Cyclooxygenase-2 inhibitor prevents radiation-enhanced infiltration of F98 glioma cells in brain of Fischer rat

Guillaume Desmarais1, Gabriel Charest1, David Fortin2, Rachel Bujold1,3, David Mathieu2 & Benoit Paquette1

1Center for Research in Radiotherapy, Department of Nuclear Medicine and Radiobiology, Faculty of Medicine and Health Sciences, Université de Sherbrooke, Québec; 2Department of Surgery, Division of Neurosurgery/Neuro-oncology, Québec, and 3Division of Radiation Oncology, Centre Hospitalier Universitaire de Sherbrooke, Sherbrooke, Québec, Canada

Abstract
Purpose: Radiation induces a neuro-inflammation that is characterized by the expression of genes known to increase the invasion of cancer cells. In Fischer rats, brain irradiation increases the infiltration of cancer cells and reduced the median survival of the animals. In this study, we have determined whether these adverse effects of radiation can be prevented with the cyclooxygenase-2 (COX-2) inhibitor meloxicam.

Materials and methods: Brain of Fischer rats treated or not with meloxicam were irradiated (15 Gy) and then implanted with the F98 glioma cells. The median survival of the animals, the infiltration of F98 cells, and the expression of inflammatory cytokines and pro-migration molecules were measured.

Results: Meloxicam reduced by 75% the production of prostaglandin E2 (bioproduct of COX-2) in irradiated brains validating its anti-inflammatory effect. Median survival was increased to control levels by the treatment of meloxicam following brain irradiation. This protective effect was associated with a reduction of the infiltration of F98 cells in the brain, a complete inhibition of radiation-enhancement of matrix metalloproteinase-2, and a significant reduction of tumor necrosis factor α (TNF-α) and tumor growth factor β1 (TGF-β1) expression. Using invasion chambers, interleukin-1β (IL-1β) stimulated by 5-fold the invasiveness of F98 cells, but this stimulation was completely inhibited by meloxicam. This suggests that a cooperation between IL-1β and COX-2 are involved in radiation-enhancement of F98 cell invasion.

Conclusions: Our results indicate the importance of reducing the inflammatory response of normal brain tissue following irradiation in an effort to extend median survival in F98 tumor-bearing rats.

Keywords: Cyclooxygenase-2 inhibitor, glioblastoma multiforme, interleukin-1β, matrix metalloproteinase, cancer cell infiltration, radiotherapy

Abbreviations: ATCC, American Type Culture Collection; TUBBS, anti-tubulinβ5; COX, cyclooxygenase; dNTP, deoxyribonucleotide; GBM, glioblastoma multiforme; H&E, haematoxylin and eosin; IL-1β, interleukin-1β; IL-6, interleukin-6; LC/MS/MS, liquid chromatography tandem mass spectrometry; MMP, matrix metalloproteinase; PBS, phosphate buffered saline; PTEN, phosphatase and tensin homolog; PGE2, prostaglandin E2; PGE2-d4, prostaglandin E2-d4; PLA2, phospholipase A2; PUM1, pumilio homolog 1; qPCR, quantitative PCR; RPL19, ribosomal protein L-19; SDS, sodium dodecyl-sulfate; TGF-β1, tumor growth factor β1; TNF-α, tumor necrosis factor α.

Introduction
Glioblastoma multiforme (GBM) is the most aggressive primary brain neoplasm, taking the lives of patients within a median of 12–15 months after diagnosis and standard treatment (Stupp et al. 2005). Because of their infiltrative nature, complete resection of GBM tumors is a virtually unreachable goal (Brandes et al. 2008). Therefore, the objective of the surgery is to maximize the extent of resection, thereby alleviating neurological deficits caused by the local mass effect, and allowing a decrease in steroids doses (Berger and Hadjipanayis 2007). Cancer cells left behind are targeted using radiotherapy and chemotherapy.

Ionizing radiation produces a therapeutic effect by eradicating tumor cells, but also causes damage to the surrounding healthy tissues. Therefore, acute and late effects of radiotherapy on the brain are common and represent a significant source of morbidity (Lawrence et al. 2010). The acute side-effects of radiotherapy include nausea, vomiting, headache, vertigo and seizures, while late effects such as cognitive disturbance can be seen (Anand et al. 2012). This narrow therapeutic index leads to an optimization of radiation dose based on the overall tolerance of healthy tissues instead of giving a high therapeutic dose that would eliminate most cancer cells (Chakravarti and Palianichamy 2008).
The short life expectancy after treatment is associated with a rapid recurrence of the glioma, typically within 2–3 cm from the resection cavity (Burger et al. 1983, Gaspar et al. 1992). Although this rapid infiltration and proliferation of glioma cells after treatment has been known for decades, the molecular mechanisms involved are still largely unknown (Mangiola et al. 2010). Therefore, to extend the life expectancy of GBM patients, it is paramount to identify what stimulates the infiltration of glioma cells in brain and then assess new therapeutic modality to prevent it.

In an animal model developed to mimic the stimulation of glioma cell infiltration in brain after irradiation, it was shown that a sublethal in vitro irradiation of rat 9L glioma cells resulted in a greater number of tumor satellites after their injection into the striatum of rat brain (Wild-Bode et al. 2001). Another study using the rat glioma F98 cells showed that irradiation of these cancer cells has only a marginal effect. Stimulation of the F98 cell infiltration was mainly induced by the irradiated healthy brain and resulted in a reduction of median survival of rats bearing this tumor (Desmarais et al. 2012). This stimulation was associated with pro-infiltration mediators released from irradiated normal brain, such as cyclooxygenase-2 (COX-2), interleukin-1β (IL-1β) and matrix metalloproteinase-2 (MMP-2) (Desmarais et al. 2012). This suggests that the rapid recurrence of GBM could be partially attributed to an inflammatory response induced by radiation in the brain. This adverse effect of radiation was also observed in other cancers. For example, irradiation of the mouse mammary gland stimulated the migration of breast cancer cells, and increased the number of circulating tumor cells and the number of lung metastases (Bouchard et al. 2013).

In the present study, we assessed the ability of the COX-2 inhibitor meloxicam to prevent the stimulation of F98 cell infiltration that is induced by irradiated brain of Fischer rats, and determined whether this inhibition of COX-2 led to an increase of the median survival of the animals. To do so, the F98 cells were implanted after irradiation of the brain. This protocol eliminated the possibility that a reduction of cancer cell infiltration would be caused in part by toxic effects of radiation on these cells. Thus, the specific effect of meloxicam on the irradiated brain was better addressed. The effect of meloxicam on the expression of inflammatory cytokines IL-1β, interleukin-6 (IL-6), tumor growth factor β1 (TGF-β1) and tumor necrosis factor α (TNF-α) in irradiated animals was also assessed.

Materials and methods

Cell culture

The murine cell line F98 was obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) and tested negative for the mouse antibody production (MAP) assay by Charles River Laboratories (Wilmington, MA, USA). Cells were grown in monolayer using Dulbecco’s Minimal Essential Medium supplemented with 10% foetal bovine serum (Gibco, Burlington, ON, Canada), 26.2 mM of sodium bicarbonate, 2 mM L-glutamine and a mix of penicillin (100 μl/ml) and streptomycin (100 μg/ml). Cells were incubated at 37°C in a humidified environment with 5% CO₂ and propagated upon confluence every 3 days.

Animals and groups studied

The experimental protocol was approved by the institutional ethical committee and complied with regulations of the Canadian Council on Animal Care. Adult male Fischer rats weighing 225–260 g were bought from Charles River laboratories (Montreal, QC, Canada). Animals were kept into our specialized facility and fed ab libitum prior to surgery and irradiation. Animals were randomly assigned to one of the following groups (n = 8–10 per group): Group 1, tumor implantation without irradiation; group 2, brain irradiation (15 Gy) and tumor implantation 1 day later; group 3, tumor implantation without irradiation + meloxicam; and group 4, brain irradiation (15 Gy) and tumor implantation 1 day later + meloxicam. Meloxicam was administered daily by intraperitoneal injection starting 48 h prior irradiation until the animals were sacrificed on day 15. No toxicity was observed in animals treated with Meloxicam. Half of each group was sacrificed 15 days post tumor implantation to measure the infiltration of F98 cells in brain. The other half was used to assess the median survival time. The protocol for animal experiments is show in Figure 1.

Tumor implantation

Anaesthesia was induced by an intraperitoneal injection of ketamine:xylazine (87:13 mg/kg). Animals were then mounted onto a stereotactic frame. A midline incision was performed and a burr hole was punctured using a 16-gauge needle. The burr hole coordinates were, using the bregma as a reference point, 1 mm anterior, and 3 mm lateral to the right. Then 1 × 10⁴ cells suspended in a volume of 5 μl of non-supplemented Dulbecco’s Minimal Essential Medium were injected into the brain with a 26-gauge needle at a depth of 6 mm from the surface of the skull. The suspension of cells was infused at a constant rate of 1 μl/min using a micro-infusion pump (WPI model UMP3, Sarasota, FL, USA).

Figure 1. Experimental protocol and animal groups. Group 1: Tumor implantation without irradiation. Group 2: Brain irradiation (15 Gy) and tumor implantation 1 day later. Group 3: Tumor implantation without irradiation + meloxicam. Group 4: Brain irradiation (15 Gy) and tumor implantation 1 day later + meloxicam. For groups 3 and 4, meloxicam was injected daily by intraperitoneal starting 48 h prior irradiation until the animals were sacrificed at day 15. Half of each group was sacrificed 15 days post tumor implantation to measure the infiltration of F98 cells in brain. The other half was used to assess to the median survival time.
The needle was then slowly withdrawn to minimize the risks of subdural and extracranial seeding. Animals were observed daily for apparition of signs of neurological deficits (hemiparesis, ataxia) and increased intracranial pressure (lethargy and cachexia).

**Brain irradiation**

Rats were anesthetized (ketamine:xylazine, 87:13 mg/kg) and positioned on a stereotactic frame adapted for use in the Leksell Gamma Knife (model 4C) (Elekta AB, Norcross, GA, USA) (Charest et al. 2009). The 14-mm collimators were used to deliver to the whole brain a single dose of 15 Gy. Rats were then brought back to the animal facility for their recovery from anaesthesia and subsequent follow-up. In human, GBM are frequently treated with 60 Gy administered in 30 fractions of 2 Gy. Daily irradiation in animals can be performed using 3% isoflurane as an anesthetic, a procedure that we have already done in mice (Bouchard et al. 2013). However, this set-up was not available to irradiate rats, and 30 daily injections of ketamine:xylazine would be fatal. The Biological Equivalent Dose (BED) was calculated to compare the fractionated protocol of 60 Gy in 30 fractions with a single dose of 15 Gy. Using α/β ratio of 3, the BED calculated for a single dose of 15 Gy was 90 Gy. This is comparable to the clinical standard of 30 fractions of 2 Gy (BED of 100 Gy).

**Brain processing**

Euthanasia was carried out by exsanguination and intracardiac perfusion of 30 ml formaldehyde 4% for histological analysis, or phosphate buffered saline (PBS) for molecular quantifications. Brain specimens were removed and kept in formaldehyde for 48 h prior coronal plane sectioning using a brain matrix (taking the implantation needle mark on the cortex as a reference point for the slicing), and finally embedded into paraffin. The blocks were cut into 5 μm thick slides and stained with haematoxylin and eosin (H&E). For biomarker quantifications, brains were snap frozen into liquid nitrogen and pulverised into a fine powder and kept at −80°C for further quantifications.

**Morphological analysis of brain tumors**

Brain sample slices, including the F98 tumor, were scanned for each animal using a Nikon 9000 film scanner to get a view of the whole brain coronal section. Then tumor morphology was registered and analyzed using the Image Pro software (Media Cybernetics, Bethesda, MD, USA). Briefly, areas of interest were highlighted prior to analysis. Tumors were divided into two distinct areas: Primary tumor and invasive tumor. Areas corresponding to the primary tumor were defined as the central region with a well-defined edge, while the invasive areas corresponded to clusters of neoplastic cells which have migrated from the primary tumor and did not display direct physical contact with the primary nodule. The following parameters were measured: Surface of the primary tumor, overall surface of the invasive tumor, and distance of infiltration of neoplastic cell clusters from the edge of the primary tumor.

**Prostaglandins E2 quantification by liquid chromatography/tandem mass spectrometry**

Brains were snap frozen with liquid nitrogen and pulsed brain tissues (~ 100 mg) were homogenized with a dounce homogenizer in 3 ml of acetone-saline solution (2:1) containing 10 ng of prostaglandin E2-d4 (PGE2-d4) which contains four deuterium atoms at the 3′, 4′, and 4′ positions (internal standard, Cayman Chemical, Ann Arbor, MI, USA) and 0.05% butylated hydroxytoluene to prevent the oxidation of prostanoids. The homogenate was transferred to a screw-top tube, vortexed for 1 min, and centrifuged (10 min, 2000 g, 4°C). The supernatant was transferred to another tube and mixed with 2 ml hexane by vortexing for 1 min. After centrifugation (10 min, 2000 g, 4°C), the upper phase containing lipids was discarded. The lower phase was acidified with 30 μl 2 M formic acid and then 2 ml chloroform containing 0.05% butylated hydroxytoluene were added. The mixture was vortexed and again centrifuged (10 min, 2000 g, 4°C) to separate the two phases. The lower phase containing chloroform was transferred to a conical centrifuge tube for evaporation with a SpeedVac Concentrator (Sarant, Nepean, ON, Canada). Samples were reconstituted in 200 μl methanol:10 mM ammonium acetate buffer, pH 8.5 (70:30) prior to liquid chromatography/tandem mass spectrometry (LC/MS/MS) analyses. All extraction procedures were performed under low light and low temperature conditions to minimize potential photooxidation or thermal degradation of eicosanoid metabolites.

Prostaglandin E2 (PGE2) was quantified by LC/MS/MS using an API 3000 mass spectrometer (Applied Biosystem, Streetsville, ON, Canada) equipped with a Sciex turbo ion spray (AB Sciex, Concord, ON, Canada) and a Shimadzu pump and controller (Columbia, MD, USA). Prostaglandins were chromatographically resolved using a Kromasil column 100-3.5C18 150 × 2.1 mm (Eka Chemicals, Valleyfield, QC, Canada). A linear acetonitrile gradient from 45–90% during 12 min at a flow rate of 200 μl/min was used. The mobile phase consisted of water buffered with 0.05% of acetic acid (A) and acetonitrile 90% with acetic acid 0.05% (B). Injection volume was 5 μl per sample which were kept at 4°C during analysis. Individual products were detected using negative ionization and the monitoring of the transition m/z 351 → 271 for PGE2 and 355 → 275 for PGE2d4 at collision energy of −25 V. Area under the curves for specific ions were used for quantifications.

**Matrix metalloproteinase-2 quantification by zymography gel**

Brain tissues pulverized into a fine powder were homogenized with a RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 % Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl-sulfate (SDS) and protease inhibitor cocktail (BD, Mississauga, ON, Canada). The homogenates were then centrifuged at (10,000 g, 4°C) for 10 min. Supernatants were collected and stored at −20°C for further analyses. Protein quantification was made with the DC protein assay (Bio-Rad Laboratories, Mississauga, ON, Canada). Zymography analysis was performed as previously reported (Lemay et al. 2013).
et al. 2011). This gel allows the distinction between matrix metalloproteinases (MMP) and proMMP which migrate according to their molecular weights. After the electrophoresis, proMMP is artificially activated without losing its propeptide. Gels are then incubated in activation buffer which allows proMMP and MMP to cleave locally the gelatin resulting in clear bands after staining with Coomassie blue. Briefly, samples were applied on a 12% polyacrylamide-SDS gel containing 0.1% gelatine and electrophoresed at 150 V during 3 h at 4°C. After removal of SDS from the gel by incubating in 2.5% Triton X-100 (30 min, 4 times), the gel was incubated at 37°C for 18 h in 40 mM Tris-HCl pH 7.5, containing 10 mM CaCl₂, 1 μM ZnCl₂, 200 mM NaCl, and stained with Coomassie blue R-250. Gels were scanned and the bands analyzed using Image J 1.34n (public domain Java image, NIH).

mRNA levels measured by quantitative polymerase chain reaction
Total RNA extractions were performed on brain tissues pulverized into a fine powder using the Absolutely RNA Microprep Kit (Stratagene La Jolla, CA, USA) as previously described (Desmarais et al. 2012), and RNA integrity was assessed with an Agilent 2100 Bioanalyzer (Agilent, Montreal, QC, Canada). Reverse transcription was performed with 2 μg total RNA in a total volume of 20 μl containing Transcriptor reverse transcriptase, random hexamers, deoxyribonucleotides (dNTP) (Roche Diagnostics, Laval, QC, Canada), and 10 units of RNAseOUT (Invitrogen, Burlington, ON, Canada) following the manufacturer’s protocol. All forward and reverse primers were individually resuspended in stock solutions (20–100 μM) containing Tris-ethylendiaminetra-cetic acid buffer and subsequently diluted as a primer pair down to 1 μM in RNase DNase-free water (IDT, Coralville, Iowa, USA). Quantitative PCR (qPCR) reactions were performed in 10 μl in 96-well plates on a Realplex2 thermocycler (Eppendorf, Mississauga, ON, Canada) with 5 μl of 2X Fast-Start Universal SYBR Green Master mix (Roche Diagnostics, Laval, QC, Canada), 10 ng (3 μl) cDNA, and 200 nM final (2 μl) primer pair solutions. The following cycling conditions were used: 10 min at 95°C; 50 cycles: 15 sec at 95°C, 30 sec at 60°C, 30 sec at 72°C. Relative expression levels were calculated using the qBASE framework (Hellemans et al. 2007) and the housekeeping genes anti-tubulinβ (TUBB5), pumilio homolog 1 (PUM1) and ribosomal protein L-19 (RPL19) for rat cDNA. In every qPCR run, a no template control was performed for each primer pair and these were consistently negative. Primer sequences are listed in the Supplementary Table 1 (available online at http://informahealthcare.com/abs/doi/10.3109/09553002.2015.1043756).

Invasion assay
F98 cells (4 × 10⁵) harvested with Cell Dissociation Solution (Sigma, St. Louis, MO, USA) were added to the upper compartment of invasion chambers (BD Biosciences, Bedford, MA, USA). The role of PGE2 and IL-1β was assessed by adding them individually at 10 ng/ml to the lower and upper compartments. In other invasion chambers, meloxicam (10 μM) was incubated with the F98 cells 30 min before the addition of IL-1β. Cells that crossed the layer of Matrigel and the porous membrane 24 h later were fixed, stained and counted under the microscope. Each experimental condition was performed in triplicate and repeated 2 times.

Statistical analysis
Data are expressed as the mean ± standard deviation mean. Statistical analyses for the median survival time were performed using non-parametric Mann-Whitney test for mean comparisons and Chi-square for distribution comparisons, while the other results was analysed using a t-test. A value of $p \leq 0.05$ was considered as significant. The non-irradiated groups were compared to the irradiated ones, and those treated with meloxicam were compared to the irradiated groups.

Results
Optimizing the dose of meloxicam
The COX-2 inhibitor meloxicam was injected daily at 0.1, 0.5 or 1 mg/kg in an attempt to prevent the stimulation of PGE2 production induced by irradiating the brain of Fischer rats. As we previously reported (Desmarais et al. 2012), an irradiation of 15 Gy has increased by 9.7-fold the production of PGE2 measured 4 h post-irradiation, and by 7.2-fold ($p < 0.05$) at day 15 (Figure 2). Treatment with meloxicam at all doses tested resulted in an important and similar reduction by 2–4 fold of PGE2. However, even with the highest concentration of meloxicam, the level of PGE2 in irradiated brain remained higher than in non-irradiated brain. The dose of 0.1 mg/kg was elected for use in all other experiments.

Meloxicam prevented the reduction of median survival induced by radiation
Brain irradiation before implantation of F98 cells reduced the median survival of rats by 28% (Table I and Figure 3, control: 25.0 days vs. brain 15 Gy = 18.2 days, $p = 0.0005$). Treatment with meloxicam completely prevented this adverse effect of
radiation. The median survival of the treated animals was similar to the one measured in the non-irradiated control group (control vs. brain 15 Gy + meloxicam, \( p = 0.44 \); brain 15 Gy vs. brain 15 Gy + meloxicam, \( p = 0.009 \)). Only a small but significant increase in median survival was observed in non-irradiated animals treated with meloxicam (control: 25.0 days vs. brain non-irradiated + meloxicam = 27 days, \( p = 0.03 \)).

### Meloxicam reduced the radiation-enhancement of cancer cell infiltration

As we previously reported (Desmarais et al. 2012), the distance of cancer cell infiltration from the edge of the tumor was multiplied by 2.7 when the brain was irradiated before implantation of the F98 cells (\( p = 0.005 \), Figure 4A), compared to non-irradiated control. Consequently, the brain surface infiltrated by cancer cells was 2.5 times larger, which is reported as the ratio surface infiltrated/primary tumor (\( p < 0.05 \), Figure 4B). In this study, we found that the increased infiltration induced by irradiating brain was partially reversed with the use of the COX-2 inhibitor meloxicam. Indeed, the infiltration distance was still 1.97-fold longer in irradiated brain of animals that were also treated with meloxicam compared to non-irradiated brains (\( p < 0.05 \), Figure 4A). This effect of meloxicam was not associated to a significantly modification of the surface of the primary tumor (data not shown).

Supporting the role of COX-2 and its bioproduct PGE2 in cancer cell invasion, addition of this prostaglandin to the F98 cells stimulated their invasiveness by 1.5-fold (\( p = 0.007 \), as assessed in vitro with invasion chambers (Figure 4C).

Migration of cancer cells requires the cleavage of components of extracellular matrix by proteases, such as the matrix metalloproteinase-2 (MMP-2) (Park et al. 2006). In irradiated brain, activity of MMP-2 was enhanced by 1.52-fold (\( p = 0.002 \)), 4 h post-irradiation, and 2.52-fold at day 15 (\( p = 0.0003 \)), as assessed with a gel zymography. This stimulation was completely prevented after treatment with the COX-2 inhibitor meloxicam (Figure 4D).

### Expression of genes involved in the production of PGE2

An important increase of PGE2 level was measured 4 h post-irradiation and was still significantly elevated on day 15 (Figure 2). As PGE2 is produced by COX-1 and COX-2, we determined whether the expression of these enzymes was stimulated in irradiated brain. A low but significant expression of COX-1 was measured 4 h post-irradiation, which decreased on day 15 but still remained elevated compared to control (Figure 5A). No increase of the mRNA of COX-2 was measured 4 h post-irradiation, but a significant stimulation was observed on day 15 (\( p = 0.004 \)) (Figure 5B). Treatment with meloxicam did not significantly modify the mRNA level of COX-1, and COX-2 measured 4 h post-irradiation. A significant reduction was measured for COX-2 only at day 15 post-irradiation (day 15, \( p = 0.03 \)) (Figure 5A and B).

Arachidonic acid is released from phospholipid membrane by the phospholipase A2 (PLA2) and then metabolized to various prostaglandins and leukotrienes via COX-1, COX-2, or 5-lipoxygenase, depending on the type of cells (Kuwata et al. 2014). Four hours after brain irradiation, the mRNA level of the isoform sPLA2 was significantly increased by 1.9-fold (\( p = 0.015 \)). This stimulation was even more important at day 15 (2.7-fold, \( p = 0.037 \)), and was largely prevented by treating the animals with meloxicam (Figure 5C). On the other hand, expression of the cytosolic form of PLA2 (cPLA2) was not significantly modified after the irradiation (Figure 5D; 4 h post-irradiation, \( p = 0.069 \)).

### Effect of meloxicam on the expression of pro-inflammatory cytokines

As COX-2 plays a central role in the inflammatory response, we investigated whether treatment with the COX-2 inhibitor meloxicam could indirectly affect expression of the pro-inflammatory cytokines IL-1β, IL-6, TGF-β1 and TNF-α that are known to stimulate the invasion of cancer cells (Huang et al. 2009, Ding et al. 2010, Liu et al. 2010, Paquette et al. 2013).

For the cytokines IL-1β, IL-6, and TNF-α, a significant increase of their mRNA was observed 4 h post-irradiation (\( p < 0.05 \), but not for TGF-β1 (\( p = 0.21 \)). These stimulations were temporary as their expression at day 15 were similar or smaller to the level measured in non-irradiated brains (Figure 6A–D). Treatment with meloxicam led to a small reduction that was significant only at 4 h post-irradiation for TGF-β1 (\( p = 0.006 \), and TNF-α (\( p = 0.024 \)).

As the highest stimulation of mRNA expression was measured with IL-1β, its ability to stimulate the in vitro invasion of F98 cells was assessed with invasion chambers. The addition of IL-1β (10 ng/ml) produced an increase in the number of F98 cells that crossed the layer of Matrigel by 5-fold (\( p = 0.015 \), Figure 6E). This stimulation of F98 invasion was completely inhibited by adding meloxicam (IL-1β vs.
Glioma cell infiltration stimulated by radiation

Our results showed that treatment with meloxicam largely prevented the stimulation of F98 cell infiltration in the brain; more importantly, the median survival time was similar to non-irradiated animals. The benefit of meloxicam treatment was also specific to irradiated animals as the COX-2 inhibitor only slightly improved the median survival of non-irradiated animals implanted with the F98 cells. Nevertheless, in some non-irradiated animals, their median survival seems to be increased by meloxicam. Since growth of the tumor may also induce some inflammation in brain, treatment with meloxicam could reduce this stimulation of F98 cell infiltration resulting in a longer median survival. Overall, our study supports the importance of inhibiting the inflammatory reaction induced by radiation in brain tissue surrounding the tumor cells to prolong the median survival of the animals.

MMP degrade extracellular matrix proteins, thereby opening routes to the infiltration of glioma cells (Nakada et al. 2003, Larkins et al. 2006, Lemay et al. 2011). We showed that treatment with meloxicam partially inhibited the stimulation of F98 cell infiltration in irradiated brain (p < 0.05), and the brain surface infiltrated by cancer cells (p < 0.05). The level of MMP-2 activity was measured by zymography in the brain of Fischer rats. The stimulation of MMP-2 activity in irradiated brain was completely prevented by meloxicam (p < 0.05).

IL-1β + meloxicam, p = 0.019). This supports that the contribution of IL-1β to the enhancement of F98 invasion can be efficiently inhibited by a COX-2 inhibitor.

**Discussion**

A neuro-inflammatory process occurs after irradiation of the brain that is associated with a stimulation of the expression of pro-inflammatory genes, some of which are known for their ability to increase cancer cell invasion (Kyrkanides et al. 2002, Moore et al. 2005, Moravan et al. 2011). The potential impact of this inflammation on the median survival of irradiated animals bearing a brain tumor was only recently appraised (Desmarais et al. 2012). Irradiation of Fischer rat brain followed by the implantation of F98 cells favored the infiltration of F98 cells, thereby resulting in a reduction in median survival time of the animals. Similar findings were observed with a sub-curative dose of radiation delivery to the F98 tumor cells implanted in Fischer rat brain with no prior radiation exposure. This adverse effect of radiation was associated with pro-infiltration mediators released from irradiated brain parenchyma, such as IL-1β, MMP-2, and PGE2, the bioactive product of COX-2 (Wild-Bode et al. 2001, Desmarais et al. 2012).

As COX-2 seems to play an important role in radiation-induced inflammation in brain and cancer cell infiltration, we investigated whether treatment with the COX-2 inhibitor meloxicam could prevent the stimulation of F98 cell infiltration. Our results showed that treatment with meloxicam largely prevented the stimulation of F98 cell infiltration in the brain; more importantly, the median survival time was similar to non-irradiated animals. The benefit of meloxicam treatment was also specific to irradiated animals as the COX-2 inhibitor only slightly improved the median survival of non-irradiated animals implanted with the F98 cells. Nevertheless, in some non-irradiated animals, their median survival seems to be increased by meloxicam. Since growth of the tumor may also induce some inflammation in brain, treatment with meloxicam could reduce this stimulation of F98 cell infiltration resulting in a longer median survival. Overall, our study supports the importance of inhibiting the inflammatory reaction induced by radiation in brain tissue surrounding the tumor cells to prolong the median survival of the animals.

MMP degrade extracellular matrix proteins, thereby opening routes to the infiltration of glioma cells (Nakada et al. 2003, Larkins et al. 2006, Lemay et al. 2011). We showed that MMP-2 levels were higher than normal into the irradiated brains, and this upregulation persisted at day 15 post-irradiation. Consequently, remodelling of ECM through an enhancement of MMP-2 activity could be an important feature explaining the enhancement of the infiltrative properties of cancer cells observed into irradiated brains. This stimulation of MMP-2 was completely blocked by meloxicam treatment. Supporting the role of meloxicam in the reduction of MMP-2 expression, it was previously reported that the
COX-1 and COX-2 are preferentially expressed by different cell types and brain areas. COX-1 is abundantly expressed in microglia, even under resting conditions (Yermakova et al. 1999), while COX-2 is predominantly observed in brain neurons (Tanaka et al. 2009), and it is also preferentially localised in neocortex, hippocampus, amygdala, and limbic cortices (Yamagata et al. 1993, Kaufmann et al. 1996). This distribution of the two COX isoforms in the brain may suggest that the relative importance of COX-1 and COX-2 in the stimulation of glioma cell infiltration might vary according to the tumor location in the brain. In our study, the F98 cells were implanted in the caudate nucleus. Administration of a COX-2 inhibitor increased the median survival of irradiated rats to the same level that was measured in the non-irradiated group. This protective effect was obtained even if the increased infiltration of cancer cells has not been completely blocked. Therefore, we cannot exclude that a COX-1 inhibitor would further inhibit radiation-enhancement of cancer cell infiltration. It also remains to be determined whether inhibition of COX-1 and/or COX-2 would be required for tumors implanted in other brain areas.

COX-1 and COX-2 are preferentially expressed by different cell types and brain areas. COX-1 is abundantly expressed in microglia, even under resting conditions (Yermakova et al. 1999), while COX-2 is predominantly observed in brain neurons (Tanaka et al. 2009), and it is also preferentially localised in neocortex, hippocampus, amygdala, and limbic cortices (Yamagata et al. 1993, Kaufmann et al. 1996). This distribution of the two COX isoforms in the brain may suggest that the relative importance of COX-1 and COX-2 in the stimulation of glioma cell infiltration might vary according to the tumor location in the brain. In our study, the F98 cells were implanted in the caudate nucleus. Administration of a COX-2 inhibitor increased the median survival of irradiated rats to the same level that was measured in the non-irradiated group. This protective effect was obtained even if the increased infiltration of cancer cells has not been completely blocked. Therefore, we cannot exclude that a COX-1 inhibitor would further inhibit radiation-enhancement of cancer cell infiltration. It also remains to be determined whether inhibition of COX-1 and/or COX-2 would be required for tumors implanted in other brain areas.

COX-1 is usually considered as a housekeeping gene whose activity is dependent solely on availability of its substrate, the arachidonic acid. However, a recent study reported that COX-1 mRNA was rapidly induced in rat cerebral cortex in a model of reversible ischemia suggesting that expression COX-1 in brain might be stimulated.

Figure 5. Expression of genes involved in the production of PGE2. (A) Meloxicam did not affect the mRNA level of COX-1 that was slightly reduced at 4 h and at day 15 post-irradiation. (B) A significant stimulation of COX-2 mRNA was observed on day 15 (p = 0.004). A stimulation that was reduced by meloxicam (p = 0.05). (C) The mRNA level of sPLA2 was significantly increased 4 h post-irradiation (1.9-fold, p = 0.015), and also at day 15 post-irradiation (2.7-fold, p = 0.037). These stimulations were largely prevented by meloxicam. (D) Brain irradiation did not significantly modify the expression of cPLA2. The mRNA levels were quantified by qPCR.
COX-2 is constitutively expressed (Yamagata et al. 1993). COX-2 expression is tightly regulated at the transcription/translation level (Masferrer et al. 1994, Smith and Dewitt 1996) and it can be induced by inflammatory cytokines, such as IL-1β (Paquette et al. 2013, Kuwata et al. 2014). In our rat model, a significant increase of IL-1β mRNA was induced in the brain 4 h post-irradiation and was followed by a significant increase in the level of TGF-β1 mRNA.

Regarding COX-2, few tissues express this cyclooxygenase under normal circumstances, except in the brain where it is expressed constitutively (A) (B) (C) (D) (E). COX-2 expression is upregulated under specific conditions (Holtz et al. 1996). Our results showed that an exposure to 15 Gy was not sufficient to induce the expression of COX-1 in Fischer rat brain in the first 15 days after irradiation, as measured by level of mRNA.

Figure 6. Effect of meloxicam on the expression of pro-inflammatory cytokines. The mRNA levels of the pro-inflammatory cytokines in the treated groups were normalized according to the control. (A) IL-1β, (B) IL-6, (C) TNF-α, and (D) TGF-β1 were quantified by qPCR. The highest stimulation of mRNA expression in irradiated brains was measured with IL-1β. Treatment with meloxicam (1 mg/kg daily) resulted to a significant reduction at 4 h post-irradiation only for TGF-β1 (p < 0.006), and TNF-α (p = 0.024). E) The stimulation of F98 cell invasion in vitro by IL-1β was completely inhibited by meloxicam (p = 0.019).
stimulation of COX-2 detected at day 15. Therefore, the significant elevation of PGE2 measured 4 h post-irradiation is not likely directly related to an increase in COX-2 expression.

An increase in the level of PGE2 can also be induced by enhancing the liberation of arachidonic acid from membrane glycerophospholipids regulated by PLA2 enzymes (Kuwata et al. 2014). The expression of two isoforms of PLA2 (cPLA2 and sPLA2) can be linked to inflammation as their expression can be stimulated by IL-1β (Xin et al. 2007, Lee et al. 2010). Indeed in rat fibroblasts, IL-1β induced the release of arachidonic acid that peaked at 6 h after treatment and then gradually declined (Paquette et al. 2013, Kuwata et al. 2014). A stimulation of the production of PGE2 was then observed that was completely inhibited by the COX-2 inhibitor NS-398 (Paquette et al. 2013). In our study, large increase of IL-1β in irradiated brain was associated with a stimulation of sPLA2 expression, but not with cPLA2. This suggests that the elevation of PGE2 observed 4 h post-irradiation might be related to a stimulation of sPLA2 expression.

The role of IL-1β in neuro-inflammation may be important since this cytokine increased the invasion of F98 cells, as measured with the in vitro invasion chamber assay. The IL-1β pathway was also related to COX-2 since the stimulation of F98 cell invasion induced by this cytokine was completely inhibited by meloxicam. Since the animals were treated daily with meloxicam, this COX-2 inhibitor could inhibit the stimulation of F98 cell infiltration induced by IL-1β in irradiated brain. Similar results were previously reported with the breast cancer cell MDA-MB-231, where a stimulation of their invasion by IL-1β was prevented by a COX-2 inhibitor (Paquette et al. 2013). In C3H/HeN mouse brain following irradiation, a COX-2 selective inhibitor significantly attenuated the level of induction of IL-1β, further supporting the link between IL-1β and COX-2 (Moore et al. 2005). This suggests that the cooperation between IL-1β and COX-2 for the stimulation of cancer cell invasion is not specific to rat glioma F98 cells.

The stimulation of glioma cell infiltration induced by radiation may involve other inflammatory cytokines. This hypothesis is supported by in vitro invasion assays where IL-6 (Li et al. 2010), TNF-α (Huang et al. 2009) and TGF-β1 (Merzak et al. 1994) increased the invasion of glioma cells. In irradiated Fischer rat brains, a small but significant increase was measured for IL-6 and TNF-α. However, unlike in C3H/HeN mouse brain (Kyrkanides et al. 2002), inhibition of COX-2 by meloxicam did not significantly attenuate the levels of these inflammatory mediators in Fischer rat brain. Although we cannot rule out that these cytokines might stimulate the infiltration of glioma cells in other models, our results support that they did not play a key role in the F98 Fischer rat glioma model.

In conclusion, a rapid infiltration and proliferation of glioma cells after radiation treatment are known for decades and seems to involved pro-inflammatory cytokines. In this study, we shown that inhibition of COX-2 prevents the stimulation of F98 cell infiltration in irradiated brain resulting in a longer median survival time of the animals. These results support that a COX-2 inhibitor could be a good candidate as adjuvant to radiotherapy in patients treated for a GBM; however, the dosage suitable for clinical use should be determined to ensure that no toxicity would be induced. Since IL-1β has been linked to the stimulation of the infiltration of cancer cells, an inhibitor targeting this cytokine could also be investigated in clinical trials.

Acknowledgements
Authors BP, DM, RB and DF are members of the FRQS-funded Centre de recherche du CHUS. The authors would like to thank Dr Leonid Volkov of the department of microscopy and cytometry of the Centre de recherche du CHUS for his contribution in the histological evaluation of normal tissues invaded by cancer cells. Meloxicam was generously provided by Boehringer Ingelheim International GmbH.

Declaration of interest
The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

References
Anand AK, Chaudhory AR, Aggarwal HN, Sachdeva PK, Negi PS, Sinha SN, Babu AG, Jena A, Rao A, Chaudhury PS. 2012. Survival outcome and neurotoxicity in patients of high-grade gliomas treated with conformational radiation and temozolomide. J Cancer Res Therapeut 8:50–56.
Burger MS, Hadjipanayis CG. 2007. Surgery of intrinsic cerebral tumors. Neurosurgery 61:279–304.
Bouchard G, Bouvette G, Therriault H, Bujold R, Saucier C, Paquette B. 2013. Pre-irradiation of mouse mammary gland stimulates breast cancer cell migration and development of lung metastases. Br J Cancer 109:1829–1839.
Brandes AA, Tosoni A, Franceschi E, Reni M, Gatta G, Vecht C. 2008. Glioblastoma in adults. Crit Rev Oncol/Hematol 67:139–152.
Burger PC, Dubois PJ, Schold SC Jr, Smith KR Jr, Odom GL, Crafts DC, Giangaspero F. 1983. Computerized tomographic and pathologic studies of the untreated, quiescent, and recurrent glioblastoma multiforme. J Neurosurg 58:159–169.
Chakravarti A, Palanichamy K. 2008. Overcoming therapeutic resistance in malignant gliomas: Current practices and future directions. Cancer Res Treatment 139:173–189.
Charest G, Mathieu D, Lepage M, Fortin D, Paquette B, Sanche L. 2009. Polymer gel in rat skull to assess the accuracy of a new rat stereotactic device for use with the Gamma Knife. Acta Neurochirurgica 151:677–683.
Desmarais G, Fortin D, Bujold R, Wagner R, Mathieu D, Paquette B. 2012. Infiltration of glioma cells in brain parenchyma stimulated by radiation in the F98/Fischer rat model. Int J Radiat Biol 88:565–574.
Ding Q, Bai YF, Wang YQ, An RH. 2010. TGF-beta1 reverses inhibition of COX-2 with NS398 and increases invasion in prostate cancer cells. Am J Medical Sci 339:425–432.
Gaspar LE, Fisher BJ, Macdonald DR, LeBer DV, Halperin EC, Schold SC Jr, Cairncross JG. 1992. Supratentorial malignant glioma: Patterns of recurrence and implications for external beam local treatment. Int J Radiat Oncol Biol Phys 24:55–57.
Hellemands J, Mortier G, De Paepe A, Speelman J, Vandesompele J. 2007. qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. Genome Biol 8:R19.
Holtz ML, Kindy MS, Craddock S, Moore RW, Pettigrew LC. 1996. Induction of PGH synthase and c-fos mRNA during early reperfusion of ischemic rat brain. Molec Brain Res 35:339–343.
Huang HC, Huang CY, Lin-Shiau SY, Lin JK. 2009. Ursolic acid inhibits IL-1beta or TNF-alpha-induced C6 glioma invasion through suppressing the association ZIP/p62 with PKC-zeta and downregulating the MMP-9 expression. Molec Carcinogenesis 48:517–531.
