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Safety and efficacy of a novel cannabinoid chemotherapeutic, KM-233, for the treatment of high-grade glioma

Christopher Duntsch[1], Murali Krishna Divi[2], Terreia Jones[3], Qihong Zhou[1], Mathangi Krishnamurthy[2], Peter Boehm[1], George Wood[2], Allen Sills[1] and Bob M. Moore II[2]

[1]Departments of Neurosurgery; [2]Department of Pharmaceutical Sciences, [3]Department of Pharmacy, The University of Tennessee Health Science Center, Memphis, TN, USA

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Corresponding Author:

Christopher Duntsch, Department of Neurosurgery, The University of Tennessee Health Science Center, 847 Monroe Avenue, Suite 427, Memphis, TN 38163, USA

Tel.: +1-901-448-4493; Fax: +1-901-448-8468; E-mail: duntsch@bellsouth.net

OBJECTIVE: To test *in vitro* and *in vivo* the safety and efficacy of a novel chemotherapeutic agent, KM-233, for the treatment of glioma.

METHODS: *In vitro* cell cytotoxicity assays were used to measure and compare the cytotoxic effects of KM-233, Δ^8-tetrahydrocannabinol (THC), and bis-chloroethyl-nitrosurea (BCNU) against human U87 glioma cells. An organotypic brain slice culture model was used for safety and toxicity studies. A human glioma-SCID mouse side-pocket tumor model was used to test *in vivo* the safety and efficacy of KM-233 with intratumoral and intra-peritoneal administration.

RESULTS: KM-233 is a classical cannabinoid with good blood brain barrier penetration that possesses a selective affinity for the CB2 receptors relative to THC. KM-233 was as efficacious in its cytotoxicity against human U87 glioma as Δ^8-tetrahydrocannabinol, and superior to the commonly used anti-glioma chemotherapeutic agent, BCNU. The cytotoxic effects of KM-233 against human glioma cells *in vitro* occur as early as two hours after administration, and dosing of KM-233 can be cycled without compromising cytotoxic efficacy and while improving safety. Cyclical dosing of KM-233 to treat U87 glioma in a SCID mouse xenograft side pocket model was effective at reducing the tumor burden with both systemic and intratumoral administration.

CONCLUSION: These studies provide both *in vitro* and *in vivo* evidence that KM-233 shows promising efficacy against human glioma cell lines in both *in vitro* and *in vivo* studies, minimal toxicity to healthy cultured brain tissue, and should be considered for definitive preclinical development in animal models of glioma.

Introduction

The historical median survival of patients with a glioblastoma multiforme (GBM) using the best radiological, surgical, and anti-tumor drug therapy available is less than one year depending on age and other prognostic factors [1-5]. Unfortunately, therapeutic adjuvants to surgery such as radiotherapy and chemotherapy provide only a minor improvement in the disease course and life expectancy and are associated with significant side effects [6-10]. Current chemotherapy regimens for newly diagnosed malignant glioma include single-agent intravenous bis-chloroethyl-nitrosurea (carmustine, BCNU), single-agent oral temozolomide, or the combination of procarbazine, chloroethyl-cyclohexylnitrosourea (lomustine, CCNU), and vincristine (PCV combination). With rare exception, to date there have been no good data proving prolongation of time to progression or survival for patients with malignant glioma when other drugs are added to the "standard" BCNU or PCV chemotherapeutic regimens, including cisplatin [11,12], carboplatin [13-15], dibromodulcitol [16], mercaptopurine [17], or 6-thioguanine [18]. The toxicity profile varies among these treatments, with myelosuppression being the most frequent dose-limiting factor [9,19]. Recent clinical trials have shown some efficacy for the oral chemotherapeutic agent temozolomide, particularly when given as an adjuvant with concurrent external beam radiotherapy [20]. However, two-year survival still remains less than 30% on this regimen, and significant drug-related toxicities occur.

Ligands of the cannabinoid receptors 1 and 2 (CB1 and CB2) have recently been shown to have varying degrees of cytoxicity against a variety of cancer cell lines. In fact, the antitumor effects of Δ^9-THC, the principal psychoactive component of marijuana, have been known since the 1970s [21]. Subsequent studies have demonstrated that several plant-derived (THC and

cannabidiol), synthetic (WIN-55, 212-2, JWH-133 and HU-210), and endogenous cannabinoids (anandamide and 2-arachidonoylglycerol) exert antiproliferative actions and induce apoptosis in various mouse, rat, and human cancer cells in culture [22-34].

The ability of cannabinoids to modulate cell survival and death pathways in neoplasias, although poorly understood at present, is significant from a drug development perspective. Specifically, clinically investigated cannabinoids, in contrast to conventional cancer chemotherapies, do not exhibit the typical toxicities associated with most chemotherapeutic agents, are well studied and tolerated by patients, and have the ability to penetrate the blood-brain-barrier (BBB). Of particular interest is the report that selective CB2 receptor-activating compounds are effective in regressing gliomas and skin carcinomas while inhibiting pain in the absence of overt signs of psychoactivity [34-37]. Given that the unwanted psychotropic effects of cannabinoids are mediated largely by CB1 receptors in the brain, a strategic approach to designing cannabinoids as chemotherapeutics would be the development of selective CB2 receptor agonists.

The renewed interest in ligands of the cannabinoid receptors as potential anti-neoplastics prompted us to investigate a series of novel classical cannabinoid (CCBs) ligands as anti-glioma agents. These compounds were designed and synthesized in an effort to produce high-affinity receptor subtype selective ligands. The lead compound in this series, KM-233, represents the first generation of C1' aryl substituted CCBs to be developed (Figure 1) [38]. This compound exhibits a significant increase in affinity for the CB2 receptor (K_i=0.91 nM) with only a modest increase for the CB1 receptor (K_i=12.3 nM) [38]. Furthermore, the introduction of the phenyl +0.ring in the C1' position resulted in an increase in the ClogP (computed LogP, a measure of differential solublity or hydrophobicity relative to the octanol/water partition coefficient) from

6.08 for Δ^8-THC to 6.27 for KM-233. The combination of increased lipophilicity and receptor affinity of KM-233 predicts that this compound should have significant transit across the BBB *in vivo* as well as good activity at the CB2 receptor on glioma cells, respectively.

We have completed *in vitro* and *in vivo* studies designed to test the efficacy and safety of KM-233 for the treatment of glioma. Using the U87 human glioma cell line for *in vitro* tissue culture studies and cytotoxicity assays, we have demonstrated that KM-233 has excellent cytotoxic effects and minimal toxicity both *in vitro* and *in vivo* when dosing is cycled on a daily basis.

Materials and methods

Cells, cell culture, and in vitro cytotoxicity assays

The human U87 glioma cell line (American Type Culture Collection, ATCC, Manassas, VA) was maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS, 1X non-essential amino acids (NEAA) and 0.01M HEPES buffer. The U87 glioma cell line was previously stably transduced with the pFB retrovirus (pFB-GFP) (Stratagene, La Jolla, CA) expressing green fluorescent protein (GFP) to enhance visual analysis. At the beginning of each experiment, the media was replaced with supplemented serum free media, to avoid the inhibitory effects of serum on cannabinoid molecules. The cultures were treated with varying amounts of drug (KM-233, THC, or BCNU) formulated with 0.5% dimethylsulfoxide (DMSO) as described in the results section. Following addition of drug, cell death was analyzed at 24, 48, and 72 h post-treatment using the CellTiter 96R Non-Radioactive Cell Proliferation assay (G5421,

Promega, Madison, WI). The percentage of viable cells present in the culture at each time point was calculated by comparing the absorbance value at 492 nm from the MTS assay for each condition with untreated control cells using a Lab Systems Multiskan Biochromatic Elisa plate reader (Vienna, VA). All described values represent the average of three data points, and the standard deviation for each average is provided. Percent viability and cytotoxicity as well as standard deviations were calculated from the absorbance values using Microsoft Excel. Inhibitory concentration, IC50, values were calculated using Graphpad Prism® software.

Organotypic brain slice culture for toxicity studies

The methods used to create organotypic brain slice culture for toxicity studies were modified from those introduced by Plenz and Kitai [39] and were published previously [40]. In brief, 1-2 days old Sprague-Dawley rat pups were decapitated and the brains were removed rapidly and then coronal slices were made on a vibratome at 500 μm for striatum and substantia nigra and 400 μm for cortex. The areas of cortex, striatum, and substantia nigra pars compacta were dissected into 0.5-1 mm size and were subsequently placed on Millicell culture insert (Millipore Corp., Billerica, MA) on a cover slip. The coverslip with the tissue was then placed into a culture tube (Nalge Nunc International, Rochester, NY), and to each tube was added 750 μl of incubation medium. The tubes were then incubated at 35°C on a carousel rotated at a speed of 0.5 RPM. The slices were used for experiments after three weeks in culture.

Immunohistochemistry

The methods were published previously [40]. In brief, the slices were fixed, dried, and stored at 4°C for later use. The slides were incubated in mouse anti-microtubule-associated protein 2 (MAP-2, 1:500, Sigma-Aldrich Corp., St. Louis, MO) overnight at room temperature; washed with PBS 3 times; incubated in Cy™2 or Cy™3-conjugated AffiniPure donkey anti-mouse IgG (1:250, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) for 4 h at room temperature in the dark. The MAP-2 immunoreactivity was visualized with a Bio-Rad confocal microscope and digital images were collected using the associated confocal software.

Generation of a mouse xenograft subcutaneous model of glioma

U87-GFP cells in exponential growth were harvested with EDTA/trypsin for 5 min at 37°C. The cells were centrifuged for 5 min at 1,000 RPM, and the pellets were resuspended in sterile PBS, counted using Trypan blue staining methods, and resuspended in PBS at a concentration of $1x10^4$/μl and placed on ice. Adult Prkd mice (roughly 6 weeks of age) were used for all studies and handled in accordance with protocols approved by the Animal Care and Use Committee at the University of Tennessee Health Science Center. To create a subcutaneous tumor model, $2x10^6$ U87 cells resuspended in approximately 200 μl PBS were injected subcutaneously into the right flank. Either of two formulations of KM-233 or the respective vehicle controls were administered directly to the subcutaneous tumor bed or systemically via intraperitoneal administration beginning on the 5th day post-inoculation of tumor. The animals were weighed daily, and monitored closely for signs of systemic or local toxicity from administration of KM-233. After 20 days of tumor incubation (15 days of KM-233 treatment) the animals were

sacrificed and the tumors were carefully dissected from the soft tissues using fluorescent microscopy.

Results

Efficacy and IC50 studies of KM-233 against U87 human glioma cells

To determine the cytotoxic effect and IC50 of KM-233 against human U87 glioma cells, we treated cells with escalating doses of KM-233 over 3 days. Cell death was analyzed at 24, 48, and 72 h post-inoculation using a cell proliferation assay. As shown in Figure 2A, as little as 3 μM KM-233 effectively killed the glioma cells over a 3 day incubation period, with maximum cytotoxic effect occurring at 5 μM. Doses of 10, 20, and 30 μM were tested and had no greater cytotoxic effect in these assays than 5 μM (data not shown). The IC50 value for KM-233 was examined in a dose escalation study and determined to be 1.429 μM. Figure 2B-F shows phase-contrast photomicrographs of representative experiments demonstrating the effects on U87 glioma of the no treatment control (Figure 2B, serum free culture media), vehicle control (Figure 2C, serum free culture media with 0.5% DMSO), and escalating doses of KM-233 (Figure 2D-F).

Comparison of the cytotoxic efficacy of KM-233 with the natural cannabinoid Δ^8-THC

Using similar cytotoxicity assays to those described above, we compared side-by-side the chemotherapeutic effects of KM-233 to the natural product THC. For these experiments a

standardized dose of 5 μM was used for both THC and KM-233 based on the results of the studies described in Figure 2A. Although the end result at 72h was similar, 5 μM of KM-233 was found to be reproducibly superior to THC over the first 48 hours (Figure 3A). Figure 3B to G include phase-contrast photomicrographs of representative experiments showing the effects of the no treatment control (Figure 3B, serum free culture media), vehicle control (Figure 3C, serum free culture media with 0.5% DMSO), and 3 μM and 5 μM of KM-233 or THC (Figure 3D-G). These experiments demonstrate that KM-233 is as efficacious as Δ^8-THC as a chemotherapeutic agent, and may have a more rapid onset of cytotoxic effects following its administration.

Comparison of cytotoxic efficacy of KM-233 with the glioma chemotherapeutic BCNU

We compared KM-233 with the commonly used anti-glioma agent BCNU in dose escalation studies. Because of its poor cytotoxicity against glioma in these studies, only the results of the higher doses of BCNU tested are included. As shown in Figure 4A, 5 μM of KM-233 was far superior to the highest dose of BCNU tested (300 μM). These results were confirmed using phase-contrast photomicroscopy of representative experiments showing the effects of the no treatment control (Figure 4B, serum free culture media), vehicle control (Figure 4C, serum free culture media with 0.5% DMSO), and 5 μM KM-233 or BCNU (Figure 4D-G).

Kinetic studies of KM-233 cytotoxicity and effects of dose cycling on efficacy

To determine the initial time point at which cytoxicity begins with KM-233, a similar MTS cytotoxicity assay was performed using 2 h increments after initiation of treatment with 10 μM and 30 μM KM-233. As shown in Figure 5, the onset of cytoxicity against the U87 glioma cell line began around 2 h after administration of drug, irrespective of whether higher doses were used. The rapid onset of cytoxicity led us to speculate whether KM-233 could be cycled to limit its toxicity without affecting its efficacy. To study the effect of cycling KM-233 on its efficacy, similar cytotoxic studies were conducted in which 10 μM KM-233 was cycled by adding it for an 8 h incubation period over 1, 2, and 3 days as compared to 3 days of continuous treatment. After each day of cycling, media containing KM-233 or the control media [media alone (NT, no treatment) or vehicle control (VC, media with 0.5% DMSO)] was replaced by fresh media supplemented with 10% serum. The average absorbance values for U87 glioma cells at the beginning of each experiment are shown for comparison (T0, Time 0). As shown in Figure 6, cycling of KM-233 for 3 days resulted in an impressive reduction in cell viability (roughly 60% on average) that was as efficacious as continuous treatment with KM-233 for 3 days when compared to nontreated or vehicle controls. Cycling for 1 and 2 days also resulted in a reduction of cell viability in a dose-dependent manner but was not as efficacious as cycling for three days (data not shown) predicting a need for multiple rounds of drug cycling to completely eradicate tumor cells.

Toxicity studies of KM-233 against organotypic brain slice culture

To test the toxicity of KM-233 on healthy brain tissue, we completed dose escalation studies using an organotypic brain slice glioma culture system. Figure 7A demonstrates baseline MAP-2

immunoreactivity in this organotypic slice culture model, which for the purposes of these experiments serves as a non-specific neuronal marker that is a sensitive indicator of neuronal integrity [41].

To test the toxicity of KM-233 on healthy cultured brain tissue, a series of experiments similar to those described in Figure 6 were initiated. KM-233 (10 μM) or the appropriate control media was added to the cultures either continuously for 3 days, or cycled once per day for 8 h for 1, 2, and 3 days followed by replacement with normal culture media after each cycle. After 3 days, the organotypic brain slice cultures were fixed and stained with MAP-2. As shown in Figure 7D to F, cycling of KM-233 for 1, 2, and 3 days was well tolerated by the brain tissue as indicated by excellent MAP-2 immunoreactivity. Continuous administration at 10 μM did result in mild neurotoxicity to the brain slice neurons as indicated by a reduction in MAP-2 expression and a pathologic distribution of MAP-2 (Figure 7G). When higher doses of KM-233 were tested, they were poorly tolerated by sensitive brain slice tissue as demonstrated in Figure 7H-K where KM-233 at 20 μM was cycled for 1, 2, and 3 days (Figure 7H-J, respectively) or administered continuously for 3 days (Figure 7K). In summary, doses that exceed the maximal *in vitro* cytotoxicity seen with human U87 glioma cells (10 μM), KM-233 appear safe when exposed to normal brain tissue in a closed environment under conditions of drug cycling for up to 3 days.

In vivo efficacy and safety of KM-233 against human U87 glioma cells in a rodent xenograft side-pocket model

To study the effect of KM-233 on human U87 glioma *in vivo* with administration of drug intra-peritoneal or intra-tumoral, tumors were induced in SCID mice by subcutaneous flank inoculation of human U87-GFP glioma cells. After 5 days of tumor cell inoculation, animals were assigned randomly to groups (n=4 per group) that included KM-233 formulation and vehicle formulation as a control. Cremophor EL formulation was selected for formulating KM-233 given its extreme hydrophobicity and increased ClogP relative to THC [38]. Formulated drug (3% cremophor EL® and 3% ethanol, 94% saline) or vehicle control was administered twice a day via intra-tumoral injection directly into the tumor bed or intraperitoneally for 15 subsequent days at a dose of 2 mg/kg. The animals were then sacrificed on the 16th day of treatment, and the tumors were dissected and weighed. As shown in Figure 8A and B, KM-233 cremophor EL® formulation is effectively reduced the average weight of the tumors of each treatment group as compared to vehicle controls regardless of administration route. Figure 8C and D show representative fluorescent photomicrographs of the average tumor body size in animals treated with KM-233 formulation compared with the respective vehicle control treated group. Clinical monitoring and daily weights were similar between groups indicating no gross deleterious effects of KM-233 when administered locally or systemically (data not shown). These data demonstrate that KM-233 was safe and effective in reducing tumor volume via both direct intra-tumoral injection and systemic administration by intraperitoneal injection.

Discussion

We have completed studies designed to test *in vitro* and *in vivo* the efficacy and safety of the novel chemotherapeutic agent KM-233, developed by Krishnamurthy et al. [38], for the

treatment of glioma. KM-233 is the lead compound from a new subclass of classical cannabinoid compounds containing a C1' aryl substituted moiety that has enhanced affinity and selectivity for the CB2 receptor over the CB1 receptor when compared to Δ^8-THC (Figure 1), while increasing its ClogP value. The combination of CB2 selectivity and good penetration of the BBB collectively combine to create a potentially attractive chemotherapeutic agent for the treatment of high-grade brain gliomas.

Using *in vitro* tissue culture studies and cytotoxicity assays, we have demonstrated that KM-233 has excellent cytotoxic effects against U87 human glioma cells that are comparable to Δ^8-THC and far superior to the commonly used anti-glioma chemotherapeutic agent, BCNU (Figures 2-4). Mechanisms associated with the poor cytotoxic efficacy seen with BCNU include reduced intracellular drug accumulation through membrane efflux pumps, drug detoxification, and alterations in drug target specificity. Additionally, variations in multidrug resistance genes [42], DNA repair activity such as O6-methylguanine-DNA methyltransferase [43], and glutathione S-transferase and intracellular glutathione content have been implicated in reduced BCNU efficacy [44]. Conversely, resistance to cannabinoid activity by glioma cells has not been described. An additional advantage of KM-233 over BCNU is that the former works through CB1/CB2 receptor activation and the latter via DNA alkylation and it is therefore possible that considerable synergy may be seen if these drugs were to be used in combination.

The cytotoxic effects of KM-233 against human glioma cells *in vitro* occur as early as two hours after administration, and dosing of KM-233 can be cycled without compromising cytotoxic efficacy and while improving safety (Figures 6 and 7). To further test whether cyclical dosing was a viable option *in vivo*, similar studies were completed using a U87-PRKD SCID mouse side-pocket xenograft model. KM-233 formulated with (Cremophor EL® 3%, 3% ethanol, 94%

saline) showed good efficacy with both intra-tumoral injection and intra-peritoneal administration (Figure 8). *In vitro* studies identical to those described above were conducted with the human glioma cell line U373 and the rat glioma cell lines C6 and F98 with similar results to as reported here for the human glioma cell line, U87, indicating that the cytotoxic effects of KM-233 occur irrespective of cell line or species tested (data not shown).

Cannabinoids are becoming attractive as a class of antiproliferative agents that are active against a wide spectrum of tumor cells in both tissue culture and rodent model studies [31,36,45,46]. These studies demonstrate that cannabinoids both inhibit the growth of tumors as well as induce apoptosis through CB1 and CB2 activation. Selective CB2 receptor activating compounds have recently emerged as a new class of chemotherapeutics and have been shown to be effective as anti-tumor agents while lacking the significant psychoactivity associated with CB1 agonists [34-37]. Interestingly, Riboni *et al*. [47]. have shown a correlation of CB2 expression with increasing grade of glioma making CB2 selective agonists even more attractive for this class of brain tumors.

The mechanisms of induction of apoptosis after only hours of CB1/CB2 activation are not clear, but are at least due in part to poorly understood complex signal transduction events that include the RAF-1/MEK/ERK signaling cascades and early accumulation of the proapoptotic sphingolipid ceramide [31,48,49]. Sanchez *et al*. [24] have shown that THC-induced apoptosis in rat C6 glioma cells may occur via CB1 receptor-independent stimulation of sphingomyelin breakdown. Additionally, Blazquez *et al*. [36] reported that cannabinoid administration inhibits the vascular endothelial growth factor (VEGF) pathway in cultured glioma cells, in glioma-bearing mice, and in two patients with GBM. The differences observed in cytotoxicity between healthy cultured brain tissue and *in vitro* glioma cell cultures maybe due to differences in CB1

and/or CB2 receptor expression and distribution, or due to differences in signal transduction events downstream from these receptors. Potential mechanisms are currently under intense study in the hope that they can be manipulated to further enhance KM-233's *in vivo* efficacy and safety as a glioma chemotherapeutic.

In summary, these studies provide proof-of-principle that KM-233 is an effective glioma-lytic agent with minimal toxic side effects to healthy cultured brain tissue when the dosing is cycled. Further studies in animal models of intracranial glioma are needed to better assess its *in vivo* safety and efficacy, and will provide important preclinical data for this and other CB2 selective agonists as potential chemotherapeutic agents for the treatment of glioma.

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Figure legends

Figure 1. Chemical structures of KM-233 and Δ^8-Tetrahydrocannabinol.

Figure 2. *In vitro* efficacy and IC50 studies for KM-233 against human U87 glioma cells. A, Cytotoxicity studies of KM-233 induced cytotoxicity against human U87 glioma cells over 3 days of incubation. B-F, representative phase contrast photomicrographs showing the effects of KM-233 on human U87 glioma cells after 72 h of incubation. B, no treatment; C: vehicle control; D, 1.0 μM KM-233; E, 3.0 μM KM-233; F, 5.0 μM KM-233. For all experiments, cells were cultured in serum-free medium (no treatment), serum-free media with 0.5% DMSO (vehicle control), or serum-free media with 0.5% DMSO and varying amounts of KM-233 over 3 days of incubation.

Figure 3. Comparison of *in vitro* cytotoxic efficacy of KM-233 and Δ^8 THC against U87 glioma cells. A, cytotoxicity studies of KM-233 and Δ^8 THC induced cytotoxicity against human U87 glioma cells over 3 days of incubation. All values represent the average of three data points, and the error bars represent the standard deviation for each group. B-G, representative phase contrast photomicrographs showing the effects of KM-233 and Δ^8-THC on human U87 glioma cells after 72 h of incubation. B, no treatment; C, vehicle control; D, 3.0 μM KM-233; E, 3.0 μM Δ^8-THC; F, 5.0 μM KM-233; G, 5.0 μM Δ^8-THC. For all experiments, cells were cultured in serum free medium (no treatment), serum free media with 0.5% DMSO (vehicle control), or serum-free media with 0.5% DMSO and 5.0 μM KM-233 or 5.0 μM Δ^8-THC over 3 days of incubation.

Figure 4. Comparison of in vitro cytotoxic efficacy of KM-233 and BCNU against U87 glioma cells. A, cytotoxicity studies of KM-233 and BCNU induced cytotoxicity against human U87 glioma cells over 3 days of incubation. All values represent the average of three data points, and the error bars represent the standard deviation for each group. B-G, representative phase contrast photomicrographs showing the effects of KM-233 and BCNU on human U87 glioma cells after 72 h of incubation; B, no treatment; C, vehicle control; D, 5.0 μM KM-233; E, 100 μM BCNU; F, 200 μM BCNU; G, 300 μM BCNU. For all experiments, cells were cultured in serum free medium (no treatment), serum free media with 0.5% DMSO (vehicle control), or serum-free media with 0.5% DMSO and 5.0 μM KM-233, or 100, 200, or 300 μM BCNU over 3 days of incubation.

Figure 5. In vitro kinetic studies of the onset of cytotoxicity induced by KM-233 in U87 glioma cells. Line graphs demonstrating the changes in cell viability over time after exposure of human U87 glioma cells to 10 or 30 μM KM-233 over 6 h of incubation. All values represent the average of three data points, and the error bars represent the standard deviation for each group. For all experiments, cells were cultured in serum-free media (no treatment), serum-free media with 0.5% DMSO (vehicle control), or serum-free media with 0.5% DMSO and varying amounts of KM-233 over 6 h of incubation.

Figure 6. Comparison of *in vitro* cytotoxic efficacy of continuously administered KM-233 and cycled KM-233. Bar graph representing the average cell viability at time 0 (T0) or at 3 days for cycled and continuously administered drug. For all experiments, cells were cultured in serum-free medium (NT, no treatment), serum-free media with 0.5% DMSO (VC, vehicle control), or

serum-free media with 0.5% DMSO and 10 μM of KM-233 cycled for 8 h in drug and 16 h in culture media over 3 days or given continuously. All values represent the average of three data points, and the error bars represent the standard deviation for each group.

Figure 7. MAP-2 immunoreactivity of organotypic brain slice cultures after treatment with various controls, continuous KM-233 at various doses, or cycled KM-233 for 3 days at various doses. A-C, photomicrographs of MAP-2 immunoreactivity of brain slice cultures after three days of incubation with normal culture media (A), serum free media (B, no treatment control), or serum free media containing 0.5% DMSO (C, vehicle control) demonstrating excellent MAP-2 expression and distribution for all three controls. D-F, photomicrographs of MAP-2 immunoreactivity of brain slice culture after three days incubation with 1 (C), 2 (D), or 3 (E) days of cycled KM-233 at 10 μM. G, photomicrographs of MAP-2 immunoreactivity of a brain slice culture after three days of continuous incubation with KM-233 at 10 μM. H-J, photomicrographs of MAP-2 immunoreactivity of brain slice culture after three days incubation with 1 (H), 2 (I), or 3 (J) days of cycled KM-233 at 20 μM. G, photomicrographs of MAP-2 immunoreactivity of a brain slice culture after three days of continuous incubation with KM-233 at 20 μM. All slice studies were done in triplicate and a representative photomicrograph for each group is shown.

Figure 8. In vivo efficacy of KM-233 prepared with two different formulations against human U87 glioma cells. Human glioma cells were inoculated into the flanks of nude mice (n=4 per group). After 5 days of tumor cell incubation, groups were treated either by direct injection of the tumor bed (A) or intraperitoneally (B) every 12 hours for 15 subsequent days with vehicle or

2 mg/kg of KM-233 formulated in 100 μl of saline containing 3% cremophor and 3% ethanol. KM-233 was efficacious at reducing the tumor burden. Panels C and D demonstrate fluorescent photomicrographs of the average size of tumors dissected from animals treated with KM-233 (C) as compared to the vehicle control (D).

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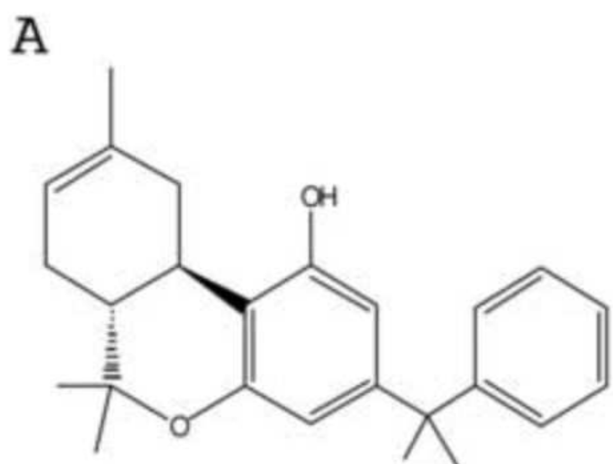

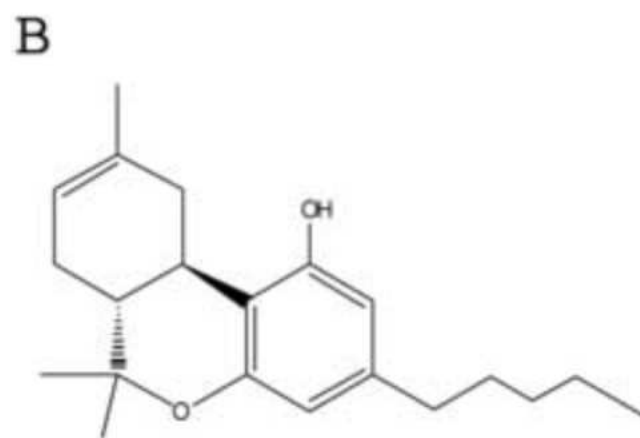

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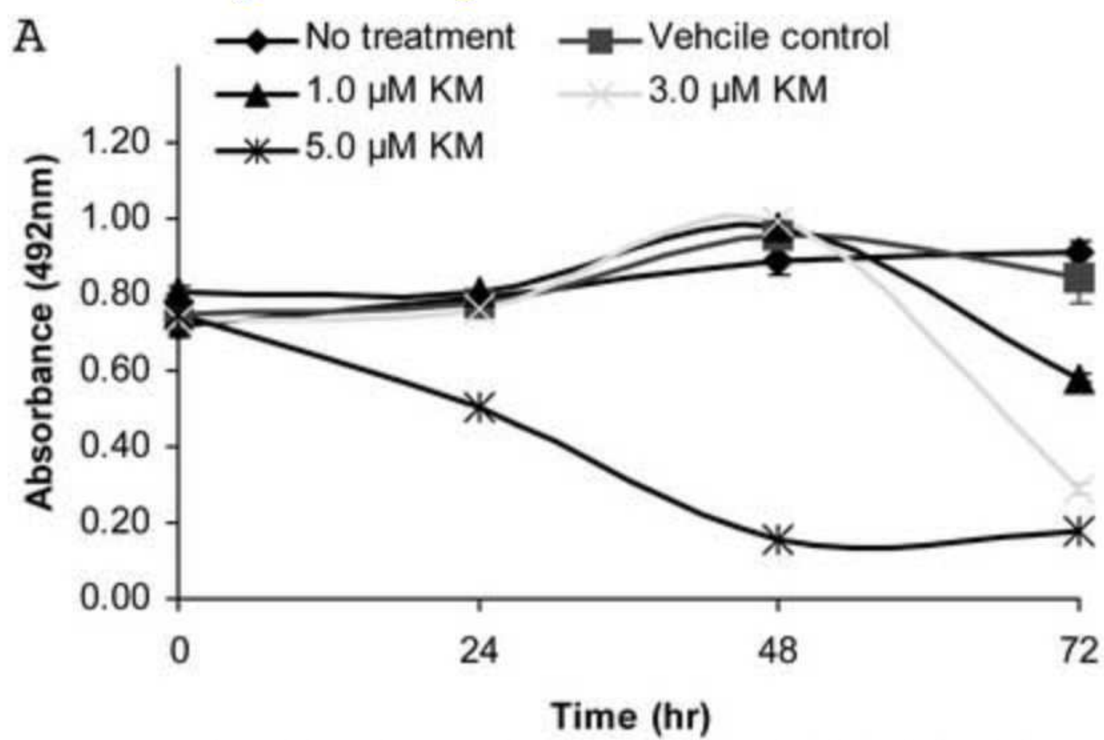

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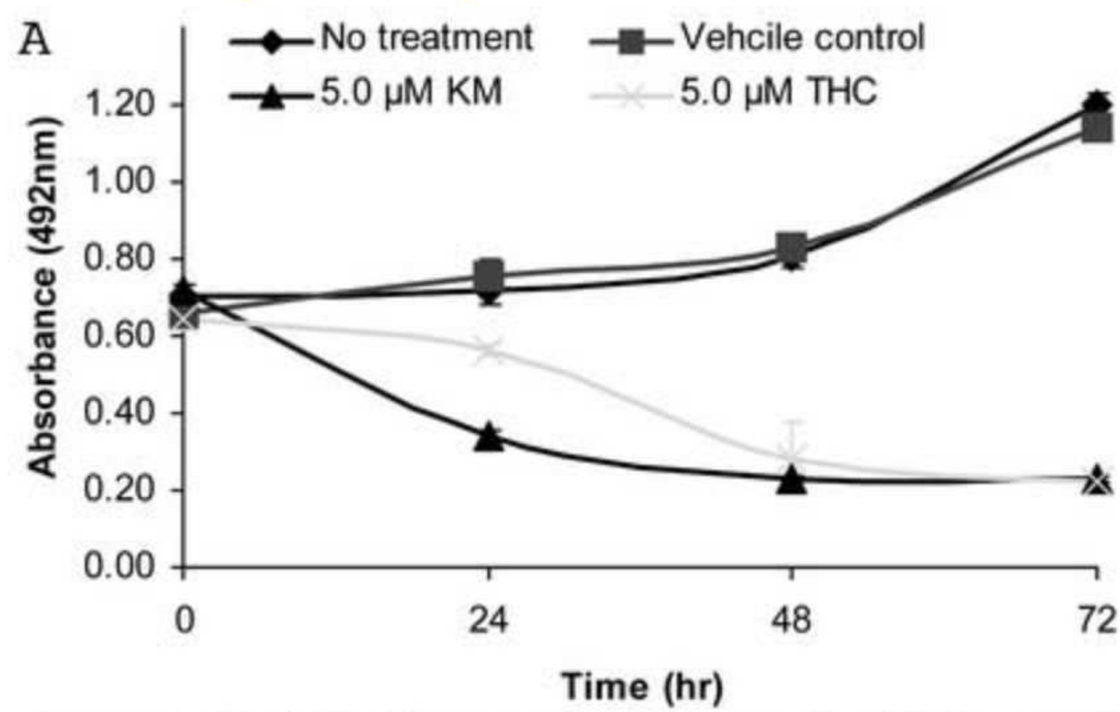

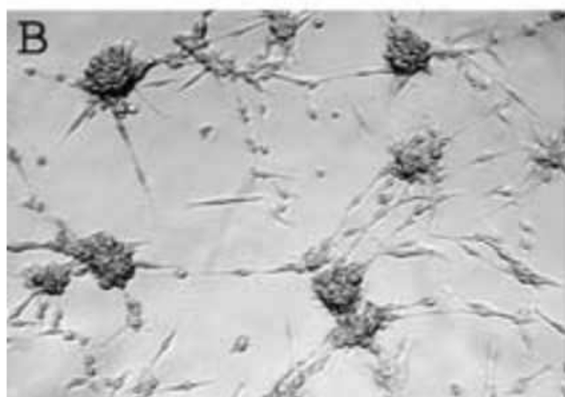

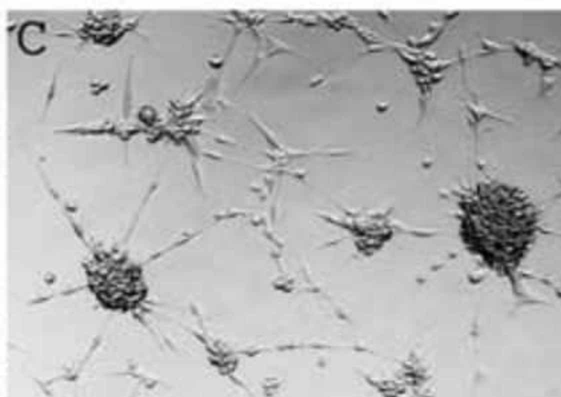

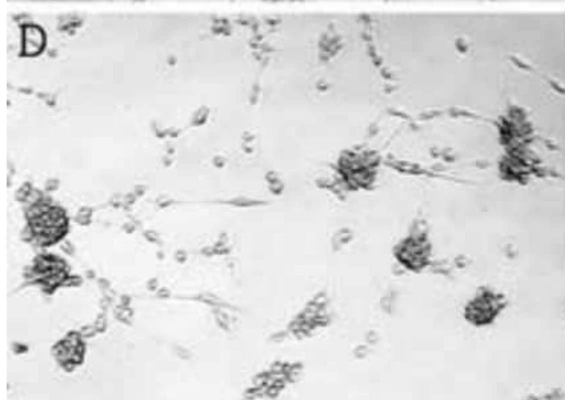

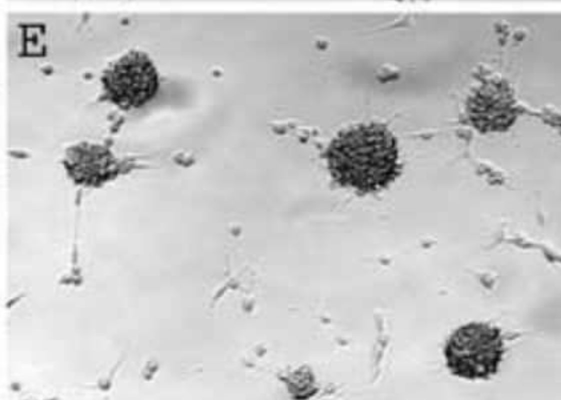

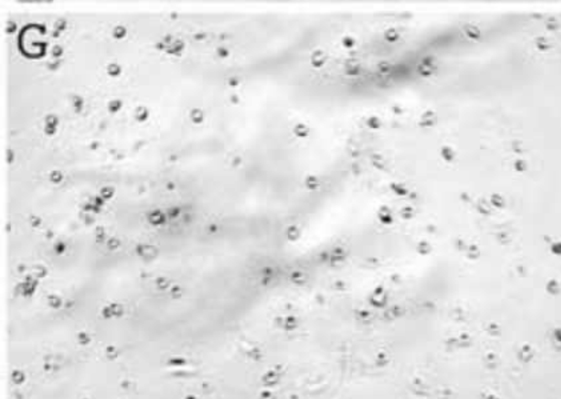

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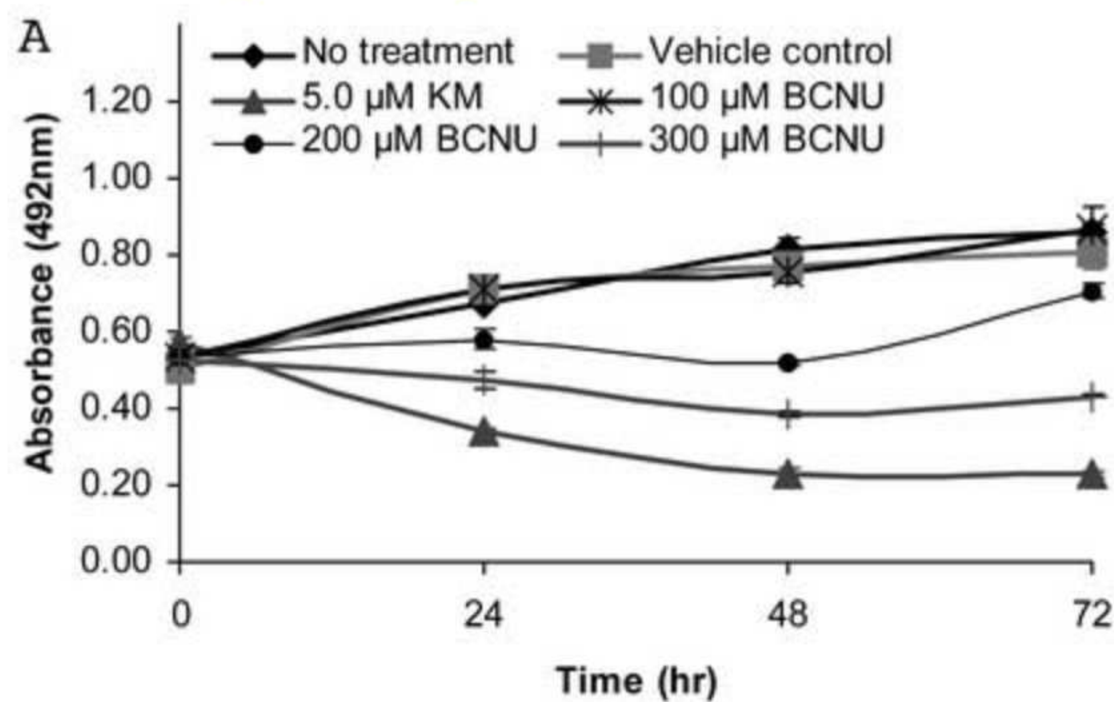

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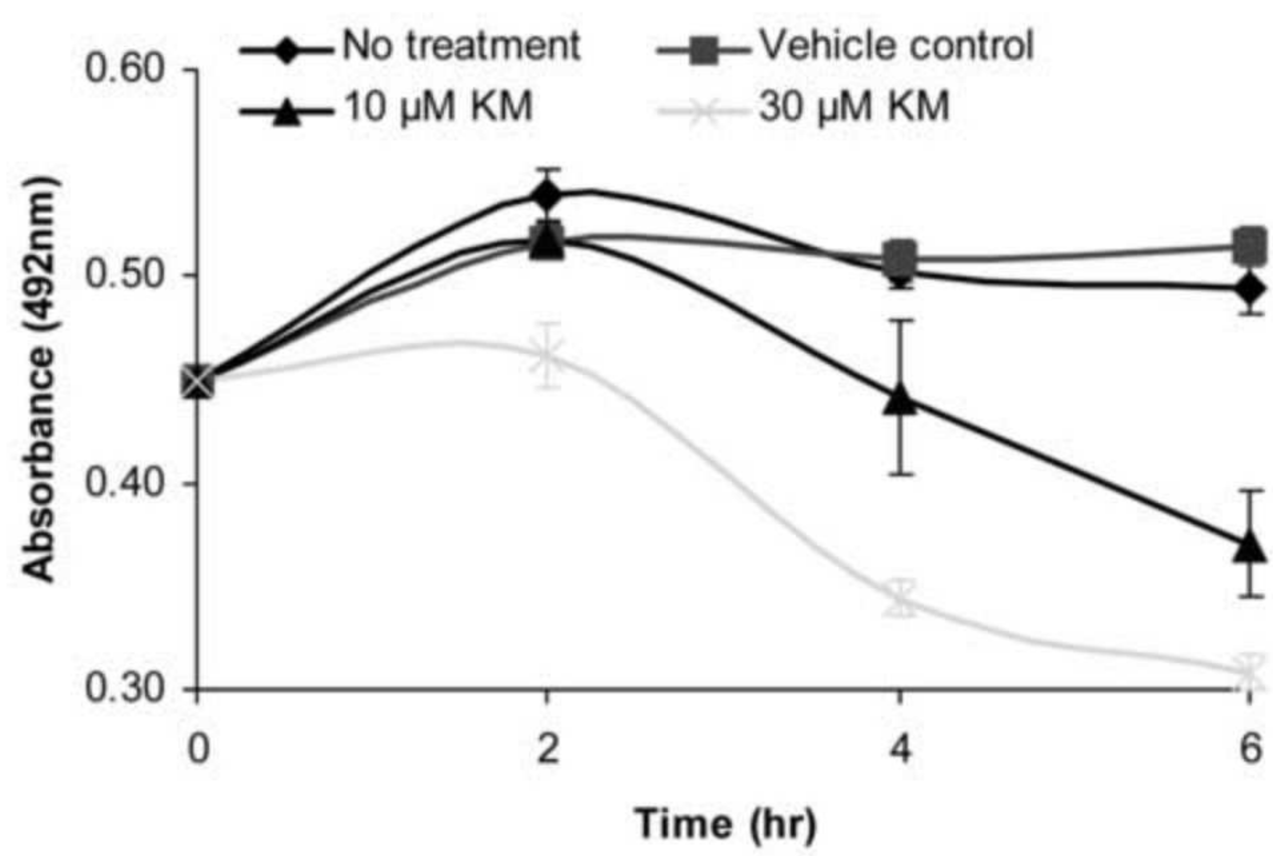

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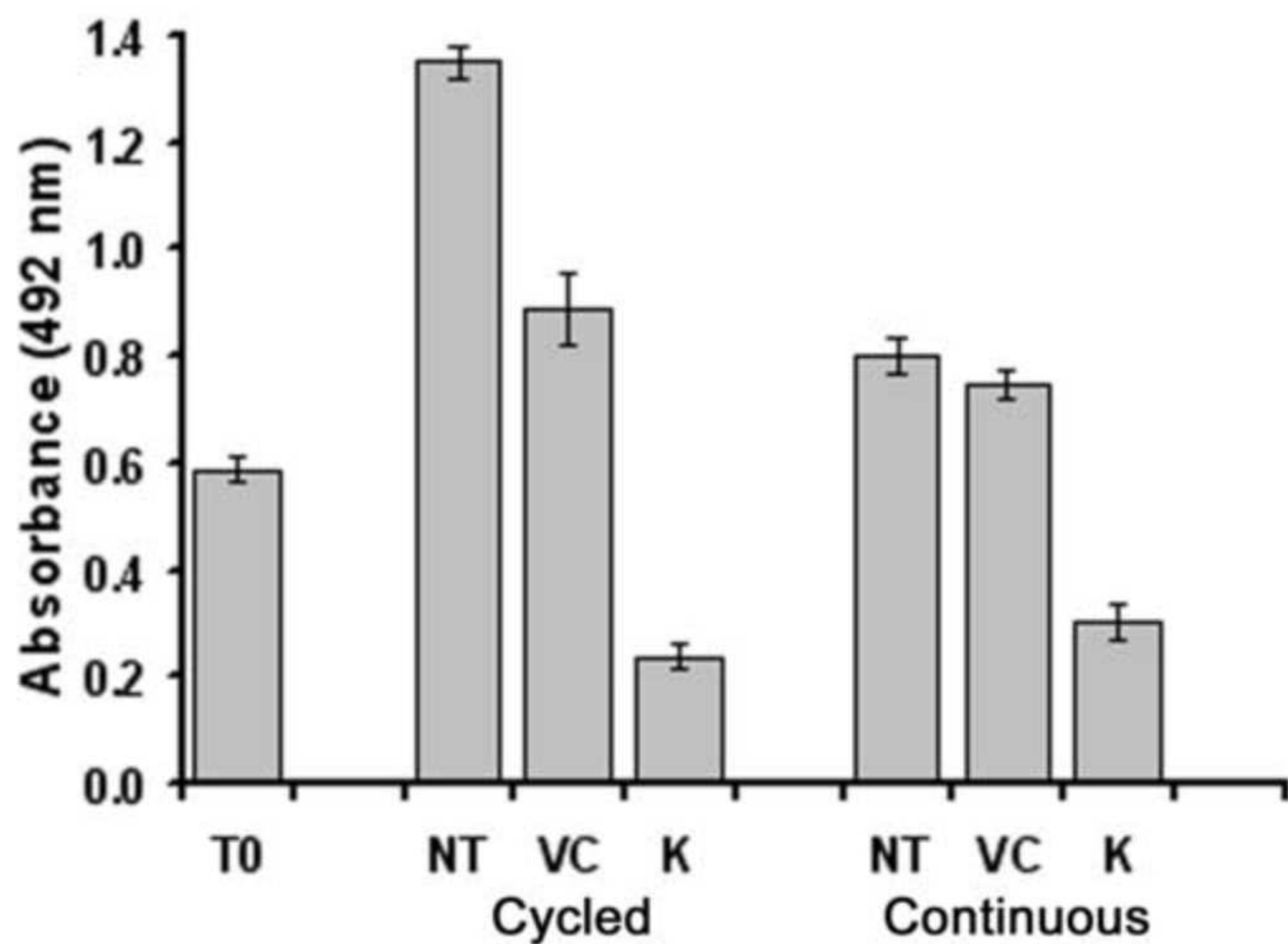

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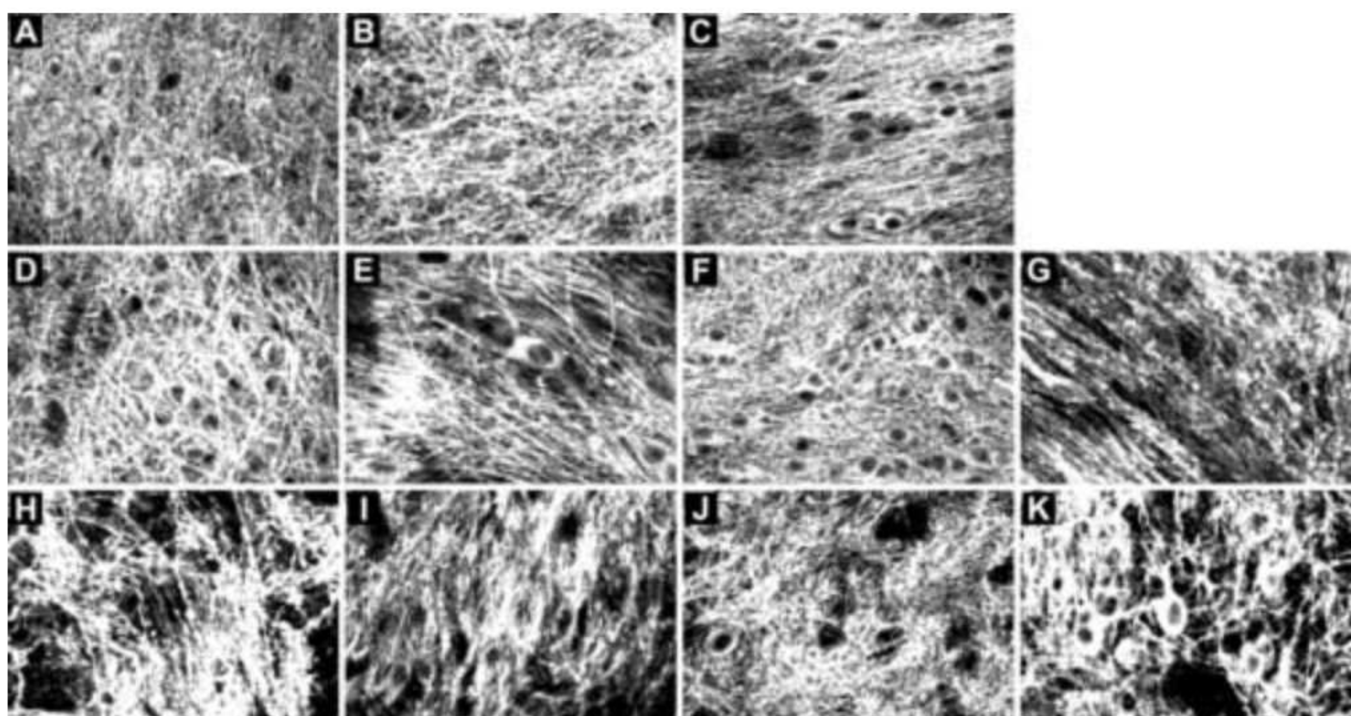

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