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N,N-Dimethylsphingosine conjugates of poly-L-glutamic acid: Synthesis, characterization, and initial biological evaluation

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ABSTRACT

Poly-L-glutamic acid (PGA) has previously been demonstrated to be an effective backbone for creating a hydrophilic prodrug of the established anti-tumor agent, paclitaxel, the active agent in Taxol; this approach has obviated the need for the toxic Cremophor excipient, used to enhance the solubility of paclitaxel in the clinical formulation. In order to form hydrophilic prodrugs of the hydrophobic pro-apoptotic sphingolipid, *N,N*-dimethylsphingosine (DMSP), PGA was condensed with DMSP, previously modified with coumarin to allow spectroscopic detection during conjugate synthesis, to yield PGA–DMSP. Conjugates with different loadings of DMSP were prepared and evaluated for in vitro cytotoxicity against two human breast adenocarcinoma cell lines. Time- and loading-dependent expression of cytotoxicity was observed, such that endpoints essentially equivalent to those observed with free-DMSP were achieved, but in a more protracted manner, consistent with prodrug behavior. PGA–DMSP was initially evaluated for toxicity in female nude mice, and administration of high net levels of DMSP, exceeding those achievable with free-DMSP, was well-tolerated. We propose that PGA–DMSP conjugates merit evaluation for anti-tumor efficacy in pre-clinical tumor models.

Dimethylsphingosine (DMSP) is both a rapid inducer of the intrinsic apoptotic pathway and a competitive inhibitor of sphingosine kinase (SpK), and is a potentially useful tool to elucidate the role of sphingosine-1-phosphate (Sp-1-P) in cell signaling.[1–3] Sp-1-P itself has been shown to be an important down-regulator of apoptosis[4–7] and up-regulator of tumor angiogenesis.[8–12] DMSP is also a promising compound for the chemotherapeutic management of various types of malignancies.[13–16] A pharmaceutical limitation of DMSP, however, is its poor solubility (~2 mg/ml) and instability in aqueous media making it difficult to formulate for therapeutic studies. One approach to overcome this problem is to prepare conjugates of DMSP with highly water-soluble biodegradable macromolecules such as polysaccharides, proteins, or polypeptides. This approach has been described for other sparingly water-soluble anticancer drugs. For example, a conjugate of paclitaxel and poly-L-glutamic acid (PGA) has shown excellent therapeutic promise in the management of patients with epithelial malignancies, and is currently in Phase III clinical trials.[17–22] Since DMSP, like paclitaxel, is highly lipophilic, we sought to enhance its water solubility by preparing conjugates with PGA. Such conjugates should also show preferential localization into tumor tissue because of the enhanced permeability and retention (EPR) effect observed in tumor vasculature.[23–25]

To test our hypothesis, we first synthesized the PGA–DMSP conjugates (Chart 2). The extent of substitution of PGA with DMSP was tracked by quantification of the fluorescence of the DMSP–Coumarin (Chart 1), since DMSP itself has a negligible spectral footprint. Further, we have previously demonstrated that substitution of DMSP with Coumarin did not affect its biological activity.[26] More specifically, the individual mono-substituted isomers or binary mixture thereof[26] were found to have 24 h IC_{50} values against three different human tumor cell lines equivalent to or slightly lower than those determined for free-DMSP.

Synthesis of PGA–DMSP was accomplished as follows: Dicyclohexylcarbodiimide was added to a solution of PGA (MW 37,500) and 2-*N,N-dimethylamino-3-[7-(diethylamino)-2-oxo-2H*-chromon e-3-carboxyl]-octadec-4-ene-1-ol (**2**, Chart 1), in dry *N,N*-dimethylformamide-containing dimethylaminopyridine. The reaction mixture was stirred at room temperature for 48 h, and then poured into diethyl ether. The precipitate that formed was filtered and washed several times with diethyl ether and then dried. It was taken up in 0.5 M $NaHCO_3$ solution and dialyzed using a membrane tube (Spectra Pore molecular porous membrane tubing, Spectrum Laboratories Inc., with a molecular weight cut-off of 12,000–14,000) against distilled water at 4 °C for 24 h. The solution was lyophilized to give the sodium salt of **3** (Chart 2) as a yellow powder.

The UV spectrum (obtained on a Perkin-Elmer spectrophotometer) of the starting DMSP–Coumarin (water: λ_{max} 422 nm) is

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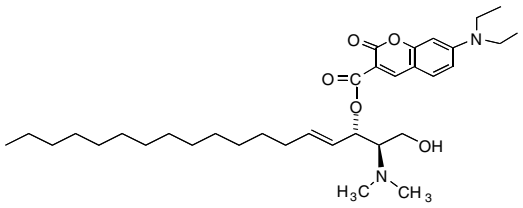

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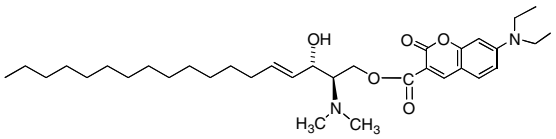

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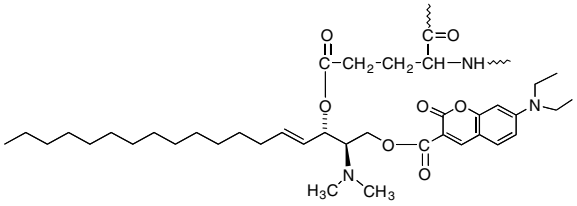
Chart 1.



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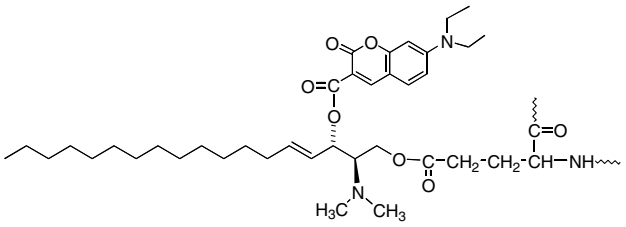

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Chart 2.

shown in Figure 1; a slight shift to longer wavelength for PGA–DMSP–Coumarin (λ_{max} 426 nm; Fig. 2) has previously been observed with paclitaxel following conjugation to PGA.[27] The amount of DMSP–Coumarin conjugated to PGA (sodium salt) was determined by UV measurements based on a standard curve generated with known concentrations of the DMSP–Coumarins in methanol for five different concentrations ranging from 4 to 25 μM. From standard plots of absorbance versus concentration, the absorption coefficient (ε) (for both compositions) was calculated as 5×10^4 mol^{-1} cm^{-1}. Based on this, the DMSP content of **3** was calculated as 20% (w/w), and the conversion of free-to-polymer-bound DMSP–Coumarin was calculated as 94% (When used in the context of the PGA conjugate, the terms DMSP–Coumarins and DMSP–Coumarin will be used synonymously). When analyzed by HPLC (C-18 reverse-phase column), the product eluted as a single peak; no evidence of free-DMSP–Coumarin was noted. Further evidence of the covalent character of the product was obtained from its ^{1}H NMR (300 MHz) spectrum in D_2O. Thus, in addition to resonances attributable to PGA and DMSP protons, the aromatic protons of coumarin were evident at 8.42, 7.37, 6.63, and 6.46 ppm (compared with 8.33, 7.66, 6.60, and 6.31 ppm for free-DMSP Coumarin). DMSP resonances included: 5.62 to 5.51 (m, 2H), 4.37 (m, 1H), 3.98 (m, 2H), 2.21 (s, 6H) and 0.989 (m, 3H); other resonances of DMSP were obscured by those of PGA. PGA resonances at 4.01, 2.1, and 1.98 were in accordance with the spectrum of pure PGA.

Conjugate **4** (Chart 2) was prepared from PGA and 2-*N,N-dimethylamino-1-[7-(diethylamino)-2-oxo-2H*-chromone-3-carboxyl]-octadec-4-ene-3-ol (**1**; Chart 1) as described for conjugate **3**.

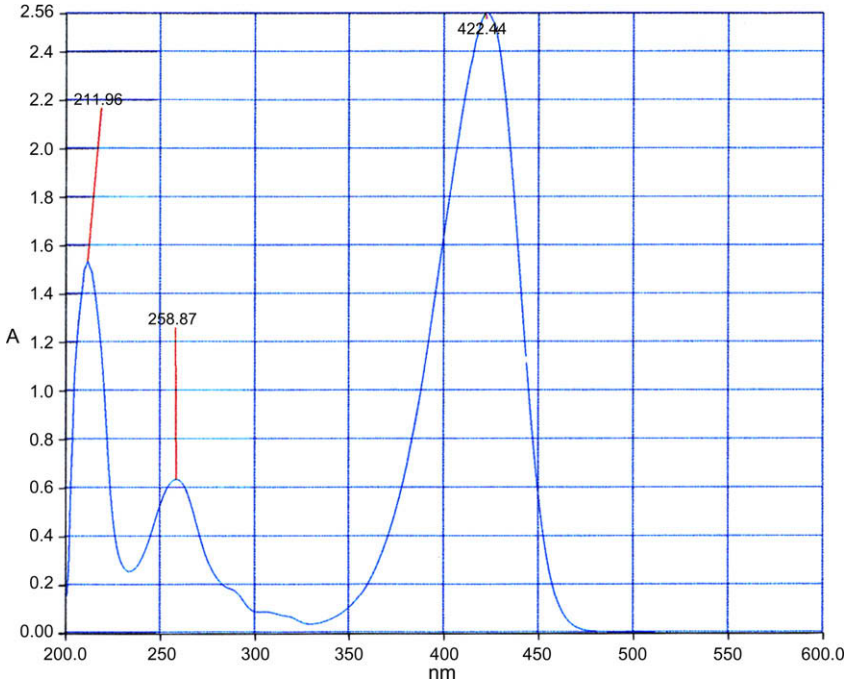

Figure 1. UV spectrum of DMSP–Coumarin, indicating a λ_{max} of 422 nm.

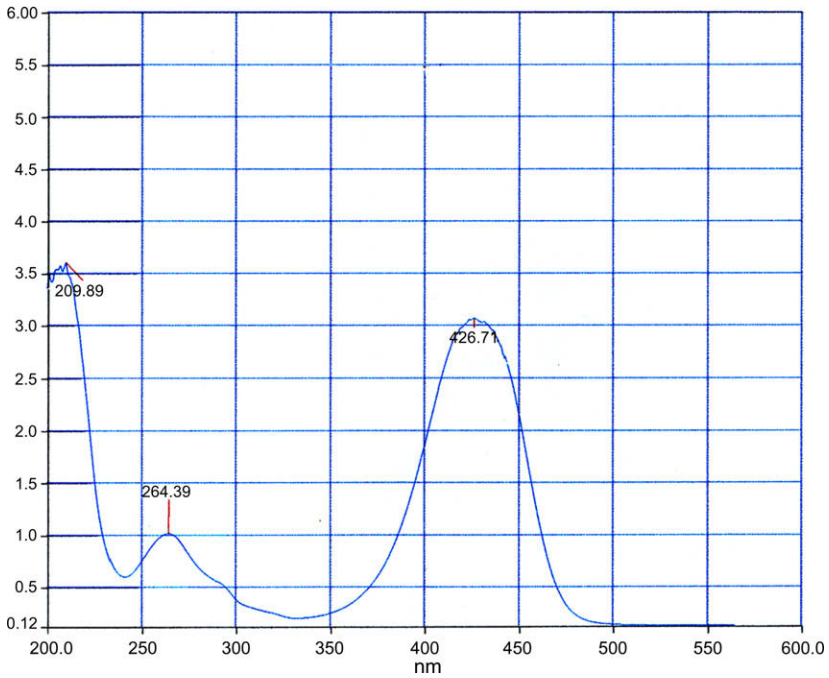

Figure 2. UV spectrum of PGA–DMSP–Coumarin in water, indicating a λ_{max} of 426 nm. Note the slight shift to longer wavelength for the conjugate compared to DMSP–Coumarin (Fig. 1).

Pilot studies indicated that PGA-DMSP conjugates **3** and **4** had indistinguishable cytotoxic activity against human tumor cell lines in vitro. Therefore, since there was no evidence for an influence on biological activity of the site of attachment of DMSP to the PGA backbone, for subsequent studies, the mixed conjugates were synthesized by direct reaction of PGA with the mixed monoesters obtained from the reaction of DMSP and coumarin acid chloride. By varying the ratio of starting materials, a series of PGA–DMSP conjugates with 7%, 13%, and 18% (w/w) loading of DMSP–Coumarin was prepared and characterized.

For cytotoxicity assays, human MCF-7 and MDA-MB-435 breast adenocarcinoma cells were used and cultured in DMEM/F-12 medium (GIBCO, Grand Island, NY) supplemented with 5% fetal calf serum (GIBCO) at 37 °C in an incubator supplied with 5% CO_2/95% air, as previously described.[28] Tumor cells were plated at 1.5–2.0 $\times$ 10^4 cells in 100 μL of medium per triplicate well in flat-bottomed 96-well microwell plates (Corning Glass Works, Corning, NY) and cultured overnight. Thereafter, they were incubated for 24, 48, or 72 h with known concentrations (based on DMSP equivalents) of free-DMSP or PGA–DMSP conjugates, the latter prepared with three different DMSP–Coumarin loadings (7%, 13%, and 18%: w/w). Remaining viable cells were stained with MTT. Re-solubilized incorporated dye was quantified by measurement of optical density in a microplate reader. Survival for treated cells was determined as a percentage of the optical density of dye in control cultures.

The results for MCF-7 cells are shown in Figure 3. The entire population of tumor cells was rapidly killed by 24 h with the highest concentration (40 μM) of either free-DMSP or PGA–DMSP with 7% loading (Fig. 3A), whereas the IC_{50} values were in the range of ~10–20 μM for either of these agents. This indicated that conjugation to the PGA backbone largely preserved the biological activity of the 7% DMSP–Coumarin formulation. The effects of PGA alone and the effects of the hydrochloride salt of coumarin, at concentrations corresponding to those in the 7% PGA–DMSP formulation, were negligible. The 13% and 18% loading formulations of PGA–DMSP demonstrated lesser activity than the 7% loading formulation at this early time-point, particularly evident at higher concentrations, however, the cytotoxic effects of the latter formulations became more aligned with that of the 7% loading formulation only by 48 and 72 h (Fig. 3B and C).

Nevertheless, at each time-point and at the higher concentrations used, there was a trend for a general rank order of cytotoxic potency as follows: free-DMSP $\geqslant$ 7% loading PGA–DMSP $\geqslant$ 13% loading PGA–DMSP $\geqslant$ 18% loading PGA–DMSP. Since the concentrations used were based on DMSP equivalents, these results are consistent with a somewhat protracted release of the DMSP–Coumarin from the PGA backbone, occurring either extra- or intracellularly. We propose that the most likely operant mechanism is one that is based on rate-limiting enzymatic processing rather than on solvolysis. Further, sphingolipids follow surface dilution kinetics;[29] delayed bioavailability with the higher loading formulations should result in a higher target cell mass at the time of release of the DMSP from the PGA backbone in these continuously growing cultures than for the free-DMSP control, rendering the released DMSP less effective. This may in part account for the trend of rank order observed above.

Generally similar results were obtained with these reagents on MDA-MB-435 cells (Fig. 4); again there were minimal distinctions between DMSP and PGA–DMSP, except for the more protracted killing with the higher loading formulations. Overall, based on the in vitro cytotoxicity assays in these human breast cancer cell lines, covalent attachment of DMSP to PGA retained its biological activity.

It should be noted that the expression of cytotoxicity induced by DMSP (or DMSP–Coumarin) likely depends most strongly on early activation of the intrinsic mitochondrial-dependent apoptotic pathway,[30] as opposed to direct inhibition of SpK; hence, inhibitory equivalence on SpK of free-DMSP and DMSP–Coumarin was not assessed in this study. Nevertheless, modulation of the sphingolipid rheostat by DMSP to favor apoptosis may contribute to later cytotoxic endpoints.

For toxicity studies, female nude mice (3 mice per group; average mouse weight ~25 g), appropriate hosts for future xenograft

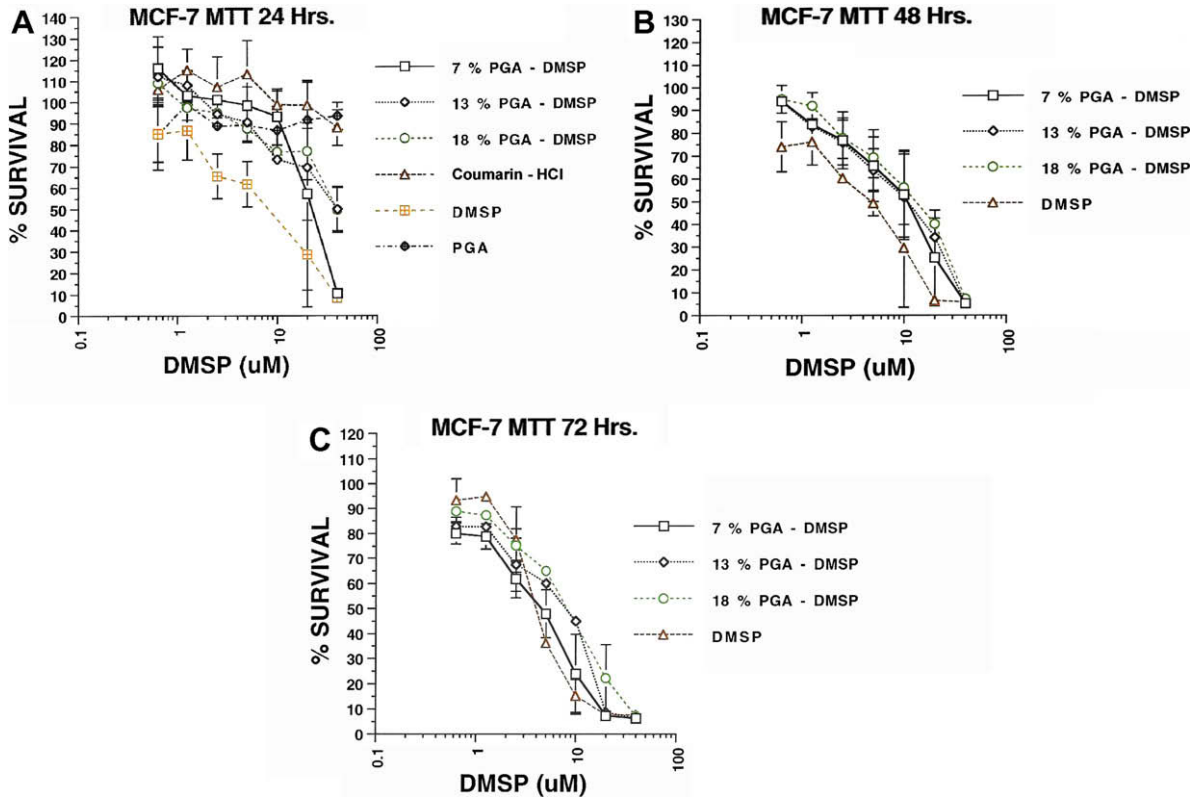

Figure 3. MCF-7 cells were subjected to incubation with a concentration range of DMSP and PGA-DMSP with 7%, 13%, and 18% DMSP–Coumarin loadings for 24 h (A), 48 h (B), and 72 h (C) prior to treatment with MTT to assess remaining cell viability. In (A) are also shown the results for PGA alone and for the hydrochloride salt of Coumarin alone, at the same concentrations as used in the corresponding concentration of the 7% PGA–DMSP conjugate. Essentially all tumor cells were killed already by 24 h with the highest concentration (40 μM) of either free-DMSP or PGA–DMSP with 7% loading (A), whereas their IC_{50} values were in the range of ~10–20 μM. The cytotoxic effects of the 13% and 18% loading formulations of PGA–DMSP demonstrated lesser activity at this early time-point, but became more aligned with that of the 7% loading formulation by 48 and 72 h (B and C, respectively). There was a modest trend for an order of cytotoxic potency of free DMSP ⩾ 7% loading PGA–DMSP ⩾ 13% loading PGA–DMSP ⩾ 18% loading PGA–DMSP at al time-points.

studies with the human breast adenocarcinoma cell models employed in the in vitro cytotoxicity assays described above, were subjected to twofold dose-escalation protocols to establish tentative maximum tolerated doses (MTDs) of PGA–DMSP. Bodyweight monitoring was employed as a surrogate for overall host well-being. The 7% DMSP loading formulation of PGA–DMSP was employed, as this appeared to achieve the most rapid effects, similar to those for free-DMSP, compared to the higher loading formulations in the in vitro cytotoxicity studies. Injections of conjugates were in minimal volumes administered intravenously (iv) in the tail vein or intraperitoneally (ip).

The starting dose was 0.10 mg (expressed in DMSP equivalents, based on DMSP–Coumarin content in PGA–DMSP; ~3.9 mg DMSP/kg) administered in 100 μL. The bodyweights in either group decreased ⩽3% on the day after the first injection, and were restored to equivalence or slightly above pre-injection weights by the time of the second injection, 4 days later.

Then each group received a dose of 0.19 mg DMSP equivalents (~7.8 mg DMSP/kg). One mouse in the iv-administration group expired inexplicably during attempted injection, but prior to the actual injection. This event was judged to be unrelated to any drug effects, so the dose-escalation protocol was continued as planned. On the following day, the bodyweights in the ip-administration group were unchanged, whereas the remaining mice in the iv group displayed ~6% bodyweight loss, which had recovered by the time of the third injection, 4 days later.

The next escalation (third dose) was to 0.39 mg DMSP equivalents (~15.5 mg DMSP/kg). A transient weight loss of ~2.5–4% was observed on the following day. A final injection was given 3 days later of 0.78 mg DMSP equivalents (~31.4 mg DMSP/kg). The iv-administration group displayed a nominal 2% bodyweight loss, but no further weight was gained over the next 2 weeks of monitoring. The ip-administration group experienced a bodyweight nadir about 3–4 days after injection, representing ~6.2% loss, but was nearly recovered to levels prior to the fourth injection 2 weeks later, at the termination of the experiment.

Based on this intensive dose-escalation protocol and the prolonged effects on bodyweight following the last injection, a tentative single-dose MTD of ~0.8 mg DMSP equivalents (~32 mg DMSP/kg) of the PGA–DMSP 7% loading formulation was assigned. We propose that, as evident in vitro, the prodrug behavior of PGA–DMSP will result in protracted release of free-DMSP in vivo; this will be assessed in future pharmacokinetic analyses. Note that even this drug level (~8 mg/ml), although not at the maximum solubility of the conjugate, was nevertheless several-fold greater than the aqueous solubility reported for free-DMSP (vendor product insert). This level also substantially exceeds the dose of free-DMSP (0.5 mg) previously observed to induce anti-tumor effects in human tumor/nude mouse xenograft models,[13,31] and is an encouragement to further pre-clinical evaluation. An MTD for free-DMSP has not been reported, presumably in part due to the previously noted limitations of poor solubility.

Studies of in vivo evaluation of PGA–DMSP in human tumor xenograft models are underway in our laboratory. The ability to spectroscopically detect the Coumarin fluorophore on the conjugate in plasma and in tumor tissue will be useful in optimizing the dosing schedule.

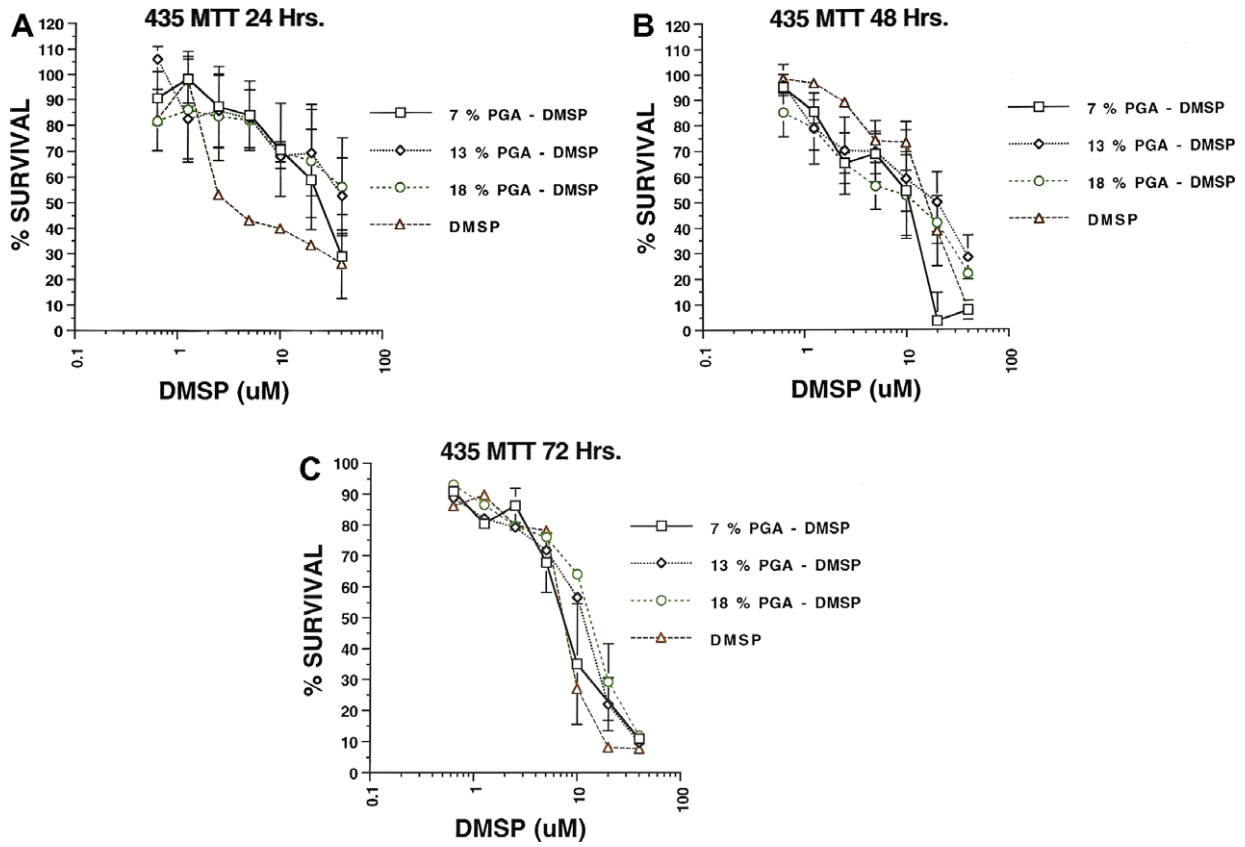

Figure 4. MDA-MB-435 cells were subjected to incubation with a concentration range of DMSP and PGA–DMSP with 7%, 13%, and 18% DMSP–Coumarin loadings for 24 h (A), 48 h (B), and 72 h (C) prior to treatment with MTT to assess remaining cell viability. As with MCF-7 cells, there were minimal distinctions between DMSP and PGA–DMSP, except for the more protracted killing with the higher loading formulations of the latter.

The in vitro cytotoxicity results in Figures 3 and 4, as well as the initial toxicity results described above, might suggest that the 7% (or lower) PGA–DMSP loading formulation should be the one selected for further evaluation. However, another view emerges when considering the possible pharmacokinetics of PGA–DMSP in a tumor-bearing host. For example, based on the in vitro data, the higher loading (13% and 18%) formulations are more stable and initially less susceptible to activation and release of free-DMSP; this might allow more extensive tumor accumulation to have occurred via the EPR effect[23–25] prior to drug release than with the lower loading formulation(s). This is one of the variables that will require exploration and optimization as the next phase of pre-clinical development is undertaken in establishing anti-tumor efficacy. It will also be of interest to compare the toxicity and efficacy profiles of these PGA–DMSP formulations with stealth liposomal formulations of DMSP that we have demonstrated to be efficacious in HER-2/neu over-expressing human breast carcinoma xenografts.[†]

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