Tubulin Polymerization Rates Produced by EMulate Therapeutics' Anti-Cancer *ul*RFE® Derived from Paclitaxel as Compared to Known Clinical Concentrations of Paclitaxel (Cremaphor/EL Suspension)

Prepared for EMulate Therapeutics by Xavier A. Figueroa, Ph.D., June 8, 2022

Executive Summary

*EMulate Therapeutics has invented and patented a technology that utilizes radio frequency energy (RFE) precisely targeted at the low and ultra-low ends of the RFE spectrum (u*lRFE®*) to specifically regulate signaling and metabolic pathways on the molecular and genetic levels – without chemicals, radiation or drugs – delivered via a simple-to-use non-invasive therapeutic system. EMulate has used its u*lRFE *technology to create anti-cancer u*lRFE *products derived from paclitaxel, the synthetic form of Taxol. One such u*lRFE *product was produced by measuring and recording solvated paclitaxel molecules in the frequency range of 0-22 kHz (AIA_rfe). EMulate's A1A_rfe product has demonstrated effectiveness in treating patients diagnosed with glioblastoma multiforme (GBM) in a feasibility (phase II) clinical trial. A modified u*lRFE *product was produced by digitally modifying the A1A_rfe to operate in the frequency range of 0-8 kHz (A1A_TxU).*

Paclitaxel produces a significant increase in the rate of microtubule growth in tubulin polymerization assays and is used clinically to effectively treat a variety of solid tumors. In laboratory assays, A1A_rfe produces an increased rate of polymerization in a tubulin polymerization assay. Recent experiments have shown improvement in the effectiveness of A1A_TxU over A1A_rfe to produce tubulin polymerization. We believe this improvement, when the emitted frequency is reduced from the 0-22 kHz range to the 0-8 kHz range, can produce better outcomes in future clinical trials in which GBM patients are treated with A1A_TxU.

Introduction

Paclitaxel is an effective chemotherapy drug that targets tubulin. Paclitaxel-treated cells have defects in mitotic spindle assembly, chromosome segregation, and cell division. Paclitaxel stabilizes the microtubule polymer, protects it from disassembly and increases microtubule growth. Chromosomes are thus unable to segregate properly during cell division. This blocks cell division and can lead to apoptosis or a cell becoming dormant [1,2]. The ability of paclitaxel to inhibit spindle function is generally attributed to its suppression of microtubule dynamics [3], but other studies have demonstrated that suppression of dynamics occurs at concentrations lower than those needed to block mitosis: paclitaxel binds to the beta-tubulin subunits of microtubules [4].

In the clinical literature [5] a meta-analysis revealed that levels of paclitaxel in blood serum were present at an initial median value of 5 µM (5000 nM) after short durations of intravenous infusions (1-6 hours) in clinical patients. By 23 hours post-infusion, the levels of paclitaxel dropped below 50 nM, indicating a relatively fast clearance rate from the blood. By 48 hours, levels of paclitaxel in blood serum are zero.

Because paclitaxel is issued systemically, but its desired effect is local to organs, it is important to focus on paclitaxel levels present in organs as opposed to blood serum levels alone. Levels of paclitaxel in rabbit studies using intravenous paclitaxel revealed a direct relationship between levels of paclitaxel in blood serum and levels of paclitaxel in organs [6]. Moreover, the clearance rate of paclitaxel from blood in rabbits was in the same range (Figure 1) as reported in a clinical population [5].

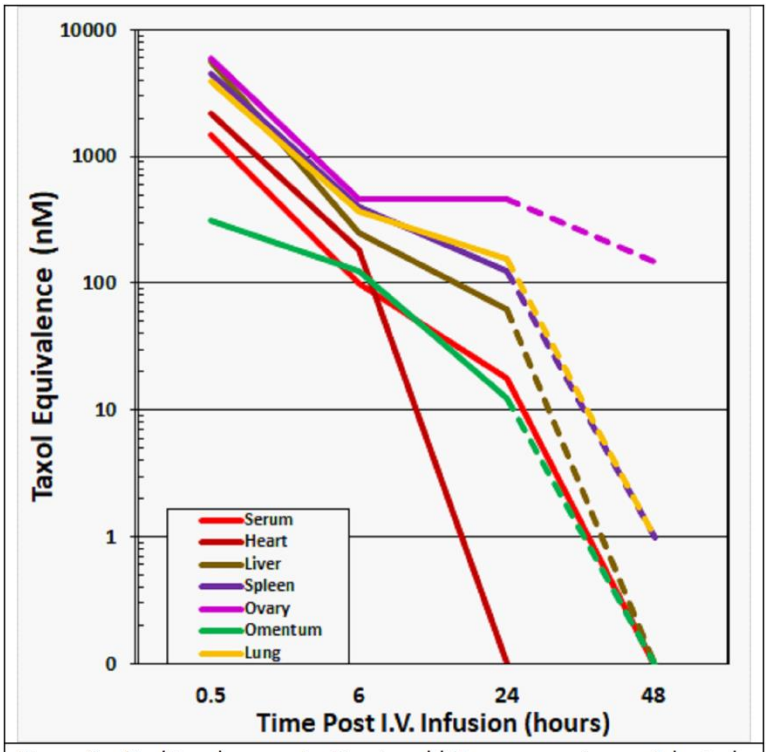

Figure 1 – Paclitaxel concentration in rabbit organs systems. Adapted from [6]. Dashed lines are extrapolated clearance rates.

In blood, Paclitaxel concentration dropped to ~ 50 nM at 12 hours and < 25 nM at 24 hours. Paclitaxel concentrations remained elevated in organ tissues like ovaries, lungs and spleen. Paclitaxel, being a hydrophobic anti-cancer drug, preferentially remains in organ tissue, which contain a high level of lipids.

The results of the meta-analysis of paclitaxel resident in blood (for clinical patients) and the paclitaxel biodistribution study in rabbits allows for a first order estimate of the relative paclitaxel concentration in organs. Organ systems retain paclitaxel for up to 24 hours at concentrations of 100-600 nM (Figure 1). (The decay rate of paclitaxel (Figure 1) in the rabbit study was measured without co-administration of cisplatin. Paclitaxel (+cisplatin) is routinely prescribed in 3-week treatment cycles and clinical experience suggests that co-administration of cisplatin can keep paclitaxel plasma levels elevated for longer periods of time than paclitaxel alone.) Data from the rabbit study suggest that standard treatments can lead to paclitaxel levels in organ tissue as high as 200-50 nM for >24 hours, but those levels are likely to drop to zero at 72-96 hours. Therefore, the 3-week infusion cycle is justified in terms of the pharmacodynamics of using paclitaxel to treat malignant tumors and to manage adverse reactions and side effects of this chemotherapy regimen. It is unfortunate that paclitaxel has almost no penetration in the brain because the blood-brain-barrier limits the entry of this high molecular weight compound [7].

Use of *ul*RFE® in Polymerization Assays

The *ul*RFE derived from paclitaxel, A1A_rfe and A1A_TxU, are both capable of increasing tubulin polymerization [8].

A tubulin polymerization assay is used as a screening method to detect the ability of compounds to inhibit or increase tubulin polymerization. Changes in the rate of tubulin polymerization based on paclitaxel dosage is well established (Figure 2). Without paclitaxel, tubulin self-assembly into microtubules is dependent on tubulin concentration (36 µM tubulin for this assay). When the tubulin

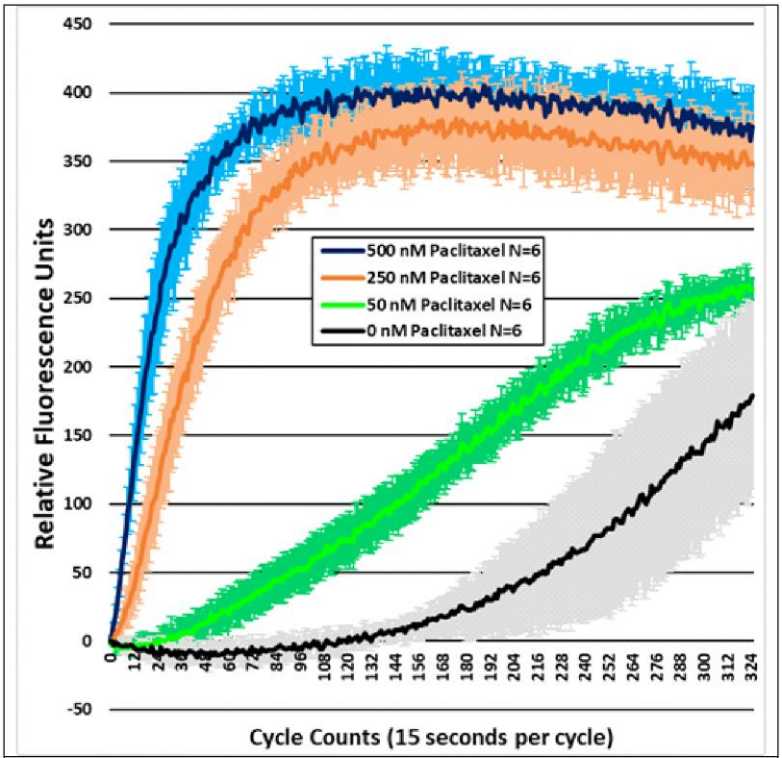

Figure 2 – Tubulin polymerization assays for physical paclitaxel. A dose response curve of 36 uM tubulin to four different concentrations of physical paclitaxel (500 nM (blue), 250 nM (orange), 50 nM (green) and 0 nM (black) [DMSO]). Error bars are standard deviations.

concentration is held constant, but the paclitaxel concentration is increased, the rate of polymerization (as measured as a change in fluorescence) increases with time. The rate of cell nucleation is shortened, and growth is immediate at 500 nM and 250 nM paclitaxel. 50 nM paclitaxel-treated samples showed a shortened nucleation phase (compared to 0 nM paclitaxel), but the nucleation phase was significantly longer than the higher dose groups. The rate of maximal polymerization was approximately 0.7 RFU/second for 500 nM paclitaxel and 0.3 RFU/second at 250 nM paclitaxel. At 50 nM paclitaxel the maximal polymerization rate (green line) is 0.06 RFU/second. The no-*ul*RFE polymerization rate (black line) was measured at 0.01 RFU/second.

Evidence from other research pathways using *ul*RFE derived from sources other than paclitaxel demonstrated that by modifying our recordings – reducing the frequency range of the *ul*RFE® from 0-22 kHz to 0-8 kHz – improved experimental outcomes and increased intended effectiveness. Therefore, when using A1A_TxU within a frequency range of 0-8 kHz instead of A1A_rfe with a frequency range of 0-22kHz in polymerization assays, we expected to see an improvement in tubulin polymerization rates.

Results

During a follow-on series of tubulin polymerization assays, polymerization effects of the A1A_rfe and the A1A_TxU were tested. Figure 3 (left graph) shows the effects of the A1A_rfe (which was used in EMulate's clinical trials). The average polymerization rate for the A1A_rfe is 0.03 RFU/seconds. The average polymerization rate for the A1A_TxU is 0.04 RFU/seconds (Figure 3, right graph). The rate of polymerization for the baseline rate of no *ul*RFE (blue line) is 0.01 RFU/seconds. Paclitaxel at 50 nM concentration was used to compare the relative effects of the paclitaxel *ul*RFE to the effects of physical paclitaxel alone on polymerization.

The profile of the tubulin polymerization growth curve in Figure 3 shows that the relative effect of A1A_rfe is approximately equivalent to 20-25 nM paclitaxel. A1A_TxU's effect on tubulin polymerization is approximately equivalent to 35-50 nM paclitaxel. This is an increase in effectiveness of about 25-50%.

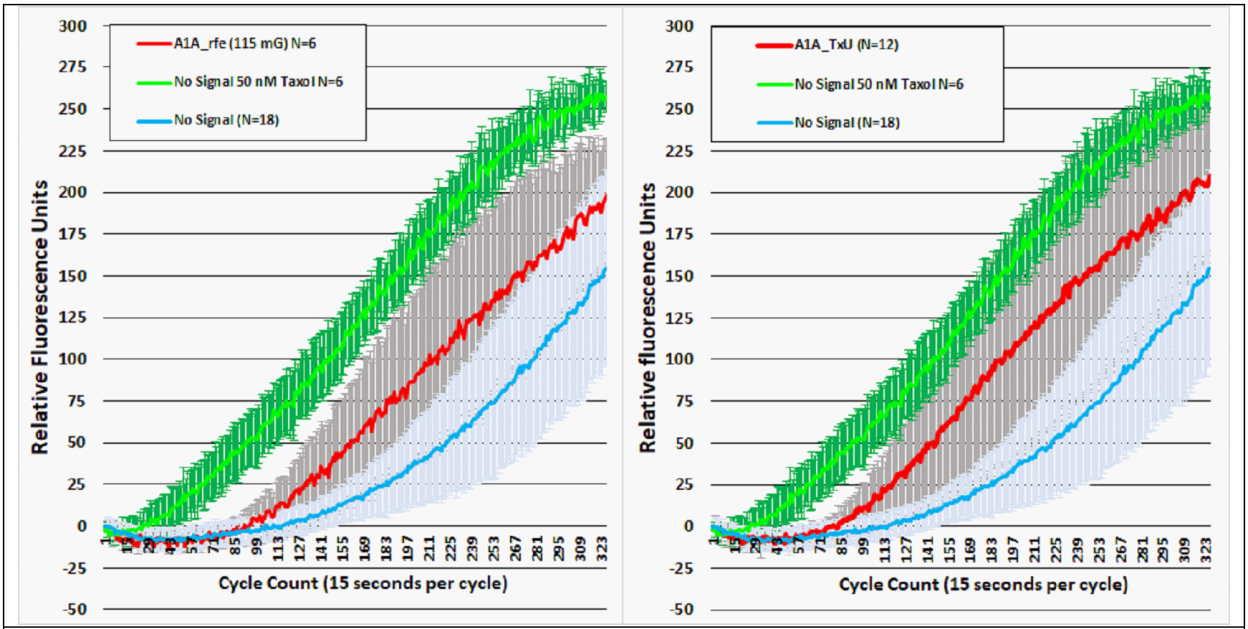

Figure 3 – Tubulin polymerization differences between the A1A_rfe and the modified A1A_TxU. Left graph, the A1A_rfe (red line) used in the EMulate clinical trials, compared to 50 nM paclitaxel treated tubulin (green line) and no *ul*RFE exposed tubulin (blue line). Right graph, modified paclitaxel *ul*RFE (A1A_TxU; red line), compared to 50 nM paclitaxel treated tubulin (green line) and no *ul*RFE exposed tubulin (blue line). Error bars are standard deviations.

Discussion

In the literature, the effective concentration of paclitaxel in blood and organ systems ranges from 5000 nM in blood serum, to, for example, 6000 nM in spleen, but for only 12-24 hours, rapidly dropping to 10-300 nM paclitaxel concentration. Paclitaxel is cleared quickly from the body and organ systems, yet even the short residence time of paclitaxel has been shown to effectively slow or stop tumor growth in lung, ovarian and other type of organ cancers.

The large bolus of paclitaxel administered, and the distribution of paclitaxel to all organ systems, can induce adverse side-effects, thus limiting the number or cycles over which the drug can be given. In contrast, the use of an A1A_TxU magnetic field emulating the effects of paclitaxel ensures an equivalent treatment level as if 35-50 nM paclitaxel were present at the site being exposed to the magnetic field. This concentration equivalence with the A1A_TxU aligns with published concentrations of active therapies of paclitaxel. Although on the low end of the concentration curve, the effect of *ul*RFE derived from paclitaxel is constantly present. There is no dilution or elimination as would occur with a physical drug and the *ul*RFE can be applied 24 hours a day, 7 days a week (Figure 4), if necessary. Additional potential clinical advantages can be observed from using paclitaxel *ul*RFE as a treatment agent instead of paclitaxel itself, such as: no significant adverse events (SAEs) have been reported to date in clinical trials using A1A_rfe to treat GBM; paclitaxel *ul*RFE can be locally administered directly to the tumor site rather than reaching the site systemically; and paclitaxel *ul*RFE can bypass the blood brain barrier (BBB). All of these clinical advantages may prove to increase overall survival in treating GBM and other solid tumors with or without concurrent chemotherapy.

This constant state of the disruptive effect of polymerization on microtubules is cumulative, as has been reported in our recurrent GBM feasibility (phase II) clinical trials (*manuscript in preparation*; Figure 5).

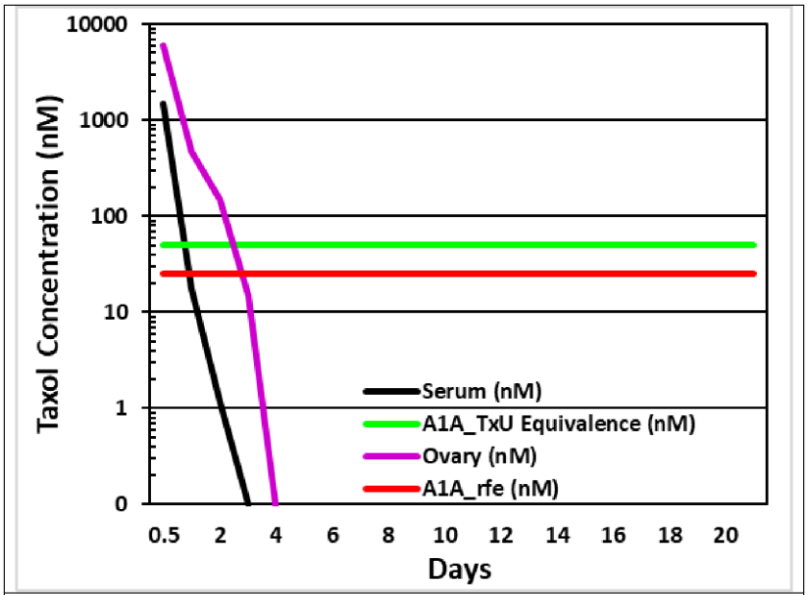

Figure 4 – Comparison of ovary and serum clearance of physical paclitaxel versus the equivalent level of A1A_rfe and A1A_TxU to a standard 3-week chemotherapy dosing program. Estimated from [6] and postulated equivalence of AIA_rfe on paclitaxel levels.

The A1A_rfe demonstrated an encouraging trend, that is, the A1A_rfe (*ul*RFE alone) demonstrates an equivalent median overall survival (Active Tx: 28.8 weeks, A1A_rfe alone: 28 weeks) and a superior progression free survival (Active Tx: 9.1 weeks, A1A_rfe alone: 17 weeks) as compared to a historical active treatment (Figure 5).

A truly encouraging trend in our recurrent GBM feasibility (phase II) clinical trials is the almost 12-week median overall survival increase (Figure 5) as compared to the historical active treatment when using A1A_rfe combined with best supportive care (BSC; any of the active treatments; Active Tx: 28.8 weeks, A1A_rfe + BSC: 40 weeks). The same trend was observed in progression free survival (PFS) measured with a 15-week increase in median PFS with a combination therapy of *ul*RFE + BCS, over the historical active treatments (Active Tx: 9.1 weeks, A1A_rfe + BSC: 24 weeks).

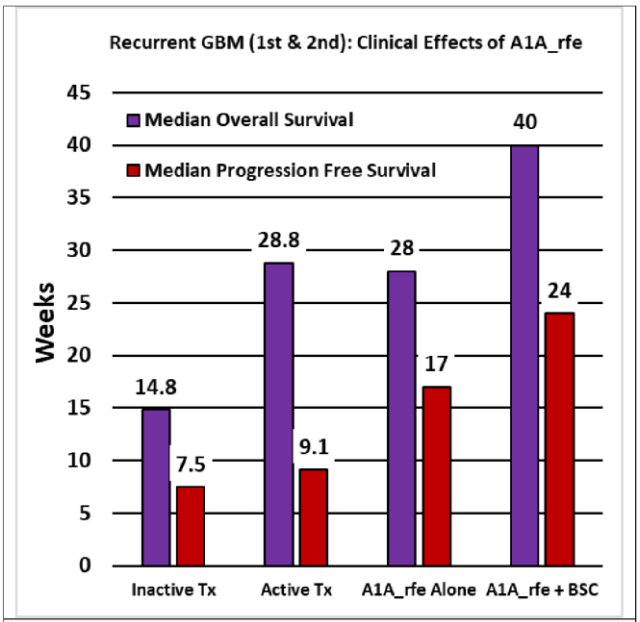

Figure 5 – Median progression free survival and overall survival from the EMulate Phase I & Phase II clinical trials for GBM as measured in weeks. ***Active therapies:*** *Temozolimide, Procarbazine, Gefitinib, Cetuximab, Bevacizumab, Bevacizumab + Irinotecan, Enzastaurin, Lomustin;* ***Inactive therapies:*** *Paclitaxel & Estramustine, Lonidamine, Cyclophosphamide, Medtronomic chemotherapy, Fenretidine, Sulfasalazine, Temozolimide & Benzylguanine. BSC: Best-Supportive-Care.*

Given the significant increase in the tubulin measures we have seen with A1A_TxU, we expect that further testing of the A1A_TxU will yield a significant improvement in tumor inhibition and clinical outcomes.

Conclusion

The observed pre-clinical and clinical effects of A1A_rfe indicate that the modified A1A_TxU will likely produce an improved outcome in animal models and in clinical trials. We fully expect to incorporate this modified *ul*RFE into our next generation therapeutic device for treating GBM, and in other solid tumor studies. We will apply the same technical improvements present in the A1A_TxU to other *ul*RFE products that EMulate develops.

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