals before the cell commits itself to replication. Before the restriction point cells are sensitive to extracellular stimuli (growth factors, cytokines). But after passing the restriction point cell become independent of growth factors, cell density and cell size.

Escape of cells from the exact regulation of cell cycle progression leads to malignant transformation (Nurse, 2002). These transformed cells, in contrast to their normal counterparts, proliferate rapidly (Blagoskonny and Pardee, 2001).

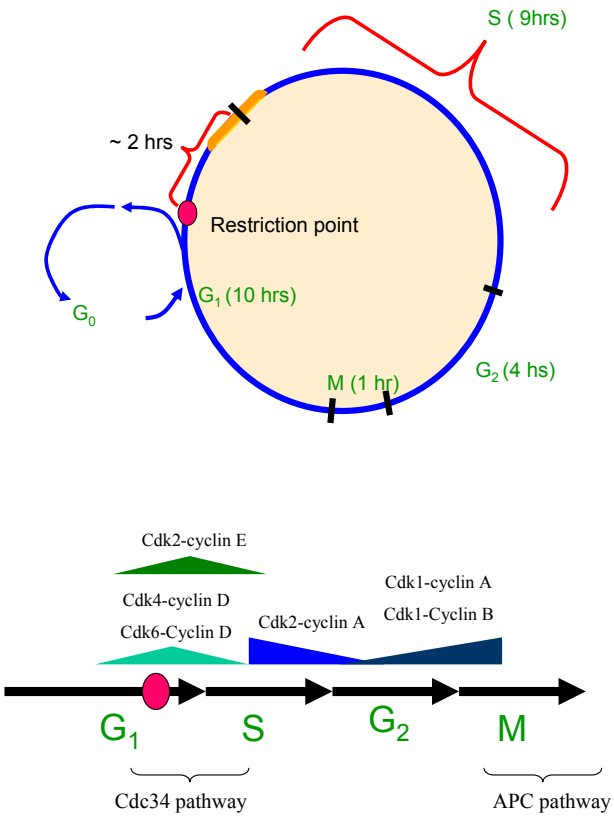

Figure 1. Top: A typical mammalian cell cycle. In virtually all cells, the cell cycle is composed of four discrete phases, being the DNA synthesis phase (S phase), the cell division phase (M phase) and the gap phases between these two: the G_1-phase between M and S phases and the G_2-phase between S and M phases. As long as growth factors are present, adherent cells will continue to proliferate. In the absence of growth factors, cells will stop dividing and enter the quiescent state (G_0). Bottom: The transition between the different phases is regulated by cyclin/cdk complexes. Different cyclins (**A**, **B**, **D**, and **E**) are present during different cell cycle phases and interact with different CDKs. Two proteolytic pathways drive the cycle in one direction because of the irreversibility of protein degradation. The Cdc34 pathway degrades S-phase CdkC inhibitor allowing entry into S phase, whereas the APC pathway regulates chromosome segregation and mitotic exit by degradation of anaphase inhibitor and mitotic CdkC cyclin subunit.

The vast majority of human neoplasias have abnormalities in one or more of its cell cycle components. Indeed, dysregulation of molecules controlling the cell cycle have been observed in more than 80% of human cancers (Dai and Grant, 2003). Although it appears that the oncogenic defects may target any major transition or

checkpoint of the cell cycle, the step strikingly deregulated most frequently is the G_1/S transition (Carnero, 2002). In particular, mutations that result in disruption of cell cycle arrest pathways are found in more than 90% of high-grade human gliomas. The most common mutation that achieves disruption of the cell cycle arrest pathways is deletion of the INK4A- ARF locus that occurs in approximately 60% of high-grade gliomas (Fulci *et al*, 2000)

The G_1/S transition and the S phase

DNA replication is one of the ultimate targets for the regulatory pathways that control cell proliferation and cell cycle progression. The onset of DNA replication at the G_1/S transition may be initiated by the synthesis of a subset of the replication machinery, by the post-translational activation of one or more pre-existing replication factors or by a change in their intracellular localization (for review, see Din *et al*, 1997). Essential DNA replication proteins are not present in cells being in the G_0 state. During the G_0/G_1 transition, however, a cascade of signal transduction events results in the synthesis of replication proteins. The transition from G_1 to S phase is modulated by an increase in the expression of cyclins D and E and their catalytic subunits cdk2, cdk4 and cdk6. In proliferating cells, the proteins directly involved in DNA replication that have been examined hitherto, are present at all stages of the cell cycle (for review, see Din *et al*, 1997). These differences between resting cells and proliferating cells make the latter potential targets for cytostatic treatment modalities (or side-effects from such treatment) as well as for teratogenic and carcinogenic factors.

Protein phosphorylation via protein kinase activities and proteolysis via proteolytic pathways have key functions in the control of cell cycle-related events (for review, see King *et al*, 1996; Wuarin and Nurse, 1996; Yew and Kirschner, 1997). Dysregulation of protein kinase and protease activities have been reported in almost all cancers. Many different protein kinases activities have been reported to be elevated in human brain tumors suggesting that protein kinase inhibitors are potential candidates for anticancer treatment. For this reason, protein kinase inhibitors and protease inhibitors have been the focus of extensive research in cancer therapy (Dai and Grant, 2003; Drexler, 1997; Oikawa *et al*, 1998; Senderowicz, 1999; Senderowicz and Sausville, 2000).

DNA synthesis: DNA replication versus DNA repair and the concept of "ongoing DNA replication"

DNA synthesis in cells is required for DNA replication, known as scheduled DNA replication or replicative DNA synthesis (RDS), and for repair mechanisms usually measured as unscheduled DNA synthesis (UDS) (Schmitz *et al*, 1999) by different methods (autoradiography, immunoenzymatic staining of 5-bromo-2'-deoxuridine and liquid scintillation counting) with similar sensitivity (Sawada *et al*, 1995). UDS corresponds to DNA damage and excision repair as it has been demonstrated in a variety of tissues and cell lines (Heyting and van't Veer, 1981; Painter ad Cleaver, 1969). The use of cultures in which DNA synthesis is blocked by hydroxyurea (HU) - which inhibits replication but not repair - together with cultures that are not blocked allows an estimation of the effect of chemicals on both types of DNA synthesis (Agrelo and Severin, 1981).

RDS is restricted to the S phase of the cell cycle (Laskey *et al*, 1989) and is controlled by short as well as long-term mechanisms. Short terms mechanism involves the direct regulation of DNA replication machinery activities such as topoisomerases and DNA polymerases, while long term mechanisms involves the regulation of signaling pathways affecting cell cycle transition. Therefore, within a cell, DNA replication might be blocked by drugs affecting essential DNA replication machinery when DNA replication is ongoing, for instance, by blocking DNA topoisomerases (e.g. etoposide) or by affecting the G_1/S transition (CDK inhibitors).

Regulation of DNA synthesis in normal rat cerebral cortex and in human brain tumours

The mammalian central nervous system (CNS) is structurally and functionally very complex. Controlled cell behavior and cell-cell interactions form the basis for the normal development and function of this organ. One fundamental part of the biology of the CNS, as well as other organs, is the regulation and function of cell cycle-related events. The controlled cell proliferation is a complex function that is essential for normal growth and development. It is regulated by multiple mechanisms (cell-cell interactions, endocrine/paracrine mechanisms, intracellular machineries) and important

components in these systems are growth factors, growth factor receptors, signal amplification/down regulation mechanisms and intracellular regulatory systems for transition as well as progression through different cell cycle phases (for review, see Weiner, 1995). Therefore, when studying the regulation of DNA replication and their modulation by potential antineoplastic drugs it is essential to use methods that preserve these interrelationships.

The rat cerebral cortex offers a good model to study DNA replication: 1) in young intact animals, cell proliferation and DNA synthesis are high and decreases during development (Altman and Das, 1966; Sung, 1969). 2) In the rat cerebral cortex, the proportions of RDS and UDS are differentially regulated during development. HU inhibits DNA synthesis (based on rates of incorporation of labeled thymidine) in immature rat brain by 90% and in the adult by 40%, indicating that 60% of DNA synthesis in the adult brain is connected to DNA repair of neurons and glia (Vilenchik and Tretjak, 1977). Moreover, in adult Wistar rats, more than half of the radioactivity incorporated into DNA after a single intracranial injection of [methyl-^{3}H]-thymidine disappears from the tissue in the following few hours (Perrone-Capano *et al*, 1982). This observation is consistent with the presence of "metabolic DNA" unrelated to replication (Perrone-Capano *et al*, 1982).

AIMS OF THE STUDY

- To develop and evaluate a method that would make it possible to determine ongoing DNA synthesis in CNS tissue under conditions preserving *in situ/in vivo* existing cell interactions and perform a simultaneous monitoring of drug effects.
- To apply such a method for investigation of the regulatory mechanisms for ongoing DNA synthesis in normal and neoplastic CNS tissue.
- To investigate drug effects on scheduled/unscheduled DNA synthesis.
- To investigate potential drug treatment strategies for human gliomas.
- To test the effect of new potential antineoplastic drug/s in the rat RG2 glioma model.

MATERIAL AND METHODS

Generation of tissue mini-units

Tissue mini-units were generated from normal rat cerebral cortex, specimens from human glioma and from rat glioma model specimens.

The basic procedure initially developed for rat cerebral cortex (Papers I and III) was performed as follow: Sprague Dawley rats from different ages (ranging from 1 postnatal day to > 60 days) were killed by decapitation. The cerebral cortex was rapidly dissected and placed in a 15 ml conical tube containing pre-warmed +37 °C Dulbeco´s modified Eagle´s medium, (DMEM). The tissue was immediately fragmented by aspirating and expelling 10 times using a Pasteur pipette. The mini-units obtained in this way were sedimented at 1 x g, rinsed twice with pre-warmed +37 ºC DMEM, transferred to an Eppendorf tube and centrifuged at 1,000 rpm during 5 sec. The supernatant was discarded and the tissue was seeded in microwells on a flat bottom 96-multiwell plate (Nunc cat # 167008, Denmark). Each microwell had previously been filled up with 50 µl of DMEM (+37 °C) containing 2 µCi/ml ^{3}H-methyl-thymidine with the drug(s) tested at the proper concentration or the appropriate vehicle for controls.

In order to standardize as much as possible the amount of tissue mini-units applied into the wells, the extreme ends (0.5 cm) of standard yellow tips (200 µl Gelb, Art. no. 100, Nerbe Plus, Seevetal, Germany) were removed and used to dispense 1µl of tissue mini-units into each microwell. We found that in this way the average amount of protein content per microwell was relatively uniform (see results). A 96-multiwell plate could be loaded in 6-8 min. The plates were then incubated at +37 °C, 5% CO_2 and 95% relative humidity, during 0 to 180 min (see results). The amount of protein per microwell was measured by collecting the content of a single microwell, washing with phosphate-buffered saline (PBS) and then homogenizing in 125 µl ethylenediaminetetraacetic acid (EDTA)-PBS to preserve the tissue/EDTA-PBS relation used during the assay. In addition we double controlled these data by measuring the average amount of protein in 4 pooled microwells (see results). This last procedure was adopted to compensate for the possible differences in the mini-unit (protein) amounts dispensed into the individual microwells and for obtaining a more accurate DNA counting. Thus, the contents of 4 microwells - representing one

experimental point - were after incubation pooled in one Eppendorf tube and placed in ice. Each of these experimental points was determined in quadruplicates. After centrifugation of the Eppendorf tubes at 1,000 rpm for 5 sec, the supernatants were discarded, the pellets were rinsed with 0.5 ml ice cold PBS pH 7.4 and then homogenized by sonication in 0.5 ml ice cold EDTA-PBS pH 7.4). After this step, the samples were stored at -80 °C since the EDTA prevents DNAase activity (Labarca and Paigen, 1980).

Modifications of this method were also used: in Paper III where flat-bottom 24 macrowell plates (Nunc Cat No. 146485, Denmark) were used for the study of the reversibility of genistein effect. In order to preserve the amount of tissue per ml/DMEM ratio used in the original method 6 µl of mini-unit tissue were seeded in each macrowell containing 300 µl DMEM. In Paper IV additional refinements were necessary in order to deal with the different consistency of tumoral samples and laboratory facilities (e.g. when tumoral specimens were obtained at the operation room and needed to be transported and processed in a nearby laboratory). Briefly, tumoral tissue specimens were placed in a 15 ml conical tube containing pre-warmed +37 °C DMEM, and transported in a thermo container at +37 °C to a nearby laboratory facility room. Due to the harder consistency of the tumoral tissue (when compared to normal rat cerebral cortex), the tumoral specimen was placed on a Petri dish and cut into small fragments with a surgical blade prior to fragmentation by aspirating and expelling using a Pasteur pipette.

Determination of ^{3}H-methyl-thymidine incorporation into DNA

From each homogenized sample preparation, 15 µl were used for protein determination while 400 µl were mixed with 1 ml ice cold EDTA-PBS containing 14 % trichloroacetic acid (TCA) and incubated 10 min on ice. The DNA was precipitated by centrifugation at 14,000 rpm for 10 min at +4 °C in an Eppendorf centrifuge. The supernatants were discarded and 1.5 ml scintillation liquid (Insta-Gel, Packard, Netherlands) was added. The Eppendorf tubes were placed in a 25 ml clear glass vial and the radioactivity was counted in an LKB 1214 Rackbeta liquid scintillation counter.

Despite quenching, the best counting reproducibility was observed when the procedure was performed directly in the Eppendorf tube placed into the counting vial.

In this way the small losses of TCA insoluble material that may occur when transferring the sample are avoided. No radioactivity was detected when microwells were incubated without tissue (data not shown) or when incubation time was 0 min (Fig. 2) indicating that unincorporated ^{3}H-methyl-thymidine does not precipitate in EDTA-PBS buffer containing TCA.

DNA synthesis assessment

The DNA synthesis rate was calculated as cpm of ^{3}H-methyl-thymidine incorporated into TCA insoluble material per mg of protein per min. The effects of the drugs were expressed as the percentage of control DNA synthesis rate.

Standard methods used in this work such as protein determination, agarose gels, and topoisomerase I and II activities are described in the respective papers.

Drugs used in this study

Protein kinase modulators

Tyrosine kinase inhibitors: Genistein is a natural compound that inhibits tyrosine kinases (Akiyama and Ogawara, 1991) and topoisomerase II (Okura *et al*, 1988). Herbymicin A inhibits the activity of several tyrosine kinases (Uehara and Fukazawa, 1991). Staurosporine is a potent, non-specific inhibitor of several tyrosine and serine/threonine kinases (Fallon, 1990; Fujita-Yamaguchi and Kathuria, 1988; Hofmann, 1997; Meijer, 1996; Nakano *et al*, 1987; Niggli and Keller, 1991; Tamaoki 1991). Calphostin C is a potent and selective inhibitor of PKC (Kobayashi *et al*, 1989; Tamaoki, 1991).

Sodium orthovanadate is an inhibitor of protein phosphatases (Gordon, 1991)

Roscovitine and olomoucine are purine analogues and Cyclin-Dependent Kinase Inhibitors (CDKIs) structurally related to adenosine-5'-triphosphate (ATP) (De Azevedo *et al*, 1997; Gray *et al*, 1999, Meijer *et al* 1997). They antagonize binding of kinases to ATP.

Topoisomerase inhibitors

Camptothecin and etoposide are selective inhibitors of topoisomerase I and II respectively, currently used for treatment of brain tumours (Parmey and Chang, 2003).

Protease inhibitors (ongoing work)

Nα-p-Tosyl-L-Lysine Chloro-methyl Ketone (TLCK) and N-Tosyl-L-Phenylalanine Chloromethyl Ketone (TPCK) are widely used serine protease inhibitors (Grabarek *et al*, 2002). Lactacystin (LC) and Clasto-Lastacystin-β-Lactone (CLC) are highly selective proteasome inhibitors (Dick *et al*, 1996; Dick *et al*, 1997; Fenteany *et al*, 1995; Fenteany and Schreiber, 1998).

Calpain Inhibitor I (Ca I I) and Calpain Inhibitor II (Ca I II) are potent inhibitors of the proteasome as well as calpains (Rock *et al*, 1994).

Cbz-leucynil-leucynil-leucinal (MG-132) and Proteasome Inhibitor I (PSI) are proteasome inhibitors with inhibitory effect on calpains (Figueiredo-Pereira *et al*, 1994; Tsubuki *et al*, 1996).

Phenylmethylsulfonilfluoride (PMSF) is an inhibitor of serine and cysteine proteases that does not affect proteasome function (Dubiel *et al*, 1992).

Detailed information of IC_{50} values of different targets for protein kinases modulators and protease inhibitors are given in table 1 of Paper III and manuscript submitted (Yakisich *et al*), respectively, (See also Davies *et al*, 2000 and "ABBREVIATIONS" (Page 8) for alternative names of the drugs used in this study).

RESULTS AND DISCUSSION

The "mini-unit system" and its application for studying biological processes

The conservation of the topological and metabolic conditions are essential for the normal function of the complex enzymatic cascades involved in proliferation, differentiation and maintenance of normal and neoplastic cells (Karin, 1992). Moreover, the activity of these enzymes may be modulated by changes in the tissue environment (Jans, 1994; Karin, 1992; Segal and Greenberg, 1996; Seger and Krebs, 1995; Stahl and Yancopoulus, 1993). Consequently, it is essential that experimental data obtained when using model systems, such as cell cultures, are interpreted with care. In particular, if those data are used to explain cell cycle progression in cells within an intact organism (Boonstra, 2003) and, even more important, if the results have potential clinical implications. For example, cell cycle studies in synchronized cells by serum starvation gave different results when compared to cells synchronized by the mitotic shake-off method (Boonstra, 2003). Thus, methods that study the regulation of cellular processes under physiological conditions will be helpful to better understand the regulation of pathways controlling important biological events.

We chose to study the regulation of "ongoing" DNA replication since it is at this level where the major targets for anticancer drugs have been found. We reasoned that by using the present method would be possible to monitor only "ongoing DNA replication" under conditions preserving the "*in vivo/in situ*" proliferative activity of the intact tissue. This hypothesis is based on the following:

- Although, in this approach, the proliferating cells are dispersed through all cell cycle phases, the short incubation pulse with ^{3}H-methyl-thymidine makes it possible to monitor only the cells that are in the S phase. These cells have passed the restriction point, accordingly, they (a) have escaped from requirement for a critical threshold of protein synthesis and become insensitive to inhibition of cell cycle progression by cycloheximide (Das, 1981; Pardee, 1989); (b) have become independent of growth factors, cell density, and cell size (Das, 1981; Doree and Galas. 1994; Pardee *et al*, 1986; Pardee, 1989; Planas-Silva and Weinberg, 1997); and (c) have become insensitive to some biochemical inhibitors (Zetterberg and Larsson, 1991).

- Due to the random variation in the length of the cell cycle characteristic of mammalian cells (Shields, 1977) and the length of cell cycle of neural cells (approximately 3 h) for developing rat brain (McAuley *et al*, 1993), it is expected that only a small fraction of the cells have crossed the G_1/S transition during the short time of the assay and incorporateed ^{3}H-methyl-thymidine into DNA and thereby produce artifacts by *in vitro* transformation.
- The contribution of mitochondrial DNA synthesis is very low since ^{14}C-thymidine incorporation into mitochondrial DNA even after 28 hrs of labeling is only 0.9-1.3% of the total ^{14}C incorporation into DNA (Merits and Cain, 1969)

Several parameters were evaluated to ensure steady state of radioactive precursor incorporation and to validate the method:

A) Content of protein in each microwell

In order to maintain a constant flow of radioactive precursor during the procedure (see discussion), the amount of mini-units (protein) applied per microwell is important. The amount of protein when measured per single microwell was found to be 61.75 μg ± 31.46 (n= 22). In addition, when we measured the protein content in 4 pooled microwells, as used for the measurement of DNA synthesis rate, the average protein content per single microwell was 61.93 μg ± 15.6 (n=84) with intra- and inter-assay variations of 22% ± 6 and 8 % ±1.8 respectively. These results indicate that the average amount of protein per microwell measured by collecting the content of 4 microwells reflects correctly the protein content per single microwell (compare the mean and SD of both procedures) and can be used as an intra-assay control.

In order to further detect the possible variation of protein content that could occur during the dispensing procedure, we also measured the average amount of protein per microwell (by collecting the content of 4 microwells) loaded with different volumes of tissue mini-units. As seen from Table 1, there is a relatively small variation of protein content within the 1-2 μl range.

Table 1. Relation between volume of tissue mini-units obtained from normal rat cerebral cortex and amount of protein loaded into the microwells calculated from 4 pooled microwell measurements.

Volume of tissue mini-units	µg of protein/microwell (mean ± S.D.)
1 µl	61.93 ± 15. 6 (n = 84)
1.5 µl	66.98 ± 14.5 (n = 50)
2 µl	78.60 ± 13.1 (n = 8)

[a] The values in brackets indicate the number of determinations.

More important Table 2 shows the average content of protein/microwell when 1 µl of mini-units prepared from specimens obtained from human gliomas was dispensed into each microwell. An around 2-fold variation of the mean values was observed when samples from different specimens were compared. However, in all cases, the average content was below 150 µg/microwell and the SDs were quite comparable to those observed for normal rat cerebral cortex

Table 2 Relation between volume of tissue mini-units obtained from human brain tumours and amount of protein loaded into the microwells calculated from 4 pooled microwell measurements

Case No.	µg of protein/microwell (Mean ± SD)
1	71 ± 28 (n = 12)
2	82 ± 25 (n = 24)
3	93 ± 22 (n = 16)
4	87 ± 26 (n = 12)
5	54 ± 17 (n = 36)
6	56 ± 25 (n = 8)
7	66 ± 28 (n = 24)
8	41 ± 18 (n = 24)

[a] The values in brackets indicate the number of determinations.

B) Linearity of incorporation of the radioactive precursor

A linear incorporation of the radioactive precursor over time was found using either ^{3}H-methyl-thymidine or L-[4,5-^{3}H]leucine (Fig. 2 top and bottom) up to 150 min (after 90 min increasing SD) followed by a tendency to plateau.

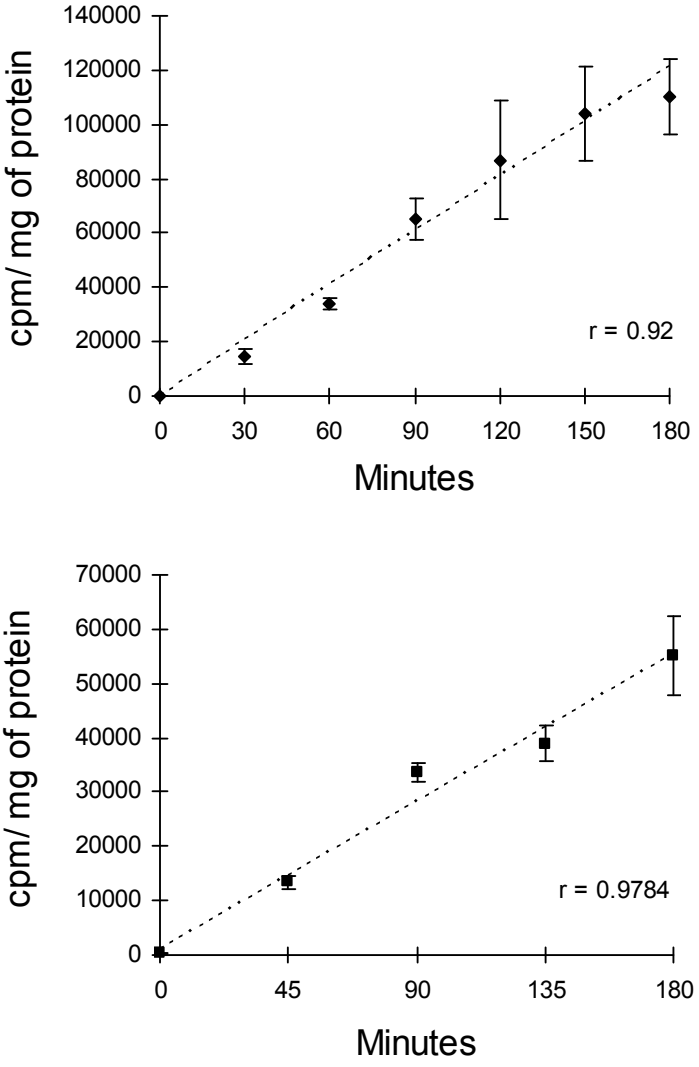

Figure 2: Incorporation of ^{3}H-methyl-tymidine (Top) into DNA or incorporation of L-[4,5-^{3}H]leucine into protein (Bottom) in mini-units from normal cerebral cortex of 3-5 postantal days old rats. Data are the mean ± S.D. of 3 independent experiments performed by quadruplicate. Dashed line shows the regression line predicted by the method of least squares. (r = correlation coefficient)

The incorporation of ^{3}H-methyl-thymidine into DNA was expressed as cpm per mg of protein instead of cpm per mg DNA for two reasons: 1) The amount of protein

correlates very well with the number of cells, specially because the amount of proteins per cell does not change significantly during short incubation times (Lathja and Toth, 1966) and 2). Protein determination by the Lowry method is a widely used technique, easy and faster to determine when compared to genomic DNA purification.

C) DNA synthesis rate in normal rat cerebral cortex.

The tissue mini-units were prepared from rats of 2 to > 44 postnatal days and incubated in DMEM containing 2 μCi/ml ^{3}H-methyl-thymidine during 90 min. The measurement of the incorporation of ^{3}H-methyl-thymidine into TCA insoluble material/ mg of protein/ min showed an age-dependent decrease (Fig. 3).

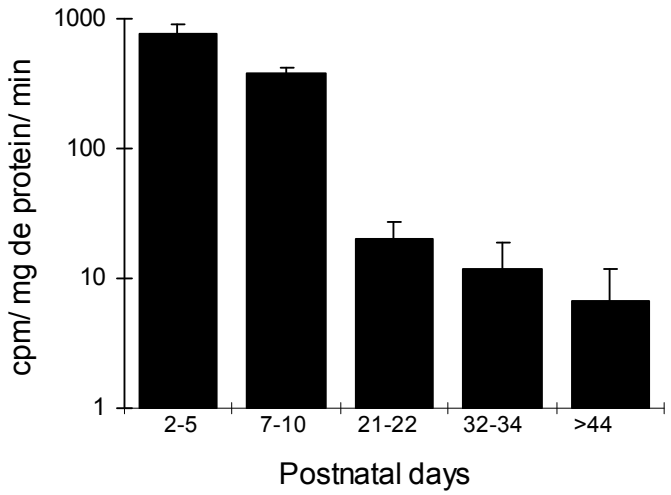

Figure 3: DNA synthesis rate in normal rat cerebral cortex. Mini-units obtained from rats of 2 to > 44 postnatal days old were incubated with 2 μCi/methyl-^{3}H-thymidine during 90 min. Data are the mean ± S.D. of 2 independent experiments performed by quadruplicate.

D) Effect of genistein on DNA synthesis rate during development of normal rat cerebral cortex

The tissue mini-units were prepared from rats of 2 to > 44 postnatal days and incubated during 90 min in DMEM containing 2 μCi/ml ^{3}H-methyl-thymidine and 100 μM genistein. This concentration of genistein was chosen since it has been shown to inhibit cell proliferation in different tissues (Barnes, 1995; Clark *et al*, 1996; Peterson, 1995). The results from our experiments showed a decreasing effect of genistein with increasing age, Fig 4.

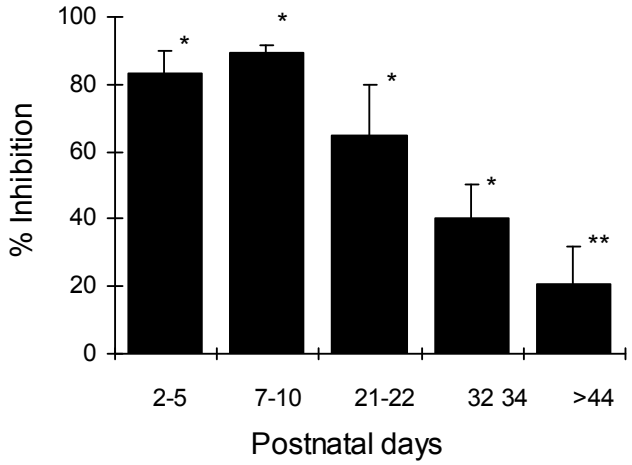

Figure 4: Effect of genistein on DNA synthesis rate during development in normal rat cerebral cortex. The mini-units were incubated with DMEM containing 2 μCi/ml ^{3}H-methyl-tymidine and 100 μM genistein during 90 min. Data are the mean ± S.D. of 2 independent experiments performed by quadruplicate. DMSO at the same concentration was used as control. * = P < 0.01 and ** P = < 0.02 vs the corresponding control (Student's *t* test).

E) Effect of sodium azide on DNA synthesis rate

Sodium azide decreased the DNA synthesis rate in a concentration (1 - 50 mM) dependent manner (Fig. 5). This indicates that the ^{3}H-methyl-thymidine incorporation into DNA is not an artifact but an active process requiring intact metabolic conditions.

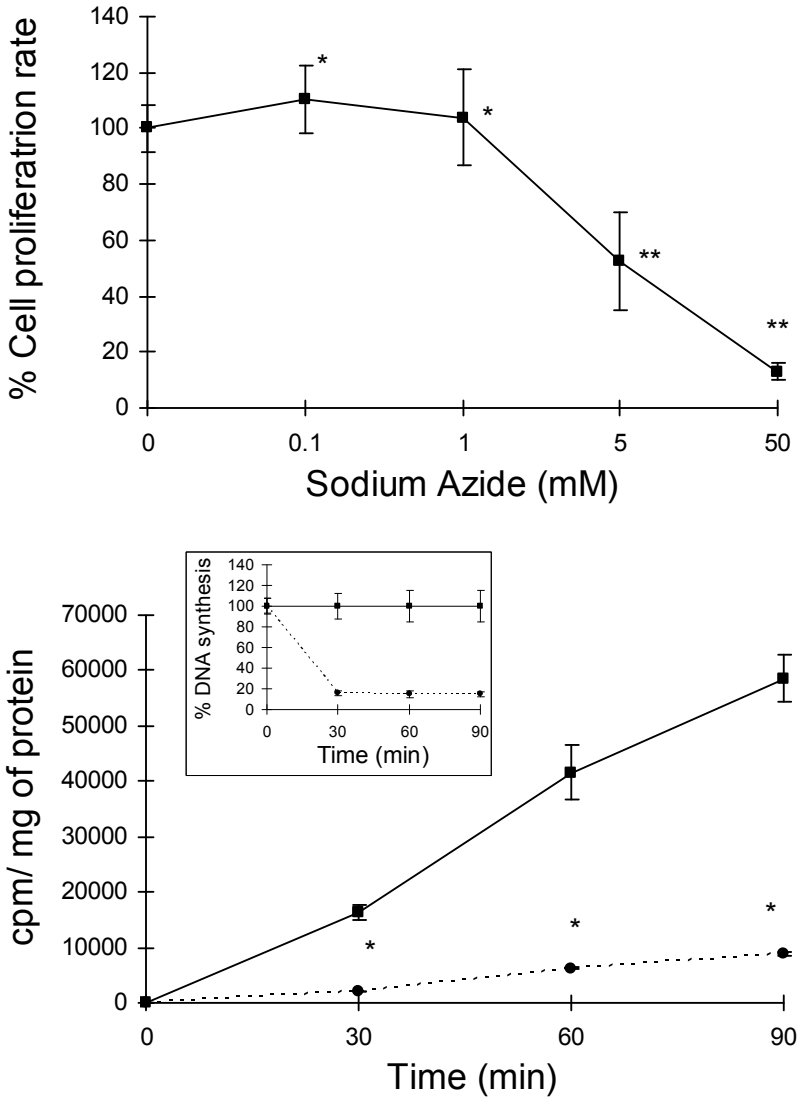

Figure 5: Concentration-dependent (top) and temporal (bottom) effects of sodium azide on DNA synthesis rate (% cell proliferation rate) in mini-units from normal rat cerebral cortex

In summary, our data show that the use of tissue mini-units in combination with a short incubation time may allow the preservation of the in vivo metabolic and proliferative behavior. This conclusion is supported by the following data: 1) The incorporation of ^{3}H-methyl-thymidine into DNA or L-[4,5-^{3}H]leucine into protein during 150 min (despite increasing SD after 90 min) of incubation proceeded linearly (Fig. 2) indicating a steady state of labelling as it has been described for the incorporation of (2-^{3}H)-mannose as well as (4,5-^{3}H)-leucine into protein (Alperin *et al*

1986). 2) The age-dependent decrease of DNA synthesis rate in rat cerebral cortex (Fig. 3) is in agreement with previous data which have shown that DNA synthesis in normal rat cerebral cortex decreases during development in intact animals (Altman and Das, 1966) and that the in vitro DNA synthesis in rat brain cortex tissue is age dependent (Sung, 1969). 3) The age-dependent inhibitory effect of genistein on the DNA synthesis rate (Fig. 4) may reflect the general decline of DNA synthesis (Altman and Das, 1966), DNA synthesis (Sung, 1969) and tyrosine kinase activity (Cudmore and Gurd, 1991; Dasgupta *et al*, 1984; Okada and Nakagawa, 1988) during brain development. 4) The concentration-dependent effect of genistein on the DNA synthesis rate has previously been reported to occur in several cell lines (Barnes, 1995; Clark *et al*, 1996; Peterson, 1995). 5) The DNA synthesis measured by this method requires integrity of the cell metabolism since sodium azide, a well known metabolism inhibitor, blocks DNA synthesis rate within 30 min (Figs. 5)

Additional advantages of the method include: (1) the sensitivity to drugs can be assessed simultaneously with the DNA synthesis rate, (2) the complete procedure can be performed within 4-6 hrs and (3) many experiments can be performed with the tissue from one tissue specimen.

This method was used to study the effect of different drugs on DNA synthesis in normal (**Papers I-III and manuscript VI**) as well as neoplastic tissues (**Paper IV and Vitali *et al*, 2002**). In addition, this method was successfully applied to study the mechanism of action of some drugs (**Papers II and III, manuscript VI**).

In **paper II** we have reported a novel effect of two drugs considered highly specific CDK inhibitors (Meijer, 1997). We found that roscovitine and olomoucine inhibit DNA synthesis probably by a direct effect on the DNA replication machinery. Additional, mechanisms of actions may be responsible for cellular effects independent of CDK inhibition. For instance, it has been reported that roscovitine is also an inhibitor of RNA synthesis (Ljungman and Paulsen, 2001).

In **paper III** we showed that the early inhibitory effect of genistein on DNA synthesis is probably mediated by topoisomerase II inhibition. We also showed that the early inhibitory effect of high (micromolar range) concentration of staurosporine on ongoing DNA synthesis is probably mediated by topoisomerase II inhibition rather than by its ability to inhibit protein kinases at low (nanomolar range) concentration (**paper III**).

In **manuscript VI** we studied the effect of roscovitine in the developing rat cerebral cortex, its effect on scheduled as well as in unscheduled DNA synthesis and we compared the effect of roscovitine on DNA synthesis in tumoral tissue versus normal adult brain tissue

Mechanism of drug effect on ongoing DNA synthesis in normal rat cerebral cortex as well as human brain tumors

After having confirmed that the tissue mini-unit method was a suitable method to study ongoing DNA replication, we focused our studies in the regulation of this important process by evaluating the effect of different drugs on normal (**Papers II-III)** as well as neoplastic tissues (**Paper IV**). We selected drugs with low molecular weight (MW) for our studies since they are more capable to cross the BBB. The studied drugs have pharmacodynamic capacities of being CDK modulators, protein kinase inhibitors, protease inhibitors or topoisomerase inhibitors.

Role of protein phosphorylation on DNA synthesis and the effect of protein kinase modulators

Abnormal protein kinase activity is common in gliomas. For instance, activation of tyrosine kinase receptors plays an important role in gliomagenesis since amplification or mutations of EGFR is found in 30-50% of GBM patients (Hurtt *et al*, 1992; Schlegel *et al*, 1994).

A panel of different protein kinase inhibitors (genistein, herbimycin A, staurosporine, calphostin C) and the protein phosphatase inhibitor sodium orthovanadate were tested as potential ongoing DNA synthesis inhibitors. Genistein, was found to strongly inhibit ongoing DNA synthesis. Conversely, the other protein kinase modulators at concentrations usually reported to inhibit DNA replication showed no effect. Only at high concentration herbimycin A and staurosporine produced a significant inhibitory effect. Experiments using as control the selective topoisomerase I and II inhibitors, camptothecin and etoposide, showed that the temporal courses of the effect of these drugs on ongoing DNA replication were similar to that observed for

genistein, suggesting that the mechanism of action of genistein was probably due to topoisomerase inhibition rather than to protein kinase inhibition. In vitro studies showed that genistein and high concentrations of stauroporine and herbimycin are inhibitors of topoisomerase II.

The highly specific CDK inhibitors (roscovitine and olomoucine) showed a strong inhibitory effect on ongoing DNA replication within less than 30 min. However, staurosporine, an even more potent CDK inhibitor failed to block DNA replication indicating that the inhibitory effect of roscovitine and olomoucine was independent of CDK inhibition.

So far, specific protein kinases that regulate "ongoing DNA replication" with no role in other cellular functions has not been identified. Although, it has been recently reported that CDKs prevent the assembly of prereplicative complexes (Pre-RCs) during S, G_2 and M phases of the cell cycle in yeast (Mimura *et al*, 2004), a role for CDKs on ongoing DNA synthesis has not been established.

The present data indicated that the mechanism of action of the protein kinase inhibitors used in this study maybe mediated by topoisomerase II inhibition rather than by protein kinase inhibition.

Our results lead us to propose that most of the protein phosphorylation machinery is involved in the regulation of DNA replication through long-term mechanisms (such as regulation of transition in the cell cycle) rather than short-term mechanisms.

Ongoing studies: role of proteolysis on DNA synthesis and the effects of protease inhibitors

In eukaryotic cells, proteins are degraded primarily by two distinct proteolytic mechanisms. Proteins that enter the cells from extracellular compartment are degraded by lysosomes. The lysosomal-mediated breakdown is primarily involved in the degradation of intracellular proteins under stressed conditions, membrane-associated proteins or extracellular proteins taken up by endocytosis. The other proteolytic mechanism, the ubiquitin-proteasome pathway is the major proteolytic system in the cytosol and nucleus of all eukaryotic cells (Hershko and Ciechanover, 1998; Rock et al, 1994). This ATP-dependent pathway was discovered more than 20 years ago and plays

an important role in cell cycle progression, regulation of cell growth and gene expression. The ubiquitin-proteasome pathway also plays an important role in the regulation of many physiological processes as well as in the development of a number of major human diseases. In this pathway, protein substrates are first marked for degradation by a poly-ubiquitin chain and then rapidly degraded by the 26S proteasome (Lee and Goldberg, 1998). Some proteins (e.g.ornithine decarboxylase) do not need to be marked in order to be degraded (Murakami *et al*, 1992).

The cell cycle progression is controlled by the proteasomal degradation of cyclins and inhibitors of cyclin-dependent kinases (Koepp *et al*, 1999; Yew, 2001). Many proteins involved in the regulation of the cell cycle are degraded by the proteasome (Sanchez *et al*, 1999; Verma *et al*, 1997; Yew and Kirschner, 1997). The ability of proteasome inhibitors to inhibit cell proliferation and selectively induce apoptosis in proliferating cell, together with their ability to inhibit angiogenesis (Drexler, 1997; Oikawa *et al*, 1998), makes these agents attractive candidates as anti-cancer drugs. The proteasome is distributed ubiquitously in the rat CNS (Mengual *at al*, 1996).

Calpains are intracellular Ca^{++} -regulated cysteine proteases that play an important role in different steps of the cell cycle (Janossy *et al*, 2004)

Given the emerging roles of different proteolytic pathways on the regulation of the cell cycle as well as the fast and transient nature of these processes, we decided to study the effect of nine different protease inhibitors (TLCK, TPCK, PMSF, MG-132, PSI, LC, CLC, Ca I I and Ca I II) on ongoing DNA replication. We observed that the general serine and cysteine protease inhibitors TLCK, TCPK and PMSF significantly inhibited DNA synthesis in a concentration-dependent manner (Fig. 6). It is important to mention that PMSF at the concentration used in this study has no inhibitory effect on the proteasome (Dubiel *et al*, 1992). The most selective proteasome inhibitors LC and CLC (1-100 μM) had no significant inhibitory effect, except for LC at 100 μM. The proteasome and calpain inhibitors Ca I I and Ca I II also lacked effect on DNA synthesis unless used at high concentrations (≥250 and ≥ 500 μM, respectively).

Similar to what we concluded regarding protein phosphorylation, we conclude here that protein degradation through the ubiquitin-proteasome pathway is involved in the regulation of DNA replication through long-term mechanisms.

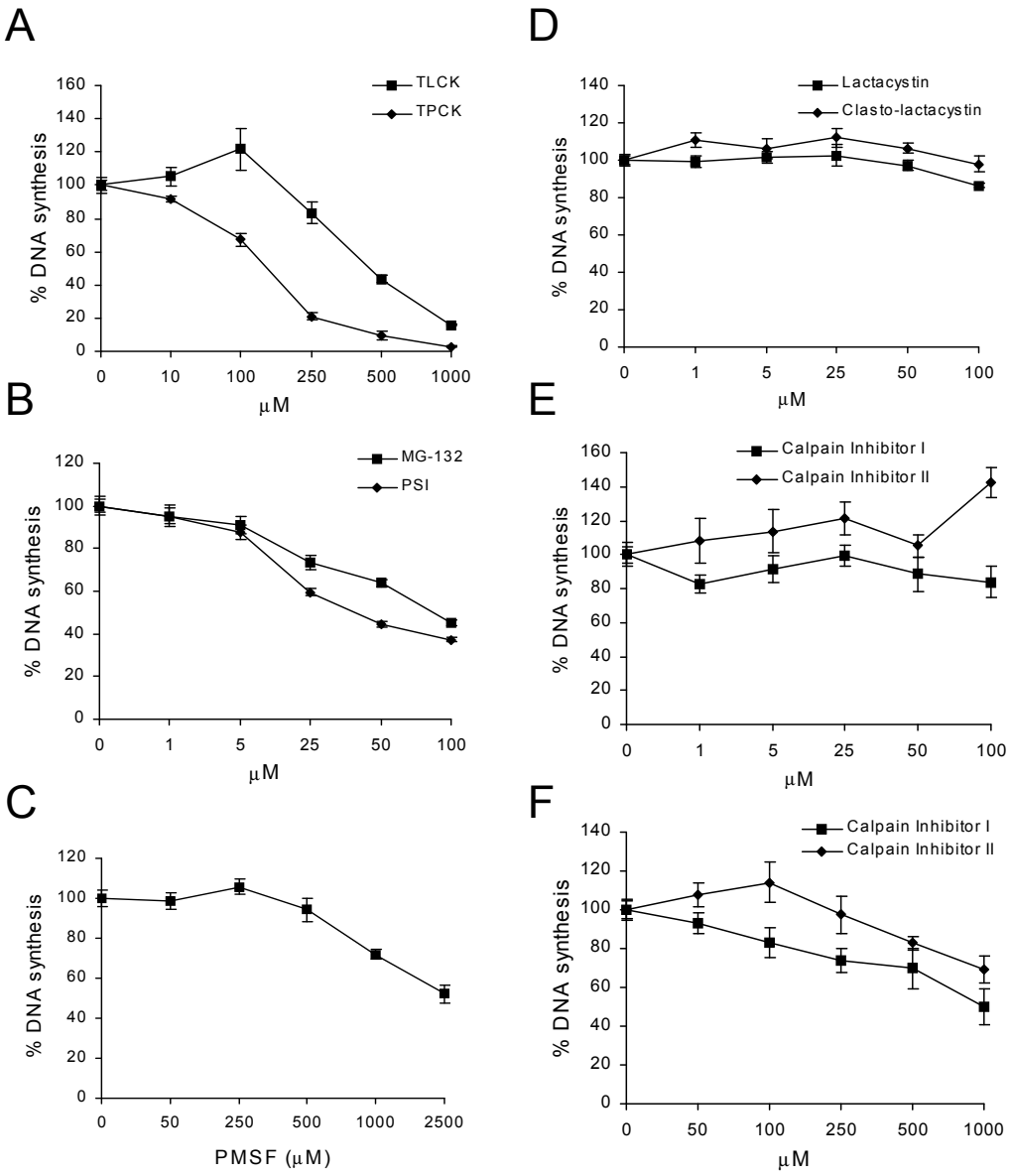

Figure 6: Effects of protease inhibitors on DNA synthesis in tissue mini-units prepared from developing rat cerebral cortex. Each point represents the mean ± SEM of two independent experiments performed by quadruplicates: A) TLCK and TCPK, B) MG-132 and PSI C) PMSF, D) LC and CLC, E and F) Ca I I and Ca I II. Control mini-units (0) in A-F were incubated with DMSO (0.1-1 % (v/v)). * = $P < 0.01$ and ** = $P < 0.05$ vs the corresponding control (Student's t test).

Taken together our observations we suggest that protein phosphorylation and proteasome- and calpain-mediated proteolysis seem to be required before the onset of DNA synthesis in order to integrate all the mitogenic and metabolic pathways as well as to contribute to the formation of a pre-replicative state of the DNA replication

machinery. When DNA replication has been initiated, the control over this process is relatively independent of protein phosphorylation and/or proteolysis or is mainly driven and regulated by different sets of proteases and PKs at present not identified.

Effect of inhibitors of ongoing DNA synthesis on normal brain tissue and human gliomas: roscovitine as a potential antineoplastic drugs

Among several drugs identified in our study as inhibitors of ongoing DNA replication in normal rat cerebral cortex, we decided to further study the potential use of these substances as antineoplastics drugs in gliomas.

Roscovitine is a purine analogue which competes with ATP for the binding site on CDKs (Meijer *et al*, 1997; Canduri *et al*, 2004) with high affinity and specificity for cdk1/cdc2, cdk2, cdk5 and cdk7 (Hardscastle *et al*, 2002). Roscovitine has been shown to arrest cells in the G_1 and G_2/M phases of the cell cycle (Meijer *et al*, 1997), cause nuclear fragmentation (David-Pfeuty, 1999; Wojciechowski *et al*, 2003) and induce apoptosis in human cell lines (Mgbonyebi *et al*, 1999; Somerville and Cory, 2000). Roscovitine inhibits the growth of several cancer cell lines including head and neck squamous cell carcinoma cells (Mihara *et al*, 2002), human breast cancer cells (Wesierska-Gadek *et al*, 2003; Wesierska-Gadek *et al*, 2004) lung and colorectal carcinoma and uterine sarcoma cell lines (McClue *et al*, 2002) at micromolar concentrations. The average IC_{50} was ~16 μM for a panel of 60 tumour cell lines (Dai and Grant, 2003).

We found that roscovitine is a potent inhibitor of ongoing DNA synthesis in the developing rat cerebral cortex (**paper II**) as well as in human gliomas (**paper IV**). However, roscovitine had little effect on DNA synthesis in adult rat cerebral cortex (**paper II, manuscript VI**) rising the possibility that roscovitine may selectively block DNA synthesis in high proliferative tissue (developing brain or human gliomas) with little effect (and toxicity) in adult brain tissue. We also found an age dependent inhibitory effect of roscovitine on normal rat cerebral cortex during development and a differential effect on scheduled and unscheduled DNA synthesis (**manuscript VI**). Moreover, in an on going pilot study we found that in tissue mini-units prepared from tumoral samples from the RG2 rat glioma model, ongoing DNA synthesis was also inhibited to a significant degree by 90 min incubation with roscovitine: 23% by 5 μM, 60% by 25 μM and 68% by 50 μM (data not shown). For comparison, tissue mini-units

obtained from normal cerebral cortex of the opposite hemisphere of the same animal was used. Fig. 7 shows the percentage of DNA synthesis inhibition induced by 50 μM roscovitine in glioma cells as well as in adult rat cerebral cortex. The result show that the proliferation rate of glioma cells are very high when compared to normal rat cerebral cortex, furthermore, roscovitine produced a strong inhibitory effect on the proliferation rate of glioma cells with no significant effect on normal tissue. Taken together, our results suggest that roscovitine preferentially inhibits DNA synthesis connected with replicative processes and, therefore, it might be an interesting antineoplastic drug for cancers arising in low proliferative tissues (e.g. adult brain).

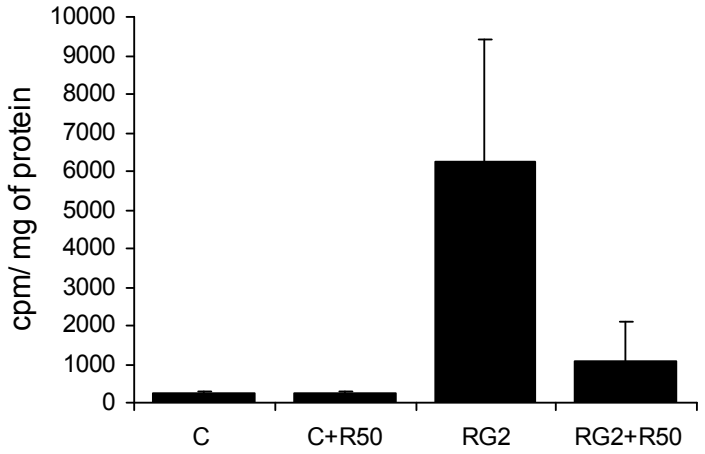

Figure 7: Effect of roscovitine on DNA synthesis in rat glioma model. The mini-units prepared from normal tissue (C) or from tumoral tissue (RG2) were incubated with DMEM plus 2 μCi/ml ^{3}H-methyl-tymidine alone (C, RG2) or DMEM + 2 μCi/ml ^{3}H-methyl-thymidine + 50 (C+R50, RG2+R50) μM roscovitine for 90 min. Data are the mean ± SD of 1 one (of two) representative experiment performed by quadruplicate.

Our data are in agreement with results obtained by comparing the effect of roscovitine in cancer cell lines versus non-proliferating cells (McClue *et al*, 2002). The average potency against a group of human tumor cells lines was 15.2 μM (range 7.9 - 30.2 μM) while the average potency against non-proliferating cells was almost two-fold lower (range 22.2 to > 100 μM).

CDK inhibitors are inducers of apoptosis in mature cerebellar granule neurons in culture. This effect occurs at relative higher concentrations: 10 μM decreased cell viability to 74.9 ± 7.9 % compared to vehicle alone (100 %) (Monaco III *et al*, 2004).

We have also performed pilot studies of the effect of roscovitine on RG2 glioma cells. Interestingly, we observed that 25-50 μM roscovitine blocked DNA synthesis (as determined by cell count). Figure 8 shows the inhibitory effect of 50 μM roscovitine on RG2 cells.

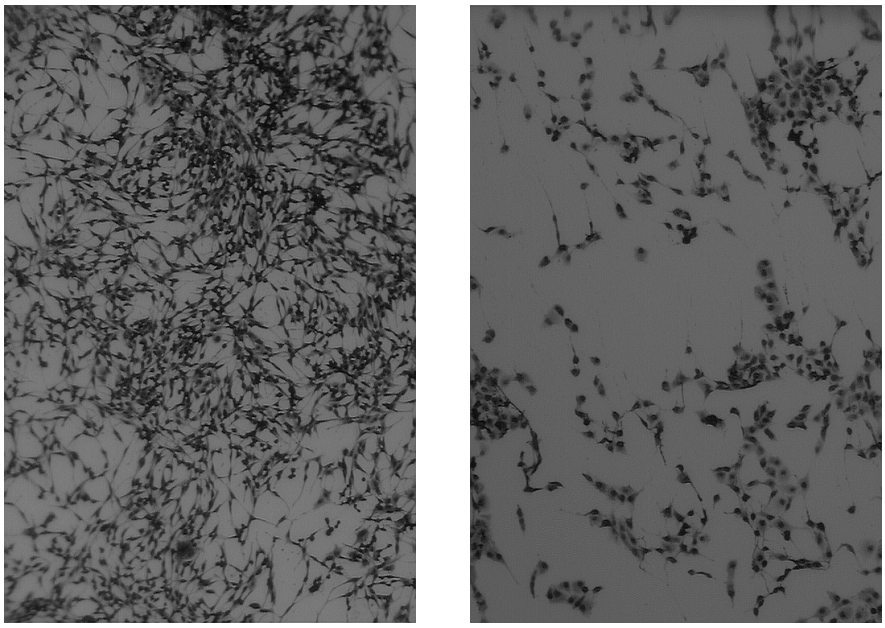

Figure 8: Effect of roscovitine (50 μM) on DNA synthesis of RG2 glioma cells. Left: mock, right: roscovitine treated cells for 48 hr

Several small-molecule cdk modulators are being discovered and tested in the clinical work. The first ATP competitive cdk inhibitors tested in clinical trials, flavopiridol and UCN-01, showed promising results with evidence of antitumor activity and plasma concentrations sufficient to inhibit cdk-related functions (Senderowicz, 2003). Roscovitine is the second-best-studied CDK inhibitor *in vivo* (after flavopiridol). The purified enantiomeric R-roscovitine is currently in phase-II clinical trials for treating breast cancer and lung carcinomas (Fischer and Gianella-Borradori, 2003). It has proven non-toxic in several animal models (Pippin *et al*, 1997; Nutley *et al*, 2000. In general, plasma concentrations of CDK inhibitors above those that inhibit cell-cycle progression *in vitro* have been shown to be non-toxic for mice, dogs and even humans (Sausville *et al*, 2000; Senderowicz, 1999; Senderowicz and Sausville, 2000; Stinson *et*

al, 1998). It is important to point out that other drugs that inhibit cell-cycle progression are currently used in humans. The statins, for example, are efficient inhibitors of the cell cycle (Jakobisiak *et al*, 1991) and have been used in humans for many years without major toxic effects. Moreover, new drug delivery methods, such as the use of intracerebroventricular infusion might allow the application of higher concentrations of antineoplastic drugs into the brain while preventing systemic toxicity. This kind of experimental studies using CDK inhibitors (flavopiridol, roscovitine and olomoucine) has been reported in rats (Osuga *et al*, 2000) and mice (Zhang *et al*, 2004).

On the other hand Roscovitine is a small lipid soluble substance able to pass the BBB. Indeed, in Sprague-Dawley rats after a single intravenous dose of 25 mg/kg the roscovitine concentrations in the brain were 30% of that observed in plasma (Vita *et al*, 2005). On the basis of its relatively selectivity and low direct cytotoxicity, roscovitine is clearly a potential useful anti- cancer drug and a definite candidate for treatment of glioblastomas offering an alternative to classic cytostatic agents.

Effect of thiol-reacting substances on topoisomerases

Topoisomerase I and II, are ubiquitous enzymes critical for DNA function and cell survival. They play a crucial role in DNA condensation, replication, transcription, and repair. The topoisomerase IIα isofform is synthesized in late G_1 and early S-phase of the cell cycle, persists throughout the G_2 and M phases and, finally, degrades as the cell enters G_1, thereby identifying the percentage of cycling cells. Expression of this protein has been suggested to provide prognostic information in adult malignant gliomas. TIIα has been related to a certain form of multidrug resistance to a number of anticancer agents in these neoplasms (Bredel, 2001).

There is substantial evidence that the glutathione/glutathione S transferase system may be a key factor in determining the chemosensitivity of some kind of human brain tumours including gliomas, medulloblastoma, meningiomas and neurinomas. There is also evidence that altered activity of topoisomerase IIα is involved in the development of an atypical multidrug resistant drug phenotype in high-grade gliomas (Bredel and Zentner, 2002).

We observed that disulfiram, a thiol-reacting drug, is a potent inhibitor of topoisomerases (**Paper V**). Since disulfiram is a relatively non-toxic drug we speculate

that thiol reacting drugs might be a new class of topoisomerase inhibitors with lesser side effects. In addition, it has been recently reported that disulfiram is potentially an attractive agent to combat multidrug resistance due to its ability to interact with proteins that mediate multidrug resistance or resistance to nucleoside analogs (Sauna *et al*, 2004).

On the other hand, we also observed that glutathione, a natural thiol-reacting substance found at high concentration in virtually all cells, is also a topoisomerase inhibitor which preferentially blocks topoisomerase II activity (**manuscript in preparation**). The current preliminary results and the observation that, in addition to its obvious link to proliferation, topoisomerase is a known determinant of sensitivity to anticancer agents in adult gliomas (Bredel, 2001) offer an underlying principle for more extensive studies on the potential utility of thiol-reacting substances as inhibitors of topoisomerases. The present results might be of importance in developing more rationale approaches to overcome drug resistance. In addition, disulfiram might also have some additional beneficial effect due to its ability to inhibit angiogenesis (Marikovsky *et al*, 2002).

CONCLUSIONS AND PERSPECTIVES

The "mini-unit system" as a tool for elucidating biological processes and for screening of new drugs

We have verified that the min-unit system is a useful tool to study biological processes such as the role of protein phosphorylation and proteolysis, especially on ongoing DNA replication. Comparing drug effect in normal rat cerebral cortex versus human brain tumor tissue we easily identified molecules having a differential inhibitory effect on ongoing DNA synthesis in tumor samples versus normal brain tissue. By comparing different pharmacological agents it was possible to elucidate the mechanism of action of a particular drug.

Because the outcome after tumor recurrence is variable, a test that could contribute to prediction of the patient outcome and/or to individualization of treatment would be useful. Cellular responses to anti-cancer agents result from the interaction between drugs, cellular targets and mechanisms of damage repair. Despite the pharmacological advances in the treatment of cancer, the clinical efficacy of chemotherapy is unpredictable in most patients. The individualization of therapy is the longstanding goal of pharmacologists; since the 50s, *in vitro* tests have been developed that identify effective drugs and avoid unnecessary toxicity (Cortazar and Johnson, 1999; Danesi *et al*, 2001). Identifying genes responsible for sensitivity to drugs and screening patients before treatment, it would make possible to create individualized chemosensitivity–chemoresistance maps to help drug identification to be combined for optimized treatments. This perspective implies that, in the future, the concept of the 'standard' protocol will be abandoned in favor of individualized cancer chemotherapy based on the genetic pattern of the disease and the host. Once we can identify which agents will be most active we will be able to investigate other factors that also have a profound influence on treatment efficacy, such as drug distribution and tumour hypoxia. Drug selection for patient treatment will therefore be strongly different in the future

The proliferation index (PI) is a measure of the proportion of cells in a tumor that have the capacity to divide and proliferate at a given point in time. A number of different methods have been developed to determine proliferation index. These include thymidine incorporation, bromodeoxyuridine (BUdR) incorporation, Ki-67 antigen

expression (including the MIB-1 clone), argyrophilic nucleolar organizer regions (AG-NOR), proliferating cell nuclear antigen (PCNA), and flow cytometry. We hypothesized that the mini-unit system, which preserves the metabolic and proliferative properties of the tumors *in situ*, may be useful in designing and interpreting studies as well as providing the patients with a more accurate prognosis. This new method might help to overcome the limitations of cell cultures for screening of drugs. Although cell cultures are very useful for different kind of studies, many potential antineoplastic agents with promising effects when tested in these *in vitro* models repeatedly failed in patients (Wolff *et al*, 1999). Attempts to overcome these limitations lead to the development of multicellular spheroid models (Sutherland, 1988). However, the heterogeneity of human gliomas and the complex growth patterns of the spheroids make difficult to interpret the drug response effect. (De Witt Hamer *et al*, 2005; Sander and Deisboeck, 2002).

Although, we limited our study to proliferation related events, such as DNA synthesis, other aspects of tumor metabolism (e.g. DNA repair) can be evaluated in order to identify new potential tumour vulnerabilities. For instance, the tissue mini-unit was successfully applied to study streptomycin effect on protein mannosylation (Alperin *et al*, 2000) during developing rat cerebral cortex.

Role of protein phosphorylation and proteolysis on ongoing DNA replication and rational approach for antineoplastic therapy

We have shown that many protein kinases and proteases that inhibit DNA replication in long term cultures have no effect on ongoing DNA replication in our system, in both rat normal brain tissue and human gliomas. This is probably because these inhibitors target protein kinases and proteases involved in transition of the cell cycle rather than proteins involved in the DNA replication machinery.

The targets of these drugs are involved not only in cell cycle progression but also in many other cellular processes. This could explain their high toxicity. We propose that a potential DNA synthesis inhibitor, used as antineoplastic drug for the treatment of human gliomas, should preferentially inhibit replicative DNA synthesis with little or no effect on DNA synthesis connected with DNA repair.

A model for the regulation of DNA replication by protein phosphorylation and proteolysis

Our data indicates that regulators of cell cycle transitions (such as protein kinases or proteases) have little or no effect on ongoing DNA synthesis. This indicates that an important shift in the set of protein kinases and proteases occurs during the G_1/S phase transitions (Figure 9). Protein kinases and proteases required for G_1/S transitions (e.g. protein kinase C, some CDKs, or the proteasome) are not longer needed once S-phase has started. This shift might be important for avoiding deleterious effect on DNA replication and can be exploited to target only proliferating tissue.

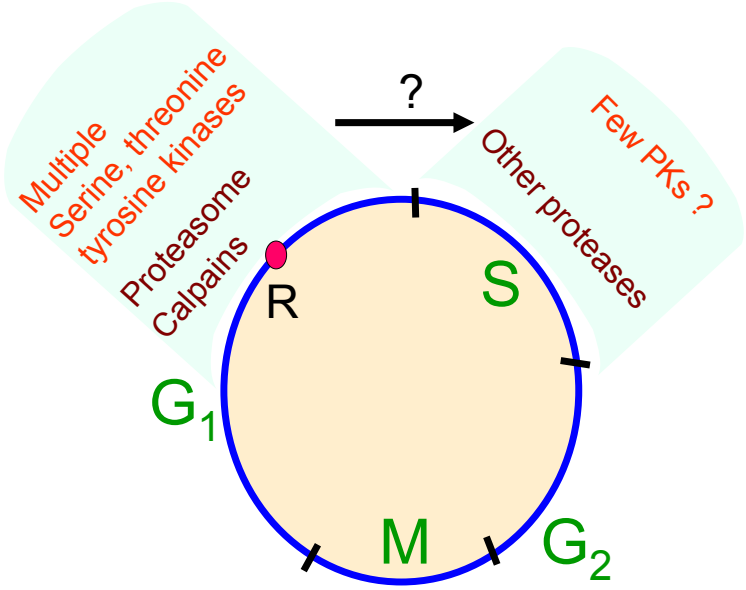

Figure 9: Proposed model for the integrated regulation of DNA replication by protein phosphorylation and proteolysis. During G_1 multiple protein kinases (e.g. serine, threonine and tyrosine kinases) as well as proteases (e.g. the proteasome and calpains) modulate DNA replication. During the G_1/S transition (arrow) a shift in the set of protein kinases and proteases by a poorly understood mechanism (?) occurs. During S phase, a different set (probably few) of protein kinases (PKs) and proteases regulates "ongoing DNA replication"

Therefore, it is important to discriminate between general DNA synthesis inhibitors that operate through mechanisms involving arrest in cell cycle progression

and specific DNA synthesis inhibitors that inhibit ongoing DNA synthesis. The latter class of inhibitors will be more selective and less toxic.

Thus, the recognition of the target of new drugs that specifically affects "ongoing DNA replication" and the detection of differences in the regulation of this process between normal and neoplastic tissue has to be studied.

Clinical implications and future directions

Since dysregulation of protein kinases and proteases are found in gliomas, protein kinase and protease inhibitors are potential antineoplastic candidates and had attracted the attention of molecular pharmacologists. The search for new antineoplastic drugs has undergone a major change: it has moved from a strategy identifying drugs that merely kill tumour cells towards a more mechanistic strategy identifying drugs acting on molecular targets that underlies cell transformation. The evidence that CDKs, their regulators and substrates are targets of genetic alteration in different types of human cancer has stimulated the search for chemical CDK inhibitors. However, many inhibitors, although highly specific, have the potential to target enzymes involved in several functions. For instance, dysregulation of protein kinase C has been found in gliomas (Baltuch *et al*, 1995; Bredel and Pollack, 1997; Sharif and Sharif, 1999). In particular, PKCα activity levels seem to be increased in malignant gliomas (Leirdal and Sioud, 1999). PKC is a family of at least 13 different members classified into four subgroups on the basis of their mode of activation (Mellor and Parker, 1998; Musashi *et al*, 2000). These PKC isoforms show considerable tissue- and cell-specificity: PKCγ expression is thought to be characteristic of normal CNS tissue, while the α, δ and ζ isoforms are encountered more abundantly in non-neuronal tissues (Wetsel *et al*, 1992). Although protein kinase C inhibitors are currently under pre-clinical and clinical investigation, yet, our incomplete understanding of the cell-and tissue-specificity of the different PKC isoforms may lead to unexpected and or undesired results in clinical practice. Indeed the PKC system plays a fundamental role in normal CNS physiology including regulation of astrocyte growth, differentiation of oligodendrocytes, prolonged potentiation of neurons, outgrowth of axons, and the release of neurotransmitters (Yong *et al*, 1994; O'Driscoll *et al*, 1995). Total PKC expression and activity levels in normal nervous tissue are significantly higher when compared with non-neuronal tissue

(Bredel and Pollack, 1997; Baltuch *et al*, 1995). The combined knowledge of the biology of tumoral as well as normal cells might lead to more efficient ways to kill tumoral cells while preserving normal cells.

The same kind of consideration should be made regarding proteasome inhibitors for treatment of human glioma. Although very specific proteasome inhibitors might be developed it has been reported that neonatal rat astrocytes are almost as sensitive to proteasome inhibitors as glioma cell lines (Wagenknecht *et al*, 2000), suggesting that proteasome inhibition does not specifically target the neoplastic phenotype of glioma cells. Thus, newly discovered antineoplastic agents need to be extensively tested before their effects are assessed in clinical trials. Despite the lack of knowledge on their mechanism of action, some of these agents might improve survival and the quality of life and represent new hope for cancer patients and their families.

One limitation of our method is that tests with the tissue mini-unit method require fresh tissue and in some cases the experiments cannot be repeated since tumoral samples are unique. Another limitation of the method is that the tissue mini-unit can not be artificially "transformed" like cell lines and therefore, some mechanistic studies are difficult to perform. However, emerging techniques might be used in the future in combination to the mini-unit system to answer important mechanistic questions. We speculate that chromatin immunoprecipitation (an emerging technique used to study DNA-protein interaction) in combination with tissue mini-units treated with pharmacological agents will be useful to study mechanistic aspect of drug effect on ongoing DNA replication. For instance, the effect of antineoplastic drugs on proteins involved in ongoing DNA replication can be easily assessed in order to look for differences in endogenous regulation or responses to pharmacological agents.

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