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ORIGINAL ARTICLE

Metronomic chemotherapy dosing-schedules with estramustine and temozolomide act synergistically with anti-VEGFR-2 antibody to cause inhibition of human umbilical venous endothelial cell growth

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Abstract

The effects of 'metronomic' or extended chemotherapy dosing schedules (ECS) are mediated through poorly understood anti-angiogenic mechanisms. ECS combined with biological anti-angiogenic agents have produced promising pre-clinical results. *Materials and Methods.* We have expanded the list of agents with an *in vitro* ECS profile to include the methylating agent temozolomide (Temodal[®]) and the anti-mitotic agent estramustine (Estracyt[®]). These agents were also combined with a specific anti-angiogenic inhibitor IMC-1C11 and a non-specific agent with anti-angiogenic properties, Compound 5h. The *in vitro* HUVEC ECS model system was optimised and cell proliferation assays undertaken. *Results.* As a single agent, estramustine inhibited endothelial cell proliferation with an IC₅₀ of 4.5 µM and was active at 10–33% of the maximum tolerated dose (MTD) from clinical schedules, whilst temozolomide had IC₅₀ of 6.6 µM and was active at 1–6% of MTD. In combination, significant synergy was seen with IMC-1C11 in combination with either drug, whilst modest additive effects were observed with Compound 5h. None of the combinations resulted in significant cytotoxicity or apoptosis. *Discussion.* The results show that ECS of temozolomide and estramustine can be significantly enhanced when combined with specific anti-angiogenic inhibitors in an *in vitro* HUVEC system.

'Metronomic' scheduling of chemotherapy is a clinical strategy whereby low-dose cycles of chemotherapeutic drugs are administered in a continuous or regularly-spaced fashion in order to inhibit tumour angiogenesis [1]. Such a strategy represents a radical departure from the traditional norms of chemotherapy based on the maximum tolerated dose (MTD) paradigm, where the drug dose cycle is increased until dose-limiting toxicity is encountered. The potential benefits of 'metronomic' dosing over traditional schedules of chemotherapy include lower risk of acquiring drug resistance, lower incidence of systemic side effects and a lower degree of bone marrow perturbation – a process which can mobilize bone marrow-derived circulating endothelial progenitor cells which may contribute towards tumour angiogenesis [2]. The 'metronomic' strategy is also

suitable for combinations of cytotoxic drugs with new molecularly-targeted anti-angiogenic agents, and there is compelling pre-clinical evidence to suggest that anti-angiogenic efficacy is enhanced when cytotoxic drugs administered in 'metronomic' schedules are combined in this manner [3,4]. Clinical trials are currently underway to confirm such pre-clinical findings [5,6]. In addition, extended oral administration of chemotherapeutic agents appears to be the optimum schedule fulfilling all the major requirements of 'metronomic' dosing, and as such, cytotoxic drugs available through the oral route are likely to be the most suitable agents for this strategy.

However, many uncertainties remain concerning the principle of 'metronomic' dosing. Issues such as identifying the most suitable cytotoxic drugs,

establishing the optimal dosing and frequency schedule, determining the biological activity of such therapy through surrogate markers, the potential development of acquired drug resistance in the long-term, all remain to be elucidated for each treatment strategy. At present there are some pre-clinical approaches that include animal and *in vitro* models of ‘metronomic’ dosing which are generally acceptable as useful for the study of this treatment strategy. The work we present in this study is based on one such approach [7]. The previously published protocols of an *in vitro* endothelial model were used to investigate the potential anti-proliferative properties of *estramustine* and *temozolomide* both individually and in combination with specific and non-specific anti-angiogenic agents. The drugs represent different types of orally bioavailable agents which are in clinical use as conventional chemotherapeutic drugs at present. Estramustine is an anti-mitotic agent consisting of a conjugate of oestradiol and normustine and is at present licensed for the treatment of advanced hormone-refractory prostatic carcinoma. Temozolomide is an alkylating agent used in the treatment of melanoma and glioblastoma. Paclitaxel, an anti-mitotic drug which has proven efficacy in metronomic regimens either singly or in combination with anti-angiogenic agents *in vitro* and *in vivo* studies [7,8] was used as a “positive control” cytotoxic agent and served as a comparison for estramustine and temozolomide.

We investigated the synergistic activity between a protracted dosing schedule and a specific anti-angiogenic agent (anti-VEGFR-2 monoclonal antibody, IMC-1C11) [9] or a non-specific one (Compound 5h) [10]. For the purposes of clarity we use the term metronomic only in a clinical context and prefer the term *extended chemotherapy dosing-schedule* (ECS) to describe the *in vitro* methodology. The role of anti-VEGFR-2 antibody in combinatorial ECS has been established in pre-clinical studies previously [11,12]. Compound 5h is a novel orally bioavailable drug belonging to the FTI group of compounds for which there is growing evidence of anti-angiogenic properties [13] through currently unknown mechanisms. The cell proliferation assays were conducted on dermal fibroblasts and human tumour cell lines for comparative purposes. In order to demonstrate the clinical relevance of all drugs in terms of possessing anti-proliferative properties at clinically achievable doses, the relevant ECS doses of each drug were correlated with its MTD using known data from conventional pharmacokinetic studies.

Materials and methods

Drugs

Purified temozolomide was kindly provided by Schering-Plough, UK; purified estramustine by Dr. B. Asp (Pharmacia, Sweden); Paclitaxel, (Sigma, UK); IMC-1C11 by Dr. D. Hicklin (ImClone, USA); and Compound 5h by Dr. I. Bell (Merck, USA). For *in vitro* experiments, all drugs were reconstituted at 10 mM by dissolving in DMSO. All drugs were diluted in culture medium immediately prior to use and the highest DMSO concentration in culture was less than 0.1% (v/v).

Cells and culture conditions

All media and tissue culture reagents used were purchased from Invitrogen (UK) unless otherwise stated. HUVECs were purchased from TCS Cellworks (UK). Human dermal fibroblasts and the human renal cell carcinoma cell line CAKI2 were purchased from the European Collection of Cell Cultures (UK); the human melanoma cell line A2058 was purchased from the American Type Culture Collection (LGC Promocell).

The HUVECs were maintained in Medium 199 with Earle’s salts and Glutamax-I supplemented with 10% (v/v) heat-inactivated FBS (HiFBS), heparin (20 units/ml; Sigma, UK) and endothelial cell growth supplement (150 µg/ml; BD Biosciences, UK). Dermal fibroblasts were cultured in DMEM with non-essential amino acids, glucose and sodium pyruvate supplemented with glutamine (2 mM; Sigma, UK) and 10% (v/v) HiFBS. CAKI2 and A2058 cell lines were maintained in 10% (v/v) HiFBS McCoy’s Modified 5A Medium with L-Glutamine and 10% (v/v) HiFBS RPMI 1640 Medium with L-Glutamine respectively. All cells were kept in a humidified atmosphere of 5% CO₂ at 37°C and harvested with a solution of 0.25% (w/v) trypsin-0.03% (w/v) EDTA when they were in logarithmic phase of growth and maintained as described above. For the *in vitro* experiments, all cells used were below Passage 6.

Cell proliferation assay

In vitro chemosensitivity testing was performed on cells grown as a monolayer on 96-well flat-bottomed tissue culture plates (Sarstedt, USA). Each drug concentration was set up in quintuplicate. Preliminary experiments were conducted to determine the optimal cell seeding density which enabled the longest period of uninterrupted growth in 96-well plates up to confluence. HUVECs were seeded at 3500 cells/well, while fibroblasts, CAKI2 and

A2058 cell lines were seeded at 1500 cells/well, 1000 cells/well and 500 cells/well respectively, each in 100 μ l of media. The longest growth period, which determined the duration of drug exposure for all cells, was 4 days. Preliminary experiments were then conducted to determine the therapeutic range of each drug. Cells were treated with estramustine (0–100 μ M), temozolomide (0–100 μ M), paclitaxel (0–2 nM), IMC-1C11 (0–50 μ g/ml) or Compound 5h (0–100 μ M). The chemotherapeutic drugs were then combined with either IMC-1C11 or Compound 5h at 25 μ g/ml and 0.1 μ M respectively. For all experiments, in order to maintain a constant concentration of the drugs, the media was replenished with fresh solutions containing the appropriate drug concentrations on a daily basis according to Bocci et al. [7].

For the cell proliferation assay, a standard (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl-2-(4-sulfophenyl)-2H-tetrazolium, inner salt) (MTS) assay (Promega, UK) was performed [14]. The assay was performed immediately prior to drug addition to determine the baseline number of cells prior to treatment, and at the end of the period of drug treatment. Briefly, 20 μ l of MTS solution was added to each well containing 100 μ l of media and the background-subtracted absorbance at 4 hours was recorded. Cell growth from baseline was expressed as percentage change in absorbance values. To calculate relative growth, the growth of control, untreated cells was taken as 100%, and the corresponding growth of treated populations of cells was expressed as a percentage of control growth [14]. The concentration of drugs required to reduce cell growth by 50% (IC_{50}) as compared with controls was calculated by nonlinear regression fit analysis of the mean values of the data points. All data represent the mean of three independent experiments.

Statistical analysis

The results of relative cell growth are expressed as mean values \pm standard error. The data was analysed using a two-sample t-test for populations of unequal variance to compare the effects of different drug doses and combinations. SPSS software version 11.0 (SPSS, Chicago, USA) was used for the statistical analysis. A confidence level of $p \leq 0.05$ was considered statistically significant.

Quantification of drug interactions

Drug interactions in terms of anti-proliferative activity were quantified by the fractional inhibition method [15] as follows:

If the fractional inhibition of cell growth or viability of each drug is expressed as i_1 and i_2 and the fractional inhibition achieved via a combination of the two drugs is expressed as i_{1+2} , the following criteria applies: (1) additive inhibition occurs when $i_{1+2} = i_1 + i_2$; (2) synergistic inhibition occurs when $i_{1+2} > i_1 + i_2$; and (3) antagonism occurs when $i_{1+2} < i_1 + i_2$.

Results

Anti-proliferative activity of estramustine, temozolomide, paclitaxel and compound 5h at low doses

The range of cell numbers, which refers to the minimum and maximum cell numbers for each cell type within the experimental 4 day period, were as follows: 3500–6400 cells/well for HUVECs; 1500–2250 cells/well for fibroblasts; 1000–2200 cells/well for CAKI2 cells; and 500–2150 cells/well for A2058 cells. The derived baseline control (i.e. untreated cells) growth rate of HUVECs, fibroblasts, CAKI2 and A2058 cells after the 4 days was therefore $80.4 \pm 3.6\%$, $49.9 \pm 2.6\%$, $120.5 \pm 7.8\%$ and $330.3 \pm 16.4\%$ respectively. The percent of growth of the untreated cells after the 4 days was normalized to 100%, and the growth of treated cells was expressed as a percentage of this base-line growth [14].

For the treated cell population, all drugs except IMC-1C11 inhibited cell proliferation. HUVECs were significantly more sensitive to the anti-proliferative effects of all drugs in a dose-dependent manner. Estramustine (Figure 1A) affected only HUVECs from 1 μ M ($p < 0.05$) to 10 μ M ($p < 0.05$), with a calculated IC_{50} of 4.5 μ M. In contrast, fibroblasts were only inhibited with drug concentrations at $> 50 \mu$ M ($p < 0.05$), giving a calculated IC_{50} of 80.9 μ M. Both CAKI2 and A2058 cell lines were only significantly inhibited at $> 100 \mu$ M ($p < 0.05$ in both cases) and an IC_{50} was not reached for either cell lines.

The same trend was observed with temozolomide, with HUVECs demonstrating significant sensitivity towards the drug at relatively low doses (Figure 1B). Only HUVECs were inhibited from 2.5 μ M ($p < 0.05$) to 10 μ M ($p < 0.05$), with a calculated IC_{50} of 6.6 μ M. In contrast, fibroblasts were only affected at $> 100 \mu$ M ($p < 0.05$), CAKI2 cell line $> 100 \mu$ M ($p < 0.05$) and A2058 cell line at $> 50 \mu$ M ($p < 0.05$). An IC_{50} was never achieved for these cells. For paclitaxel (Figure 1C), only HUVECs were inhibited at 150 pM ($p < 0.05$), giving a calculated IC_{50} of 228 pM. Fibroblasts and A2058 cell line were only affected at > 500 pM ($p < 0.05$ in both cases), giving IC_{50} values of 985 pM and 768 pM respectively. CAKI2 cell line was only

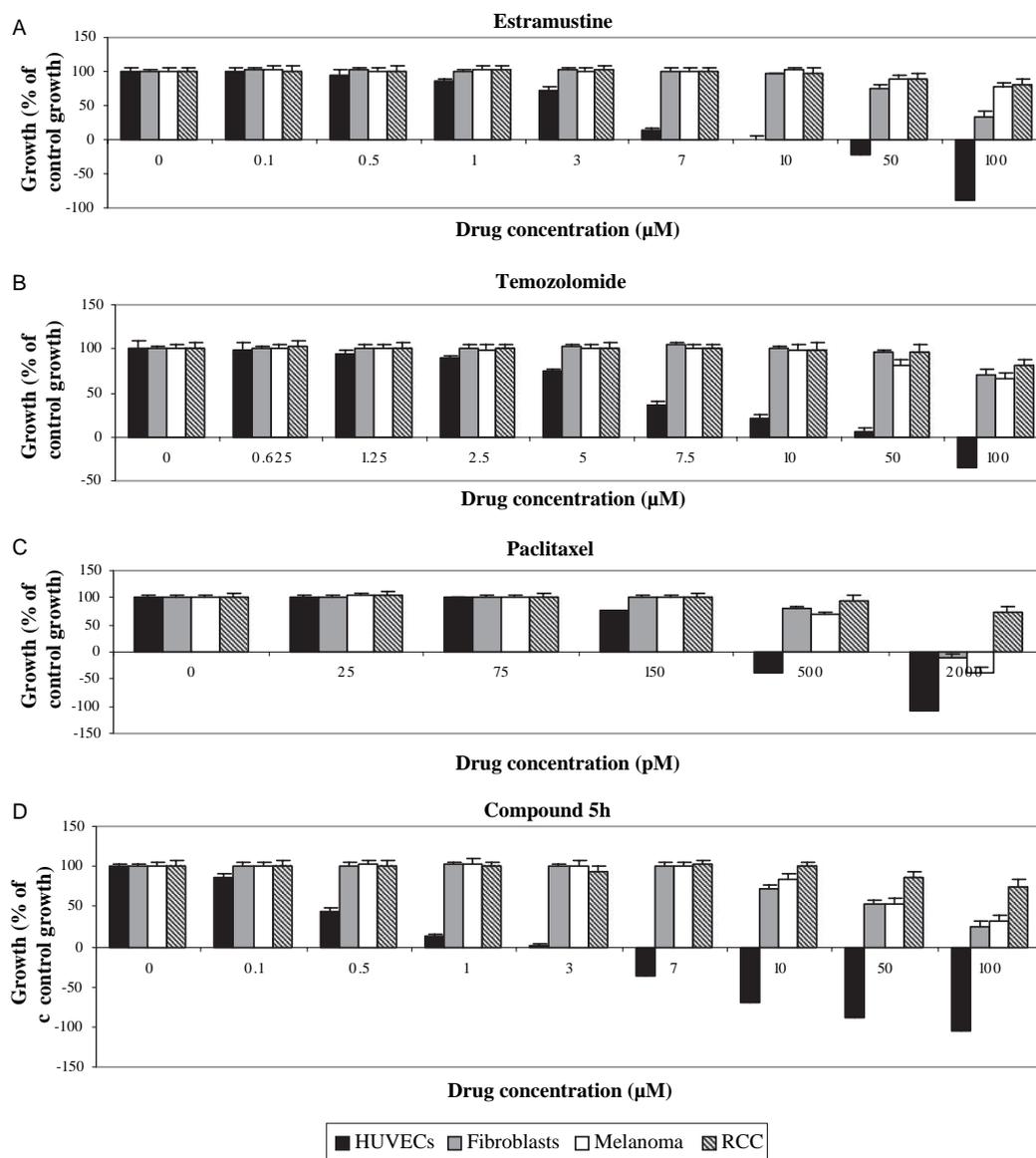


Figure 1. Effect of continuous and protracted (i.e. 4-day) exposure of Estramustine (A), Temozolomide (B), Paclitaxel (C), and Compound 5h (D) on *in vitro* cell growth to determine therapeutic drug ranges. The growth of untreated control cells was taken as 100% and the growth of treated populations of cells was expressed as % of this. Columns and bars represent mean values \pm SE, respectively. Negative growth figures indicate that cell number after treatment was lower than cell number prior to treatment. For relevant statistical analyses, refer to main text.

significantly affected at 2 nM ($p < 0.05$) and an IC_{50} was not reached.

The findings that the ECS therapy with the conventional cytotoxic agents significantly more affected the low proliferative HUVECs compared to the highly proliferative CAKI2 and A2058 cells was not unexpected. As the investigated doses are very low they should not cause conventional cytotoxicity. The “metronomic” effects observed in the HUVECs may be mediated by the potent and endothelial specific angiogenesis inhibitor, thrombospondin-1 [16] hence the tumourostatic effects are not due to the doses actually killing the cancer cells.

Compound 5h (Figure 1D) showed endothelial-specific growth inhibitory activity, with only HUVECs significantly affected from 0.1 μ M ($p < 0.05$) to 7 μ M ($p < 0.05$), with a calculated IC_{50} of 0.44 μ M. In contrast, fibroblasts and A2058 cell line were only inhibited at >10 μ M ($p < 0.05$ in both cases), giving IC_{50} values of 54 μ M and 58.7 μ M respectively. CAKI2 cell line was only inhibited at >50 μ M ($p < 0.05$) and an IC_{50} was not reached.

The monoclonal anti-VEGFR-2 antibody IMC-1C11 did not cause any significant inhibition of cell growth within the range of drug concentrations used (0–50 μ g/ml).

Synergy of anti-proliferative activity of estramustine, temozolomide and paclitaxel at low doses in combination with IMC-1C11 or compound 5h

Although IMC-1C11 did not cause any significant inhibition of cell growth within the range of drug concentrations used (0–50 µg/ml), the effect of combination of the low dose chemotherapeutic agents with IMC-1C11 at a concentration of 25 µg/ml was investigated. The rationale for choosing this dose was provided by a previous study undertaken by Klement et al. [12] who demonstrated that addition of IMC-1C11 (25 µg/ml) alone had no significant effect on HUVECs but when combined with the low dose cytotoxic agent, vinblastine, striking anti-mitotic effects were observed.

For estramustine, addition of 25 µg/ml IMC-1C11 (Figure 2A) increased the growth inhibition of HUVECs, and there was synergistic inhibitory activity between 1–3 µM of estramustine ($p < 0.05$). Although synergy was not demonstrated with 25 µg/ml IMC-1C11 and 7 µM of estramustine, HUVEC growth was inhibited by 92.3% (Figure 2A). The addition of 0.1 µM Compound 5h (Figure 3A) to 7 µM of estramustine enhanced HUVEC growth inhibition, but this increase was lower than the expected combined inhibition of the two drugs based on single-drug schedules; hence neither synergy nor additive inhibitory activity was demonstrated between estramustine and Compound 5h. None of the other cell types were inhibited by single or combined drug schedules within the range of drug doses tested.

For temozolomide, addition of 25 µg/ml IMC-1C11 (Figure 2B) increased HUVEC growth inhibition significantly, and synergistic inhibitory activity was demonstrated across all doses ($p < 0.05$ for all cases). A similar trend was observed with the addition of 0.1 µM Compound 5h to 1 µM and 5 µM of temozolomide (Figure 3B), where significant synergy of growth inhibitory activity was demonstrated. However, there was no significant synergistic or additional inhibition at 7.5 µM of temozolomide with the addition of Compound 5h. Growth inhibition was not exhibited in the other cell types, either on single or combined drug schedules.

For paclitaxel, the addition of 25 µg/ml IMC-1C11 (Figure 2C) significantly increased the growth inhibition of HUVECs ($p < 0.05$ for all cases), with synergy being demonstrated across all doses. Addition of 0.1 µM Compound 5h (Figure 3C) resulted in additive inhibition rather than synergistic inhibition at 25 pM and 75 pM of paclitaxel respectively. Neither single-drug nor combined drug schedules incurred any growth inhibition in any of the other cell types within the range of drug doses tested.

In summary, modest but statistically significant synergy of endothelial-specific growth inhibitory activity was demonstrated between all cytotoxic agents and IMC-1C11 at low cytotoxic drug doses. Synergy was also demonstrated between temozolomide and Compound 5h, whereas paclitaxel and Compound 5h showed additive inhibitory activity rather than true synergy.

Discussion

We have shown that two orally bioavailable drugs belonging to different classes of cytotoxic compounds possess anti-endothelial properties at ECS dosing. *Estramustine* (an anti-mitotic agent) demonstrated an IC_{50} of 4.5 µM for HUVEC, but significant anti-proliferative effects were present at 1 µM. Pharmacokinetic data from Phase I clinical trials of oral and intravenous dosing schedules of estramustine suggest that levels of 1–3 µM are much lower than the peak plasma concentrations attained from maximum tolerated doses [17,18]. In a Phase I trial of weekly-administered intravenous estramustine phosphate (EMP) carried out by Hudes et al. [18], the MTD was determined to be 2500 mg/m². At a clinically well-tolerated dose of 2000 mg/m², the peak plasma concentrations (C_{max}) of the active metabolites *estramustine* and *estromustine* exceeded 10 µM each. Even at a low dose of 500 mg/m², the corresponding C_{max} value of estramustine exceeded 2 µM. The results from the study also suggested that weekly intravenous EMP at 2000 mg/m² generated similar plasma concentrations of estramustine and estromustine as daily oral EMP at between 560–840 mg per day, which are the oral doses typically recommended for this drug. As such, these data and the findings from the present study suggest that estramustine may be suitable for use in a 'metronomic' schedule at 10–33% of its MTD.

Temozolomide (an alkylating agent) demonstrated an IC_{50} of 6.6 µM for HUVECs, but significant anti-proliferative effects were present at 2.5 µM. In Phase I clinical trials of temozolomide, daily oral 5-day schedules of the drug demonstrated a maximum tolerated dose of 250 mg/m²/day [19]. At 50 mg/m²/day and 225 mg/m²/day, after 5 days of treatment, the C_{max} values attained were 16 µM and 63.9 µM respectively. Consequently, there is some preliminary evidence from the present study to support the use of temozolomide in a 'metronomic' protocol at 1–5% of the MTD. In regard to the use of temozolomide in an extended continuous schedule, Brock et al. [20] conducted a Phase I clinical trial which compared a 7-week daily oral schedule of temozolomide at a dose of 75 mg/m²/day for 7 weeks

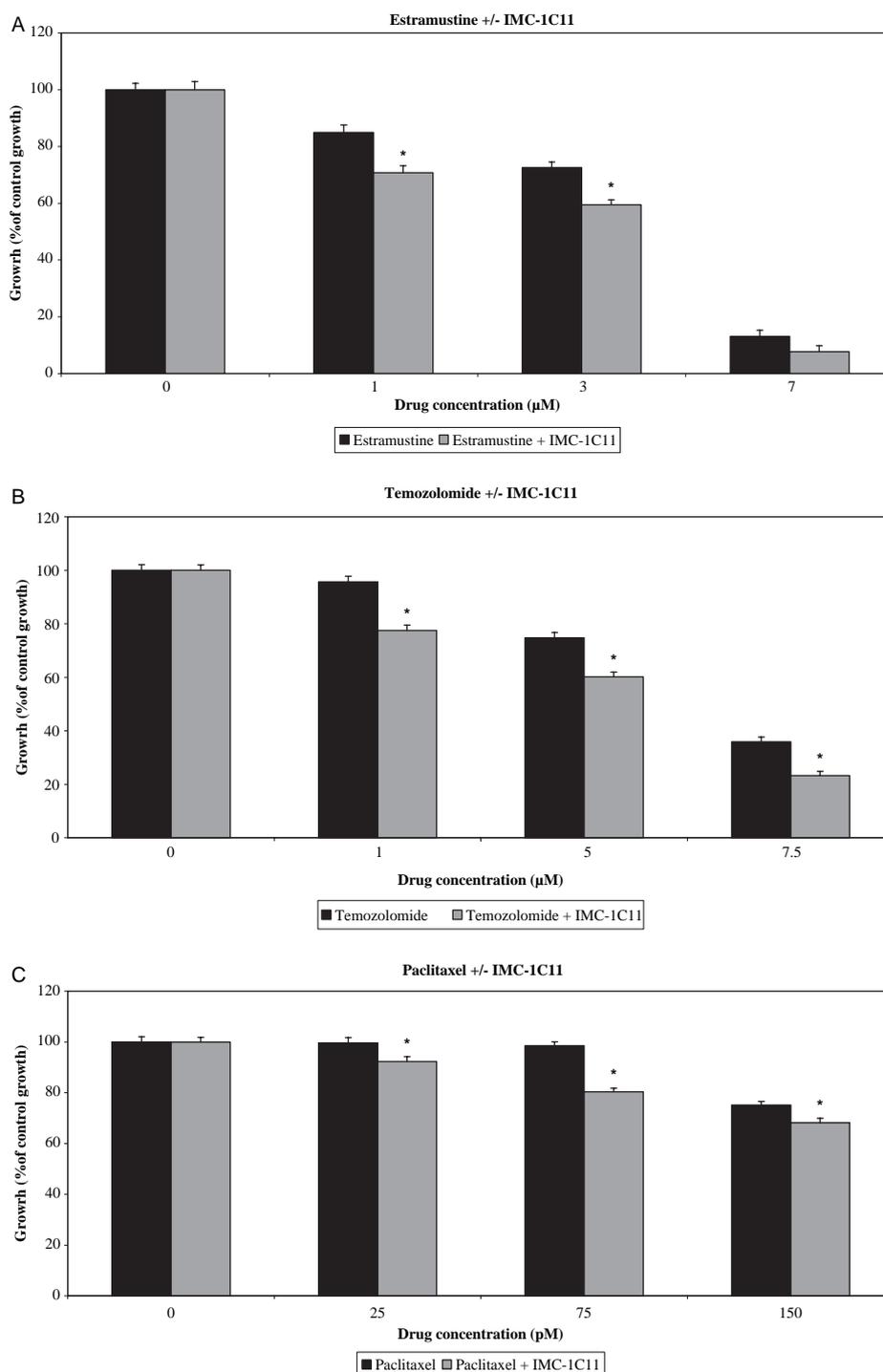


Figure 2. Effect of low-dose Estramustine (A), Temozolomide (B) and Paclitaxel (C) in combination with IMC-1C11 (25 μg/ml) on *in vitro* HUVEC growth compared with single-drug schedule, following continuous, 4-day exposure. The growth of untreated control cells was taken as 100% and the growth of treated populations of cells was expressed as % of this. Columns and bars represent mean values \pm SE, respectively. * $p < 0.05$ comparing combinational regime to single-drug schedule (synergistic effect).

with a traditional 5-day regimen which comprised of a dose of 200 mg/m²/day repeated every 28 days in the treatment of patients with advanced melanoma or glioma. Although overall response rates in terms of tumour regression were similar, the extended dosing schedule resulted in a lower incidence of

toxicities and a 2.1-fold greater drug exposure over a 4-week period. The anti-angiogenic effects of therapy were not assessed.

Paclitaxel demonstrated an IC₅₀ of 228 pM for HUVECs, which was considerably higher than the corresponding value obtained by Bocci et al. [7]

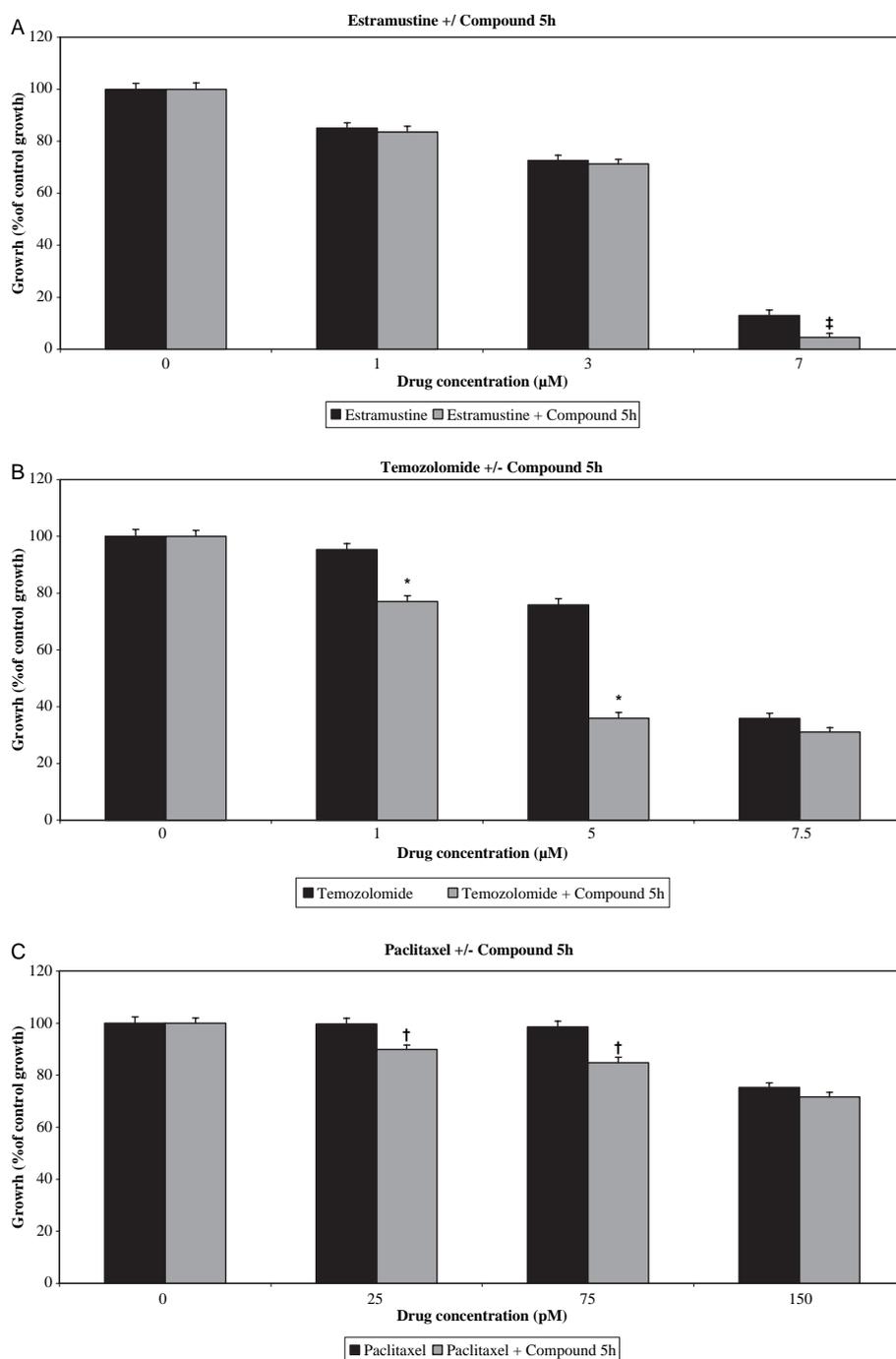


Figure 3. Effect of low-dose Estramustine (A), Temozolomide (B) and Paclitaxel (C) in combination with Compound 5h (0.1 μM) on *in vitro* HUVEC growth compared with single-drug schedule, following continuous, 4-day exposure. The growth of untreated control cells was taken as 100% and the growth of treated populations of cells was expressed as % of this. Columns and bars represent mean values \pm SE, respectively. * $p < 0.05$ comparing combinational regime to single-drug schedule (synergistic effect), † $p < 0.05$ comparing combinational regime to single-drug schedule (additive effect), ‡ $p < 0.05$ comparing combinational regime to single-drug schedule.

which was 96 pM. Nevertheless, the same therapeutic specificity towards endothelial cells was demonstrated up to 1 nM. Previous pharmacokinetic studies involving paclitaxel have shown that 6-hour infusions and 24-hour infusions, each administered once every 3 weeks, resulted in an MTD of 250 mg/m² and corresponding C_{max} values of 13 μM

and 1 μM respectively [21]. Consequently, the data suggest that paclitaxel may be used at less than 1% of the MTD as part of a combined 'metronomic' protocol.

Preliminary studies using LDH release and annexin-V:FITC binding demonstrated no significant cytotoxicity or induction of apoptosis when studying

the optimal drug concentrations (data not shown; available on request) although further investigations are required to clarify the exact mechanisms of action. Bocci et al. [7] found that ECS scheduling with several cytotoxic agents including paclitaxel caused significant apoptosis in endothelial cells after 6 days of treatment. However, Browder et al. [22] showed that low drug doses inhibited growth without inducing apoptosis when combining cyclophosphamide and TNP-470.

We have also presented evidence that suggests that the anti-endothelial effects of ECS chemotherapy can be further enhanced by the addition of molecular inhibitors of angiogenesis, such as anti-VEGFR-2 monoclonal antibodies or drugs which can potentially interfere with VEGFR-2 signalling pathways, such as FTIs (e.g. Compound 5h). Expression of the target for the anti-VEGFR-2 antibody (i.e. VEGFR-2) was confirmed on the HUVECs by flow cytometry (data not shown; available upon request) and our findings are in agreement with others. [23,24]. Synergy of activity was demonstrated between all cytotoxic drugs studied and the anti-VEGFR-2 antibody. This finding correlates with the findings of Klement et al. [10] involving the use of low-dose vinblastine and monoclonal anti-VEGFR-2 antibody to treat xenografts of neuroblastoma cell lines in SCID mice. Both vinblastine and anti-VEGFR-2 antibodies alone caused significant but transient tumour regressions, but the combination therapy resulted in full and sustained regressions lasting more than 6 months. The rationale for the combination was that since inhibition of VEGFR-2 can potentially prevent cells under cytotoxic stress from utilizing survival pathways to elude apoptosis, addition of anti-VEGFR-2 antibody to schedules comprising of low-doses of cytotoxic agents can be synergistic.

We also found that Compound 5h, a novel, orally bioavailable 3-aminopyrrolidinone FTI, demonstrated endothelial-specific anti-proliferative properties at very low doses and it appeared to have a wide endothelial-specific therapeutic window. FTIs were initially synthesized to inhibit the function of the *ras* protein, but their exact mechanism of action has since been the subject of much controversy, and may involve non-*ras* related processes [25]. It is thought that one of the downstream pathways activated by the VEGF/VEGFR-2 system may involve the *ras*-dependent MAPK signalling pathway [26], and this provides *ras*-inhibiting drugs such as FTIs with an anti-angiogenic rationale. Our study provides some evidence that FTIs may possess anti-endothelial properties, as previously shown by others [27,28].

However, the effect of this non-specific anti-endothelial agent in combination with ECS chemotherapy is not as successful as that of combining ECS with a specific anti-angiogenic inhibitor in our *in vitro* model.

It has to be noted that the use of *in vitro* models based on HUVECs or any other endothelial cell type to represent tumour endothelium is not without its problems. There is a valid argument in using models based on cells of microvascular origin (e.g. human microvascular endothelial cells) to reflect more accurately the microvascular nature of angiogenesis *in vivo*. Furthermore it is patently obvious that the simplified demonstration of inhibitory effects on an endothelial cell model *in vitro* fails to take into account the anatomical complexity of even suboptimal tumour-related vasculature, which includes pericytes and other potential targets for anti-angiogenic effects. No *in vitro* model is truly representative of the *in vivo* process, and they should be regarded as approximations rather than true representations. Nevertheless, *in vitro* chemosensitivity assays do play crucial roles in the development of novel therapeutic strategies, especially within clinical trial settings [29]. Our work demonstrates that in two classes of chemotherapy agents, the non-specific effects of ECS chemotherapy scheduling can be significantly enhanced when combined with an agent that targets the angiogenic pathway specific for the studied system. The combination however with a non-specific inhibitor of angiogenesis, even if this agent is effective as a single agent in the system studied, has unpredictable results. This may be related to the model used or the type of chemotherapy agents combined. In conclusion, further research needs to be undertaken in order to define the molecular mechanisms of action of the different anti-angiogenic agents to determine true synergistic responses.

In summary, based on the ECS protocol proposed by Bocci et al. [7], we have demonstrated a readily available, easily accessible, rapid and technically simple *in vitro* system of screening potential cytotoxic drugs either alone or in combination for their anti-endothelial effects within an ECS schedule. Although *in vitro* assays of the type we have used cannot determine the mechanism of action, they can however afford some insight into what may be a clinically relevant combination of anti-angiogenics. In particular, they raise the issue that unless we improve our understanding of the mechanism of actions of the non-specific agents, we are bound to perpetuate the same problems of empiricism that have marked our use of MTD chemotherapy and its combinations.

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