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Destruction of WiDr multicellular tumor spheroids with the novel thymidylate synthase inhibitor 1843U89 at physiological thymidine concentrations

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Abstract. The activity of a novel thymidylate synthase inhibitor, 1843U89, against WiDr human colon carcinoma multicellular tumor spheroids was investigated. Continuous exposure of the spheroids to 3 n*M* 1843U89 for 10 days resulted in spheroid disruption, whereas 100 n*M* methotrexate (MTX) was required for similar effects. Short-term treatment experiments demonstrated that a 3-day exposure to 100 n*M* 1843U89 caused spheroid disruption 9 days after drug removal. A 4-day exposure to 10 n*M* 1843U89 caused spheroid disruption 8 days after drug removal. In contrast, treatment with 10 or 100 n*M* 1843U89 for 6–48 h or treatment with 1 n*M* 1843U89 for up to 5 days caused only growth delay. Continuous exposure of spheroids to 30 n*M* 1843U89 in the presence of 0.05–0.3 μ*M* thymidine was as effective in causing spheroid disruption as treatment in the absence of thymidine, but treatment in the presence of 0.7–3.0 μ*M* thymidine caused partial reversal of spheroid disruption. The results of these experiments suggest that 1843U89 should have potent solid tumor activity in humans but should be less effective in mice due to differences in circulating thymidine levels (0.1 vs 1 μ*M*, respectively).

Introduction

The in vitro multicellular tumor spheroid is a tumor model of intermediate complexity between conventional monolayer cultures in vitro and solid tumors in vivo that simulates many parameters of in vivo tumors. These parameters include cell-cell contacts, a viable rim and necrotic core, similar growth kinetics, low internal oxygen tensions, pH gradients, tumor marker expression, antigenic heterogeneity, and concentration gradients of critical metabolites such as glucose [1]. These characteristics, along with in vitro convenience, make multicellular tumor spheroids a suitable system for evaluating the effects of novel chemotherapeutic drugs as well as other therapeutic modalities.

Dividing cells require thymidylate for DNA synthesis; consequently, thymidylate synthase (TS) is a major target in cancer chemotherapy. The clinically established combination of 5-fluorouracil (5-FU) with folinic acid, which has TS as its target, is an effective therapy for metastatic colon carcinoma [2]. N^{10}-Propargyl-5,8-dideazafolate (CB3717), a folate analog TS inhibitor, has demonstrated activity in humans but was withdrawn from clinical trials for non-mechanism-based liver and kidney toxicities [3, 4] ICI-D1694, a second-generation analog of CB3717 that was not expected to express these toxicities, is now in the clinic [5]. We are investigating the potential of a novel TS anticancer agent, 1843U89, which should not express the toxicities seen with CB3717 [6, 7]. 1843U89 is a potent inhibitor of human TS and is transported well into human cell lines, which results in growth inhibition of a variety of human cancer cell lines at values below 1 n*M* [6, 7]. However, circulating thymidine levels of 1–2 μ*M* in mice interfere with the antitumor activity of 1843U89 and other TS inhibitors in the rodents [7–10]. Circulating levels in humans are approximately 0.1 μ*M* [8], and it is generally assumed that this lower thymidine level will not reverse TS inhibitor activity, but direct in vitro evidence for this has been difficult to obtain [9]. In the present study, WiDr (human colon carcinoma) multicellular spheroids were used as a model to demonstrate that 1843U89 causes spheroid disruption in the low monomolar range. Thymidine addition to the cultures did not alter the effect at concentrations of thymidine up to and including 0.3 μ*M*; however, at higher levels (0.7 μ*M* and above), partial reversal was noted. These results provide strong evidence that the antitumor effect of TS inhibitors will not be blocked by circulating thymidine levels in humans.

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Materials and methods

Chemicals. Methotrexate [MTX, (+) amethopterin] and thymidine were obtained from Sigma Chemical Company (St. Louis, Mo.) and was prepared as 0.1- and 1-mM stock solutions in Dulbecco's phosphate-buffered saline (PBS, Gibco Laboratories). (S)-2-{5-[[(Dihydro-3-methyl-1-oxobenzo(F)quinazolin-9-yl)methyl]amino]-1-oxo-2-isoindolinyl}glutaric acid (1843U89) was synthesized as described elsewhere [6]. Stock solutions were 0.1 and 1 μ*M* in PBS. Thymidine phosphorylase was purified from *Esherichia coli* at Wellcome Research Laboratories [11]. [^{14}C]-Thymidine (50.53 mCi/mmol) was obtained from Amersham International and prepared as a 75-μ*M* stock solution in PBS. All solutions were sterile-filtered through a 0.22-μm filter (Millipore Millex-GV) before they were added to the WiDr cells and culture medium at the desired concentration.

Cell culture. The human colon carcinoma WiDr was obtained from the American Type Culture Collection and adapted to grow on 10 n*M* leucovorin as the folate source [12]. Cell monolayer and spheroid cultures were maintained in folate-free RPMI-1640 (Life Technologies, Inc., Grand Island, N. Y.) supplemented with 10% dialyzed fetal bovine serum (JRH Biosciences, Lenexa, kan.) and 10 n*M* 6-*R,S*-calcium leucovorin (Burroughs Wellcome Co., N. C.). Monolayer cultures were maintained in logarithmic phase growth under a 5% CO_2 atmosphere and incubated at 37° C.

Spheroid formation. Spheroid culture methods were adapted from previously reported techniques [1, 13–15]. Monolayer cells were harvested using 0.05% trypsin/0.53 m*M* ethylenediaminetetracetic acid (EDTA) in growth medium. Siliconized Techne spinner flasks were seeded with 2×10^6 cells in 250 ml growth medium and stirred continuously at 20 rpm. The WiDr cells developed into small spheroids within 7–10 days. Culture medium was changed in the flask every 7 days.

Growth assessment. Spheroid size was measured as the cross-sectional area with an Artek 980 counter attached through the camera side port of a Nikon TMS inverted microscope. A linear relationship between diameter and square root of the cross-sectional area was determined, and the diameter of the individual spheroids was interpolated from this data. Measurements of spheroid area were compared with those recorded on day zero (day of placing spheroids in individual wells), and growth was expressed as a percentage of the day-zero value.

Treatment of WiDr spheroids with drugs. Drug treatment experiments were performed on individual spheroids that were transferred from the spinner flasks to multiwell plates coated with agarose plugs as described previously [15]. In these experiments, individual spheroids were directly pipette-transferred in fresh growth medium from the spinner flasks to 24 multiwell tissue-culture plates (Costar, Cambridge, Mass.) precoated with 0.25 ml of 0.56% low-melt preparative-grade agarose (Bio Rad) after 7–10 days' growth. The spheroids were exposed to drugs (1843U89 or MTX) continuously for a 2-week period. Typically, drugs were added at day zero (day of spheroid transfer from spinner flasks to multiwell plates); medium and drug were changed weekly.

In several of the time exposure experiments (6 h and 1, 2, and 3 days), the spheroids were treated with drug in the spinner flasks after 7–10 days' growth. They were subsequently transferred to multiwell plates with agarose plugs and washed twice with fresh medium (day 0 release from drug). Medium was replaced once a week and spheroid growth was monitored for 20 days. In other time exposure experiments (3, 4, and 5 days), the spheroids were exposed to drug in multiwell plates, with agarose plugs. Drug was removed and the spheroids were washed twice with fresh drug-free medium and resuspended in drug-free medium. Medium was replaced once a week after drug removal. These two methods gave similar results for the 3-day exposure.

In experiments using [^{14}C]-thymidine, the growth medium, [^{14}C]-thymidine, and 1843U89 were replaced fresh every 48 h. Spent

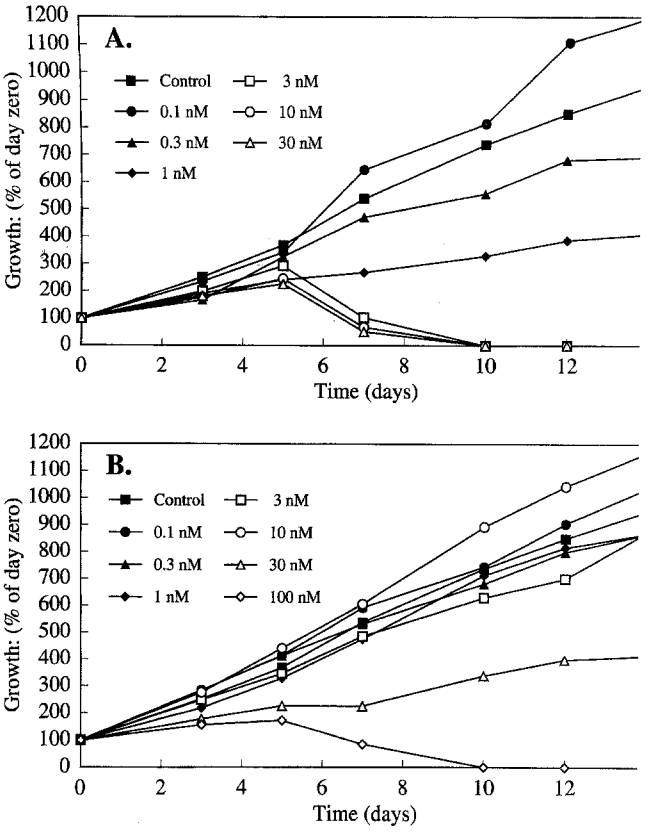

Fig. 1A, B. Effect of 1843U89 (**A**) and methotrexate (**B**) on spheroid growth. Individual spheroids were transferred from spinner flasks after 7–10 days' growth to agarose-coated multiwell plates. Spheroids were exposed to various concentrations of drug and were measured every 2 days. The values represent the mean spheroid area expressed as a percentage of that recorded on day 0. Each data point represents measurements of a minimum of two spheroids

medium was saved and the radioactive content was quantitated and confirmed to be [^{14}C]-thymidine by high-performance liquid chromatography (HPLC). Spent medium samples were deproteinized with $HClO_4$ and trioctylamine/freon (TOA/F). A 0.1 vol. of 4.4 *M* $HClO_4$ was added to the medium, mixed well, and centrifuged for 10 min at 8800 *g*. The supernatant was removed and 2 vol. of a TOA/F mixture (26 g TOA, d = 0.809 to 147 ml final volume of freon) were added. The sample was well mixed and centrifuged for 5 min at 2200 *g*. The supernatant was drawn off and filtered through 0.45-μ*M* Millipore filters. A filtrate volume of 300 μl was chromatographed on a Rainin Microsorb C18 5 μ column using a binary gradient mobile phase and a flow rate of 1 ml/min. Mobile-phase components were: A, 140 m*M* NH_4OAc in water (pH = 5.5); and B, 140 m*M* NH_4OAc/20% ACN (pH = 5.5). Initial conditions were 93% A/7%B. This condition was maintained for 300 s following an injection, and then a 900-s linear gradient going from 93%A/7%B to 70%A/30%B was initiated. The 70%A/30%B mobile phase was held for 400 s. At the end of the 1600-s data-acquisition time, there was a 600-s purge with 100% B followed by a 1200-s reequilibration to initial conditions. This resulted in retention times of 1350 s for thymidine and 550 s for thymine. Thymidine was detected by UV absorbance at 265 nm with a Waters Lambda-Max 480 spectrophotometer and by radioisotope detection with a Radiomatic Flo-One HS detector and Flo-Scint IV scintillant. Peaks were identified by their retention times.

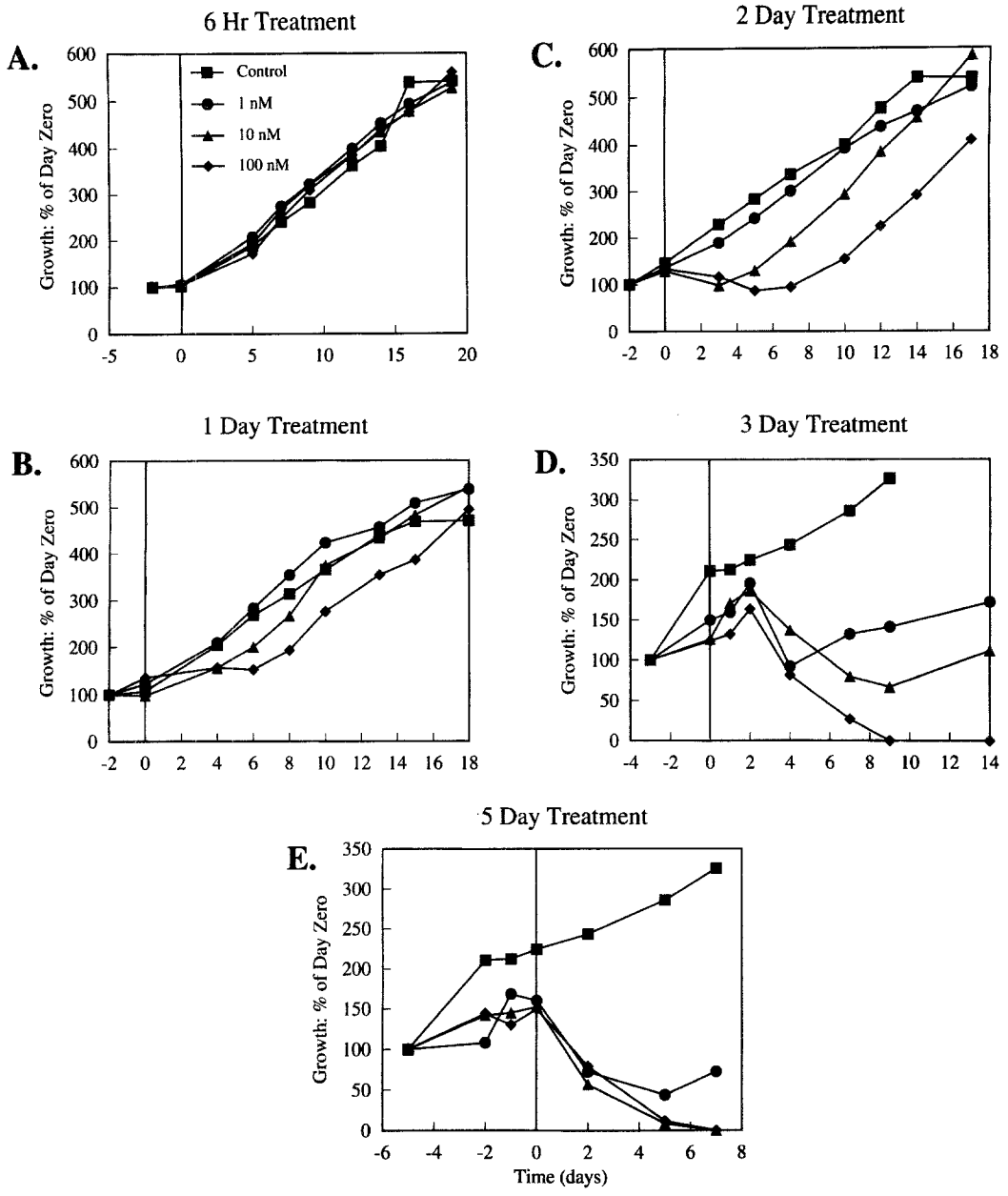

Fig. 2A–E. Effect of dosing length and concentration of 1843U89 on spheroid regrowth. Spheroids were exposed to drug for 6 h and for 1, 2, 3, and 5 days, after which the spheroids were washed with drug-free growth medium. Following drug removal, fresh medium was added and the spheroid's size was monitored. The values represent the mean spheroid area expressed as a percentage of that recorded on day 0. Each data point represents measurements of a minimum of two spheroids

Results and discussion

Comparison of the effects of MTX and 1843U89 on WiDr spheroids

WiDr spheroids were continuously exposed to 1843U89, a novel TS inhibitor, for a 2-week period. The results of this experiment are shown in Fig. 1A. The graph reveals partial inhibition of spheroid growth at concentrations of 0.3–1.0 n*M* (62% inhibition at 1 n*M*). At concentrations of 3 n*M* and above, complete spheroid disruption was observed by day 10. For comparative purposes, WiDr spheroids were exposed continuously for a 2-week period to MTX. The results are shown in Fig. 1B. The graph reveals no significant inhibition of spheroid growth at concentrations of up to 10 n*M* MTX; 30 n*M* MTX produced 66% inhibition. At a concentration of 100 n*M* MTX, complete spheroid disruption was observed by day 10. Thus, in this solid tumor model, 1843U89 was 30 times as potent as MTX.

We have previously determined by light microscopy of normal and treated spheroids that drug-induced spheroid disruption such as that described herein is due to massive cellular disruption throughout the spheroid [15]. Similar microscopy experiments also reveal the same extensive cell death in 1843U89-treated spheroids (data not shown).

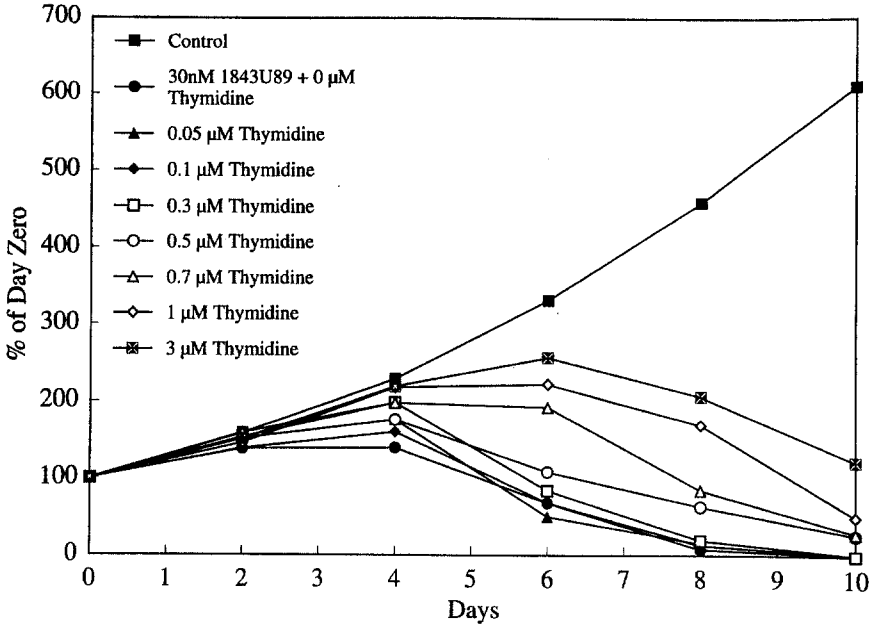

Fig. 3. [^{14}C]-Thymidine reversal of 1843U89 toxicity in spheroids. Individual spheroids were exposed to 30 n*M* 1843U89 with and without 0.05–3.0 μ*M* [^{14}C]-thymidine. Medium and drug were changed every 48 h. Each data point represents measurements of a minimum of three spheroids. The values represent the mean spheroid area expressed as a percentage of that recorded on day 0

Table 1. Percentage of recovery of [^{14}C]-thymidine in spent medium after 48 h exposure to spheroids

THY [μ*M*]	Day 2	Day 4	Day 6	Day 8	Day 10	Average
0.05	96	100	100	92	96	96.8+3.3
0.1	86	91	95	103	110	97.0+10
0.3	79	85	88	93	103	89.6+9.0
0.5	82	85	90	85	99	88.2+6.7
0.7	80	87	90	101	106	92.8+10.6
1	81	83	90	99	109	92.4+11.7
3	75	95	93	102	99	92.8+10.6

Effect of dosing length of 1843U89 on WiDr spheroid regrowth

The time-dependent effects of 1, 10, or 100 n*M*, 1843U89 on spheroid disruption or regrowth were investigated. The results are shown in Fig. 2A–E. After 6 h treatment with 1843U89, there was no inhibition of growth at any concentration. After a 24-h treatment, growth delay lasting for 5 days after treatment was seen only at 100 n*M*, after which regrowth equal to control rates was observed. After 48 h treatment, inhibition of growth was seen at both 100 and 10 n*M*. Treatment with 1843U89 for 72 h resulted in inhibition of growth at 1 n*M*, the lowest concentration tested. Treatment with 10 n*M* drug for 72 h produced a more prolonged inhibition of growth in comparison with the 48-h treatment. At 100 n*M* 1843U89, complete spheroid disruption was seen by the 9th day after drug removal. After 5 days' treatment, complete spheroid disruption was seen at both 10 and 100 n*M* and a more prolonged inhibition of growth was seen at 1 n*M* in comparison with the 72-h treatment. The data show that as the length of exposure to 1843U89 was increased, there was not only inhibition of spheroid growth but also complete disruption of spheroid structure and loss of viability.

In contrast, inhibitors of de novo purine biosynthesis caused reversible inhibition of spheriod growth without spheroid disruption. Spheroid growth resumed following drug removal, even after 15 days of complete growth inhibition in the presence of purine synthesis inhibitors [15].

Reversal of 1843U89 toxicity with thymidine

It has been shown that 20 μ*M* thymidine will totally reverse the toxicity of concentrations of up to 10 μ*M* 1843U89 in WiDr cells grown in monolayer culture [6, 7]. To investigate the effect of thymidine on 1843U89 toxicity in the three-dimensional array, spheroids were continuously exposed for 10 days to 30 n*M* 1843U89 with and without various concentrations of [^{14}C]-thymidine. A concentration of 30 n*M* 1843U89 was chosen to insure spheroid disruption in the absence of thymidine. Figure 3 shows that in the presence of ≥0.7 μ*M* thymidine, partial reversal of inhibition of cell growth and spheroid disruption occurred, whereas at levels of 0.3 μ*M* thymidine and below, no significant effect of thymidine was observed. To insure that the thymidine levels in these experiments remained constant, [^{14}C]-thymidine was used and growth medium was changed every 48 h, with fresh [^{14}C]-thymidine and 1843U89 being added. The spent medium was analyzed by HPLC for thymidine and thymine content. At all concentrations ranging from 0.05 to 1 μ*M* thymidine,

Table 2. Spheroid size after 10 days' continuous exposure to 1843U89 and [^{14}C]-thymidine

Thymidine (μM)	1843U89 (nM)	Spheroid size on day 10 (% day 0)
0	0	611
0	30	0
0.1	30	0
0.3	30	0
0.5	30	26
0.7	30	29
1	30	50
3	30	121

Individual spheroids were transferred to agarose-coated multiwell plates and exposed to 30 nM 1843U89 and several concentrations of [^{14}C]-thymidine. Medium was changed every other day, with fresh 1843U89 and [^{14}C]-thymidine being added. The values represent the mean spheroid area expressed as a percentage of that recorded on day 0

97%–100% of the detectable radioactivity recovered from the spend medium coeluted with thymidine. When the spent medium was treated with thymidine phosphorylase prior to HPLC analysis, the peak moved to the position of thymine, verifying the identity of the thymidine peak. Thus, the thymidine was indeed stable in the medium over the course of the experiment. The quantitative thymidine recovery from spent medium is shown in Table 1. The results show that 88%–97% of the thymidine added to fresh medium was recovered in the spent medium; thus, the spheroids did not deplete the added thymidine. The [^{14}C]-thymidine experiments in toto show that thymidine levels in the medium were maintained throughout the experiment. This observation is important, since others have shown that thymidine is depleted when similar experiments are performed with monolayer cells [9], making interpretation of these monolayer experiments difficult.

Humans have circulating thymidine levels of approximately 0.1 μM and mice have circulating thymidine levels of approximately 1 μM [8]. Whereas thymidine does not compete with 1843U89 for TS binding, it circumvents the block by replacing thymidylate by salvage. Figure 3 and Table 2 show that at or below 0.3 μM thymidine, there was no reversal of antitumor activity. Thus, in humans, circulating thymidine levels would not be expected to block 1843U89 chemotherapy. However, since thymidine levels of 0.7 μM and above cause partial reversal, the data predict that circulating thymidine in mice would adversely affect antitumor activity in this species. Thus, the mouse is not a suitable model for evaluation of 1843U89 or, possibly, other compounds that have TS as their sole mode of action. Indeed, it has been observed that efficient tumor growth inhibition by 1843U89 or other TS inhibitors in mice requires depletion of circulating thymidine [7, 9, 10]. It is interesting in this regard that Howell et al. [16] have reported that in humans, 0.3–0.4 μM circulating thymidine is effective in MTX toxicity rescue. Thus, it appears that thymidine levels in this range are critical for both antitumor activity and toxicity rescue. In summary, the data obtained with WiDr spheroids suggest that multiday dosing with 1843U89 at plasma levels of 10–100 nM should have solid-tumor activity in man.

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