

# Supplementary Materials: Targeting Protein Kinase Ck2: Evaluating Cx-4945 Potential for GL261 Glioblastoma Therapy in Immunocompetent Mice

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## S1. Supplementary Materials and Methods

### S1.1. Cell Viability Assay

GL261 cells were plated at 5000 cells per well in 96-well multiwell plates (Sigma Aldrich, Madrid, Spain). Cells were allowed to adhere for 24 h before drugs were added to the medium at increasing concentrations: for Temozolomide (TMZ), 0  $\mu$ M, 0.8  $\mu$ M, 4  $\mu$ M, 20  $\mu$ M, 100  $\mu$ M, 200  $\mu$ M, 500  $\mu$ M, 1000  $\mu$ M, 5000  $\mu$ M and 10,000  $\mu$ M, apigenin (APG) and 4,5,6,7-Tetrabromobenzotriazole (TBB): 0  $\mu$ M, 0.8  $\mu$ M, 4  $\mu$ M, 20  $\mu$ M, 100  $\mu$ M, 200  $\mu$ M and 500  $\mu$ M, and 5-(3-Chlorophenylamino)benzo[c][2,6] naphthyridine-8-carboxylic acid (CX-4945): 0  $\mu$ M, 0.2  $\mu$ M, 2  $\mu$ M, 5  $\mu$ M, 20  $\mu$ M, 50  $\mu$ M, 100  $\mu$ M, 200  $\mu$ M and 500  $\mu$ M. Controls in each plate included cell culture RPMI (Roswell Park Memorial Institute) medium and dimethyl sulfoxide (DMSO) (0.4% for CX-4945, TBB and APG, and 0.8% for TMZ).

Drug-treated and control wells were run in triplicate. After 72 h of drug exposure, cell viability was measured using 2,3-bis-(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide (XTT) Assay (Sigma Aldrich, Madrid, Spain) as per the manufacturer's instructions. DMSO-treated wells were considered as "100% viability" for each treatment plate. In the case of combined TMZ and CX-4945 treatment, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Sigma Aldrich) was used for cell viability assay. Two compound combinations were used: CX-4945 30  $\mu$ M plus TMZ 1 mM, and CX-4945 50  $\mu$ M plus TMZ 1.5 mM, which were compared to control (medium and DMSO) and TMZ or CX-4945 alone. Controls included RPMI medium and DMSO (0.8%).

### S1.2. Therapeutic Agent Preparations (CK2 Inhibitors and TMZ)

CX-4945 sodium salt (Glix Laboratories, Southborough, MA, USA) was dissolved in 0.4% DMSO (cell experiments), or in phosphate buffer 25 mM pH 7.2 (in vivo studies), as described in [1]. APG (Sigma-Aldrich) and TBB (Calbiochem, Merck KGaA, Darmstadt, Germany), used for cell experiments, were dissolved in 0.4% DMSO. TMZ (Sigma-Aldrich) was dissolved in 0.8% DMSO for cell experiments, and in 10% DMSO in saline solution (0.9% NaCl) for in vivo experiments.

### S1.3. Tissue Homogenization and Protein Extraction

Tissue samples were weighted and 250  $\mu$ L of cold lysis buffer for each 100 mg of tissue was added (cold lysis buffer: 20 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.2% sodium deoxycholate, 2 $\times$  proteases inhibitor EDTA free (Roche, Madrid, Spain)), 1 $\times$  phosphatases inhibitors (Sigma-Aldrich): Phosphatase inhibitor cocktail 2 (Reference P5726), Phosphatase inhibitor cocktail 3 (Reference P0044) and 1% triton-x-100 (Sigma-Aldrich). Samples were homogenized with a 20 G needle 10 times and with a 26 G needle 10 more times. Sonication (Fisher Sonic Dismembrator Model 300, Thermo Fisher Scientific, Waltham, MA, USA) was performed five times for 5-s intervals at 30% amplification. After remaining 30 min on ice, the lysate was centrifuged at 25,000 $\times$  g for 20 min at 4  $^{\circ}$ C. Supernatants were used for WB and CK2 activity analysis.

### S1.4. Western Blot Analysis

GL261 cells were lysed as described in [2]. Protein concentration was determined by Bradford method [3] and equal amounts of protein (25  $\mu$ g) were loaded on 11% Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE), blotted on polyvinylidene fluoride (PVDF) Immobilon-P membranes (Millipore, Darmstadt, Germany), and immunodetected with the



**Table S4.** Average  $\pm$  standard deviation (AV  $\pm$  SD) for tumor volume (mm<sup>3</sup>) and body weight (g) for mice before starting metronomic therapy: CX-4945, TMZ, CX-4945 and TMZ, and control mice (day 10 post-inoculation). No significant differences ( $p > 0.05$ ) were found between the different groups ( $n = 6$ ) neither for tumor volumes, nor for mice body weight. Student's *t*-test applied.

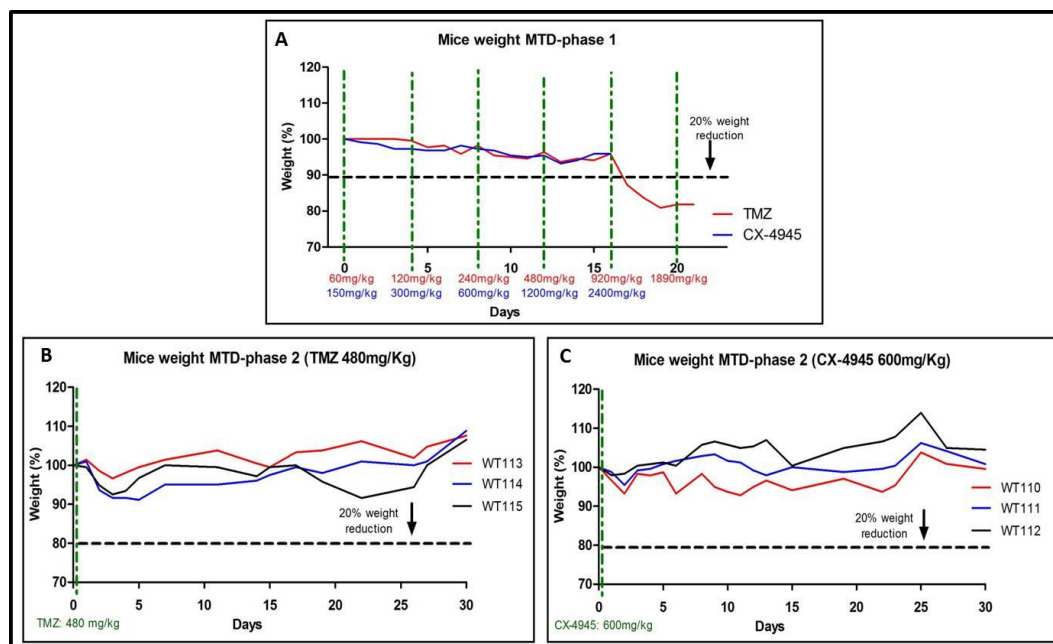
| DAY 10 (Tumor Volume and Weight) |        |       |       |       |       |       |       |                |
|----------------------------------|--------|-------|-------|-------|-------|-------|-------|----------------|
| CX                               | Mice   | C1144 | C1158 | C1147 | C1148 | C1149 | C1150 | AV $\pm$ SD    |
|                                  | Weight | 20.6  | 20.6  | 20.6  | 20.4  | 19.9  | 20.8  | 20.5 $\pm$ 0.3 |
|                                  | Volume | 5.3   | 5.1   | 6.5   | 7.5   | 2.3   | 3.0   | 5.0 $\pm$ 2.0  |
| CX+TMZ                           | Mice   | C1151 | C1152 | C1153 | C1154 | C1155 | C1156 | AV $\pm$ SD    |
|                                  | Weight | 21.9  | 20.7  | 21.0  | 20.1  | 22.5  | 24.1  | 21.7 $\pm$ 1.4 |
|                                  | Volume | 5.9   | 7.7   | 3.1   | 5.2   | 4.6   | 2.9   | 4.9 $\pm$ 1.8  |
| TMZ                              | Mice   | C1166 | C1167 | C1168 | C1169 | C1170 | C1171 | AV $\pm$ SD    |
|                                  | Weight | 19.7  | 20.7  | 22.0  | 21.1  | 17.1  | 21.0  | 20.3 $\pm$ 1.7 |
|                                  | Volume | 4.9   | 5.3   | 5.8   | 6.9   | 5.3   | 8.0   | 6.0 $\pm$ 1.2  |
| CONTROL                          | Mice   | C1157 | C1145 | C1160 | C1161 | C1162 | C1165 | AV $\pm$ SD    |
|                                  | Weight | 20.9  | 20.2  | 21.1  | 21.1  | 21.1  | 20.0  | 20.7 $\pm$ 0.5 |
|                                  | Volume | 5.2   | 4.1   | 6.9   | 3.6   | 10.1  | 7.1   | 6.2 $\pm$ 2.4  |

**Table S5.** Doses for CX-4945 and TMZ administration in maximum tolerated dose (MTD) calculation experiments. The final volume administration and doses were adjusted to actual animal weights.

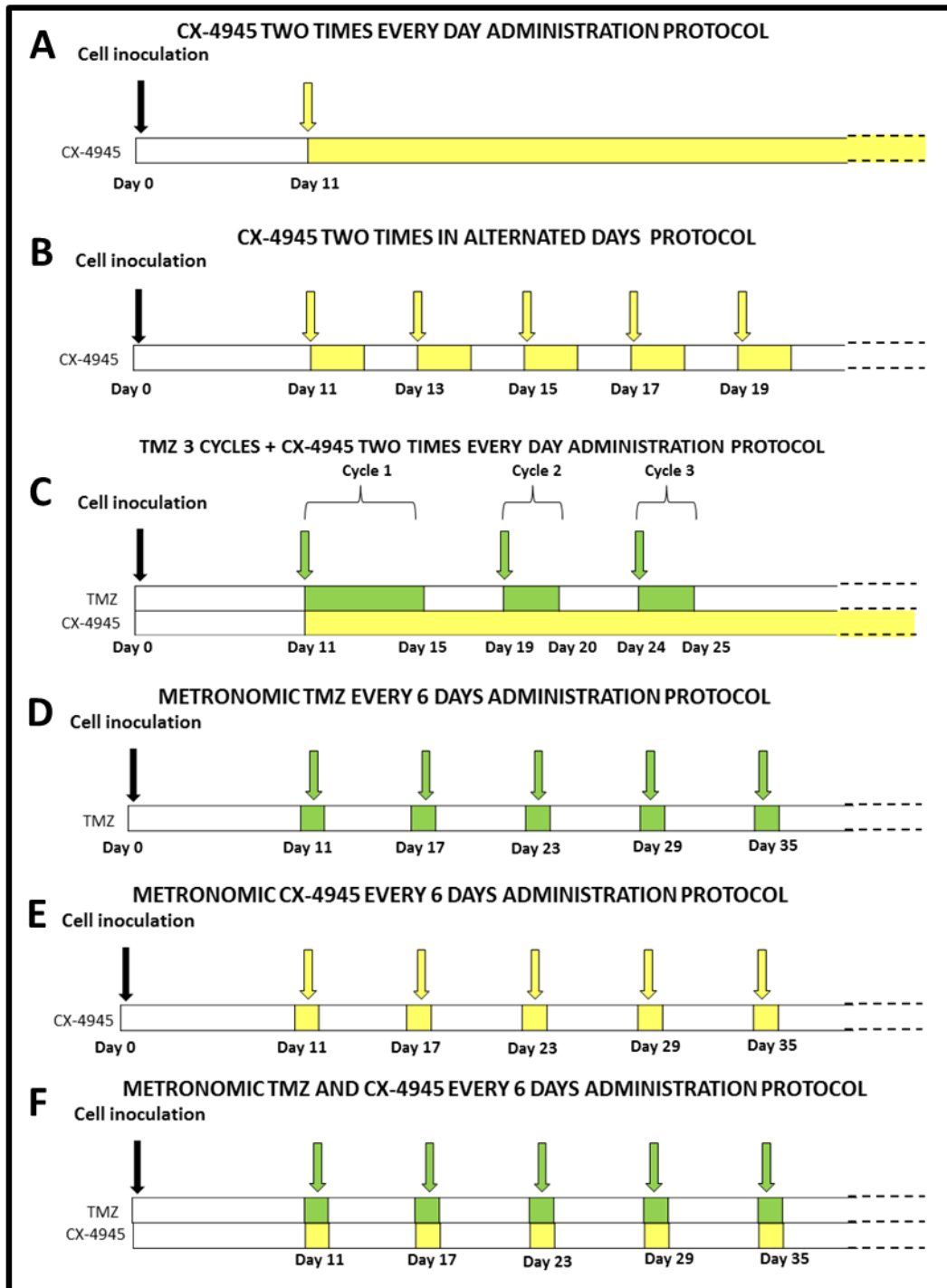
| Day             | 0   | 4   | 8   | 12   | 16   | 20   |
|-----------------|-----|-----|-----|------|------|------|
| CX-4945 (mg/Kg) | 150 | 300 | 600 | 1200 | 2400 | 4800 |
| TMZ (mg/Kg)     | 60  | 120 | 240 | 480  | 920  | 1890 |

**Table S6.** Symptoms and signals guidance to decide the MTD. Adapted from [4]. If at least two parameters for endpoint are detected, there is indication of adverse side effects and further dose increasing is discouraged.

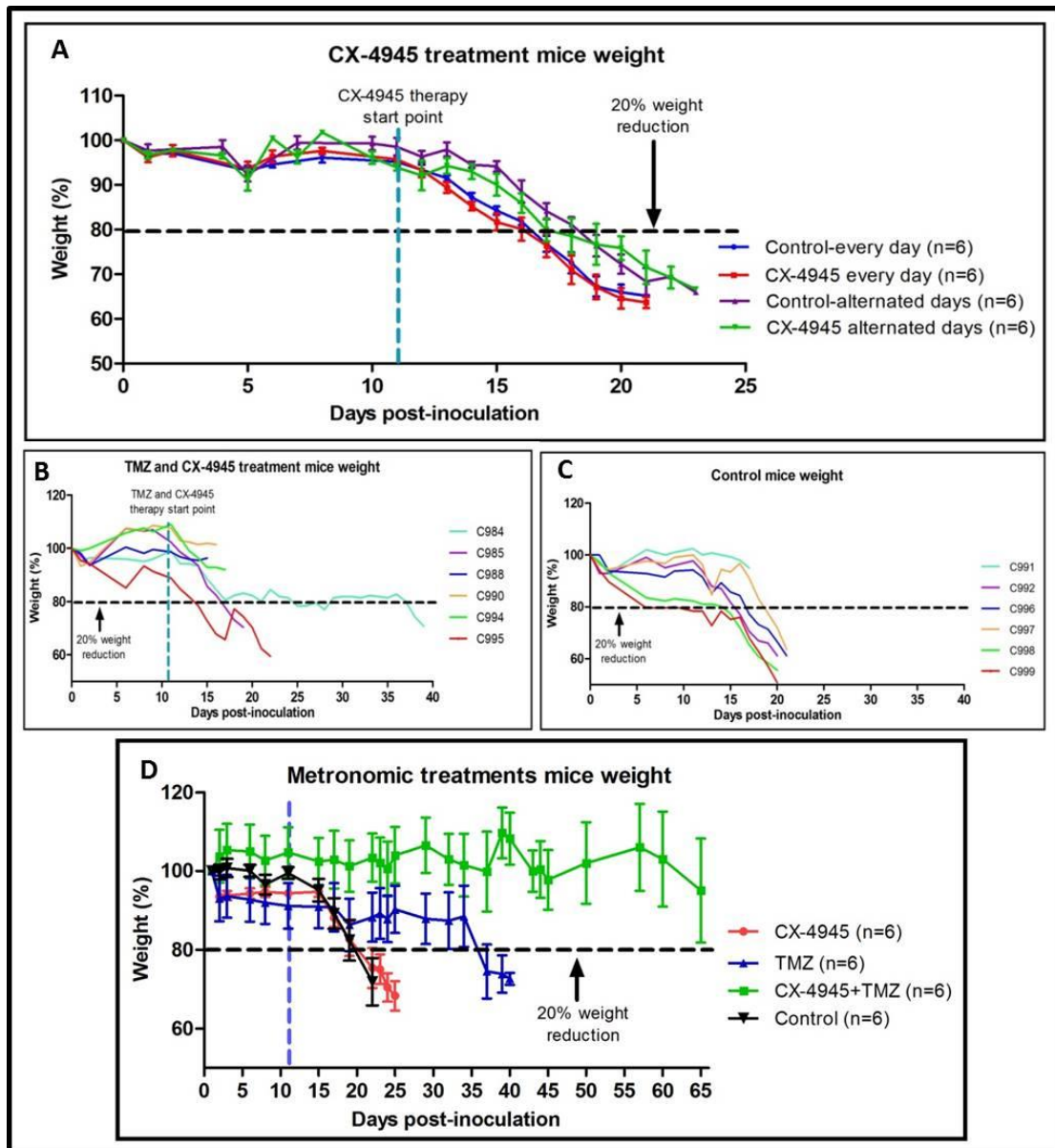
| Parameter for Endpoint                                     | Means of Verification   |
|--|---|
| Weight loss of above 20% regarding the previous register   | Scale readings  |
| Marked piloerection  | Piloerection detected during animal observation                                   |
| Animal shows subdued behaviour patterns even when provoked | Apathic behaviour during weighting procedure, in comparison with control animals. |
| Intermittent or persistent tremors                         | Observation of animals before and after weighting procedure                       |



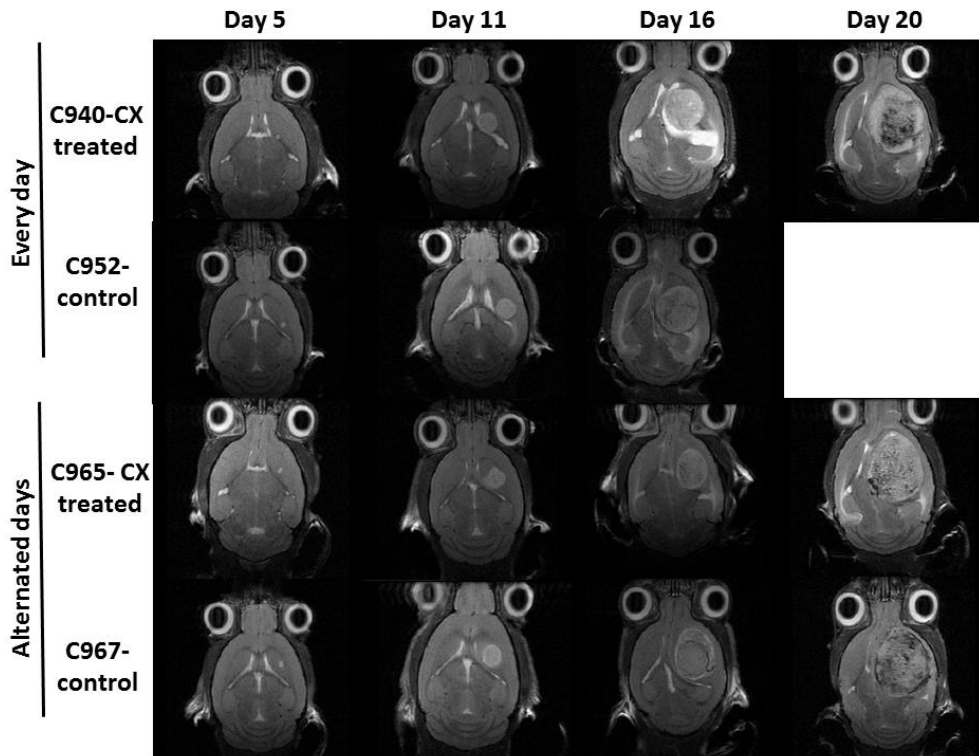
**Figure 1.** Mice body weight (maximum tolerated dose (MTD) studies). **(A)** Body weight of mice treated with increasing doses of Temozolomide (TMZ) (red line) and CX-4945 (blue line); **(B)** Body weight of mice treated with TMZ single dose (480 mg/kg) ( $n = 3$ ) and **(C)** Body weight of mice treated two times a day with CX-4945 (600 mg/kg total dose) ( $n = 3$ ). In all cases, the weight is expressed in %, considering 100% as the initial weight, and the dashed black line indicates the 20% weight reduction point. See main article text for further details.



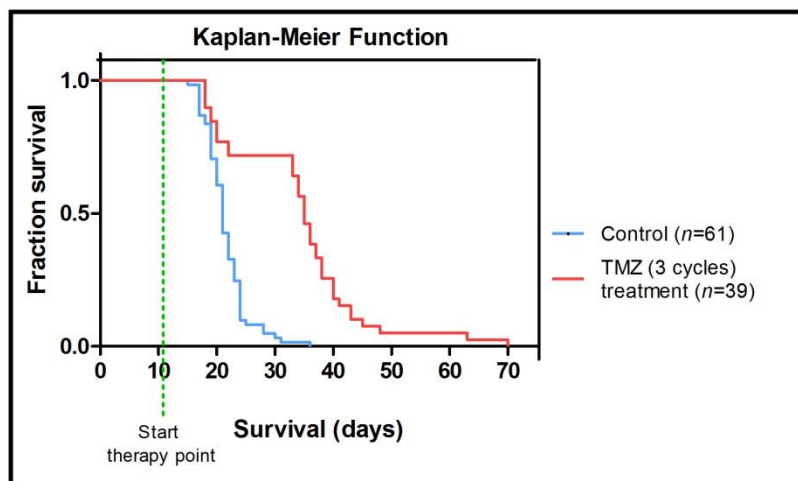
**Figure 2.** Therapy administration scheme protocols (A) for every day CX-4945 (150 mg/kg) administration (75 mg/kg at 8 h and 75 mg/kg at 16 h) (B) for alternated days CX-4945 (150 mg/kg) administration protocol (75 mg/kg at 8 h and 75 mg/kg at 16 h) (C) for TMZ + CX-4945 administration protocol. TMZ 60 mg/kg was administered at days 11–15, 19–20 and 24–25 post-inoculation and 150 mg/kg of CX-4945 (75 mg/kg at 8 h and 75 mg/kg at 16 h) (D) for metronomic TMZ (60 mg/Kg) every 6 days protocol (E) for metronomic CX-4945 (75 mg/kg at 8 h and 75 mg/kg at 16 h) every 6 days protocol and (F) for metronomic TMZ + CX-4945 every six days administration protocol. TMZ 60 mg/kg and 150 mg/kg of CX-4945 were administered: CX-4945 75 mg/kg at 8 h, TMZ 60 mg/Kg at 12 h and CX-4945 75 mg/kg at 16 h). In all cases, treatment started at day 11 post-inoculation.



**Figure 3.** Weight averages of treated and control mice. (A) Weight (average  $\pm$  SD) of mice treated two times every day with CX-4945 ( $n = 6$ , red line) and control vehicle ( $n = 6$ , blue line), and for mice treated two times a day in alternated days with CX-4945 ( $n = 6$ , green line) and control vehicle ( $n = 6$ , purple line). The dashed blue line indicates CX-4945 therapy start point. No differences were observed between groups ( $p > 0.05$ ); (B) Weights of individual mice treated with a combination of TMZ cycles (5-2-2) [5] and CX-4945 two times every day ( $n = 6$ ) until death or euthanasia for ethical reasons. The dashed blue line indicates TMZ and CX-4945 therapy start point. Case C984 was considered an outlier according to Grubbs' and Dixon's tests ( $p < 0.05$ ); (C) Body weight of each control mice (controls of mice represented in B) until death or euthanasia for ethical reasons. Administration of vehicles: phosphate buffer two times a day (CX-4945 vehicle) and 10% DMSO solution in 0.9% NaCl (TMZ vehicle) in 3 cycles, were performed. In all cases, the weight is expressed in %, assuming that at day 0 the initial weight corresponds to 100%. The dashed black line indicates the 20% weight reduction point; (D) Weight (average  $\pm$  SD) of mice treated with CX-4945 metronomic treatment ( $n = 6$ , red line), of mice treated with TMZ metronomic treatment ( $n = 6$ , blue line), of mice treated with CX-4945 and TMZ metronomic treatment ( $n = 6$ , green line) and control mice ( $n = 6$ , blue line). The dashed blue line indicates therapies start point. Significant differences were observed between all groups (control vs. treated groups and different treatments between them ( $p < 0.05$ )).



**Figure 4.** Magnetic resonance imaging (MRI) images of CX-4945 treated mice. Follow up of tumor volume evolution by T2w MRI axial images of CX-4945- treated tumor bearing mice (at days 5, 11, 16 and 20 post-inoculation). C940: treated with CX-4945 every day, C952: control of every day treatment (phosphate buffer CX-4945 vehicle administration), C965: CX-4945 treated in alternated days and C967: control for alternated days (vehicle administration). CX-4945 dosage was 150mg/kg split into two times per day (75 mg/kg 8 h and 75 mg/kg 16 h). MRI is not displayed for C952 day 20 because this mouse was found dead the day 17 post-inoculation. C940 was euthanized the day 20 post-inoculation for ethical reasons, and C965 and C967 were euthanized the days 21 and 20, respectively. Cxxxx corresponds to a unique alpha-numeric animal identifier code in the GABRMN group.



**Figure 5.** Survival Kaplan-Meier curve for 3 cycles of TMZ vs. control. Control mice ( $n = 61$ , blue line) and TMZ treatment ( $n = 39$ , red line). Survival rate average was  $21.5 \pm 3.7$  days for control mice and  $33.9 \pm 11.7$  days for TMZ (3 cycles) treated mice. Significant differences were found between groups ( $p < 0.05$ ) when



comparing control mice with TMZ treated mice. The dashed green line indicates the therapy start point. Results for control and TMZ treatment extracted from [5] and unpublished data.

## References

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