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**Re.: Expediente No. 03 89.905
Fase Nacional Solicitud de Patente PCT
a nombre de
EAST CAROLINA UNIVERSITY
Código 02 - 01 - 444**

En relación con la solicitud contenida en el expediente de la referencia y, en particular, con el Concepto Técnico No. 671, contenido en su último oficio proferido dentro del mismo, por medio del presente escrito y dentro del término conferido, me permito manifestarle:

1. Con respecto a la excepción a la patentabilidad, con el presente escrito acompaño:

- 1.1 Un nuevo capítulo de reivindicaciones, debidamente revisado y del que se ha omitido el método terapéutico.

Consideramos que, con fundamento en las modificaciones propuestas, se superan las observaciones efectuadas.

Le solicito se sirva agregar este escrito y su anexo al expediente y conceder el privilegio solicitado.

De Usted Atentamente,

GERMAN CASTILLO GRAU
T.P. No. 2978 del C.S.J.

REIVINDICACIONES

1. Un compuesto que comprende eritropoyetina y un agente citotóxico para la fabricación de un medicamento para el tratamiento o reducción de toxicidad pulmonar inducida por la quimioterapia.
2. El compuesto de acuerdo con la reivindicación 1 en donde el agente citotóxico es un agente antineoplástico.
3. El compuesto de acuerdo con cualquier reivindicación anterior en donde la eritropoyetina es para administración en una cantidad de 1 U/kg a 2000 U/kg.
4. El compuesto de acuerdo con cualquier reivindicación anterior en donde la eritropoyetina es para administración en una cantidad de 1 U/kg a 200 U/kg.
5. Eritropoyetina para el tratamiento o reducción de toxicidad pulmonar inducida por la quimioterapia inducida por un agente citotóxico y para administración simultáneamente con, antes o después del agente citotóxico.
6. Eritropoyetina de acuerdo con la reivindicación 5 en donde el agente citotóxico es un agente antineoplástico.

7. Eritropoyetina de acuerdo con la reivindicación 5 o la reivindicación 6, y para administración en una cantidad de 1 U/kg a 2000 U/kg.
8. Eritropoyetina de acuerdo con la reivindicación 5 o la reivindicación 6, y para administración en una cantidad de 1 U/kg a 200 U/kg.
9. Eritropoyetina de acuerdo con cualquiera de las reivindicaciones 5 a 8 para administración simultáneamente con el agente citotóxico.
10. Eritropoyetina de acuerdo con cualquiera de las reivindicaciones 5 a 8 para administración antes del agente citotóxico.
11. Eritropoyetina de acuerdo con cualquiera de las reivindicaciones 5 a 8 para administración después del agente citotóxico.

Hemotoxicity by Prolonged Etoposide Administration to Mice Can Be Prevented by Simultaneous Growth Factor Therapy¹

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ABSTRACT

In this study, we determined *in vivo* interactions between hemopoietic growth factors and etoposide (VP-16) to assess whether normal blood cell production could be maintained during chemotherapy if hemopoietic growth factors were simultaneously administered. Groups of mice were treated for 7 consecutive days with four different doses of VP-16 in combination with three different doses of erythropoietin (EPO) or granulocyte colony-stimulating factor (G-CSF). In total, 12 combinations of VP-16 plus EPO and 12 combinations of VP-16 plus G-CSF were thus evaluated. Intricate dose-response surfaces of the effects of the different treatments on colony-forming units-erythroid, reticulocytes, hematocrit, colony-forming units-granulocyte/macrophage, and absolute neutrophil count were obtained, which revealed that: (a) simultaneous EPO administration was able to maintain reticulocyte production and to protect mice from VP-16 induced anemia; (b) simultaneous G-CSF administration was able to maintain granulocyte production and to protect mice from VP-16 induced neutropenia; (c) VP-16 dose escalation was feasible when EPO or G-CSF were simultaneously administered; and (d) no increased myelotoxicity on erythroid or granuloid progenitors was observed when EPO or G-CSF was simultaneously administered with VP-16. These results suggest that *in vivo* either individual hemopoietic progenitors can become resistant against VP-16-induced cell death by appropriate simultaneous growth factor administration or that the loss of overall cell amplification, induced by VP-16, can be compensated by extra amplification of surviving progenitors. Furthermore, these data indicate that a strict separation in time of cytostatic drug and growth factor treatment is not necessarily the optimal schedule with respect to the reduction of hemotoxicity.

INTRODUCTION

Hemopoietic growth factors are currently being intensively investigated to assess their role in hastening the restoration of reduced peripheral blood cell numbers in chemo- or radiotherapeutic settings. These growth factors are, in the vast majority of cases, administered to patients after they have been treated with anti-neoplastic agents, thus separating the cytotoxic and the cytokinetic therapy in time. This strategy is pursued because very little is known about the *in vivo* interactions of growth factors and cytostatic drugs. Concern exists about possible enhanced hemotoxicity when growth factors and cytostatic drugs are administered simultaneously, because growth factors may increase the sensitivity of hemopoietic progenitors for cytostatic drugs. Up to now, however, this approach has not been investigated satisfactorily, although there are reasons to believe that the simultaneous administration of specific hemopoietic growth factors and certain cytostatic drugs may well be beneficial in terms of normal blood cell formation, thus allowing chemotherapeutic dose escalation.

Theoretically, it can be argued that a certain dose of a cytostatic drug kills a certain percentage of a specific hemopoietic progenitor

cell pool. If a growth factor is administered simultaneously, amplification of the fraction of progenitors which was not killed is increased, which may result in increased peripheral blood cell production. This argument assumes that the sensitivity of the remaining progenitors for the cytostatic drug is not increased by the growth factor.

A second indication for a possible beneficial effect of simultaneous treatment with growth factors and cytostatic drugs comes from the fact that many cytotoxic agents, acting with different molecular mechanisms, induce the appearance of apoptotic characteristics in target cells (1, 2). In addition, it has also been shown that many growth factors prevent dividing cells from apoptosis (3-5). The question arises whether *in vivo* toxicity, resulting from apoptosis-inducing cytostatic agents, can be prevented by appropriate, apoptosis-preventing growth factors. There are indications from several groups that *in vitro* this is indeed possible. It has been demonstrated that the induction of apoptosis in leukemic cells, treated with several cytotoxic agents (Adriamycin, 1- β -D-arabino-furanosylcytosine, and vincristine), could be prevented by GM-CSF,³ G-CSF and interleukin 6 (6). Collins *et al.* (7) analyzed the *in vitro* effect of a prolonged simultaneous incubation of BAF-3 cells with VP-16 or cisplatin and interleukin 3. Interleukin 3 was able to protect cells from apoptotic cell death induced by these drugs (7). If these *in vitro* results can be extrapolated to *in vivo* situations, it would open the possibility to protect normal hemopoietic progenitors during chemotherapy.

In the present study, therefore, we assessed normal hemopoietic cell production in mice treated with several doses of VP-16 simultaneously with two widely used growth factors, EPO and G-CSF. It has been shown that continuous administration of VP-16 results in superior responses in patients (8). In the murine system, the VP-16 dose killing 10% of the mice after a 5-day treatment has been shown to be approximately 0.2 mg/day (9). These reports led us to design a multifactorial experiment in which mice were treated with 4 doses of VP-16 (0, 0.05, 0.1, and 0.2 mg/day) for 7 days. Concomitantly, rhEPO or rhG-CSF, each in three doses, was infused. With this approach, we were able to prevent anemia and neutropenia and could increase VP-16 doses significantly without inducing hemotoxicity. By a detailed statistical analysis of the data, we were able to construct equi-response curves of each cellular parameter. These curves illustrate the intricate behavior of the different parameters.

MATERIALS AND METHODS

Mice. Female C57Bl/6 mice, aged 12-16 weeks were used in all experiments.

Growth Factors. Recombinant human EPO, a gift from Boehringer Mannheim (Almere, the Netherlands) was administered at doses of 10 and 25 units/day/mouse. Recombinant human G-CSF, a gift from Amgen (Thousand Oaks, CA) was administered at doses of 0.25 and 2.5 μ g/day/mouse.

VP-16. VP-16 (etoposide; Vepesid) was obtained from Bristol-Myers Squibb and was administered at doses of 0.05, 0.1, and 0.2 mg/day/mouse.

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³ The abbreviations used are: GM-CSF, granulocyte-macrophage colony-stimulating factor; G-CSF, granulocyte-CSF; EPO, erythropoietin; rh, recombinant human; CFU-E, colony-forming units-erythroid; VP-16, etoposide.

Table 1 Summary of the statistical analysis of the EPO + VP-16 experiment

This is a summary of the significance (NS, not significant) and the sign of all coefficient estimates of the regression model which was used for the statistical analysis. Thus it indicates whether a certain cell stage was significantly affected by low EPO or VP-16 doses alone (b and d term), whether this effect was saturated at high EPO or VP-16 doses alone (c and e term), and whether in the combination of EPO and VP-16 significant interaction occurred. As an example, the hematocrit was increased by low EPO doses. At high EPO doses no saturation was observed. VP-16 reduced the hematocrit at low doses, at higher VP-16 doses this linear inhibitory effect was saturated (i.e., had reached its maximum). Furthermore VP-16 had specifically inhibitory effects on EPO-stimulated erythropoiesis.

	b (EPO)	c (EPO ²)	d (VP-16)	e (VP-16 ²)	f (EPO × VP-16)
Hematocrit	+	NS	-	+	-
Reticulocytes	NS	+	-	+	-
CFU-E/femur	NS	NS	NS	NS	NS
CFU-E/spleen	+	NS	NS	NS	-

Osmotic Pumps. In order to ensure constant serum levels, growth factors and VP-16 were appropriately diluted and administered by s.c. implanted osmotic pumps (Alza, Palo Alto, CA). EPO and VP-16 were administered by the same pump. G-CSF, however, appeared to be inactivated when mixed with VP-16; therefore, two pumps were implanted in the G-CSF/VP-16 mice.

Cell Suspensions and Assays. Femoral and splenic single cell suspensions were made using standard procedures. Hemopoietic progenitors were cultured with the methylcellulose method of Iscove and Sieber (10). CFU-GM/burst-forming unit-crythrocyte cultures were supplemented with 100 ng/ml recombinant rat stem cell factor (a gift from Amgen), 10 ng/ml recombinant murine GM-CSF (a gift from Behringwerke, Marburg, Germany) and 2 units/ml rhEPO. This resulted in optimal colony formation in our assay. CFU-E cultures were supplemented with 500 milliunits/ml EPO. Reticulocytes and neutrophils were counted on, respectively, brilliant crystal blue or May-Grünwald-Giemsa-stained blood smears using light microscopy. The WBC was determined with a Coulter cell analyzer.

Statistical Analysis. Data as given in the figures are the means of 2-4 mice/datapoint. The EPO/VP-16 and G-CSF/VP-16 experiments were designed in a multifactorial way (3 EPO/G-CSF doses × 4 VP-16 doses). The statistical evaluation of this type of experiment involves a regression analysis to determine whether and how much a certain parameter significantly affects the shape of the three-dimensional dose-response surface. This type of analysis is far more powerful than a pairwise comparison of selected datapoints and thereby reduces the number of experimental animals needed to detect significant effects.

We have used this strategy previously in experiments investigating the effects of stem cell factor plus EPO (11) and G-CSF plus EPO (12). In the present study, we could not expect simple linear growth factor dose-response relationships. Therefore the regression model had to be specified in a meaningful way. For both EPO and G-CSF, we have published information showing dose-response saturation characteristics on a logarithmic scale (13, 14). As the regression model analyzes the biological effect (response) of a given G-CSF or EPO dose, these given EPO and G-CSF doses were logarithmically transformed. Thus, EPO doses were coded 0 units = 0, 10 units = 1, and 25 units = 1.4. Similarly, G-CSF doses were coded as 0 μg = 0, 0.25 μg = 1, and 2.5 μg = 2. This coding expresses that the biological "distance" (response) from 0 to 0.25 μg G-CSF is similar as from 0.25 to 2.5 μg G-CSF. The EPO coding analogously acknowledges that the effect from 0 to 10 units EPO is stronger than from 10 to 25 units EPO. This coding protocol is similar as previously published analyses (11, 12).

With the data obtained in the EPO/VP-16 experiment for the number of each cell stage (Y), a regression was performed based upon the model: $Y = a + b \times \text{EPO} + c \times \text{EPO}^2 + d \times \text{VP-16} + e \times \text{VP-16}^2 + f \times (\text{EPO} \times \text{VP-16})$. With the data obtained in the G-CSF/VP-16 experiment, a similar regression was performed, based upon the model $Y = a + b \times \text{G-CSF} + c \times \text{G-CSF}^2 + d \times \text{VP-16} + e \times \text{VP-16}^2 + f \times (\text{G-CSF} \times \text{VP-16})$. These models are identical as the ones we have previously

published (11, 12). They assess whether: (a) there is a significant linear effect of low EPO/G-CSF doses ("b" term); (b) this linear D/R effect is saturated at higher EPO/G-CSF doses ("c" term); (c) there is a significant effect of low VP-16 doses ("d" term); (d) this linear VP-16 D/R effect reaches saturation at high doses ("e" term); and (e) there is an interaction between EPO and VP-16 ("f" term).

Estimates for the regression coefficients and the 95% confidence intervals were determined. A summary of the results of the regression analysis is given in Tables 1 and 2. For each cell stage, it is indicated whether the regression coefficient of a term differed significantly from 0 ($P < 0.05$). Positive values of the linear terms (EPO/G-CSF and VP-16) should be interpreted as stimulatory activity; negative values of these terms indicate inhibitory effects. A negative or positive value of a quadratic term (EPO²/G-CSF² and VP-16²) indicates flattening of a linear effect, which is saturation of the linear response. A negative value of the interaction term (EPO/G-CSF × VP-16) indicates that VP-16 has stronger inhibitory activity in stimulated than in nonstimulated hemopoiesis. All analyses were performed using SAS PROC REG.

Visualizing the Regression Analysis. The regression analysis evaluated the shape of the complete three-dimensional dose-response surface mathematically. The dose-response surface, as described by the regression model, was graphically depicted in a two-dimensional fashion by filling in all possible coded EPO/G-CSF and VP-16 combinations in the regression model and by calculating the predicted Y value. The contour plots thus obtained give a quantitative description of the behavior of each cell stage in response to EPO, G-CSF, and VP-16. They evaluate at which dose ranges VP-16 dose-escalation was feasible and facilitate interpretation of Table 1. These contour plots show so-called "equi-response curves," dose combinations which resulted in similar cell counts.

RESULTS

Simultaneous EPO and VP-16 Administration for 7 Days. VP-16 administration during 7 days induced a dose-dependent reticulocytopenia and anemia (Fig. 1). Reticulocytes were undetectable at a dose of 0.1 mg/day or more. This was also reflected in the hematocrit which was maximally reduced to about 37% at a dose of 0.1 mg/day or more. Simultaneous EPO administration dose-dependently increased both the number of reticulocytes and the hematocrit at VP-16 doses of 0.05 and 0.1 mg/day. At a VP-16 dose of 0.2 mg/day, however, no protective effect of EPO was observed anymore. The corresponding contour plots, which were created by the regression analysis, illustrate the dose ranges of combined EPO and VP-16 administration which are able to maintain normal values (Fig. 2). It is evident from the slopes of the graphs in Fig. 1 that, although EPO was

Table 2 Summary of the statistical analysis of the G-CSF + VP-16 experiment

This is a summary of the significance (NS, not significant) and the sign of all coefficient estimates of the regression model which was used for the statistical analysis. Thus it indicates whether a certain cell stage was significantly affected by low G-CSF or VP-16 doses alone (b and c term), whether this effect was saturated at high G-CSF or VP-16 doses alone (d and e term), and whether in the combination of G-CSF and VP-16 significant interaction occurred. For an example see legend to Table 1.

	b (G-CSF)	c (G-CSF ²)	d (VP-16)	e (VP-16 ²)	f (G-CSF × VP-16)
Granulocytes/ml	NS	+	-	+	-
CFU-GM/femur	+	-	NS	+	NS
CFU-GM/spleen	NS	+	+	-	-

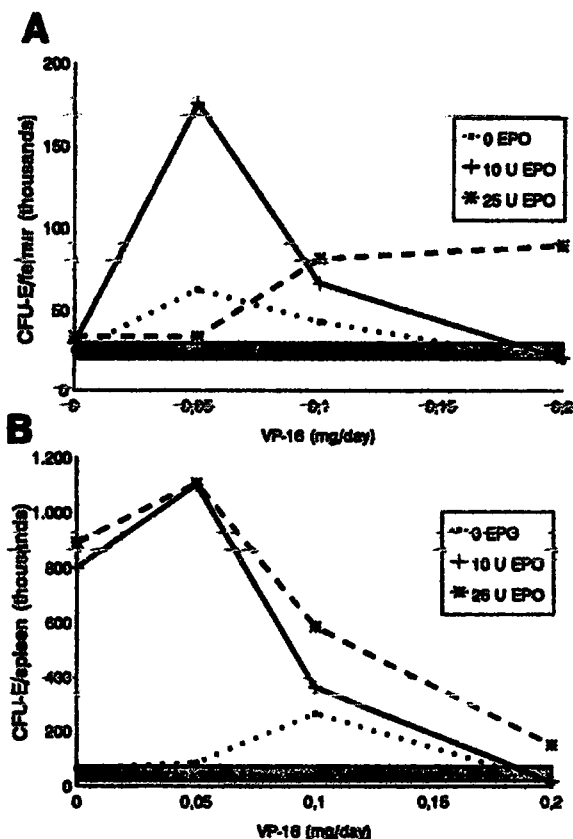


Fig. 3. Effect of simultaneous administration of EPO and VP-16 for 7 days on CFU-E numbers/marrow (A) and spleen (B). Shaded area represents normal range.

combination with high-dose G-CSF, however, CFU-GM numbers were affected but only at the highest VP-16 dose. Importantly however, CFU-GM numbers were never found to be below normal values.

Fig. 8 further illustrates the general interactions between G-CSF and VP-16 on CFU-GM numbers in marrow and spleen. It is clear that the behavior of CFU-GM is primarily regulated by G-CSF. With varying VP-16 doses, CFU-GM numbers change only slightly.

DISCUSSION

In this study, we wanted to investigate the *in vivo* interactions between hemopoietic growth factors and cytostatic drugs. We demonstrate that the anemia and neutropenia induced by a prolonged VP-16 treatment of mice could be prevented by concomitant administration of EPO or G-CSF. This prophylactic use of growth factors did not result in increased myelotoxicity on immature progenitors and allowed dose escalation. VP-16 inhibited erythropoiesis between the CFU-E and the reticulocytes; CFU-E were unaffected, but reticulocytes were absent. Simultaneous EPO administration could normalize the production of reticulocytes from CFU-E at several VP-16 doses. Granulopoiesis was also affected by VP-16, which became apparent in a severely reduced absolute neutrophil count. This was not due, however, to reduced CFU-GM numbers, which remained unaffected. It appears that VP-16 at the doses used inhibits granulopoiesis at some stage between the CFU-GM and the mature granulocytes. Simultaneous G-CSF administration could restore this defective production and was able to prevent neutropenia. Granulopoiesis appeared to be more sensitive to VP-16 than erythropoiesis because, at the lowest VP-16 dose, maximal inhibitory effects were already observed. No effects on platelet count were detected (data not shown).

There are several possibilities which may explain the protection from VP-16-induced hemotoxicity by EPO and G-CSF. The first option takes into account only populations of cells. A certain dose of VP-16 may eliminate a fixed percentage of a specific hemopoietic compartment. If the surviving cells are stimulated by simultaneous EPO or G-CSF therapy, this will increase their amplification and may compensate for the loss of cells induced by VP-16. This implies that the sensitivity for VP-16 of the surviving fraction of cells is not or not strongly increased. In this respect, it is important to consider the *in vivo* effect of EPO and G-CSF on their target cells. It is unknown to what extent apoptosis regulates *in vivo* blood cell production (15, 16). If EPO and G-CSF would act primarily by preventing premature cell death, saving these cells by exogenous growth factor administration will rapidly increase their number without necessarily affecting their cycling activity, *i.e.*, their sensitivity for a cell cycle-specific drug. Another mechanism of growth factor activity, however, could be that *in vivo* they act by increasing the cycling activity of (dormant) cells (17, 18). This would increase the sensitivity of target cells for cell cycle-specific drugs but not necessarily for less S-phase-specific drugs, like VP-16, which can induce apoptosis in quiescent thymocytes (19).

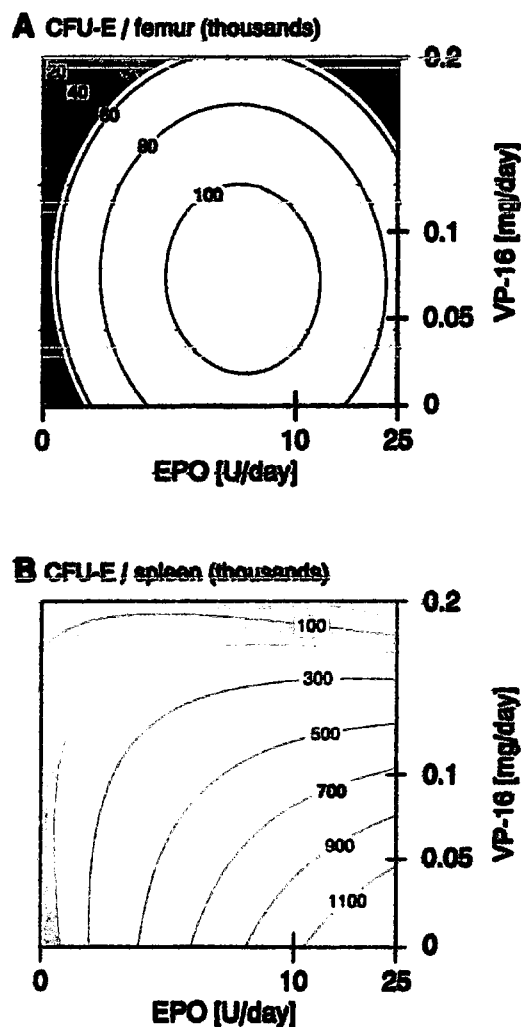


Fig. 4. Contour plot, resulting from the regression analysis of the data of Fig. 3, showing equal-response curves of the marrow (A) and spleen (B) CFU-E during combined administration of EPO and VP-16. Shaded area indicates dose ranges at which normal values (± 1 SEM) can be maintained.