Comparison of Corticotropin-Releasing Factor, Dexamethasone and Temozolomide: Treatment Efficacy and Toxicity in U87 and C6 Intracranial Gliomas.

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Running title: Comparison of hCRF and DEX efficacy in glioma models.

Key words: glioma, corticotropin-releasing factor, dexamethasone.

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Abstract.

Treatment of cerebral tumors and peritumoral brain edema remains a clinical challenge and is associated with high morbidity and mortality. Dexamethasone (DEX) is an effective drug to treat brain edema, but is associated with well-described side effects. Corticorelin acetate (Xerecept) or human corticotrophin releasing factor (hCRF) is a comparatively new drug and was evaluated in two orthotopic glioma models (U87 and C6), by a direct comparison with dexamethasone and temozolomide.

In vitro mono- and combination-treatments showed a variable response in 6 different glioma cell lines. In vivo studies showed a dose-dependent effect of hCRF (0.03 and 0.1 mg/kg/q12h) on survival of U87 intracranial xenograft-bearing animals [median survival: control 41 days (95% CI 25-61 d); “low-hCRF” 74.5 d (95% CI 41-88 d); “high-hCRF” >130 d (95% CI not reached)]. Dexamethasone treatment had no effect on survival, but significant toxicity was observed. A survival benefit was observed with TMZ and TMZ + hCRF - treated animals, but with significant TMZ toxicity. C6-bearing animals showed no survival benefit, but similar treatment toxicities. The difference in hCRF-treatment response between U87- and C6-intracranial gliomas can be explained by a difference in receptor expression. RT-PCR identified CRF2r mRNA in U87-xenografts; no CRF-receptors were identified in C6-xenografts.

HCRF was more effective than either dexamethasone or temozolomide in the treatment of U87 xenografts, with long-term survivors and only mild toxicity. HCRF therapeutic efficacy appears to be dependent on tumor hCRF-receptor expression. These results support further clinical assessment hCRF therapeutic efficacy and levels of CRFr expression in different human gliomas.
Translational Relevance

Tumor-associated edema is a major negative factor for patients with brain tumors, and is responsible for high morbidity and mortality in this patient group. Dexamethasone has been the most effective and commonly used drug to treat brain edema for decades, but is associated with well-known side effects. Recently, bevacizumab has shown remarkable transient responses in patients with high-grade gliomas, both on MRI and in clinical performance that is at least partially related to its anti-edematous effect. In this study we evaluated the efficacy and toxicity of human corticotropin-releasing factor (hCRF) in the treatment of two orthotopic intercranial brain tumors (C6, rat; and U87, human) in a SCID-mouse model. Comparisons were also made with dexamethasone and temozolomide treatment regimens.

Three important observations were made: 1) hCRF was more effective than either dexamethasone or temozolomide in the treatment of U87 intracranial xenografts; 2) hCRF treatment was associated with significantly less toxicity in comparison to either dexamethasone or temozolomide treatment; 3) hCRF efficacy was dependent on hCRF receptor expression in the tumor (e.g., C6 gliomas showed no response to hCRF and had no measurable levels of CRF mRNA on RT-PCR). These results support the development of hCRF-releasing formulations to optimize the therapeutic antitumoral effect in human subjects with brain tumors, since several clinical trials have shown that hCRF is safe and enables reductions of steroid-dosing.
Introduction.

Peritumoral brain edema is a significant cause of morbidity and mortality and there have been relatively few advances in brain edema treatment since the introduction of dexamethasone in the 1970s. Relatively new treatments which have demonstrated a significant impact on tumor-associated brain edema include human corticotropin-releasing factor (hCRF) (1-4) and anti-angiogenic therapy (5, 6), although the latter (bevacizumab) has recently been associated with potentiation of tumor cell invasion and rapid progression following the cessation of therapy (7-10). This study focuses on hCRF monotherapy in two xenograft mouse models, and provides a direct comparison between hCRF, dexamethasone and temozolomide monotherapy.

Human corticotropin-releasing factor (hCRF) is a 41 residue neuropeptide, initially isolated from sheep hypothalamic extracts in 1981 by Vale et al (11). It is produced in the hypothalamus and is an important component in regulating the hypothalamic-pituitary-adrenal (HPA) axis. CRF is the predominant regulator of adrenocorticotropic-hormone (ACTH) formation and release by the pituitary (12). In addition to its primary location in the hypothalamic paraventricular nucleus, it has also been identified in cerebral cortical interneurons, the limbic system, brain stem and spinal cord (13).

It has been demonstrated that hCRF also possesses anti-edema properties. As an anti-edematous agent hCRF prevents vascular leakage induced by inflammatory mediators that selectively act on postcapillary venules and veins in skin (14), from alveolar capillaries (15-18), and from muscle capillaries (19). The anti-edematous effects of hCRF on systemic vessels appears to be mediated in part through activation of CRF2 receptors, whereas in the brain and in brain tumors, this effect is mediated through both CRF1 and CRF2 receptors (20). These observations suggest that hCRF acts throughout the microcirculation to preserve endothelial cell integrity. Several possible mechanisms of hCRF action have been reviewed in the literature (21), but the exact mechanisms are not yet fully established.
HCRF has been proposed as a new treatment for peritumoral brain edema. Strong pre-clinical data supported hCRF as an effective anti-brain edema agent, which has substantially less toxicity than dexamethasone (22). The first study of exogenous CRF administration and toxicity assessment in patients was reported by Chrousos at al (23). More recently, Xerecept (Corticorelin acetate or hCRF) has been shown to be a well-tolerated drug based on data from ongoing clinical trials involving nearly 200 patients. The side effects of clinical treatment with hCRF in i.v. single doses (1-5 µg/kg) and continuous i.v. infusions of up to 2,000 µg / 24 hours / patient were not associated with any significant side effects. Only at much higher i.v. single doses (up to 30 µg/kg) did significant symptoms develop, including hypotension, tachycardia, arrhythmias and mental 'absences'.

Ongoing clinical trials involving nearly 200 patients that have received hCRF indicate that subcutaneous (sc) administration of the drug, often for extended periods, is well tolerated (24-26). The evolving clinical efficacy and safety data supports the use of hCRF as a dexamethasone-sparing treatment (if not an alternative to dexamethasone) for the management of symptomatic peri-tumoral brain edema (4). In contrast to the well known systemic side effects associated with chronic Dexamethasone administration, which can be even more debilitating than the primary disease process, hCRF has demonstrated much less toxicity (27, 28), with the primary effect being transient local reactions at or near injection site.

The evolving clinical efficacy and safety data indicate that Xerecept might provide a dexamethasone-sparing treatment (if not an alternative to dexamethasone) for the management of symptomatic peri-tumoral brain edema (24, 25). In addition, our previous preclinical studies indicated that hCRF may have a direct anti-tumor effect (1, 29), as well as a well-documented anti-edematous effect (1-4). In this study, we assess the efficacy and toxicity of hCRF (Xerecept, Celtic Pharma, New York) (30) treatment, both in vitro and in two orthotopic glioma animal models. First, we assessed hCRF and dexamethasone (DEX) for additive effects in cell culture viability assays involving BCNU and temozolomide treatment of six different glioma cell lines. Then we focused on a
comparison of hCRF and dexamethasone monotherapy and a comparison between temozolomide (TMZ) monotherapy and TMZ plus hCRF combined therapy.
Materials and Methods.

Cell lines, transduction and FACS sorting. RG2 and C6 rat glioma, and U87 and LN229 human glioma cells were obtained from the American Type Culture Collection (ATCC). The cell lines were maintained in 75 cm$^2$ flasks with MEM (RG2 and U87) or DMEM (C6 and LN229) media plus fetal bovine serum (10%), penicillin (100 U/ml) and streptomycin (100 µg/ml). All cells were maintained in a humidified atmosphere (5% CO$_2$/95% air) at 37°C.

To monitor intracranial glioma growth by bioluminescence imaging, we transduced U87 and C6 cells with a retroviral reporter vector containing a Firefly luciferase-1RES-Green Fluorescent protein (GFP) cassette previously developed in our laboratory (31). C6 and U87 cell lines were stably transduced as previously described (32). After transduction, the cells were expanded in culture for several days and prepared for subsequent FACS analysis and cell sorting as previously described (33).

Therapeutic drugs. Human corticotropin-realizing factor (hCRF) (Corticorelin acetate, Xerecept®) was generously provided by Celtic Pharmaceutical Development Services America, Inc., New York, NY. Dexamethasone (Dex) and BCNU were obtained from the MSKCC pharmacy. Temozolomide (TMZ) was generously provided by the Developmental Therapeutics Program, National Cancer Institute (Rockville, Maryland).

In vitro cytotoxicity assays. Cytotoxicity assays were performed using wild-type and FLuc-transduced cell lines, as previously described (34). Briefly, cells (5000 per well) were seeded into 96-well microplates and incubated for 4, 24 and 72 hours incubation in control (non-treated) condition and with different doses of BiCNU and TMZ, both alone and in combination with low and high doses of DEX (0.01 µM and 1µM) and hCRF (0.01 nM and 1 nM), treated simultaneously. The in vitro exposure times for hCRF were chosen based on
unpublished data from Celtic Pharma. The exposure time for BCNU was chosen based on previously published work (35, 36). Total number of experiments was at least 6 for each cell line. WST-1 reagent (Roche, Mannheim, Germany), was used to measure cell viability after drug incubation. Measurement of the absorbance of the samples against a background control as blank was done using Packard ELISA reader (Packard Bioscience Company, IL). EC50 was assessed using Excel and Sigma Plot programs.

**Semi-quantitative reverse transcription-PCR**

Expression level of CRF receptors (CRF1 and CRF2) was determined by semi-quantitative reverse transcription-PCR. Briefly, total RNA from cultured cells and tumor xenografts were isolated using TRIzol reagent (Invitrogen, Carlsbad, CA) and treated with RNase-free DNase I (Ambion, Austin, TX) according to the manufacturer’s instructions. The first strand of cDNA was synthesized by GoScript Reverse Transcriptase (Promega, Madison, WI). PCR was performed using the following primer sets, which are located in the conserved domains of CRF1, CRF2 and β-actin gene from human, mouse and rat species:

CRF1: (F) 5´- cctgtggcctttgtcctc-3´; (R) 5´- tggggccctgtagatgta -3´;

CRF2: (F) 5´- cctactgcaacacgacctt -3´; (R) 5´- tagcagccttccacaaaca -3´;

β-actin: (F) 5'- ggctggccgggacctgac -3'; (R) 5'- tactcctgcttgctgat -3'.

**In vivo studies**

Male rnu/rnu mice were obtained from NCI (Bethesda, MD) and all studies were performed under a Memorial Sloan Kettering Cancer Center IACUC-approved protocol. Xenografts were established in 3 month-old mice by intracranial injection of 5 x 10^4 of either U87FLuc or C6FLuc reporter cells, using a stereotactic device as previously described (29).

Eight treatment groups of 10 athymic rnu/rnu mice per group, each bearing a U87 FLuc or a C6 FLuc xenograft, were developed as outlined in **Table 1**. Treatment with hCRF, dexamethasone, TMZ or a combination of TMZ plus hCRF was initiated three weeks after tumor cell implantation and lasted for 7 weeks.
For animal **groups 1** and **2**, the stock clinical grade solution of hCRF (Xerecept®) (1 mg/ml) was diluted with 0.9% sodium chloride solution to the required “low” and “high” concentrations prior to each injection (**Table 1**). For animal **groups 3** and **4**, clinical grade, i.v. injectable Dexamethasone (DEX) (1 mg/ml) was diluted with 0.9% sodium chloride solution to the required concentrations. Treatment with either hCRF or DEX was administered by subcutaneous (s.c.) injections every 12 hours for 7 weeks, starting 2nd week after implantation. For animal **group 5**, animals were treated with a single daily dose of Temozolomide (TMZ). TMZ solution was freshly prepared daily and administered by intraperitoneal (i.p.) injection 5 of 7 days each week, for 7 weeks. TMZ powder was dissolved in pure DMSO and further diluted 1:5 with 0.9% sodium chloride solution to the desired concentration. For animal **groups 6** and **7**, the effects of combined TMZ and “low” or “high” hCRF were studied (**Table 1**). The preparation and administration of the drugs were the same as described above, respectively. For animal **group 8** (control), animals were treated with a single daily dose of drug vehicle. All surviving animals were euthanized on day 150.

**In vivo bioluminescence imaging** was performed 7 days after implantation of the xenografts and repeated weekly for the duration of the experiment, using an IVIS-200 Imaging System (Caliper, CA). Imaging was performed 10 minutes after i.p. injection of D-luciferin (2 mg per animal; Xenogen), with mice lying in the prone position. Five mice were imaged at the same time with a field of view of 25 cm. An imaging time of 3 minutes with medium binning and an f-stop of 1 were used initially; this was sequentially reduced as the xenografts grew and saturation levels of BLI signal intensity were approached. Measurements of signal intensity were obtained from region of interest analysis using Living Image software (Xenogen). The images displayed in each data set were normalized to the appropriate color intensity scale. BLI intensity was expressed as total photons per region of interest per 1 second of imaging.
**Statistical analyses.** Results are reported as Mean ± SD. We applied analysis of variance (ANOVA) to the EC\textsubscript{50} results for each drug (BCNU and TMZ) and each cell line (C6, LN229, RG2 and U87) separately, and included date of experiment as an effect in the model for quality control. We compared each of 4 treatments low- and high-dose dexamethasone and low- and high-dose hCRF vs BCNU or TMZ alone and adjusted for multiple comparisons within each experiment (combination of drug and cell line) using Dunnett’s method. We considered p<0.05 after adjustment for multiple comparisons to be statistically significant.

In order to account for possible differences in global levels or calibration of BLI measurement between animals, we used the change from the average of week 1 and week 2 to the average of week 5 and week 6 as a summary value. Survival was measured from time of administration of treatment until death, sacrifice, or end of study. Animals that were moribund at time of sacrifice were considered the same as animals that died, and others were considered as censored observations in survival analysis. Using parallel factorial designs, we applied analysis of variance to change in BLI measurements and the Cox proportional hazard model to survival data. We also included each animal’s change in BLI as a potential predictor of survival in the Cox model. Analysis showed that there was no significantly different effect on survival between high versus low dose of hCRF, DEX, or TMZ + hCRF. We combined high and low dose of each treatment in further analyses.
Results.

Effects of hCRF and dexamethasone on BCNU and TMZ cytotoxicity in cell culture. Cytotoxicity studies were performed in 6 glioma cell lines (U87, U87FLuc, C6, C6FLuc, Ln229, RG2) with increasing doses of hCRF (0.0001 to 1000 nM) and dexamethasone (0.001 to 10 µM). Minimal loss of cell viability (less than 20%) was observed following a 3 day exposure to 1 nM hCRF and 1 µM dexamethasone, and less than 10% loss of cell viability following a 3 day exposure to 0.01 nM hCRF and 0.01 µM dexamethasone. On the basis of these results, the above concentrations of “high” and “low” hCRF and dexamethasone, respectively, were selected for the in vitro drug-combination cytotoxicity studies.

BCNU and TMZ, both alkylating drugs extensively used to treat high grade gliomas, were assessed as monotherapy and in drug-combination cytotoxicity studies with either hCRF or dexamethasone. Combining BCNU with either hCRF or dexamethasone had a variable effect; whereas, there were no significant differences in cytotoxicity between TMZ treatment alone and combined treatment with hCRF or dexamethasone (data not shown). In a second experimental series, the EC\textsubscript{50} of BCNU (Table 2A) and TMZ (Table 2B) was assessed in 4 different cell lines. Although the EC\textsubscript{50} values varied over a ~3-fold range for each of the two drugs, the rank-order of sensitivity was quite different for the four cell lines. A comparison of the results, accounting for multiple comparisons, demonstrated only a few statistically significant effects. In the TMZ experiments, the addition of hCRF significantly lowered the EC\textsubscript{50} values in RG2 cells and a nonsignificant trend toward a lower EC\textsubscript{50} was observed in U87 cells. There was no significant effect of dexamethasone on TMZ EC\textsubscript{50} values with any of the cell lines.

The in vitro studies showed little evidence of a consistent additive effect when hCRF or dexamethasone was combined with BCNU or TMZ treatment. Despite these inconsistent results, we decided to study and compare the effect of hCRF, dexamethasone and TMZ monotherapy in two orthotopic glioma xenografts in immunodeficient mu/nu mice, and we also performed one combined therapy study involving TMZ and hCRF. We chose a human (U87) and a rodent (C6)
glioma cell line for comparison. Both cell lines were transduced with a reporter gene that constitutively expressed firefly luciferase (U87FLuc and C6FLuc). Importantly, there were no significant differences in cytotoxicity observed between U87 and U87FLuc cells and between C6 and C6FLuc cells (Table 2). The intracranial xenografts generated from these reporter-transduced cell lines allowed us to monitor tumor growth non-invasively using bioluminescence imaging (BLI).

**hCRF treatment of intracranial gliomas.** A dose dependant effect of hCRF on the survival of animals bearing U87 FLuc intracranial xenografts was observed (Figure 1A). Both “low” (0.03 mg/kg/q12h) and “high” doses (0.1 mg/kg/q12h) of hCRF increased survival compared to controls, median survival from 41 days (95% CI 25-61 d) to 74.5 days (95% CI 41-88 d) (p<0.05) and >130 days (95% CI not reached) (p<0.05), respectively. In contrast, hCRF had no effect on the survival of animals bearing intracranial C6 FLuc xenografts (Figure 2A).

U87 FLuc intracranial xenografts treated with hCRF usually showed a BLI signal intensity pattern that paralleled survival (Figure 3). For example, a rapid decrease in BLI signal intensity that remained low for the duration of the experiment was associated with a long survival, whereas, an increasing signal was usually associated with a shorter survival (Figure 3B). HCRF treatment had no effect on the pattern of increasing BLI signal intensity of C6FLuc gliomas, consistent with the absence of any survival benefit in these animals (Figure 2). Further analysis of treatment effects on overall survival (OS), adjusting for experiment and change in BLI effects, showed that hCRF (low or high doses) significantly prolonged OS (p<0.0001).

Very little or no toxicity was observed in animals treated with hCRF alone. The only minor complication was a random non-specific skin infection that probably reflects the systemic effects of corticosteroids on the immune system. In addition, hCRF-treated animals bearing U87FLuc gliomas continued to gain weight, similar to control animals (Figure 1B). No difference in animal weight was noted between control and “low” or “high” hCRF-treated animals.
Dexamethasone treatment of intracranial gliomas. Dexamethasone at both low (0.3 mg/kg) and high doses (1 mg/kg) was not effective in prolonging animal survival compared to control non-treated animals bearing either U87FLuc (Figure 1A) or C6FLuc (Figure 2A) gliomas. Similarly, the patterns of increasing bioluminescence intensity were indistinguishable from that of non-treated control animals (Figure 3C). Further statistical analysis showed that animals treated with hCRF had significantly longer survival than DEX-treated animals (p=0.02), whereas no differences between “low” and “high” doses of Dex were noted. Treatment-related toxicity was greater in the dexamethasone-treated animals and included significant weight loss (Figure 1B and Figure 2B), more severe skin infections and some necrosis at the injection sites.

TMZ treatment of intracranial gliomas. A significant effect of TMZ alone (p=0.008) or in combination with different doses of hCRF (p=0.003) on the survival of animals bearing U87FLuc xenografts was observed (Figure 1C), but not on the survival of animals bearing C6FLuc xenografts (Figure 2C). The temporal profiles of the bioluminescence images were similar to the survival data in both U87FLuc (Figures 1B and 4B, respectively) and C6FLuc (data not shown) xenograft-bearing animals. Many of the animals treated with TMZ showed a decrease and stabilization of the BLI signal at a low or moderate level for most of the treatment and post-treatment period, followed by a rapid increase in the last two weeks of the animal’s life. However, TMZ monotherapy and TMZ combined with hCRF was accompanied with high toxicity. There was a substantial loss of weight (up to 40%), which tended to recover during the post-treatment period if the animal survived. This weight loss is reflected in the photographic images of the animals at week 9, just after completing the course of TMZ therapy (Figure 4B and 4C). A very high incidence (27/30) of skin neoplasms was observed in animals treated with TMZ and there were rare occurrences (2/30) of internal abdominal tumors. Spontaneous tumorigenesis was not observed in any of the other treatment groups.

CRF1 and CRF2 receptor expression. U87-FLuc and C6-FLuc cell lines and xenografts were examined for CRF1 and CRF2 mRNA levels by RT-PCR
(Figure 5). U87 cultured-cells and xenografts express measurable levels of CRF2, but not CRF1 mRNA transcript. In contrast, neither CRF1 nor CRF2 transcripts could be detected in the C6 cultured-cells and xenografts.
Discussion.

We previously reported a dose-dependent decrease in vasogenic peritumoral brain edema following treatment of immunocompetent Fischer 344 rats, bearing RG2 intracranial gliomas, with human corticotropin-releasing factor (Xerecept, Corticorelin acetate) (29). We also reported a similar result in a single-dose hCRF study involving Sprague-Dawley rats bearing W256 intracranial gliomas (1, 17). Both studies demonstrated a significant anti-edematous effect in tumor and peritumoral brain tissue by proton density-weighted and T1-weighted contrast-enhanced MRI, and by ex vivo measures of tissue water content. The initial study also reported a significant survival advantage for hCRF-treated animals compared to dexamethasone-treated as well as to control animals (29).

In this study, an immunocompromised animal model harboring either an intracranial human (U87) or a rat (C6) glioma xenograft was studied, and therapeutic efficacy was evaluated in terms of animal survival. We focused on a comparison of hCRF and dexamethasone (Dex) monotherapy; although a comparison between temozolomide (TMZ) monotherapy and TMZ plus hCRF combined therapy was also performed. Interestingly, a highly significant, dose-dependent effect of hCRF monotherapy on survival was observed in animals bearing U87 gliomas, but not C6 gliomas. These results were highly consistent with tumor growth, regression and regrowth patterns visualized by sequential-weekly bioluminescence reporter gene imaging of the xenografts prior-to, during and following hCRF treatment. Interestingly, dexamethasone treatment had little or no effect on either the survival or bioluminescence imaging profiles of animals bearing either U87 or C6 gliomas, and this may reflect in part the toxic side-effects of twice-daily s.c. injections of dexamethasone. TMZ monotherapy and TMZ plus hCRF combined therapy of U87 gliomas yielded similar survival profiles that were significantly longer than control animals. Again, no survival advantage was observed in C6 gliomas.

To further explore the dichotomy in hCRF treatment response between U87 and C6 gliomas, we evaluated the levels of CRF1 and CRF2 receptor mRNA in
the two cell lines and xenografts. This investigation was based on our previous study which showed that significant levels of CRF1 mRNA (0.25±0.01 pg/µg total RNA) were detectable in W256 cells, and presumably reflects CRF1 receptor expression on tumor and/or endothelial cell membranes (22). Blockade of CRF receptors with alpha-helical CRF-(9-41) analog abolished the growth inhibitory and differentiation inducing effects of hCRF. Together, these findings suggested that W256 cells express functional rat CRF receptors in vitro and that these receptors are likely to mediate the effects observed following exposure to hCRF. Our current results suggest that C6 cells and xenografts express neither CRF1 nor CRF2 receptors, whereas U87 cells and xenografts express CRF2, but not CRF1 receptors. This difference between the two cell lines and xenografts could contribute to the difference in hCRF treatment response that was observed in this study. In addition, these results suggest that CRF1 and/or CRF2 receptor expression is required for the anti-tumor and anti-edematous effects of hCRF.

It was previously shown that CRFR1 is expressed in tumor cells and that urocortin and corticotropin-releasing factor (UCN/CRF), both members of the CRF family, reduce tumor cell growth via CRFR1 (37, 38). Multiple malignancies have been reported to have high levels of CRFR1 and CRFR2 expression, and to be sensitive to the suppressive effects of CRF and its agonists. Graziani et al. reported that UCN/CRF inhibited the growth of adenocarcinoma Ishikawa cells in a concentration-dependent manner, and that this effect was mediated by CRFR1 (38). It was reported that UCN inhibited the proliferation of melanoma cells in vitro and in vivo, also through CRFR1 (39) and in human mammary cancer cells, CRF acted on CRFR1 to inhibit the proliferative effects of estrogens on MCF7 cells in both a paracrine and autocrine manner (40). Activation of CRFR2 was observed to suppress angiogenesis and rearrange the vasculature. It was also reported that CRFR2 agonists inhibited hepatocellular carcinoma tumor angiogenesis in vitro and reduced tumor microvessel density in vivo. Our results are consistent with these observations.
Reports from other animal and human studies confirm that the anti-edematous effects of hCRF- and dexamethasone-treated animals are similar. Patients treated with hCRF and decreasing doses of dexamethasone demonstrate clinical improvement in neurologic symptoms and less steroid-associated myopathy and insulin dependence (41). The substitution of a less toxic, but equally effective drug for dexamethasone to treat tumor-associated cerebral edema has been a long-sought challenge for clinicians. Our pre-clinical studies add to the body of data that indicates that hCRF has the potential to meet this challenge. We observed higher therapeutic efficacy with hCRF than with either dexamethasone or temozolomide monotherapy in U87 glioma xenografts. Also, no toxic effects or signs of discomfort were observed in mice who have received hCRF treatment in low or high dose for 2-6 months. In comparison, animals who have received dexamethasone or temozolomide therapy exhibited significant toxicity, manifested by a decrease in weight, increased irritability, local skin necrosis and even spontaneous tumorigenesis.

The current clinical development program for hCRF has focused on the treatment of peritumoral brain edema in patients with metastatic and primary brain tumors requiring dexamethasone to control symptoms. Three major clinical trials (0303, 0501 and 0302) have established two important points: 1) hCRF is safe in man, and 2) hCRF enables significant reductions or elimination of steroid-dosing in patients with cerebral tumors, with no apparent impairment of neurocognitive status (26). Blinded independent review of magnetic resonance imaging (MRI) scans from patients receiving hCRF demonstrated that most of these patients experienced prolonged periods of stable disease, and a minority have achieved a measurable level of tumor regression (24). In a phase 1 clinical trial, 10 of the 15 patients who received hCRF had improvement in neurological symptoms or physical findings, with little or no toxicity (4). It is notable that there was a measurable decrease in steroid related side-effects, especially myopathy and the appearance of Cushingoid features. One patient, who had lymphoma-related pruritus and a long-standing rash resistant to steroids, noted improvement while receiving hCRF (25). In this preclinical study, we also noted
differences in toxicity and efficacy of hCRF, dexamethasone and temozolomide treatment of the glioma-bearing animals.

Overall we have shown therapeutic efficacy and low toxicity of hCRF in the treatment of a human-derived glioma in an orthotopic nude mouse model. Our results are consistent with the requirement of CRF-receptor expression in the tumor cells for hCRF therapeutic efficacy. Notably, hCRF treatment was less toxic in comparison to that with Dexamethasone or with Temozolomide. These results support the development of novel hCRF-releasing formulations or platforms for extended constant administration and studies to optimize the therapeutic antitumoral effect. Further clinical studies will need to evaluate whether long-term hCRF treatment (or the rapid cessation of treatment) is associated with increased glioma cell invasion and rapid tumor progression, as has been described for bevacizumab.
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### Table 1.

<table>
<thead>
<tr>
<th>Treatment Groups</th>
<th>Dose (mg/kg)</th>
<th>Route</th>
<th>Volume (ml)</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. hCRF (high dose)</td>
<td>0.1</td>
<td>s.c.</td>
<td>0.05</td>
<td>twice daily</td>
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<td>2. hCRF (low dose)</td>
<td>0.03</td>
<td>s.c.</td>
<td>0.05</td>
<td>twice daily</td>
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<tr>
<td>3. DEX (high dose)</td>
<td>1</td>
<td>s.c.</td>
<td>0.1</td>
<td>twice daily</td>
</tr>
<tr>
<td>4. DEX (low dose)</td>
<td>0.3</td>
<td>s.c.</td>
<td>0.1</td>
<td>twice daily</td>
</tr>
<tr>
<td>5. TMZ</td>
<td>40</td>
<td>i.p.</td>
<td>0.2</td>
<td>daily, 5 days per week</td>
</tr>
<tr>
<td>6. TMZ + hCRF (high dose)</td>
<td>40 + 0.1</td>
<td>i.p. + s.c.</td>
<td>daily, 5 days per week + twice daily</td>
<td></td>
</tr>
<tr>
<td>7. TMZ + hCRF (low dose)</td>
<td>40 + 0.03</td>
<td>i.p. + s.c.</td>
<td>daily, 5 days per week + twice daily</td>
<td></td>
</tr>
<tr>
<td>8. Control (vehicle)</td>
<td>-</td>
<td>-</td>
<td></td>
<td>daily</td>
</tr>
</tbody>
</table>
Table 2

A. hCRF and dexamethasone effects on BCNU EC$_{50}$

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>BCNU alone</th>
<th>BCNU + low hCRF (0.01nM)</th>
<th>BCNU + high hCRF (1.0nM)</th>
<th>BCNU + low DEX (0.01mM)</th>
<th>BCNU + high DEX (1.0mM)</th>
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</thead>
<tbody>
<tr>
<td>U87</td>
<td>226 ± 56 (9)</td>
<td>264 ± 91 (9)</td>
<td>193 ± 54 (9)</td>
<td>311 ± 65 (9)*</td>
<td>260 ± 59 (9)</td>
</tr>
<tr>
<td>C6</td>
<td>414 ± 43 (8)</td>
<td>419 ± 33 (8)</td>
<td>388 ± 59 (8)</td>
<td>346 ± 94 (8)</td>
<td>304 ± 102 (9)*</td>
</tr>
<tr>
<td>Ln229</td>
<td>234 ± 43 (7)</td>
<td>261 ± 58 (7)</td>
<td>219 ± 36 (7)</td>
<td>239 ± 47 (7)</td>
<td>201 ± 91 (7)</td>
</tr>
<tr>
<td>RG2</td>
<td>140 ± 22 (6)</td>
<td>259 ± 96 (6)</td>
<td>173 ± 60 (6)</td>
<td>329 ± 165 (6)*</td>
<td>147 ± 72 (6)</td>
</tr>
</tbody>
</table>

B. hCRF and dexamethasone effects on TMZ EC$_{50}$

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>TMZ alone</th>
<th>TMZ + low hCRF (0.01nM)</th>
<th>TMZ + high hCRF (1.0nM)</th>
<th>TMZ + low DEX (0.01mM)</th>
<th>TMZ + high DEX (1.0mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>U87</td>
<td>4.8 ± 3.5 (5)</td>
<td>3.1 ± 1.8 (5)</td>
<td>3.8 ± 2.8 (5)</td>
<td>4.5 ± 3.3 (5)</td>
<td>3.9 ± 3.2 (5)</td>
</tr>
<tr>
<td>C6</td>
<td>2.9 ± 1.1 (5)</td>
<td>3.6 ± 1.7 (5)</td>
<td>2.9 ± 1.0 (5)</td>
<td>4.5 ± 3.0 (5)</td>
<td>3.8 ± 3.5 (5)</td>
</tr>
<tr>
<td>Ln229</td>
<td>3.1 ± 0.6 (6)</td>
<td>3.2 ± 0.5 (6)</td>
<td>3.3 ± 0.6 (6)</td>
<td>3.4 ± 1.0 (6)</td>
<td>3.5 ± 1.0 (6)</td>
</tr>
<tr>
<td>RG2</td>
<td>9.8 ± 2.8 (5)</td>
<td>6.9 ± 1.4 (5)*</td>
<td>6.2 ± 0.3 (5)*</td>
<td>7.8 ± 2.1 (5)</td>
<td>7.9 ± 1.6 (5)</td>
</tr>
</tbody>
</table>

* Assays were performed after 24 hour exposure to the drugs. Significant difference compared to BCNU or TMZ alone; p<0.05 after adjustment for multiple comparisons using Dunnett’s method.
**Figure Legends.**

**Figure 1.** Time profiles of survival (A, C) and body weight (B, D) of animals bearing orthotopic U87-FLuc intracranial xenografts. The treatment period and end of the experiment (euthanasia of all surviving animals) is indicated by the bracket and arrow, respectively. Control animals received vehicle injections (black dashed line in Panels A and C). Treated animals in Panel A received either “low dose” hCRF (0.03 mg/kg twice daily; red dotted line), “high dose” hCRF (0.1 mg/kg twice daily; red solid line), “low dose” dexamethasone (0.3 mg/kg twice daily; blue dotted line), or “high dose” dexamethasone (1 mg/kg twice daily; blue solid line). The corresponding mean weight profiles of surviving animals in Panel B utilize a similar color code where solid symbols refer to “high dose” and “open symbols refer to “low dose”. Temozolomide (TMZ) treated animals are shown in Panel C. Animals received monotherapy (40 mg/kg daily, 5 of 7 days; green solid line), or combination therapy with “low” and “high” doses of hCRF (40 mg/kg of TMZ + 0.03 mg/kg of hCRF, orange dashed line; or 40 mg/kg of TMZ + 0.1 mg/kg of hCRF, orange solid line, respectively). The corresponding mean weight profiles of surviving animals in Panel B utilize a similar color code where solid symbols refer to “high dose” and “open symbols refer to “low dose”.

**Figure 2.** Time profiles of survival (A, C) and body weight (B, D) of animals bearing orthotopic C6-FLuc intracranial xenografts. Control animals received vehicle injections (black dashed line in Panels A and C); treated animals in Panel A received either “low dose” hCRF (0.03 mg/kg twice daily; red dotted line), “high dose” hCRF (0.1 mg/kg twice daily; red solid line), “low dose” dexamethasone (0.3 mg/kg twice daily; blue dotted line), or “high dose” dexamethasone (1 mg/kg twice daily; blue solid line). The corresponding mean weight profiles of surviving animals in Panel B utilize a similar color code where solid symbols refer to “high dose” and “open symbols refer to “low dose”. Temozolomide (TMZ) treated animals are shown in Panel C. Animals received monotherapy (40 mg/kg daily, 5 of 7 days; green solid line), or combination therapy with “low” and “high” doses of
hCRF (40 mg/kg of TMZ + 0.03 mg/kg of hCRF, orange dashed line; or 40 mg/kg of TMZ + 0.1 mg/kg of hCRF, orange solid line, respectively). The corresponding mean weight profiles of surviving animals in Panel B utilize a similar color code where solid symbols refer to “high dose” and “open symbols refer to “low dose”.

Figure 3. Bioluminescence imaging time-course of representative control and treated animals bearing U87 FLuc orthotopic gliomas. The profiles of control (vehicle-treated) (A), hCRF-treated (B) and dexamethasone-treated (C) animals are shown.

Figure 4. Bioluminescence imaging time-course of representative control and temozolomide -treated animals bearing U87FLuc orthotopic gliomas. The profiles of control (vehicle-treated) (A), TMZ monotherapy (B) and TMZ + hCRF combination therapy (C) are shown.

Figure 5. Expression of CRF1 and CRF2 gene transcripts in cultured-U87 and C6 parental cells (columns 1 and 5) and reporter-transduced cells (columns 2 and 6) and in subcutaneous xenografts from U87 and C6 parental cells (columns 3 and 7, and reporter-transduced cells: columns 4 and 8). Normal mouse brain tissue, which expressed both CRF1 and CRF2 mRNA, was used as a positive PCR control. Expression levels of CRF1 and CRF2 were normalized by the β-actin gene.

Figure S1 (for manuscript review purposes only). Bioluminescence imaging time-course of representative control and treated animals bearing C6FLuc orthotopic gliomas. The profiles of control (vehicle-treated) (A), hCRF-treated (B) and dexamethasone-treated (C) animals are shown.

Figure S2 (for manuscript review purposes only). Bioluminescence imaging time-course of representative control and Temozolomide-treated animals bearing C6FLuc orthotopic gliomas. The profiles of control (vehicle-treated) (A), TMZ monotherapy (B) and TMZ + hCRF combination therapy (C) are shown.
References.

Figure 1.
Figure 3
Figure 4
Figure 5
Comparison of Corticotropin-Releasing Factor, Dexamethasone and Temozolomide: Treatment Efficacy and Toxicity in U87 and C6 Intracranial Gliomas

Maxim A Moroz, Ruimin Huang, Tatiana Kochetkov, et al.

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