

BACHELOR'S DEGREE FINAL PROJECT

Montelukast as a pharmacological intervention to protect the brain from radiation-induced neurotoxicity

Main scope: Pharmacology and Therapeutics

Secondary scopes: Physiology and Physiopathology and Cell Biology

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ABBREVIATIONS

BBB	blood brain barrier
CA	cornu ammonis
CC	corpus callosum
CIR	cranial irradiation
CNS	central nervous system
CRT	cranial radiotherapy
CSF	cerebrospinal fluid
CysLT ₁ R	cysteinyl leukotriene receptor 1
DG	dentate gyrus
GCL	granule cell layer
GPR17	G protein coupled receptor 17
HC	hippocampus
IL-1	interleukin-1
IR	irradiation
LT	leukotriene
LTD4	leukotriene D4
MTK	montelukast
Olig2	oligodendrocyte transcription factor 2
OL	oligodendrocyte
RT	radiation therapy
TNF	tumour necrosis factor

1. ABSTRACT

Radiation therapy is one of the most effective tools in the treatment of malignant tumours and is applied to both adults and children who suffer from primary or metastatic brain tumours. However, in the treatment of paediatric malignancies is responsible of severe late effects, such as hormonal imbalances, growth retardation and cognitive decline. Alleviating these late effects could have a huge impact in the quality of life of the increasing number of children who survive their cancer.

Our aim was to investigate the possible protective effect in the brain of montelukast (MTK). Female mice received a double dose of 4 Gy to the whole brain separated by 12 h in between on postnatal day 21 (P21) and were sacrificed 12 days later. For cell count we looked at oligodendrocytes ($\text{Olig}2^+$ cells) in the corpus callosum (CC) and at proliferating cells ($\text{Ki}-67^+$ cells) in the hippocampus (HC). A difference after irradiation (IR) was observed in the $\text{Olig}2^+$ count, where non-IR mice showed higher numbers of oligodendrocytes compared with IR animals ($P < 0.01$). IR also affected the density of these cells ($P < 0.05$), but in no case was there a significant difference between IR NaCl and IR MTK groups. In the HC there was a significant reduction of MTK cells versus NaCl control cells ($P < 0.05$). Therefore, further studies are needed. In conclusion, we show that MTK does not protect neither against $\text{Olig}2^+$ cell loss in the CC nor $\text{Ki}-67^+$ loss in the HC after IR and that MTK lowers the number of proliferating cells in the HC regardless IR.

RESUM

La radioteràpia és una de les eines més efectives en el tractament de tumors malignes i s'aplica tant a adults com a infants que pateixen tumors primaris o metastàtics. No obstant, en el tractament pediàtric és responsable d'efectes retardats severs com descompensacions hormonals, retard del creixement i declivi cognitiu. Alleugerar aquests efectes podria tenir un gran impacte en la qualitat de vida del creixent nombre de nens que sobreviuen a la malaltia.

El nostre objectiu era investigar el possible efecte protector en el cervell del montelukast (MTK). Ratolins femella van rebre una doble dosis de 4 Gy a tot el cervell separada per 12 h entremig, en el dia postnatal 21 (P21) i van ser sacrificades 12 dies després. Pel comptatge de cèl·lules vam mirar el oligodendròcits ($Olig2^+$ cells) en el cos callós (CC) i les cèl·lules proliferatives (cèl·lules Ki-67 $^+$) a l'hipocamp (HC). Es va observar una diferència després de la irradiació (IR) en el comptatge d' $Olig2^+$ la qual mostrava que els ratolins no irradiats tenien un major nombre d'oligodendròcits comparat amb els animals irradiats ($P < 0.01$). La IR també va afectar la densitat d'aquestes cèl·lules ($P < 0.05$), però en cap cas va haver-hi una diferència significativa entre els grups IR NaCl i IR MTK. A l'HC hi havia una reducció significativa de cèl·lules MTK versus cèl·lules NaCl dels grups de control ($P < 0.05$). Per tant, es requereixen més estudis. Com a conclusió, mostrem que el MTK no protegeix ni contra la pèrdua de cèl·lules $Olig2^+$ en el CC ni contra la pèrdua de cèl·lules Ki-67 $^+$ en l'HC després de la radioteràpia i que el MTK disminueix el número de cèl·lules proliferant en l'HC independentment de la IR.

2. INTEGRATION OF THE THREE DIFFERENT SCOPES

This experimental project integrates three different scopes: pharmacology and therapeutics, physiology and physiopathology and cell biology.

As will be seen below, this project is divided in two parts: a bibliographic review and an experimental part. The experimental part consists in testing mice to check whether the drug montelukast is able to protect the brain from irradiated neurotoxicity, for that was necessary the knowledge of its distribution and mechanism of action thus pharmacology and therapeutics is the main scope.

Physiology and physiopathology scope is shown in the description of the structures and the cells from the brain. Finally, cell biology is important to understand how radiotherapy affects tumour and normal cells.

3. BACKGROUND

3.1. Brain tumours in childhood

Cancer is rare before the age of 20 (1), but out of all paediatric tumours, primary tumours of the central nervous system (CNS) are the second most common cancer in childhood (0 -14 years) comprising over 20% of cases and the third most common cancer type in adolescence (>10% of cases) in Europe (2).

Children diagnosed with CNS malignancies traditionally have had poor survival rates. Improvements in neurosurgical technique, supportive care, radiotherapy (RT) and the use of combination chemotherapy have improved 5-year survival for these patients. However, these treatments are associated with risk of long-term morbidity (3). Up to 80% of paediatric brain tumour survivors experience significant, life-long sequelae from treatment which do not only concern health and psychological aspects but also social challenges (4); for most of them, this cognitive deterioration leads to the need for special education (5).

3.2. Treatments

Treatment of brain and other CNS tumours depend on the histology, location, size, and other prognostic factors. Whenever possible, surgery is performed to remove as much of the tumour as possible. Subsequent chemotherapy and/or RT depend on the type of tumour, and optimal therapy requires coordinated efforts of paediatric specialists in fields such as neurosurgery, neuropathology, radiation oncology, and paediatric oncology (6).

3.2.1. Surgery

Most surgery procedures are performed with the intention to procure tissue to establish the histological diagnosis, to alleviate mass effect on the surrounding structures, and to achieve maximal cytoreduction in anticipation of subsequent adjuvant therapy (7).

3.2.2. Chemotherapy

Traditionally, the types of chemotherapeutic agents used in patients with brain tumours have included microtubule disrupting drugs (e.g., vincristine) which interfere in microtubule dynamics and stop cell division process (8), and alkylating agents which exert cytotoxic effects by transferring alkyl groups to DNA, thereby damaging the DNA and interfering with DNA transcription and cell division (9). Within the latter group are included platinum analogues (cisplatin and carboplatin), also nitrosoureas (e.g., carmustine, lomustine), cyclophosphamide, and ifosfamide, and more recently temozolomide. Inhibitors of topoisomerase I and II (camptothecins, etoposide) interfere with the action of these enzymes and impede the changes in the DNA helix (9), and have also been used either as single agents or in combination with alkylating drugs (10).

The main limitation of these drugs is their limited ability to cross the blood brain barrier (BBB). The concentrations within the CNS depend on multiple factors, including the permeability of the BBB to the chemotherapeutic agent, the extent to which the drug is actively transported out of the brain, and the drug volume of distribution in the brain parenchyma (11).

If they get through the BBB there is a risk of side effects as it has been demonstrated that many chemotherapeutic agents affect the precursor cells that contribute to hippocampal neurogenesis (the generation of new neurons) (12).

3.2.3. Radiotherapy

Despite the late effects of RT, it remains a major treatment modality for primary and metastatic neoplasms located in the CNS for both adults and children (13) because it is one of the most effective tools in the treatment of malignant tumours (14) (15). However, cranial radiotherapy (CRT), particularly when used in very young children, can result in neurocognitive deficits. For this reason, treatment protocols for patients with CNS tumours have been modified so that children aged younger than three years usually receive chemotherapy first in order to delay or reduce the radiation (6).

The success of RT depends on the total radiation dose and this one is limited by the tolerance of normal tissues surrounding the tumours since the purpose is to keep them healthful (16). Normal tissues show a narrow range of radiosensitivity while cells from human tumours show a very broad range of D_0 (dose required to reduce the fraction of cells surviving to 37%) values, hence, if the dose is too low no cells will be affected and, conversely, if the dose is too high all healthy and tumour cells will die (17). Ionizing radiation induces different kind of damage to the DNA (18). Normal cells have evolved sensors and pathways to respond to each one of these injuries but tumour cells cannot because they have their recombinational repair pathway altered (19). Besides, the radiosensitivity of cells varies considerably as they pass through the cell cycle and there seems to be a general tendency for cells to be more sensitive at or close to mitosis (20) (21).

The major reason why in the immature brain of both humans and rodents irradiation (IR) has more devastating and long-lasting effects than to older brains it is mainly due to its high content of progenitor cells, and that IR induces both an acute loss of progenitors through apoptosis and a perturbed microenvironment incompatible with normal proliferation and differentiation leading to a limited potential for repopulation (22).

3.3. Effects of irradiation

CRT during childhood can cause adverse side effects such as hormonal imbalances, growth retardation and persistent impaired learning and memory function. Risk factors are age at treatment, radiation quality, dose per fraction, volume treated, total dose and gender, where girls suffer more severe late effects than boys, although the reason for this difference is yet unknown (23) (24).

Referring to the neurological side-effects (Figure 1), it is possible to differentiate between acute (during radiation), early-delayed (up to 6 months post-irradiation), and late-delayed (more than 6 months to several years post-irradiation) (25). Acute brain injury is rare with current RT techniques. Early delayed brain injury can involve transient demyelination with somnolence, which is mainly seen in paediatric population but can

also affect adult patients in the first 2 months after therapy. Both of these injuries are usually reversible and resolve spontaneously. In contrast, late delayed brain injury which includes vascular abnormalities, demyelination, reduced neurogenesis, and white matter necrosis are irreversible and progressive (26) (27).

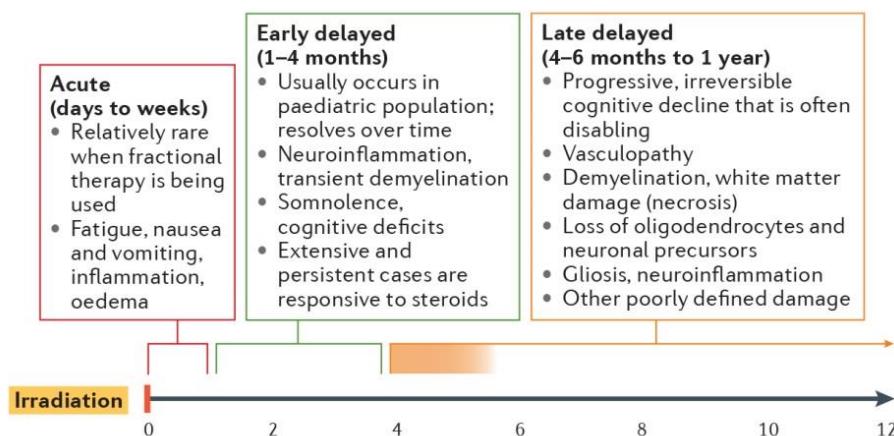


Figure 1. Symptoms of radiation-induced CNS. CNS manifestations after therapeutic intracranial IR follow this generally accepted time course (26).

3.4. Oligodendrocytes

OLs are the myelinating glial cells of the CNS (28). Most of them are located in white matter where their primary role is to form myelin. The myelin sheath is a membrane that surrounds axons and promotes the fast and efficient conduction of electrical impulse along those axons. The myelination of the developing brain is essential for the normal functioning of the mature CNS; disruption of CNS myelin lead to severe functional deficits and frequently a reduction in life span (29). Moreover, OLs accomplish distinct functional roles such as maintenance of axonal integrity and participation in signalling networks with neurons (30).

Due to the combination of a high metabolic rate with its toxic by-products, high intracellular iron, and low concentration of the antioxidative glutathione, OLs are particularly vulnerable to oxidative damage (31). For these reasons, OLs are thought to be the most radiation-sensitive glial cell type in the CNS. In rodents, the number of OLs reduces throughout the brain within a few hours of IR. Oligodendrocyte precursor cells

are widely distributed throughout the CNS (32) and they are rather susceptible to IR (20) (21). The majority (75%) of cells capable of division in the adult brain are oligodendrocyte precursor cells, this means they will be proliferating while getting IR. Loss of these cells is presumed to decrease the replacement of OLs (26). Although most major tracts are significantly myelinated by early childhood, later-maturing myelin sheaths continue to myelinate into the second and third decades of life and there is a significant remodelling of grey and white matter (33), pointing that OLs are still needed.

3.5. Corpus callosum

The corpus callosum (CC) is the largest white matter commissure and it is situated in the centre of the human brain (Figure 2). Therefore, its role is essential to the integration of the information between left and right cerebral hemispheres. In the human brain, the development of the CC is almost completed within the first four years in a child's life (34). However, its volume continues to increase through adolescence driven by myelination of the interhemispheric axons that compose the CC (35). Over adulthood, the structure may expand and decrease in size in different regions, strongly suggesting an ongoing process of remodelling (36).



Figure 2. Schematic illustration of a sagittal rodent brain with the CC outlined in gray and below it the HC in black (23).

The CC is affected in children subjected to CRT, with the greatest effects observed in the most posterior subregions (isthmus and splenium). In the normal brain, CC continues to develop in an anterior to posterior pattern well into the second decade of life but in the irradiated brain, depending on the exposure, there are subregion-specific changes. Damage to the CC interferes with the function of interhemispheric pathways (37).

3.6. Hippocampus

The hippocampus (HC) is an elongated structure in the medial temporal lobe (38) with a laminated structure. It consists of four parts: the dentate gyrus (DG); the cornu ammonis (CA) (subdivided in CA1, CA2, CA3 and CA4); the presubiculum; and the subiculum (39).

Neurogenesis occurs throughout life mainly in two regions, the subventricular zone in the lateral walls of the lateral ventricles and in a narrow band of tissue in the DG called the subgranular zone (SGZ) (Figure 3) (40). Neural stem cells in the DG are self-renewing cells which generate neurons, astrocytes, oligodendrocytes, and produce new dentate granule neurons (the principal cell type which constitutes the principal layer in the DG (41)) in all vertebrates studied, including humans (42).



Figure 3. A representative picture of the dentate gyrus of a mouse that shows the GCL outlined in discontinuous lines and the SGZ painted in black (picture taken in 5x).

The study of the epileptic patient H.M. who followed surgical removal of most of both of his medial temporal lobes as an experimental procedure to stop his seizures build the main theories about memory organization (43) . H.M. was incapable of forming new memories; he could not convert immediate memory into stable long-term memory, but surprisingly he could recall events and faces from before his surgery. This finding implied that the medial temporal lobe is not the ultimate storage site for previously acquired knowledge but memory can be supported by the neocortex and become independent of the first structure. With H.M. we also learn the term declarative memory which is what is meant when the word “memory” is used in everyday language and is dependent on the medial temporal lobe (44). The representations of facts (semantic memory) and events (episodic memory) that can be recalled consciously and verbalized constitute the

declarative memory (19). Many studies have identified that the HC plays a critical role in episodic memory, our capacity to remember everyday events in order (45); and also is involved in particular aspects of the acquisition of semantic knowledge (46). But still the major finding thanks to H.M. is the absolute need of a functioning HC to be able to consolidate new memories.

3.7. Montelukast

Montelukast (MTK), 1-((1(R)-(3-(2-(7-chloro-2-quinolinyl)-(E)-ethenyl)phenyl)-3-[(2-(1-hydroxy-1-methylethyl)phenyl)propyl]thiol]-methyl)cyclopropane acetate blocks the pro-inflammatory action of leukotriene D₄ (LTD₄) and it is approved for the maintenance treatment of asthma and to relieve symptoms of seasonal allergies. It acts by inhibiting neutrophil infiltration, balancing oxidant-antioxidant status, and regulating the generation of inflammatory mediators (47). LTD₄ is a pro-inflammatory mediator that promote vasoconstriction and increase vascular permeability; it is present in the CNS and it has been reported that causes BBB disruption and brain edema via the cysteinyl leukotriene receptor 1 (CysLT₁R) (48).

Probably due to its once daily dosing schedule (10 mg/day), safety and efficacy profiles MTK is the most prescribed CysLT₁R antagonist in Europe and the USA (49) and it has the same efficacy as pranlukast 225 mg twice daily and zafirlukast 40 mg twice daily. Moreover, there is no evidence of interaction with food unlike zafirlukast (50) (51).

Until a few years ago, only minimal distribution of MTK across the BBB had been described by the Food and Drug Administration (50), but in a study from Marschallinger *et al.* (52) they detected in a human subject taking 10 mg MTK per day the drug in the serum and in the cerebrospinal fluid (CSF). In addition, a re-analysis of the original CNS pharmacology data of MTK from the Food and Drug Administration indicates a significant BBB penetrance of the drug. Most remarkably, while in plasma and other organs MTK levels strongly decreased within 24h, the amount of drug in the brain increased and it was even higher than in plasma, suggesting the existence of an active transport mechanism through the BBB. This data proposes that the standard dose 10 mg per day in humans is sufficient to reach a therapeutic dose in the CSF.

In this same studio from Marschallinger *et al.* (52) they demonstrated that MTK reduces neuroinflammation, restores BBB integrity and increases neurogenesis, specifically in the brain of old rats antagonizing the CysLT1R and G protein-couple receptor 17 (GPR17). Although both receptors are found in the CNS, CysLTR1 expression was primarily found both *in vitro* and *in vivo* on non-neuronal cells, while GPR17 was predominantly present on cells of the neuronal lineage, suggesting that the MTK induced effects on neurogenesis might most likely be mediated through inhibition of GPR17 and not CysLTR1.

4. AIM OF THE WORK

The following project will investigate if a pharmacological intervention using MTK protect the developing brain from radiation-induced neurotoxicity.

Specific aims of the thesis

- I. To investigate MTK's effects on:
 - a) Survival of OLs in the CC after CIR.
 - b) Proliferation of cells in the GCL after CIR.

5. MATERIAL AND METHODS

The practical part of this project was realized as my Erasmus internship at the Department of Pharmacology in the Institute of Neuroscience and Physiology at the Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden. I joined the group of “Neurotoxicity in cancer survivors” whom principal investigator is Marie Kalm (PhD, Postdoc) and it is integrated also by Martina Boström (PhD, Postdoc), Erik Fernström (MD, doctoral student), Yohanna Eriksson (Msc, doctoral student) and Rita Grandér (BMA).

5.1. Animals

Female C57BL/6J mice were used (Charles River Laboratories, Sulzfeld, Germany). Animals were kept on 12-h dark/light cycle with food and water *ad libitum*. All experiments were approved by the Gothenburg committee of the Swedish Animal Welfare Agency (72-2015).

5.2. Montelukast treatment

Montelukast sodium powder (Sigma, St. Louis, MO, USA), dissolved in ethanol for maximum solubility and then further diluted with a 0.9% saline (NaCl) solution, was administered daily per intraperitoneal injection (i.p.) at a dose of 10 mg kg^{-1} of body weight for 14 days to young mice ($N = 4 - 9$ mice/group), starting 2 days before cranial irradiation (CIR) (the timeline of the study is presented in Figure 4). The animals were treated with MTK at a dose that pharmacologically resembles the one that is approved for its use in humans and that is enough to reach a therapeutic effect in the CSF (52). Age-matched group control mice received i.p. injections of the vehicle solution (10% ethanol in 0.9% saline solution) for 14 days, also starting 2 days before CIR. Weight and general appearance of the mice were recorded daily during the course of the experiment.

Criteria for early termination of treatment were obvious signs of pains, apathy and loss of weight more than 15%.

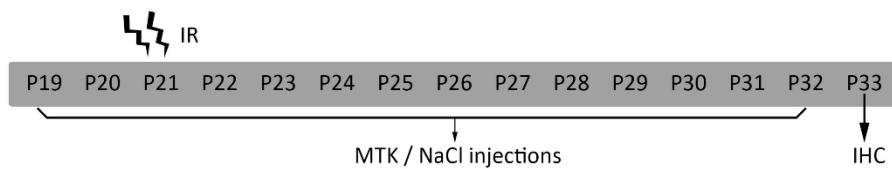


Figure 4. Outline of the experimental design. The animals were IR on postnatal day 21 (P21). The mice were injected during all the days of the experiment with either MTK or NaCl and sacrificed 12 days after IR.

5.3. Irradiation procedure

For the IR procedure, a linear accelerator (Varian Clinic 600 CD; Radiation Oncology Systems LLC San Diego, CA, USA) with a 4 MV nominal photon energy and a dose rate of 2.3 Gray (Gy) per minute was used.

Mice were postnatal day 21 (P21) when irradiated which is equivalent to a young child (53). They were anesthetized with isoflurane (0.6 - 4%) via a mask during the procedure and then placed in prone position on a polystyrene bed. To obtain an even radiation dose throughout the underlying tissue, the head was covered by a 1 cm tissue equivalent material. The whole brain was irradiated with an IR field of 2 x 2 cm. The source to skin distance was ~ 99.5 cm. A dose of 4 Gy was administered twice, with an interval of 12 h between fractions. A double dose of 4 Gy is clinically relevant and similar to a total dose of 18 Gy (54). This dose represents a clinically relevant dose, equivalent to the total dose used in treatment protocols for prophylactic CIR in selected cases of childhood acute lymphatic leukaemia (18 GY). The doses used for paediatric brain tumours are often higher, up to 55 Gy. The sham control animals were anesthetized but not subjected to irradiation (55). After IR the pups were returned to their dams. Animals were euthanized 12 days post IR for immunohistochemistry.

5.4. Tissue preparation

Animals were deeply anesthetized with sodium pentobarbital (Pentothal®; Electra-box Pharma, Tyresö, Sweden) before being transcardially perfused with a 6% formaldehyde solution buffered with sodium phosphate at pH 7.4 and stabilized with methanol (Histofix™; Histolab Products AB, Sweden). The brains were immersion-fixed in Histofix for 24 h after perfusion; this was changed to 30% sucrose solution containing 100 mM phosphate buffer, pH 7.5. The right hemisphere was cut into 25 µm sagittal sections in a series of 12, using a sliding microtome manufacturer (SM2010R, Leica Microsystems Wetzlar, Germany). The sections were stored in a cryoprotection solution, containing 25% ethylene glycol and 25% glycerol, at 4°C until staining.

5.5. Immunohistochemistry

All staining was performed using free-floating staining protocols. Sections were rinsed three times in Tris-buffered saline (TBS) and incubated in 0.6% hydrogen peroxide (H₂O₂) in TBS for 30 min to block endogenous peroxidases and minimize unspecific staining. Sections were then rinsed three times in TBS and incubated for 30 min in 3% donkey serum (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA) dissolved in TBS containing 0.1% Triton X-100 (Merck KGaA, Darmstadt, Germany) to block nonspecific binding. The sections were incubated overnight at 4°C with primary antibodies (Table 1). The next day, the sections were washed in TBS and a biotinylated secondary antibody was added (donkey anti-rabbit, 1:1000, Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA) and incubated at room temperature for 60 min. Sections were then rinsed three times with TBS and incubated with Avidin-Biotin-Peroxidase (10 µl/ml TBS of A and B, Vectastain Elite ABC Kit, Vector Laboratories, Burlingame, CA, USA) for 1 hour to let the ABP complex attach to the secondary antibodies. The sections were washed three times and then the staining was developed in 3,3'-diaminobenzidine (DAB, Saveen Werner AB, Malmö, Sweden), diluted in TBS with hydrogen peroxide (H₂O₂) and nickel chloride (NiCl₂) for enhancement of the reaction. To stop the reaction, the sections were washed several times in tap water and then placed in TBS until mounting from 0,1M phosphate buffer, pH 7.5, on glass slides.

Sections were left to dry overnight and then cover slipped with X-Tra-Kitt (Medite GmbH, Burgdorf, Germany).

Antibody	Dilution	Company
Rabbit anti-Olig2	1:500	Abcam
Rabbit anti-Ki-67	1:1000	Abcam

Table 1. List of primary antibodies.

5.6. Olig2 and Ki-67 markers

Oligodendrocyte transcription factor 2 (Olig2) is a basic helix-loop-helix transcription factor necessary for oligodendroglial development (56). It is one of the most consistent markers of the OL lineage, being expressed at all stages and having a clear role in OL progenitor cells specification in most regions of the CNS. However, its function during later stages of development is yet unclear (32).

Ki-67 is an excellent marker to determine the growth fraction of a given cell population because its expression is strictly associated with cell proliferation. The Ki-67 antibody is reactive with a nuclear structure present during all active phases of the cell cycle (G_1 , S , G_2 , M) and is absent from resting cells (G_0) (14).

5.7. Cell counting and volume assessment

For quantification of Olig2⁺ cells in the CC, analyses were performed blinded on coded slides. Olig2⁺ cells were counted throughout the CC in all sections containing a clearly separated/divided dorsal and ventral HC (see Figure 5 for a representative picture), using Stereo Investigator program (MBF Bioscience Inc., Williston, VT, USA). The CC was visualised at 5x and then traced at 10x magnifications on sagittal cut sections using a Leica DM6000B microscope (Leica Microsystems, Wetzlar, Germany). The GCL volume was measured as the area between the hilus and the molecular layer. The inner length of the GCL was measured as the border between the GCL and the hilus. Ki-67⁺ cells were

counted only in the SGZ, defined as ranging from three cell layers into the GCL to two layers into the hilus. Micrographs were taken with a Microfire camera (Optronics, Goleta, CA, USA). All immunopositive cells in the CC and in the GCL were counted in every 12th section in the right hemisphere in 20x magnification, resulting in 4-7 sections per animal. Area (mm²), volume (mm³), total Olig2⁺ cells, total Ki-67⁺ cells and density (cells/mm³) were analysed. Total volumes were calculated according to the Cavalieri principle, using the following formula: $V = SA \times P \times T$, where V is the total volume, SA is the sum of area measurement, P is the inverse of the sampling fraction and T is the sections thickness. The total number of cells was obtained by multiplying the number of counted cells with the sampling fraction.

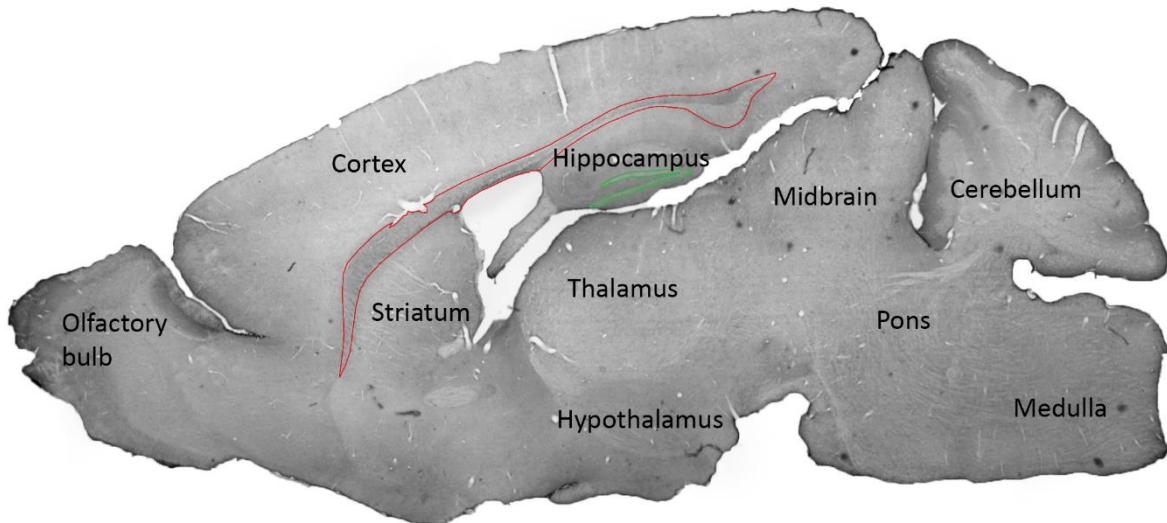


Figure 5. A representative picture of a sagittally cut section of a mouse brain chosen for counting (image taken in 1.25x magnification). The CC is outlined in red and the GCL in green.

5.8. Statistical analysis

All results are plotted as mean \pm standard error of the mean (SEM), with $P < 0.05$ considered statistically significant. Statistical analyses were performed using GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA, USA). Statistical comparisons were performed with an unpaired, two-way analysis of variance (two-way ANOVA). Significant

differences were analysed using a multiple comparison adjustment of P values (Tukey's post-hoc test for two-way Anova).

6. RESULTS

Survival of cells in the CC in the young mouse brain following CIR. Oligodendrocytes in the CC: Olig2 was used as a marker of OL in the CC. Statistical comparison by two-way Anova revealed significant changes after IR in the Olig2⁺ count, where non-IR animals showed higher numbers of OLs compared with IR animals ($F_{1,21} = 10.44, P = 0.004$, Figure 6b). Post-hoc Tukey revealed no significant differences between groups. Overall IR decreased the number of OLs by 43% in NaCl treated animals and by 50% in MTK animals. There was no significant effect from MTK in the total number of cells.

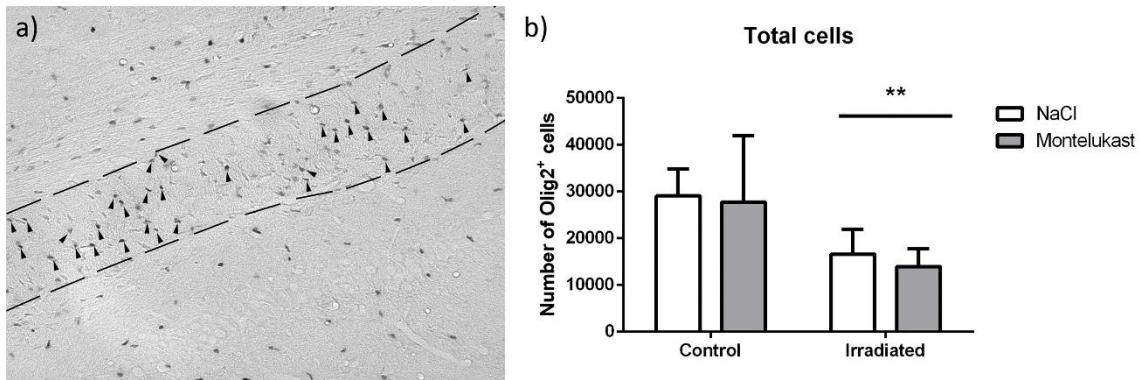


Figure 6. (a) Olig2 immunostaining in the CC of a mouse (black arrowheads point toward representative Olig2⁺ cells) (image taken in 20x magnification). (b) Number of Olig2⁺ cells in the CC. Data are shown as mean \pm S.E.M (N = 4-9 mice/group). Two-way Anova results are indicated on the graphs: ** $P < 0.01$ for irradiation.

Volume of the CC following CIR. The CC was traced in all mice. Two-way Anova results show no difference in volume between groups ($F_{1,21} = 1.669, P = 0.2105$ for irradiation; $F_{1,21} = 0.0001086, P = 0.9918$ for drug treatment; overall statistical comparisons by two-way Anova followed by post-hoc Tukey test; Figure 7a).

Density of the CC following CIR. Analysis of data displays a significant difference ($F_{1,21} = 7.226, P = 0.0138$, Figure 7b) where IR animals have lower Olig2⁺ cells/mm³ than control animals. The irradiated NaCl group had 33% less OLs compared to the NaCl control group. For the MTK treated group the difference was 45%. Post-hoc Tukey did not show any significant difference between groups revealing that MTK offers no benefit over NaCl.

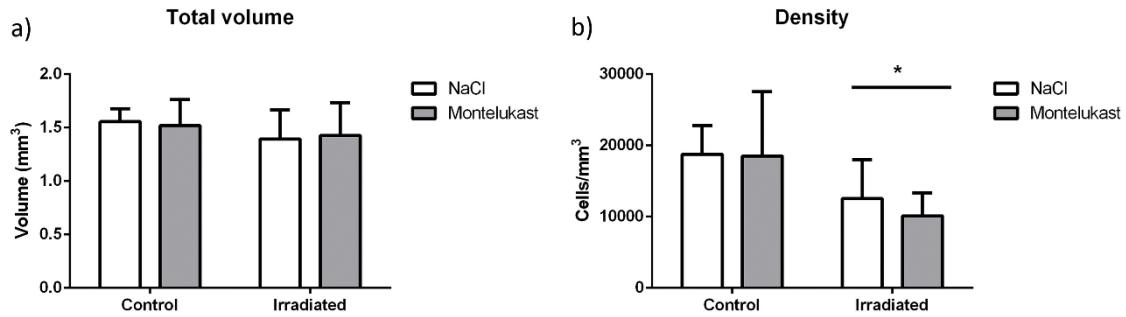


Figure 7. a) Shows total volume (mm^3) of the CC. (b) Shows a quantifications of Olig2^+ cells/ mm^3 in the CC. All data shown as mean \pm S.E.M (N = 4-9 mice/group). Two-way Anova results are indicated on the graphs: * $P < 0.05$ for irradiation.

Proliferation of cells in the GCL in the young mouse brain following CIR. *Proliferating cells in the DG:* marker Ki-67 was used followed by quantification of labelled cells. Analysis of the total cells shows a significant difference ($F_{1,22} = 5.434, P = 0.0293$, Figure 8b) between NaCl and MTK. Post-hoc Tukey analysis revealed a significant difference specifically between MTK control and NaCl control. There was a difference of 52% less Ki-67⁺ cells in MTK control mice than in NaCl control group.

Volume of the GCL following CIR. After measuring the volume of the GCL the double-way Anova shows a significant difference in the irradiated group ($F_{1,21} = 7.621, P = 0.0114$, Figure 8c). Post hoc analysis reported a difference between control and irradiated MTK treated mice. The reduction for MTK group was of 33% whereas for NaCl was just of 8%.

Density of the GCL following CIR. The density of the Ki-67⁺ cells in the GCL follows the same pattern as for total number of cells, even if the differences did not reach a significant level ($F_{1,22} = 0.06932, P = 0.7948$ for drug treatment; $F_{1,22} = 3.654, P = 0.0690$ for irradiation; statistical comparisons by two-way Anova followed by post-hoc Tukey test; Figure 8d). Interestingly, the density was a 30% higher after IR in the treatment with MTK whereas in the case of NaCl there was a decline of 28%.

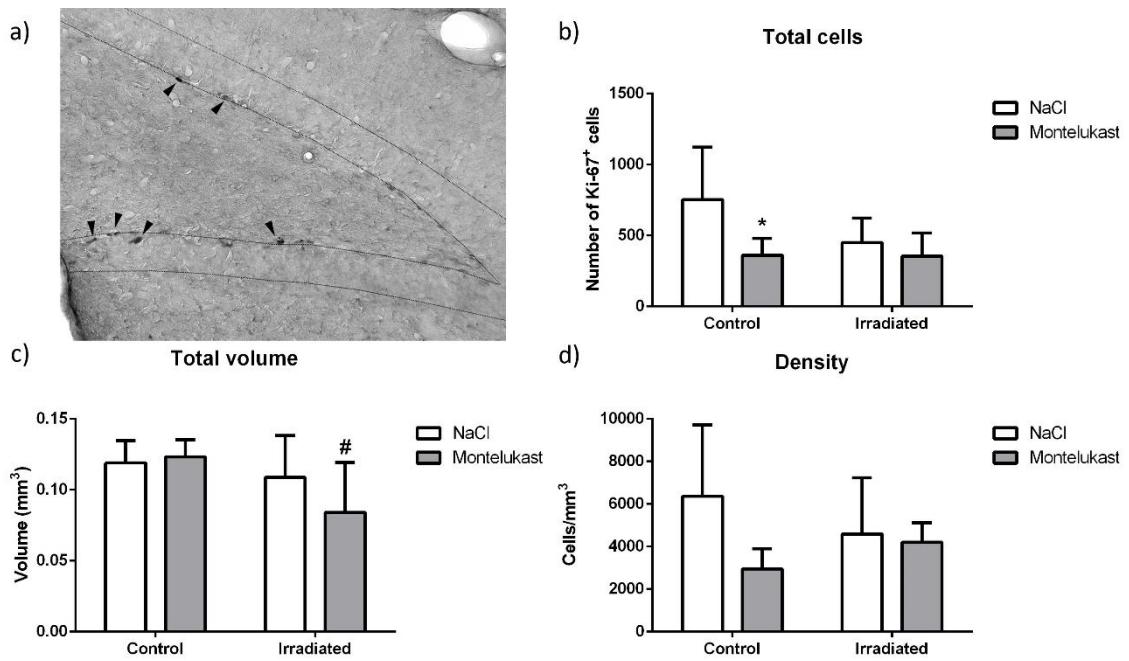


Figure 8. (a) Ki-67 immunostaining in the DG of a mouse (black arrowheads point toward representative Ki-67⁺ cells) (image taken in 10x magnification). (b) Number of Ki-67⁺ cells in the GCL. (c) Shows total volume (mm³) of the GCL. (d) Shows a quantifications of Ki-67⁺ cells/mm³ in the GCL. Two-way Anova showed a significant effect of the variable drug treatment in the number of Ki-67⁺ cells ($F_{1,22} = 5.434, P = 0.0293$) and a significant difference for irradiation in the total volume ($F_{1,22} = 7.621, P = 0.0114$). Data are shown as mean \pm S.E.M (N = 4-9 mice/group). Post-hoc Tukey test results are indicated on the graphs: * $P < 0.05$ control MTK vs control NaCl; # $P < 0.05$ irradiated MTK vs control MTK.

7. DISCUSSION

OLs and the myelin sheaths they form belong to the most vulnerable cellular components of the CNS. Due to the combination of a high metabolic rate with its toxic byproducts, high intracellular iron, and low concentration of the antioxidative glutathione, OLs are particularly vulnerable to oxidative damage. Hence, oxidative damage is a common contributor to OL loss under many pathological conditions (31). Further, DNA damage is induced directly by ionization of DNA or indirectly by generation of free radicals, resulting in apoptosis and loss of cells (22). After brain radiotherapy, oxidative stress is thought to result from an inflammatory response where IR activates microglia and causes immune cells to infiltrate the brain. These cells then generate reactive oxygen species which in turn activate more microglia and activate more immune cells that can maintain or increase the level of oxidative stress (27). The susceptibility of the myelin membrane to oxidative impairment may account for the predilection of radiation injury for white matter (13). OL loss can also occur as a result of exposure to inflammatory cytokines (31), which are shown to increase acutely in response to CRT (5). All this together explains the reduction of cells seen after IR in the CC (Figure 6b).

The DG is also known to be affected by IR, in particular the proliferating cells in the SGZ of the GCL, leading to a limited capacity for repopulation or regeneration. This may occur in at least three ways: (i) through cell death (apoptosis or mitotic catastrophe), (ii) through decrease proliferation and (iii) through perturbed precursor cell proliferation (57). Loss of proliferating neural stem and progenitor cells results in impaired growth and a smaller GCL volume than in non-irradiated brains (Figure 8c) (58).

Leukotrienes (LT) levels are typically increased in brains affected by neurodegenerative diseases. The elevated levels of 5-lipoxygenase and LTs cause post-inflammatory brain damage by mediating the generation of reactive oxygen species, resulting in a higher expression of inflammatory mediators such as tumour necrosis factor (TNF) alpha and interleukin-1 (IL-1) beta. This might consequently result in reduced neurogenesis, as TNF alpha and IL-1 beta signalling is known to inhibit neural stem cell proliferation and neurogenesis (59). Taking into account the mechanism of action of MTK, the anti-

inflammatory effect by MTK could potentially protect against OL cell death, therefore it was expected to see a higher number of Olig2⁺ cells in the IR animals treated with MTK than in NaCl animals. Although there is no significant difference, there are 16% and 21% less cells in MTK IR mice compared with NaCl IR in the CC and GCL respectively (Figure 6b) so it seems like the treatment is not working for preventing cell death. Surprisingly, we also observed differences between NaCl and MTK control animals in the GCL counting (Figure 8b). Apparently, MTK was inhibiting proliferation of cells. This correlates with another finding from the group (unpublished data) where they found a reduction in the number of microglia cells in both control and irradiated MTK animals compared with NaCl animals (Figure 9). Taken together, these findings indicate that MTK at the dose of 10 mg kg⁻¹ actually has a somewhat toxic effect on certain cells in the brain of young mice. Another explanation could be that leukotrienes are important for the developing brain and hence affect the proliferation if they are inhibited.

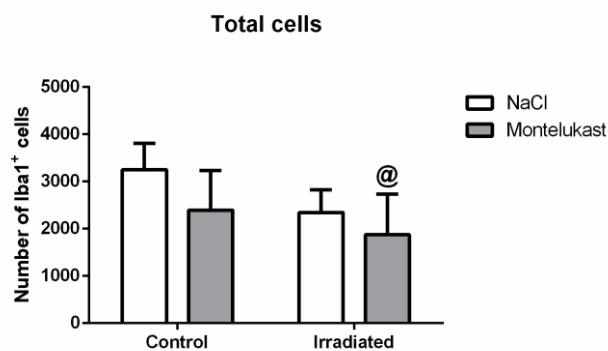


Figure 9. Number of Iba1⁺ cells in the granule cell layer. Two-way Anova showed a significant effect of the variables irradiation and drug treatment in the number of Iba1⁺ cells ($F_{1,24} = 6.814, P = 0.0153; F_{1,24} = 5.873, P = 0.0233$; respectively). Data are shown as mean \pm S.E.M (N = 5-9 mice/group). Post-hoc Tukey results are indicated on the graphs: @ $P < 0.05$ for IR MTK versus control NaCl. Kalm M., Boström M., Eriksson Y. (2017). Unpublished data.

In this occasion, it seems that MTK is also being toxic for microglia in control animals because MTK mice should be more or less at the same level as NaCl mice and in the IR group the levels of microglia should be higher because they are the key regulators of inflammation in the brain. However, preclinical safety data from the European Medicines Agency showed that signs of toxicity in animals were increased excretion of

saliva, gastrointestinal symptoms, loose stools and ion imbalance. These occurred at dosages which provided more than 17-fold the systemic exposure seen at the clinical dosage. None of our treated mice showed any of these physiological symptoms. Furthermore, no deaths occurred following a single oral administration of MTK at doses up to 5.000 mg kg^{-1} in mice, the maximum dose tested (60).

In other neuroprotective agents it has been reported a bell-shaped dose-effect relationship against brain ischemia where above some doses the drug is ineffective. For example, it was observed that pranlukast had its maximal effect in improving neurological deficiency and inhibiting infarct volumes at 0.03 and 0.1 mg kg^{-1} (61). Also, for MTK it was showed its effectiveness at doses of 0.1 and 1.0 mg kg^{-1} in focal cerebral ischemic mice (62). Although the exact reasons are unclear it has been proposed that higher doses might cause cerebral vasoconstriction, reduction of blood pressure, and hypoglycaemia (61). In any case, in the study of pranlukast there are no analysis of its presence in serum, brain and CSF, being assumed (owing to previous experiments) that due to an accumulation of arachidonic acid and its products, such as LTC₄, after ischemia a BBB dysfunction occurs which helps pranlukast going through. The same situation happens for the following experiment with MTK. Increased vascular permeability and BBB disruption after irradiation have been well documented. Data from Kalm *et al.* (63) shows that BBB permeability is increased 24 h after a single dose of 8 Gy to the young mouse brain. In our case it was a dose of 4 Gy administered twice, with an interval of 12 h to avoid severe side-effects (55), and it has been reported that the increase in BBB permeability has a slow and delayed response, from weeks to months depending on the radiation dose, for fractionated RT compared to the single-dose IR (64) (65) . It is unclear to know when exactly the permeability increase of the BBB occurred, but even if it took place during the course of the experiment we must consider that in the article from where we extrapolated the dose the BBB was also disrupted because of neuroinflammation and ageing. In behalf of that, we chose to administer the 10 mg kg^{-1} body weight dose considering that the data demonstrate that MTK crosses the BBB at a therapeutic dose, and that age-dependent differential BBB integrity does not affect the capacity of MTK to enter the brain (52).

It is possible that this previously mentioned bell-shaped dose-effect is also the case of MTK and with the dose of 10 mg kg^{-1} used in this project we are beyond this bell-shaped curve so maybe we should try a lower dose. Furthermore, we are using young mice and this dose was extrapolated from a study performed on adult rats so there are some differences to consider, being the most important the species and then the contrast in weight and age.

Moreover, in our experiment MTK was i.p. injected but it is and orally active agent and the evidence for its BBB penetrance with the used dose is based on oral administration; although pharmacokinetics of substances administered i.p. are more similar to those seen after oral administration than to parenteral route because they may undergo hepatic metabolism before reaching the systemic circulation (66). Thus, it would be advisable to assess the effect of MTK after the oral administration.

Measuring the weight of the animals on a daily basis as a routine control we found out that, after IR, the ones treated with MTK did not lose as much weight as the NaCl mice (Figure 10) so MTK seems to be protecting against weight-loss after RT. It cannot be due to an increasing in the eating because control MTK are in the same weight as control NaCl, and at this age the mice are not weaned. Nonetheless more experiments would be required to investigate this point further.

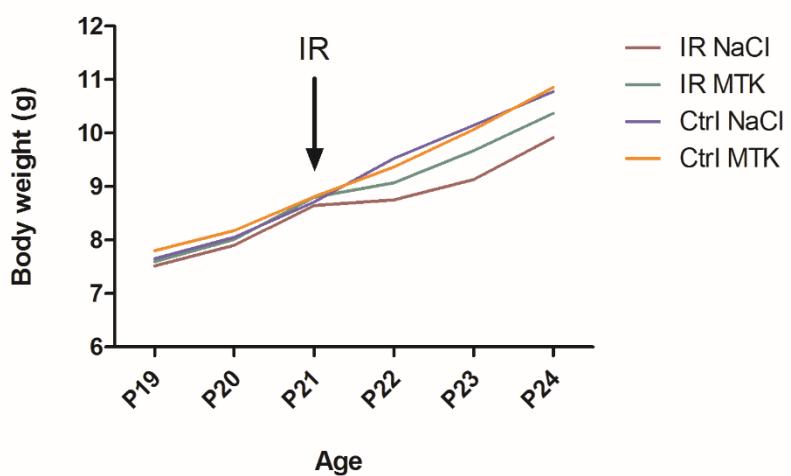


Figure 10. The mice were treated with either NaCl or montelukast for 14 days, starting 2 days before cranial radiotherapy. The weight was measured on a daily basis.

8. CONCLUDING REMARKS

This thesis concludes that montelukast does not protect the brain from radiation-induced neurotoxicity.

Specific conclusions to given aims:

- I. MTK does not protect against OL loss in the CC after IR.
- II. MTK does not protect proliferating cells in the GCL after IR.
- III. MTK lowers the number of proliferating cells in the GCL regardless of IR.
- IV. MTK protects against acute weight-loss after IR in young mice.

Finally, it would be necessary to perform more experiments using a lower dose with no toxic effects for the mice. For that, first it would be required to test MTK serum levels of young mice with a dose de-escalation to check that still pharmacologically resembles the plasma concentration after oral administration approved for its use in humans.

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