MN58b: an effective choline kinase inhibitor in the treatment of rat brain gliomas

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Introduction: Increased choline metabolism has been proposed as a hallmark of cancer, since high total choline (tCho) is consistently observed in both pre-clinical tumor models as well as in human tumors[1]. Due to increased choline kinase (ChoK) activity, elevated phosphocholine (PC) is generally observed in proliferating cancer cells and *in-situ* tumors by MRS. Thus, inhibition of ChoK using specific ChoK inhibitors, such as MN58b[2, 3], appears to be a promising treatment strategy in tumors. We have recently developed a high yield, two-step synthesis of MN58b. In this study we tested the efficacy of MN58b in the treatment of intracranial F98 gliomas. The therapeutic effects were assessed using *in-vitro* assays as well as *in-vivo* using ^{1}H MR Spectroscopy.

Materials and Methods: MTT assay: F98 rat glioma cells were plated in quadruplicate in 96-well plates at 7.5x10^{4}cells/ml and incubated overnight. Culture medium was replaced with media containing varying concentrations of MN58b. After 24h, the medium was removed and 20µl of 5mg/ml Thiazolyl Blue Tetrazolium Bromide (MTT) in sterile PBS was added and the cells were incubated for 2h. 150µl DMSO was added and absorbance read at 550 nm.

ChoK Activity Assay: F98 rat glioma cells plated in 6-well dishes were treated with 0.5 µCi/ml of ^{14}C-labeled choline for 1hr following addition of varying concentrations of MN58b. After 2hrs of treatment, cells were washed and fixed in 16% ice-cold trichloroacetic acid. Each sample was washed 3x in diethyl ether, lyophilized, and resuspended in water for thin layer chromatography separation ($NaCl/CH_3OH/NH_4OH$ 50:70:0.5 and autoradiographed using a Fujifilm FLA-7000.

PCA extracts of tumor cells and MN58b treatment: F98 cells were seeded (1x10^5/mL, 150cm^2 flasks) and incubated overnight, followed by 200 µl of 10-20 µMMN58b and sham (H_2O) treatment. Cells were trypsinized, washed and an aliquot removed for counting and protein determination. Pelleted cells were homogenized in 3 vol of 6% PCA, centrifuged (13,000 rpm, 30', 4°C), neutralized with 3 M KOH, and lyophilized. Lyophilized samples were dissolved in 500 µl of D_2O with 0.5 mmol TSP (as internal reference) and the pD was adjusted to 7.0.^{1}H MRS was performed at 11.7T on a 55mm vertical bore spectrometer(Varian, Palo Alto, CA) using the following parameters: 45° pulse, TR = 8 s, SW=6,000Hz, NP=64K and NT=128.

Tumor cell culture and implantation: Intracranial tumors were induced by stereotactic injection of F98 cells (5x10^4 cells in a 10 µl suspension) implanted into syngeneic Fischer (F344) rats as described previously[5]. MRS experiments were performed 2-weeks post inoculation.

***In-vivo* Spectroscopy:** *In-vivo*^{1}H MR spectra were obtained from the brains of six normal rats and five rats bearing an intracranial tumor. Three of the five tumor bearing animals were treated with MN58b (2mg/kg i.p. daily for 5 days) and MRS was repeated to evaluate the effects of treatment. *In-vivo* experiments were performed on a 9.4T horizontal bore scanner (Varian, Palo Alto, CA) equipped with 25 G/cm gradients. A 35 mm i.d. quadrature birdcage coil (M2M, Cleveland, OH) was used. Multi-slice spin echo and T_2-weighted images were acquired to localize the tumor and to plan the MRS voxel. Single voxel^{1}H MRS on a 3x3x3 mm^{3}voxel was performed using a PRESS sequence (TR = 3000 ms, TE= min (12.45 ms), NT = 128, SI = 4K and SW = 4000 Hz. Water suppression was performed using the VAPOR technique. An unsuppressed water spectrum was also acquired (8 averages) to compute metabolite to water ratios.

Data quantification: *Ex-vivo* F98 cell extract MRS data were analyzed using MestReNova (Mestrelab Research) to look for changes in PC and GPC. *In-vivo* MRS data were analyzed using LC-model to measure concentration [arbitrary units (AU), relative to water] of the metabolites (tCho, lipid and lac) in untreated and treated brain tumors.

Table: Effects of MN58b on Cho, Lac and Lipid in F98 brain tumor.

F98 Brain Tumor	tCho (mean±SEM)	Lac (mean±SEM)	Lipid (mean±SEM)
Untreated (n=5)	1.15±0.35	3.62±1.61	3.28±1.41
Treated (n=3)	0.70±0.23	1.71±0.89	11.84±3.08

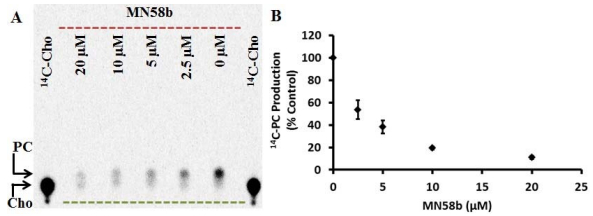


Fig. 1: *In-vitro* ChoK Activity of MN58b in F98 tumor cells.

Fig. 2

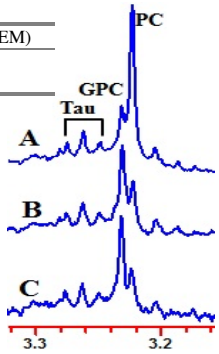
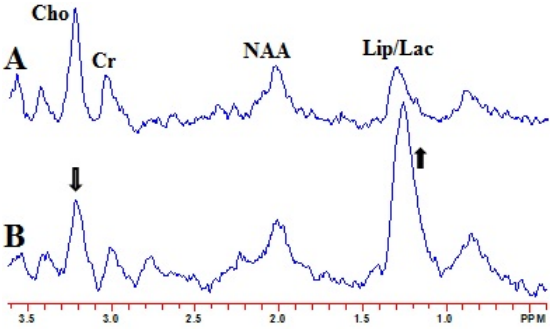


Fig. 3



Results: The IC_{50} of MN58b was determined to be 20 µM for proliferation using the MTT assay and 3.2 µM for choline kinase activity as measured by the conversion of ^{14}C labeled Cho to PC using the ChoK activity assay PC (Rf = 0.14) was quantified and the IC_{50} estimated using a sigmoidal fit (Fig 1B).Treatment of F98 cells with 10-20 µM MN58b led to a dose dependent reduction in PC as measured by ^{14}C PC production (Fig. 1A) and by *in-vitro* MRS of cell extracts (Fig. 2). *In-vivo* MRS also showed a decrease in tCho after MN58b treatment and a concomitant increase in Lip/Lac resonance (Fig.3, Table 1) indicating effective treatment response.

Fig.2 *In-vitro* NMR of F98 cell extracts. Sham treated cells show high PC (A). 10µM Mn58b treated show high GPC and low PC (B). 20µM MN58b treated cells show further changes in GPC and PC (C).

Fig.3: *In-vivo* MRS from untreated and *MN58b* treated F98 brain tumors. Untreated tumor (A) showing high tCho and low lip/lac. MN58b treatment resulted in reduced tCho and increased lip/lac level (B).

Discussion: These results demonstrate the potential of ChoK inhibition for the treatment of gliomas. MN58b was well tolerated with no acute toxic effects on the animals. *In-vivo*^{1}HMRS showed a significant MN58b-induced decrease in tCho indicating inhibition of ChoK (Fig. 3). Increased fatty acyl chain resonances have been correlated with malignancy in human brain tumors[4-6] and an increase in polyunsaturated fatty acids in mobile lipids[7, 8] along with a decrease in lactate[9, 10] is typically observed in tumors after treatment. The increased lipid resonance at 1.3 and 2.8 ppm (Fig. 3) suggests induction of apoptosis in the tumor after MN58b treatment. Both *in-vitro* and *in-vivo* data demonstrated the inhibition of ChoK. Choline kinase inhibitors, such as MN58b may potentially be a used as a new strategy for treatment of brain tumors. However, future studies confirming these results with larger cohorts of animals will be necessary to further validate these results.

References: [1] Glunde K, et al. Nat Rev Cancer. 2011;11:835-48. [2] Lacal JC. IDrugs. 2001;4: 419-26. [3] Al-Saffar NM et al. Cancer Res. 2006;66:427-34. [4] Opstad KS, et al., NMR Biomed 2008;21:677-85. [5] Kuesel AC, et al., Anticancer Res 1996;16:1485-9. [6] Kuesel AC, et al., NMR Biomed 1994;7:149-55. [7] Griffin JL, et al., Cancer Res 2003;63:3195-201. [8] Hakumäki JM, et al., Nature Med. 1999;5:1323-7. [9] Lee SC, et al., NMR Biomed. 2009;22:259-65. [10] Lee SC, et al., NMR Biomed. 2010;23:624-32.