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ORIGINAL ARTICLE

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A phase I study of the GM-CSF antagonist E21R

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Abstract *Purpose:* E21R is a competitive inhibitor of GM-CSF. This is the initial clinical study to investigate the safety, toxicity and pharmacokinetics of escalating doses of E21R. *Patients and methods:* Cohorts of three patients received doses of 10, 30, 100, 300, 600 and 1000 μg/kg per day given subcutaneously daily for 10 days. Eligible patients had solid tumours known to express GM-CSF receptors (breast, prostate, colon and lung cancer, and melanoma). No bone marrow involvement or concomitant steroids were permitted. A total of 22 patients received doses ranging from 10 to 1000 μg/kg per day. There were 18 males and 4 females with a median age of 60 years (range 33 to 81 years). Eight patients had an ECOG performance status of 0, seven a performance status of 1, and seven a performance status of 2. There were ten patients with colon cancer, four with prostate cancer, three with lung cancer, three with melanoma and two with breast cancer. *Results:* E21R was in general well tolerated and the maximum tolerated dose was not reached. The most severe toxicities were WHO grade 3 injection site erythema in one patient and grade 2 in two patients, grade 2 lethargy in three patients and grade 2 muscle aches and soreness, grade 2 joint pains and grade 2 thirst in one patient each. The primary pharmacokinetic parameters were dose-independent. Dose-dependent transient eosinophilia was noted from day 3. A fall in PSA levels was recorded in two patients with prostate cancer during their initial cycles of E21R, but they subsequently rose again. Serum from patients treated at 600 and 1000 μg/kg per day antagonized GM-CSF-mediated TF-1 cell proliferation in vitro. *Conclusion:* E21R can be safely given at doses up to 1000 μg/kg per day.

Keywords E21R · GM-CSF · Phase I · Pharmacokinetics · Prostate

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Introduction

Granulocyte macrophage colony-stimulating factor (GM-CSF) is produced by activated T cells, macrophages and endothelial cells. It stimulates both the production of haematopoietic cell lineages and the effector function of mature myeloid cells [9, 10]. It is involved in the pathogenesis of leukaemia and chronic inflammation. For example, in juvenile myelomonocytic leukaemia (JMML), the leukaemic cells produce GM-CSF and are dependent on it for their growth. GM-CSF binds to a high-affinity receptor that has a specific alpha chain, but a beta chain that is shared with receptors for interleukin-3 (IL-3) and IL-5.

E21R is a GM-CSF antagonist in which amino acid 21 has been changed from glutamic acid to arginine [9]. It is produced in *Escherichia coli* and therefore is non-glycosylated. E21R has no demonstrated in vitro agonist activity and antagonizes the receptor binding

and biological activity of GM-CSF. Cells responsive to GM-CSF express the alpha and beta chains of the GM-CSF receptor and bind GM-CSF with high affinity. In contrast, E21R, which is specifically defective in binding the beta chain, binds with 100-fold lower affinity, equivalent to binding the GM-CSF receptor alpha chain alone. E21R antagonizes GM-CSF function by selectively binding to the GM-CSF receptor alpha chain, but also directly induces apoptosis in cells carrying the GM-CSF receptor [12]. The low-affinity binding of E21R and the fact that GM-CSF is able to elicit maximum biological activity with only 10% receptor occupancy explains why at least a 1000-fold excess of E21R is required to block GM-CSF function [9].

In vitro, E21R inhibits both the spontaneous proliferation of leukaemic cells from patients with JMML and the GM-CSF-stimulated proliferation of cells from patients with acute and chronic myeloid leukaemia (AML, CML) [9, 13]. E21R also induces apoptosis of cells from patients with AML, CML and JMML depending on its relative concentration compared to GM-CSF and the absence of IL-3 receptors, which are present on normal myeloid cells, but not on the majority of leukaemic cells [12, 13]. In irradiated SCID/NOD mice, E21R both inhibits the engraftment of human JMML cells and causes regression in engrafted mice [14].

Because GM-CSF is highly species-specific, preclinical toxicology studies were performed in baboons. In baboons treated with doses up to 1 mg/kg per day subcutaneously for 21 days, no serious toxicity was recorded. There was an early reversible neutrophilia and eosinophilia. Biochemistry showed a drop in cholesterol and phosphate and a rise in chloride. Histologically there was an infiltrate of lymph nodes with neutrophils and eosinophils and eosinophilic infiltrates in the duodenal submucosa. Serum concentrations were proportional to dose and the terminal half-life over multiple subcutaneous injections was 1.5–1.75 days. Antibodies to E21R were detected on day 11 but neutralizing antibodies were only seen in one of six baboons.

The aim of this phase I study was to investigate the safety, toxicity and pharmacokinetics of escalating doses of E21R in patients with solid tumours.

Patients and methods

Eligible patients

Patients were required to have histologically or cytologically proven breast cancer, non-small-cell and small-cell lung cancer, prostate cancer, colorectal cancer or melanoma, all of which are solid tumours known to express GM-CSF receptors. Patients were required to have received conventional therapy and have relapsed or progressed. At least 4 weeks was required to have elapsed between the prior chemotherapy or radiotherapy and study entry and patients must have recovered from all of the reversible toxicities. No more than 25% of the bone marrow could have been irradiated. Patients were required to have a WHO performance status of 0, 1 or 2 and adequate haematological, liver and kidney function, haemoglobin 100 g/l, neutrophils 2.0×10^9/l, platelets 100×10^9/l, bilirubin less than 1.5 times the upper limit of normal, ALT less than 2.5 times the upper limit of normal, and calculated creatinine clearance >60 ml/min. Patients were excluded if they had bone marrow involvement by the tumour, a serious concomitant medical illness, a prior reaction to GM-CSF or were receiving corticosteroids. Patients needed to be geographically available for follow-up. The study was approved by the Royal Adelaide Hospital Ethics Committee and all patients gave written informed consent.

Treatment plan

Cohorts of three patients received escalating doses of E21R according to the following schedule: 10, 30, 100, 300, 600, 1000 μg/kg injected subcutaneously daily for 10 days. The initial lower three doses were given as a bolus, but with the higher dose levels in the last three dose cohorts a portable infusion pump was used. Dose-limiting toxicity was defined as any grade 3 or 4 non-haematological toxicity, or any toxicity necessitating discontinuation of therapy. If a patient experienced a dose-limiting toxicity at any level at least one further patient was enrolled at that level and if a second patient had a grade 3 or 4 toxicity that defined the maximal tolerated dose (MTD). At the dose recommended for phase II trials a total of six patients were entered. With small patient cohorts the analysis was planned to be descriptive.

Pharmacokinetic sampling

Blood samples to measure the concentrations of E21R, surfactant protein A (SP-A), surfactant protein B (SP-B) and to detect the presence of anti-E21R antibodies were taken immediately prior to dosing on days 1, 3, 7, 10 through 20, and 28. At the completion of the first and tenth injections, additional samples were taken at 1, 3, 6, and 12 h after injection, and further samples were taken at 24-h intervals for 10–12 days after the last dose. The blood samples (10 ml) were allowed to clot at room temperature for 2 h before being centrifuged for 10 min and the serum removed for storage at –70°C.

Serum concentrations of E21R were determined using a quantitative human GM-CSF ELISA kit from R&D Systems (Minneapolis, Minn.). Following the manufacturer's protocol and using the recombinant human GM-CSF standard provided with the kit, we were able to demonstrate full recognition of the GM-CSF E21R analogue (data not shown). Absorbance was measured at 450 nm with a 570-nm reference using a BioRad 3550 microplate reader (BioRad, Hercules, Calif.). The Microplate Manager program (BioRad) was used to generate a standard curve and to compute the concentration of E21R in each sample. All samples were assayed in triplicate, either neat or diluted to within the calibration range of the assay (7.8–500 pg/ml) using the serum diluent provided. The lower limit of quantification of the assay was 7.8 pg/ml.

For each patient, the equation describing a two-compartment open model with first-order absorption was fitted to the entire serum E21R concentration-time profile from the first dose on day 1 to the last quantifiable serum concentration, in some cases up to day 28, using the non-linear least squares regression program Kinetica 2000 (InnaPhase Corporation, Philadelphia, Pa.). All data were weighted according to $1/(Y^2$ observed) and preliminary analysis showed that neither a one- nor a three-compartment open model improved the fit to the data. A more detailed pharmacokinetic analysis is the subject of a further report. Individual values for the pharmacokinetic parameters total systemic clearance (CL), volume of distribution at steady-state (V_{ss}) and terminal half-life ($t_{1/2}$) were determined from the coefficients and exponents of the equation. Maximum concentration (C_{max}) and minimum concentration between doses (C_{min}) were determined by direct observation of the data. The area under the concentration-time curve at steady-state was calculated using the linear trapezoidal method during the last inter-dosing interval ($AUC^{ss}{}_{\tau}$). The average serum concentration (C_{av}) was calculated as $AUC^{ss}{}_{\tau}$ divided by the dosing interval (24 h). Dose-dependency of the pharmacokinetic parameters was investigated using linear regression analysis (Prism v 3.02; Graph-

Pad Software, San Diego, Calif.). All data are shown as means ± SD.

Measurement of E21R biological activity

The presence of biologically active E21R in serum samples was determined using a GM-CSF-dependent erythroleukaemia cell line, TF-1, as previously described [1]. Briefly, TF-1 cells (1×10^4/well) were incubated with growth factors and/or serum samples for 48 h before being pulsed with [^{3}H]thymidine (ICN Biomedicals, Seven Hills, NSW, Australia) at 1 μCi/well for 5 h. The cells were harvested and cell-associated radioactivity was determined in a Packard TriCarb liquid scintillation counter. Recombinant human GM-CSF and IL-3 were produced in *E. coli* as previously described [1, 9].

Detection of anti-E21R antibodies

The level of antibodies towards E21R in patient serum was determined using a sandwich ELISA. Plates were coated with E21R at 20 ng/ml in coating buffer (81 m*M* Na_2CO_3, 205 m*M* $NaHCO_3$, pH 9.5) at 37°C for 2 h. Non-specific binding sites were blocked using 1% (w/v) bovine serum albumin (BSA) in coating buffer at 37°C for 90 min. Plates were washed using phosphate-buffered saline (PBS) containing 0.05% (v/v) Tween 20 (PBST). Serum samples were initially diluted 1/20 into 1% (w/v) BSA in PBS (BSA/PBS) then twofold serially diluted in the E21R-coated plates containing BSA/PBS to a final dilution of 1/5120. Plates were incubated for 90 min then washed three times in PBST. Captured human antibodies were detected using biotin-labelled goat anti-human IgG or IgM, or biotin-labelled mouse monoclonal antibodies specific for human IgG_1, IgG_2, IgG_3 or IgG_4, from Zymed Laboratories (San Francisco, Calif.) diluted in PBST. Plates were incubated for 90 min then washed three times in PBST. Biotin-labelled antibodies were detected using streptavidin-biotinylated horseradish peroxidase complex from Amersham Pharmacia Biotech (Castle Hill, NSW, Australia) diluted in PBST. Plates were incubated for 90 min then washed three times in PBST. The assay was developed using a FAST OPD peroxidase substrate set from Sigma-Aldrich (Castle Hill, NSW, Australia) and the absorbance measured at 490 nm using a BioRad 3550 microplate reader (BioRad, Hercules, Calif.) after the reaction had been stopped by the addition of H_2SO_4 to 0.6 *M*. Antibody responses for each subject were graded according to the maximum increase in the dilution factor of any serum sample, relative to the pretreatment serum sample, required to generate a nominated optical density, usually 1.5. Subjects were graded (–) when no increase in any sample was observed, (+/–) when the maximum increase was up to fourfold and (+) when the maximum increase in the sample dilution factor was greater than fourfold.

Measurement of surfactant protein-A and -B in serum

SP-A and SP-B were measured by ELISA inhibition assays using antibodies raised against alveolar proteinosis-derived SP-A and mature SP-B, as previously described [4]. Briefly, in order to free the SP-A and SP-B from any associated plasma or surfactant components, aliquots were first treated with EDTA, SDS, and Triton X-100. Serial dilutions of the samples in PBST containing 0.25% BSA (w/v) were incubated in an ELISA plate with aliquots of the respective antibody. Free antibody was captured using a second ELISA plate coated with purified SP-A or SP-B (1 ng/ml), and the amount quantified using alkaline phosphatase-conjugated IgG against rabbit immunoglobulins and 15 m*M* disodium *p*-nitrophenyl phosphate in 1.0 *M* diethanolamine and 0.5 m*M* $MgCl_2$ as a substrate. After 1 h the absorbance of the substrate was measured at 405 nm using a Dynatech MR5000 reader (Dynatech Laboratories, Chantilly, Va.). The AssayZap program (Biosoft, Ferguson, Mo.) was used to generate a standard curve and to compute the concentration of SP-B in each sample. All samples were assayed in duplicate at four serial dilutions. Standards, assayed in quadruplicate, were included in each ELISA plate at eight serial dilutions (ranging from 7.8 to 1000 ng/ml, $r > 0.99$). The antibodies used do not react with any other known antigens and the assays have a coefficient of variation of about 6%.

Assessment of efficacy and toxicity

A history, physical examination and toxicity evaluation were performed on days 1, 3, 7, 10, 15 and 28. Blood tests for CBE and differential, electrolytes, renal function, liver function, calcium, phosphate and cholesterol were performed on days 1, 3, 7, 10, 15, 21 and 28 with bone marrow biopsies on days 1, 10 and 28. Scans and radiographs were performed pretreatment and on day 28 to evaluate the response to treatment.

Results

Clinical and pharmacokinetic

Entered onto this study were 22 patients, 18 males and 4 females with a median age of 60 years. Most patients had been heavily pretreated (Table 1). The E21R was well tolerated at all dose levels. The major toxicity was WHO grade 3 injection site erythema in one patient and grade 2 in two patients. A biopsy of one of these sites simply showed an inflammatory infiltrate of lymphocytes, eosinophils and polymorphs with dermal oedema consistent with a drug reaction. The most severe reaction was in a patient who had a history of reaction to a number of drugs. All skin reactions disappeared spontaneously within days. Grade 2 lethargy was recorded in three patients and there was one patient with each of grade two muscle aches and soreness, grade 2 joint pains and grade 2 thirst (Table 2).

There were no statistically significant changes recorded in peripheral blood neutrophils or monocyte counts, but a dose-dependent transient eosinophilia was noted on day 3 (Fig. 1). There was a non-significant trend towards increased neutrophil counts with increasing E21R dose. Bone marrow biopsies revealed no changes in cellularity, maturation or myeloid progenitor cell content. No significant biochemical changes were recorded, but there was a minor lowering of serum cholesterol by day 10.

Of the 22 patients, 10 recorded stable disease on repeat imaging at the completion of a cycle of treatment. Two patients with prostate cancer, one at the initial and the other at the final dose level, showed falls in their PSA measurements and received a second cycle of treatment. The first patient's PSA fell from a baseline of 610 to 150 μg/l by day 10 then rose to 370 μg/l by day 28 and continued to rise through cycle 2. The second patient showed a drop from 140 to 98 μg/l by day 15, returning to 100 μg/l by day 28 and continuing to rise through a subsequent cycle of E21R.

Table 1. A phase I study of GM-CSF antagonist (E21R) demographics (*SD* stable disease, *PD* progressive disease)

Trial no.	Primary site	Dose (μg/kg)	ECOG performance status	Age (years)	Gender	Best response	No. of prior regimens		Survival from day 1 (months)
							Chemotherapy	Radiotherapy	
1	Breast	10	2	56	Female	SD	5	10	6
2	Colon	10	2	60	Female	SD	2		4
3	Prostate	10	0	63	Male	SD			4
4	Colon	30	2	48	Male	PD	4		2
5	Lung	30	1	55	Male	PD	1	5	11
6	Colon	30	1	65	Male	SD	2		7
7	Colon	100	0	61	Male	PD	2		17
8	Melanoma	100	1	76	Male	PD	2		4
9	Colon	100	1	55	Male	PD	2	2	3
10	Colon	300	1	40	Male	PD	2		7
11	Prostate	300	0	73	Male	SD			29+
12	Lung	300	0	72	Female	SD		1	29+
13	Prostate	600	2	72	Male	PD	1	1	1
14	Colon	600	0	60	Male	SD	2		11
15	Melanoma	600	0	72	Male	SD	1		27+
16	Colon	600	1	57	Male	SD	2	1	8
17	Lung	1000	2	81	Male	PD		5	5
18	Rectum	1000	1	55	Male	PD	2	1	2
19	Melanoma	1000	0	33	Male	PD	1		23+
20	Colon	1000	2	59	Male	PD	2		2
21	Prostate	1000	0	58	Male	SD			11
22	Breast	1000	2	58	Female	PD	3	1	1

Table 2. Adverse events listed as related to therapy

Trial no.	Dose level (μg/kg)	Adverse event	Worst toxicity grade
1	10	Lethargy	2
3	10	Lethargy	2
3	10	Muscle aches and soreness	2
8	100	Injection site erythema	2
8	100	Lethargy	2
10	300	Thirsty	2
10	300	Injection site erythema and induration	2
15	600	Injection site erythema and induration	3
19	1000	Injection site erythema and induration	2
19	1000	Joint pains	2
21	1000	Injection site erythema	2
22	1000	Injection site erythema and induration	2

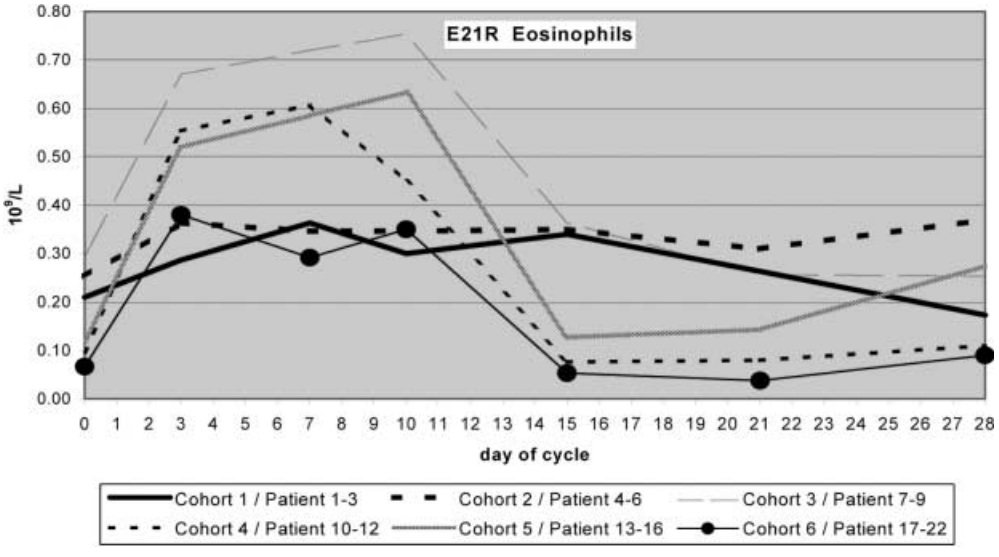


Fig. 1. Average eosinophil count per cohort after E21R treatment. The eosinophil count is averaged over the patients within each cohort over the days following the initial dose in each cohort

Table 3 summarizes the results of the pharmacokinetic analysis. For four patients in the two low-dose cohorts (01, 03, 05 and 06) estimates of terminal half-life are not reported, as concentrations of E21R were below the limit of quantitation during the apparent terminal phase. This resulted in an underestimate of the true terminal half-life in these four patients (23.3 ± 1.3 h) compared to the remaining patients (69.9 ± 24.0 h). There was considerable interpatient variability in clearance (3.5-fold), volume of distribution (11-fold) and half-life (3-fold), although no significant relationship between these parameters and E21R dose was observed ($r^2 < 0.05$, $P > 0.4$). In contrast, the pharmacokinetic analysis revealed a strong and significant relationship between E21R dose and AUC^{ss}_{τ} ($r^2 = 0.74$, $P < 0.0.0001$), C_{max} ($r^2 > 0.67$, $P < 0.0001$), C_{min} ($r^2 > 0.55$, $P = 0.0001$) and C_{av} ($r^2 > 0.74$, $P < 0.0001$). The C_{max} for E21R ranged from 15.8 ± 5.4 ng/ml in cohort 1 up to 995.2 ± 420 ng/ml in cohort 6. The C_{min} for E21R, measured between doses, ranged from 1.07 ± 0.48 ng/ml in cohort 1 up to 162.7 ± 83.5 ng/ml in cohort 6.

Biological activity of E21R

We tested the ability of serum from subjects in cohorts 5 and 6 to antagonize GM-CSF- and IL-3-mediated proliferation of the growth-factor-dependent erythroleukaemic cell line, TF-1. Data from a representative subject are shown in Fig. 2. No TF-1 proliferation was observed in the presence of 50% human serum alone but the addition of exogenous GM-CSF or IL-3 was able to stimulate TF-1 cell proliferation. The level of GM-CSF- but not IL-3-mediated proliferation was markedly inhibited in serum samples containing high levels of E21R (> 100 ng/ml) consistent with the presence of active GM-CSF antagonism.

E21R was purified from blood collected 3 h after the day-3 injection in five subjects (16, 18, 20, 21, 22). The E21R was affinity-purified from 20 ml serum using the 4D4 anti-GM-CSF monoclonal antibody [10], quantified by ELISA and tested for its ability to antagonize GM-CSF-mediated TF-1 cell proliferation [9]. All E21R samples recovered from serum exhibited full antagonistic activity compared to a reference preparation of E21R (data not shown).

Induction of anti-E21R antibodies

Of the 22 subjects, 7 mounted an observable IgG (6 subjects) or IgM (2 subjects) antibody response towards the injected E21R (Table 4) with a maximum response observed around days 15–20. Responses were for the most part low-titre IgG responses (Table 4). Induction of antibodies appears to be partly correlated with dose as anti-E21R antibodies were observed in 6 of 13 subjects receiving at least 300 μg/kg per day of E21R but in only 1 of 9 subjects receiving < 300 μg/kg per day of E21R. In three of the subjects (20, 21, 22) in whom an IgG response towards E21R was observed, an equivalent response could be detected towards recombinant wild-type GM-CSF, indicating that the antibody specificities were not restricted to the leader peptide of E21R or the Arg^{21} substitution (data not shown). We observed no inhibition of GM-CSF-mediated TF-1 cell proliferation in serum samples containing anti-E21R antibodies from subjects 16, 18, 20, 21 and 22 (Fig. 2). Serum samples containing anti-E21R antibodies from subjects 4, 10, 18, 20, 21 and 22 were unable to compete for

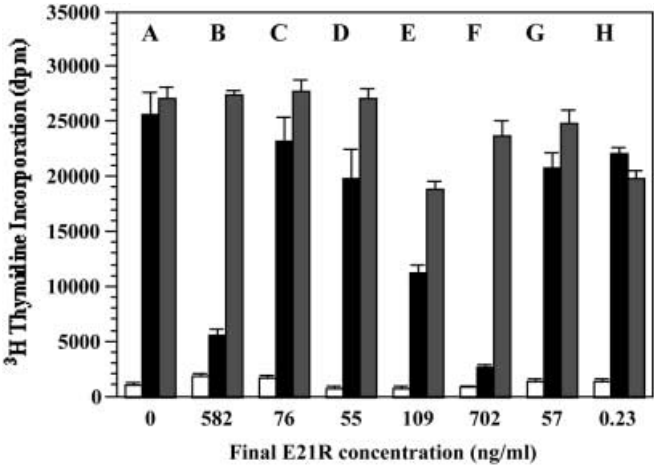


Fig. 2. Proliferation assay. Proliferation of TF-1 cells in the presence of serum from subject 22. TF-1 cells were incubated with 50% (v/v) serum from subject 22 in the presence of no exogenous cytokines (*open bars*), 0.03 ng/ml GM-CSF (*black bars*) or 0.1 ng/ml IL-3 (*grey bars*). Serum samples were collected on day 1 preinjection (*A*), day 1 6 h after injection (*B*), day 3 preinjection (*C*), day 7 preinjection (*D*), day 10 preinjection (*E*), day 10 6 h after injection (*F*), day 11 (*G*), and day 20 (*H*). The final concentration of E21R in each sample is indicated along the *x*-axis. Each value represents the mean of triplicate determinations, and the error bars represent SEM

Table 3. Pharmacokinetics

	Clearance (ml/min)	V_{ss} (l)	Half-life (h)[a]
Mean	147	45.7	69.9
SD	54	30.2	24.0
Range	76–268	13.7–149.3	37.8–112.9

[a]Patients 01, 03, 05 and 06 not included

Table 4. Summary of anti-E21R antibody responses in 22 subjects injected with E21R. Three subjects (3, 16 and 21) received a second cycle of treatment. The second cycle induced no anti-E21R antibodies in subjects 3 and 16 but did slightly enhance the anti-E21R antibody response of subject 21. The IgG response in subject 4 was shown to be primarily of the IgG_1 subclass while in subjects 20 and 21 the IgG response was of both IgG_1 and IgG_3 subclasses

Subject	*n*	IgG	IgM
1, 2, 3, 5, 6, 7, 8, 9, 11, 12, 13, 14, 15, 17, 19	15	–	–
4, 10, 16, 22	4	+/–	–
18	1	–	+/–
20	1	+	–
21	1	+	+/–

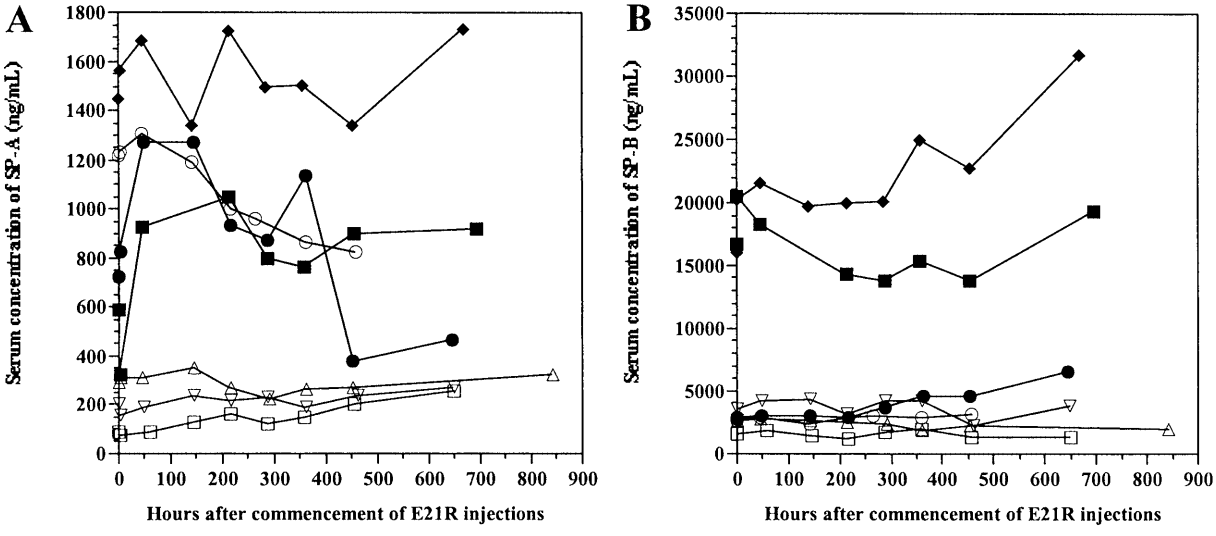
radiolabelled GM-CSF binding to the GM-CSF receptor expressed on transfected CHO cells (data not shown). Despite the presence of antibody responses towards the injected E21R, these results provide no evidence that the antibodies are able to neutralize the biological activity of GM-CSF or presumably E21R.

Surfactant proteins in the presence of E21R

Inhibition of endogenous GM-CSF activity is associated with pulmonary alveolar proteinosis due to defective surfactant clearance by alveolar macrophages [16, 20] and increased serum levels of SP-A and SP-B [5, 6]. We determined the serum levels of SP-A and SP-B to determine whether E21R treatment might be associated with impaired alveolar macrophage function.

Most subjects (18 of 22) showed no change in SP-A or SP-B levels during or after E21R treatment, although for some subjects the levels did fluctuate markedly (Fig. 3). Four subjects did show changes in SP-A or SP-B levels that may have been related to E21R treatment. Subject 18 had elevated SP-A levels prior to E21R treatment that declined slightly after E21R treatment commenced. The SP-A levels in subject 13 rose markedly during E21R treatment and had returned to pretreatment by day 16, while the SP-B levels started to increase after E21R treatment ceased (Fig. 3). Three of the four subjects in this study (subjects 5, 12 and 17) who had changes in SP-A or SP-B had a primary tumour in the lung. We observed that two of these patients had markedly elevated SP-A and SP-B levels (subjects 12 and 17) with modest increases in SP-A (subject 12) or SP-B (subject 17) after E21R treatment commenced (Fig. 3).

Fig. 3A, B. Serum SP-A (**A**) and SP-B (**B**) levels from seven representative subjects 2 (□), 5 (▽), 12 (■), 13 (●), 17 (◆), 18 (○) and 19 (△). Each data point is the mean of duplicate measurements



Discussion

In general, E21R was well tolerated at all dose levels tested in this study. We can conclude that the MTD for E21R administered for 10 days by the subcutaneous route is at least 1000 μg/kg per day, and probably higher. Injection site reactions of mild to moderate severity were relatively common and probably dose related, but were transient and did not prevent repeat dosing. Lethargy was recorded as an adverse event in four patients, but lethargy is a common feature of advanced cancer, and the event was attributed to the study drug in only one case.

Pharmacokinetic analysis demonstrated that the primary pharmacokinetic parameters (clearance, volume of distribution at steady-state and half-life) of E21R, which determine the steady-state concentration, time to reach steady-state, and degree of fluctuation during the interdosing interval were dose-independent.

Since E21R has been shown in vitro to induce apoptosis of normal bone marrow $CD34^+$ and $CD38^+$ cells, which express GM-CSF receptors, its administration could been expected to have an adverse effect on haematopoiesis [13]. In the event, serial blood counts, bone marrow examinations and progenitor assays showed no changes.

E21R has no GM-CSF agonist activity and it also induces apoptosis of eosinophils, and so the eosinophilia on day 3 requires explanation [9, 13, 14, 15]. One possibility is that E21R induces eosinophil egress from the bone marrow and/or peripheral demargination. There could be a novel GM-CSF/E21R function possibly the result of signalling through the GM-CSF receptor alpha chain as has been shown in certain non-haematopoietic cell lines [3].

Previous studies have demonstrated the induction of anti-GM-CSF antibodies in patients receiving recombinant human GM-CSF. In one trial, 4 of 16 patients with Hodgkin's disease receiving GM-CSF at 100–400 μg/m^2 per day for up to 10 days developed anti-GM-CSF

antibodies [8]. In a second study, patients with metastatic colorectal carcinoma received subcutaneous GM-CSF at 250 μg/m^2 per day for 10 days with the treatment cycle repeated every 4 weeks for four cycles [17]. No anti-GM-CSF antibodies were detected following the first treatment cycle, but after two cycles of treatment, 50% of patients had developed anti-GM-CSF antibodies and after four cycles of treatment, 95% of patients had developed anti-GM-CSF antibodies. The presence of anti-GM-CSF antibodies was correlated with altered GM-CSF pharmacokinetics and a reduction in the biological response to GM-CSF. In the present study, we observed mostly low-titre non-neutralizing, anti-E21R antibodies in 7 of 22 subjects after a single 10-day treatment cycle (Table 4). Only three subjects in the present study received a second treatment cycle with no dramatic impact upon the anti-E21R antibody response (Table 4). If multiple cycles of E21R treatment are utilized in future studies, it will be important to monitor the development of anti-E21R antibodies and assess their impact upon E21R pharmacokinetics and function.

Autoantibody inhibition of endogenous GM-CSF activity in patients with idiopathic alveolar proteinosis (AP) is associated with a decrease in the ability of alveolar macrophages to clear surfactant and results in excess surfactant accumulation in the airspaces [16, 20]. In a recent study, 5 of 14 patients with idiopathic AP showed a sustained improvement in pulmonary function following treatment with 5 μg/kg per day recombinant human GM-CSF for 6–12 weeks [18]. Patients with idiopathic AP have greatly increased circulating levels of SP-A and SP-B in a manner that reflects disease severity, but no change in plasma levels of SP-A or SP-B are associated with GM-CSF therapy [11, 18]. GM-CSF null mutant mice show a progressive accumulation of surfactant lipids and proteins in the alveolar space of the lung paralleling human pulmonary alveolar proteinosis [7, 19]. We speculated that the presence of a GM-CSF antagonist or the induction of anti-GM-CSF antibodies in subjects receiving E21R might provoke an AP-like response. In the event we did observe some changes in SP-A and SP-B levels (subjects 12, 13, 17 and 18) that appeared to be correlated with E21R treatment, but no clinically apparent pulmonary toxicity was observed in any of the subjects in this study and intermittent pulse oximetry showed no change during E21R treatment. There is still a possibility that this could be a more cumulative effect that will only become apparent with repeated dosing.

Although it was not possible to demonstrate directly its GM-CSF antagonist activity in patients, serum concentrations were achieved at which patients' serum could antagonize GM-CSF-mediated TF-1 cell proliferation (Fig. 2). The PSA responses were observed at the lowest and highest doses. The dose of 1000 μg/kg was identified as a safe dose for phase II trials. A transient haematological and clinical effect of E21R has been reported in a child with end-stage JMML [2]. Initially, other phase II studies will be conducted in CML, AML and rheumatoid arthritis.

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