EXHIBIT 3

Efficacy and immunogenicity of novel erythropoietic agents and conventional rhEPO in rats with renal insufficiency

HC Tillmann[1], B Kuhn[1], B Kränzlin[1], M Sadick[2], J Gross[3], N Gretz[1] and J Pill[4]

[1]Medical Research Center, University of Heidelberg, Klinikum, Mannheim, Germany; [2]Department of Radiology, University of Heidelberg, Klinikum, Mannheim, Germany; [3]ENT Clinics, Charité, Berlin, Germany and [4]Roche Diagnostics GmbH, Mannheim, Germany

Recombinant human erythropoietin (rhEPO) is used to treat anemia in chronic renal insufficiency. Erythropoietin (EPO) immunogenicity can lead to EPO-resistant anemia. Conjugating proteins with polyethylene glycol (PEG) can prolong elimination half-life and diminish protein immunogenicity. We investigated the efficacy of new erythropoietic agents, synthesized by single (Ro 50-3821) and multiple (MIX) integrations of PEG and succinimidyl butanoic acid with rhEPO, in rats with chronic renal insufficiency. Sprague–Dawley rats with surgically induced renal insufficiency received Ro 50-3821 or MIX subcutaneously (s.c.) over 4–12 weeks compared to rhEPO and NaCl. Hemoglobin and antibody levels served as primary efficacy and safety variables. Dosing intervals and dose–response characteristics were investigated. Ro 50-3821 (2.5 μg/kg once weekly) increased hemoglobin levels by 7 g/dl after 4 weeks compared to 1 g/dl in NaCl controls ($P<0.05$). MIX (2.5 μg/kg once weekly) and rhEPO (0.25 μg/kg three times weekly) increased hemoglobin levels by 3 g/dl. Ro 50-3821 administered for 12 weeks (0.75 μg/kg once weekly) increased hemoglobin levels (from 13 to 19 g/dl) more effectively than rhEPO (0.75 μg/kg once weekly, decline from 13 to 11 g/dl, $P<0.05$). No antibodies against Ro 50-3821 were detected after 12 weeks of treatment. Antibodies against rhEPO were seen in 69% of animals ($P<0.00001$). Ro 50-3821 increased hemoglobin levels with once weekly s.c. dosing. Multiple pegylated EPO is less effective. In rats, rhEPO failed to increase hemoglobin levels with once weekly long-term dosing. Antibody formation following rhEPO may explain this finding. Therefore, Ro 50-3821 may provide important clinical advantages compared to unpegylated EPO. It can be administered in longer dosing intervals and has a lower risk of unfavorable immunological responses.

Kidney International (2006) **69,** 60–67. doi:10.1038/sj.ki.5000006

KEYWORDS: renal insufficiency; erythropoietin; Ro 50-3821; long-acting erythropoietic agents

Correspondence: *J Pill, Roche Diagnostics GmbH, Sandhofer Strasse 116, 68305 Mannheim, Germany. E-mail: johannes.pill@roche.com*

Received 20 January 2005; revised 24 June 2005; accepted 21 July 2005

Renal anemia is a common complication in patients with chronic renal failure (CRF).[1,2] There is a direct relationship between the decline in renal function and the severity of the anemia.[3] This anemia significantly contributes to morbidity-causing symptoms such as lack of energy, breathlessness, dizziness, angina, poor appetite, and decreased exercise tolerance.[4,5] The decreased production of erythropoietin (EPO), a hormone mainly produced by the kidney, has been identified as the main cause of anemia in CRF.[6] Up to the late 1980s, iron and folate were the main treatments for anemia in these patients. If these treatments failed, patients were dependent on blood transfusions, with their associated risks of transmission of infectious diseases.

In 1985, the cloning of the human gene for EPO was achieved by Lin *et al.*[7] Production of recombinant human EPO (rhEPO) followed and, soon after, the efficacy of rhEPO treatment in dialysis patients was first demonstrated in clinical trials.[8,9] Since then, rhEPO has become an integral part in the treatment of patients with CRF. It has been shown to correct anemia to acceptable levels, with improvements in the quality of life of patients and a reduced risk for comorbidity.[10–13] In patients not yet on dialysis, it has been proposed that EPO may slow progression of renal disease, but this is still a matter of debate.[14] In a relatively limited number of predialysis patients with nonsevere anemia, early initiation of EPO significantly slowed the progression of renal disease and delayed the initiation of renal replacement therapy.[15]

However, in approximately 5–10% of patients, therapy with rhEPO does not lead to an effective stimulation of erythropoiesis.[16] The reasons are diverse[17] and include absolute or functional iron deficiency,[18] as well as a lack of compliance if EPO has to be injected at least three times per week in patients with CRF.[19] Antibody production against EPO, resulting in EPO-resistant anemia,[20–22] may play a role in a small number of patients. Less frequent dosing schemes have been a therapeutic goal in anemia-correcting therapy in patients with CRF.[23] This may be achieved by increasing the half-life of the used therapeutic agent.[24,25] Altering the dosing schemes has been employed successfully in patients with cancer or chronic hepatitis C treated with pegylated

interferon-alpha-2b, which may present a way of improving therapeutic efficacy.[26] In patients with chronic renal dysfunction, pegylated interferon-alpha-2b can also be administered safely and with a favorable pharmacokinetic profile.[27] In addition, pegylated proteins may reduce the immunogenicity of the parent protein.[28,29] So far no *in vivo* characterization of pegylated EPO pharmacodynamics is available. We therefore investigated the therapeutic efficacy, possible dosing schemes, and the immunogenicity of different erythropoietic agents synthesized by integration of polyethylene glycol (PEG) in comparison to conventional rhEPO in Sprague–Dawley rats with CRF.

RESULTS

Efficacy (protocol 1)

Ro 50-3821 and MIX in doses of 2.5 μg/kg body weight (bw) administered once per week as well as conventional recombinant rhEPO (0.25 μg/kg bw administered three times per week) increased mean hemoglobin levels after 4 weeks of treatment. The largest effect was seen in rats receiving Ro 50-3821 2.5 μg/kg bw, in which hemoglobin levels rose from 13.4 ± 1.4 to 19.5 ± 1.8 g/dl ($P < 0.05$ in comparison to NaCl control). Hemoglobin levels following conventional rhEPO therapy remained significantly lower than after Ro 50-3821 and MIX in doses of 2.5 μg/kg bw ($P < 0.05$ for both comparisons). After 4 weeks of isotonic saline, Ro 50-3821, or MIX in doses of 0.25 μg/kg bw, hemoglobin levels were unchanged (Figure 1).

Red blood count (RBC) and hematocrit measurements correlated strongly with changes in hemoglobin levels. Reticulocyte counts were not influenced by low-dose Ro 50-3821, MIX, or saline. rhEPO increased reticulocyte counts over the first week of treatment, but reticulocyte counts receded to baseline levels after 4 weeks of treatment. In contrast, Ro 50-3821- and MIX- (2.5 μg/kg bw once weekly) treated animals continued to show increased reticulocyte counts after 4 weeks of treatment. These changes were statistically significant compared to NaCl controls as well as rhEPO therapy (Figure 2).

Blood pressure levels were alike in all treatment groups. None of the drugs in the administered doses significantly increased systolic blood pressure. Similarly, no significant differences in white blood count, serum electrolytes, renal function parameters, and liver function tests were seen after treatment with either of the study drugs.

Dosing scheme (protocol 2)

rhEPO and Ro 50-3821 in doses of 0.75 μg/kg bw administered once weekly, as well as Ro 50-3821 at a dose of 0.25 μg/kg bw, were associated with a moderate increase in hemoglobin levels compared to saline controls (Table 1). These changes, however, were not significant. Only conventional rhEPO administered three times per week yielded a substantial increase in hemoglobin levels from 12.9 ± 1.1 g/dl at baseline to 17.0 ± 1.3 g/dl ($P < 0.05$ compared to saline and all other treatment groups).

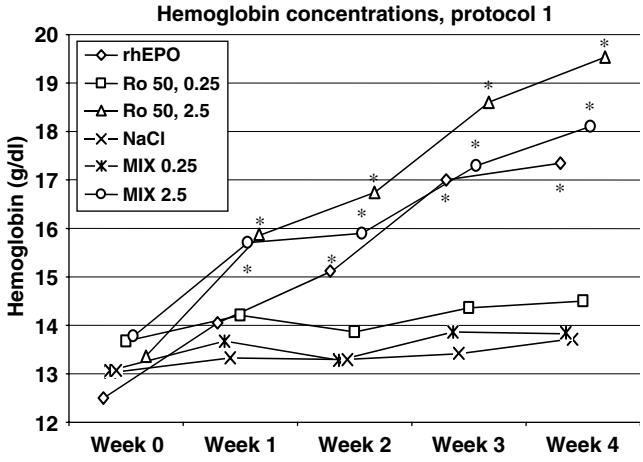

Figure 1 | Mean hemoglobin levels after respective weeks of treatment. All doses are expressed as μg/kg bw. Pegylated EPO derivatives (Ro 50-3821 and MIX) and physiologic saline were administered once weekly. Conventional rhEPO (0.25 μg/kg) was administered three times per week. For the number of animals in each dosing group, refer to Table 3. *$P < 0.05$ compared to saline control.

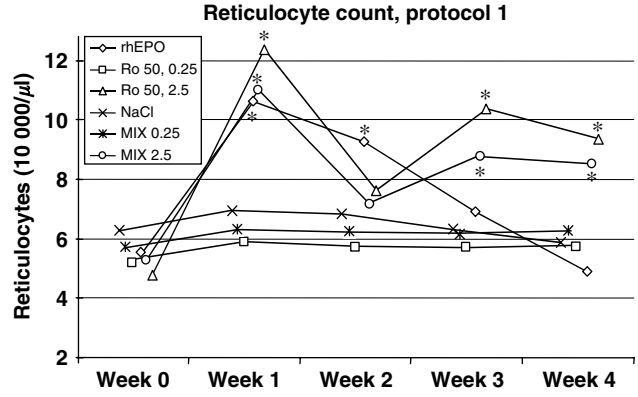

Figure 2 | Mean reticulocyte counts after respective weeks of treatment. All doses are expressed as μg/kg bw. Pegylated EPO derivatives (Ro 50-3821 and MIX) and physiologic saline were administered once weekly. Conventional rhEPO (0.25 μg/kg) was administered three times per week. For the number of animals in each dosing group, refer to Table 3. *$P < 0.05$ compared to saline control.

Systolic blood pressure levels ranged from 134 to 146 mmHg during protocol 2. No significant differences between treatment groups were noted. Similarly, there were no significant differences in white blood count, serum electrolytes, renal retention parameters, and liver function tests after treatment with either of the study drugs.

Long-term efficacy and safety (protocol 3)

In this protocol investigating the long-term erythropoietic properties of three different Ro 50-3821 doses as well as conventional rhEPO, the two high-dose Ro 50-3821 arms (2.5 and 7.5 μg/kg bw once weekly) were terminated after 8 weeks

Table 1 | Hemoglobin concentrations, protocol 2

Medication	Week	Hemoglobin (g/dl)	Medication	Week	Hemoglobin (g/dl)
rhEPO 0.25	0	12.9±1.1	Ro 50-3821 0.25	0	13.1±1.2
rhEPO 0.25	1	14.9±1.3	Ro 50-3821 0.25	1	14.9±0.9
rhEPO 0.25	2	16.2±1.3	Ro 50-3821 0.25	2	14.5±1.0
rhEPO 0.25	3	16.8±1.6	Ro 50-3821 0.25	3	15.0±1.2
rhEPO 0.25	4	17.0±1.3	Ro 50-3821 0.25	4	15.2±1.3
rhEPO 0.75	0	12.7±0.8	Ro 50-3821 0.75	0	13.6±0.6
rhEPO 0.75	1	14.6±0.8	Ro 50-3821 0.75	1	15.2±0.8
rhEPO 0.75	2	14.7±1.3	Ro 50-3821 0.75	2	14.7±0.9
rhEPO 0.75	3	15.1±1.0	Ro 50-3821 0.75	3	15.1±0.8
rhEPO 0.75	4	15.2±1.0	Ro 50-3821 0.75	4	15.7±0.9
NaCl	0	12.9±1.1			
NaCl	1	14.2±0.5			
NaCl	2	13.8±0.5			
NaCl	3	14.0±0.6			
NaCl	4	13.8±0.7			

Values are presented as mean±s.d. Medication doses are expressed as μg/kg bw. Low doses of rhEPO and Ro 50-3821 were administered three times weekly; physiologic NaCl controls as well as high doses of rhEPO and Ro 50-3821 were administered once weekly.

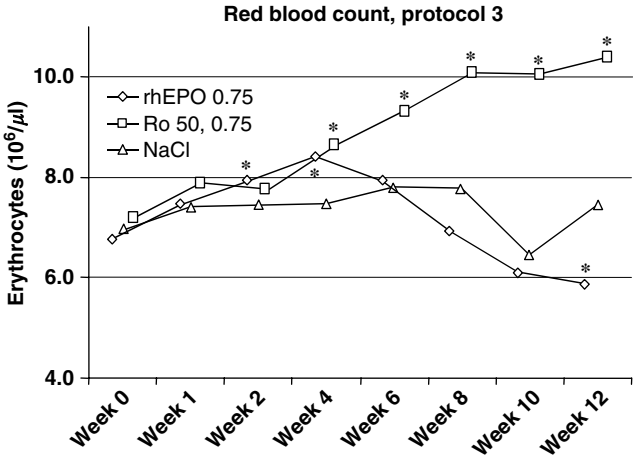

Figure 3 | Mean RBC after respective weeks of treatment. All doses are expressed as μg/kg bw. All medications were administered once weekly. For the number of animals in each dosing group, refer to Table 3. *$P<0.05$ compared to saline control.

instead of the initially planned 12 weeks. This was done due to the steadily increasing hematocrit levels impairing animal well-being. The lower-dose Ro 50-3821 and rhEPO arm (0.75 μg/kg bw once weekly) as well as the NaCl control arm were terminated as originally planned. Inferential statistics were determined only on these study arms. The results of the prematurely terminated study arms are presented with descriptive statistics only.

Erythropoiesis

Ro 50-3821 (0.75 μg/kg bw administered once weekly) steadily increased hemoglobin levels from 13.4±0.5 g/dl prior to study medication to 16.0±1.2 after 4 weeks and 18.7±2.1 g/dl after 12 weeks of treatment ($P<0.05$ compared to saline controls). In contrast, rhEPO (0.75 μg/kg bw administered once weekly) failed to increase hemoglobin levels over the period of 12 weeks. After a temporary increase from 12.9±1.1 g/dl at baseline to 15.9±1.9 g/dl following 4 weeks of treatment, hemoglobin levels dropped to 11.3±4.6 g/dl after 10 weeks and 11.2±3.6 g/dl after 12 weeks of treatment ($P<0.05$ compared to Ro 50-3821 0.75 μg/kg bw).

Similarly to hemoglobin concentrations, the erythrocyte count rose steadily under Ro 50-3821 0.75 μg/kg bw and became significantly higher than in rats receiving saline control solution after 4, 8, 10, and 12 weeks of treatment. Again, rhEPO failed to produce a lasting effect on RBC (Figure 3).

Antibody response

Ro 50-3821 (0.75 μg/kg bw administered once weekly) did not lead to a positive antibody response in any of the 13 animals after 12 weeks of treatment. Within this time frame, rhEPO administered in the same dosing scheme had led to significant antibody production in over 64% of all rats ($P<0.0001$ compared to Ro 50-3821 and NaCl; Table 2). This effect was already detected after 8 weeks of treatment. In a 'worst-case scenario', where animals that died during the study were counted towards the group of antibody-positive animals, 71% of animals showed a positive immunological reaction against rhEPO.

Safety parameters

Systolic blood pressure rose in all treatment groups between weeks 2 and 4 of treatment. An increase of 32 mmHg was measured for the high-dose Ro 50-3821 (7.5 μg/kg bw) group by week 4. rhEPO increased systolic blood pressure by 22 mmHg, whereas the low-dose Ro 50-3821 (0.75 μg/kg bw) as well as the control animals yielded an increase in systolic blood pressure of 17 mmHg by week 4. In all groups treated for 12 weeks, blood pressure levels decreased after week 4 and reached levels comparable to baseline by week 12.

Again, there were no significant differences in white blood count, serum electrolytes, renal function parameters, and liver function tests after treatment with either of the study drugs.

DISCUSSION

Erythropoietic efficacies of Ro 50-3821 and MIX, synthesized by single and multiple linkage of rhEPO with PEG, respectively, were investigated and compared to the efficacies of rhEPO and saline control in the first protocol of this study. Ro 50-3821 was more effective than MIX in stimulating erythropoiesis. A Ro 50-3821 dose of 2.5 μg/kg bw administered once weekly was superior to MIX at the same dose and rhEPO in the recommended dose for treatment of anemia in CRF (0.25 μg/kg bw administered three times per week[30]). The increase following rhEPO is in line with earlier findings in rats and other species.[31,32] Ro 50-3821, the more effective of the agents tested, showed comparable erythropoiesis-stimulating efficacy to rhEPO in the second protocol. Both

Table 2 | Antibody response, protocol 3

	Week 12		Week 10		Week 8	
Dose (μg/kg bw)	Antibodies present	Antibodies not present	Antibodies present	Antibodies not present	Antibodies present	Antibodies not present
Ro 50-3821 0.75	0	13	0	13	0	13
Worst case	0	13	0	13	0	13
rhEPO, 0.75	9*,‡	5	9*,‡	4	9*‡	4
Worst case	9*,‡	5	10*,‡	4	10*,‡	4
NaCl (0.9%)	0	11	0	11	0	11
Worst case	1	11	1	11	1	11
Ro 50-3821 2.5					0	7
Ro 50-3821 7.5					1	8

Medication was administered once weekly. Ro 50-3821 2.5 and Ro 50-3821 7.5 study arms were terminated after 8 weeks. All others continued for 12 weeks. Inferential statistics were calculated only for the latter three study arms. *$P<0.05$, **$P<0.0001$ compared to controls. ‡$P<0.0001$ compared to Ro 50-3821 0.75.
Worst case displays a scenario in which animals that died are added to the category 'antibodies present'.

compounds were administered three times per week in a dose of 0.25 or once weekly in a dose of 0.75 μg/kg bw (Table 1). This may suggest maximal stimulation of erythropoiesis already with the low dose of both drugs under the conditions used. In protocol 3, the long-term arm of this study, Ro 50-3821 was superior to rhEPO in augmenting the parameters of erythropoiesis (hemoglobin levels, RBC, and hematocrit) at a dose of 0.75 μg/kg bw administered once weekly. After 12 weeks of treatment, hemoglobin levels were 18.7 mg/dl following Ro 50-3821 compared to 11.2 mg/dl in rats receiving rhEPO ($P<0.05$). In our rats, rhEPO also failed to increase other parameters of erythropoiesis over this prolonged period of time. A decline and/or time-limited increase in RBC count in dogs and rats has been described following rhEPO.[33] With an extended duration of exposure, neutralizing antibodies have been suggested to blunt the pharmacological action of rhEPO. No other clinical or postmortem observations were made in this study to explain the reduced rhEPO response. Ro 50-3821 was clearly effective in boosting erythropoiesis in rats with chronic renal insufficiency. In dosing schemes with multiple injections per week, rhEPO had shown a beneficial effect on erythropoiesis in chronically renal insufficient rats[31] and humans.[9,10] Therefore, it may be hypothesized that rhEPO may have matched the erythropoiesis-stimulating effect of Ro 50-3821 if it had been administered more frequently. In a clinical setting, however, more frequent dosing, especially if unpleasant, usually corresponds to decreased compliance. Decreased compliance, in turn, may limit treatment success.[34] In addition, it is easily conceivable that frequent injections impair patients' quality of life.[35] Therefore, a less difficult and less unpleasant dosing scheme in patients with CRF may entail increased compliance with treatment and enhance the quality of life.

Another important aspect in choosing a drug for a patient is the safety profile of the drug. In this animal model of CRF, neither of the investigated drugs seemed to be particularly problematic. Blood pressure was largely unchanged. It is unlikely that the observed increase in protocol 3 at week 4 is attributable to the erythropoietic agents, as control animals showed a comparable increase at that time point only. Blood chemistry, white blood cell and thrombocyte count, liver function tests, and renal retention parameters were largely unaffected by the examined drugs. Since no effects on any but the red blood cell line were noted, the novel erythropoietic agents seem to exert specific qualities. All changes of safety parameters observed were within the range seen in saline-treated control animals.

During protocol 3, one animal from the rhEPO group was found dead, and nine animals had to be killed (four from the middle-dose and four from the high-dose Ro 50-3821 groups, and one from the control group). Paralyses, cramps, a bad general condition, deteriorating renal function, and a decrease or a reduced increase in body weight were observed. These findings are probably due to uremia, which in turn might be aggravated by rheological changes due to the very high hematocrit in these animals.[36,37] A distinct interindividual variation is immanent in this animal model, caused by variability of five-sixths nephrectomy. Owing to the complexity of the test situation, the assessment of toxicity findings and deaths of animals demands caution.

Postmortem tissue examinations revealed extramedullar erythropoiesis in all middle- and high-dose Ro 50-3821-treated rats and in two of the 12 rhEPO-treated rats, but in none of the animals treated with the low-dose Ro 50-3821. This exaggerated pharmacological response substantiates the proposal that subcutaneous (s.c.) Ro 50-3821 at doses of 2.5 and 7.5 μg/kg bw leads to an extremely pronounced stimulation of erythropoiesis with its untoward consequences.

However, in the light of available data, it seems legitimate to propose that the tested Ro 50-3821 has a safety profile at least as favorable as that of conventional rhEPO. This proposal is fuelled by the promising results on antibody formation following administration of the various compounds. Antibodies were found in the high-dose group of Ro 50-3821 in one out of nine animals and in rhEPO-treated animals in nine out of 14 animals. The much more pronounced antibody formation with rhEPO, within all inherent limitations of animal models in this context, suggests reduction of immunogenicity by the modification used. It has been suggested that antibody formation following

s.c. rhEPO is related to the used vehicle and is only seen after s.c. injection of epoetin alpha in Caucasians. However, recently it has been recognized that other EPO derivatives may lead to antibody formation and also that other ethnic groups may be afflicted.[38] The entire extent and clinical significance of anti-EPO antibodies is still unclear and a matter of debate.[39]

Although not a major clinical concern in the treatment of patients with CRF, Ro 50-3821 may help to avoid cases of rhEPO resistance[20–22,40] and decrease the incidence of pure red cell aplasia[40] further.

The findings presented in this study together with those described elsewhere[41,42] show that Ro 50-3821 provides an important therapeutic benefit compared to currently used treatment regimens in patients with CRF.

Differences in the binding characteristics compared to those of rhEPO have been described for Ro 50-3821.[41] A prolonged, dose-dependent, and potent enhancement of erythropoietic activity was shown for Ro 50-3821 in the first human studies,[43] indicating the relevance of the findings in animal experiments for humans.

Furthermore, the long-term costs associated with three injections per week are substantially higher than for single weekly injections.[44]

All these aspects will play an important role in future health care considerations.

CONCLUSIONS

In rats with CRF, Ro 50-3821, rhEPO conjugated with a single PEG molecule, is capable of correcting anemia. It increases crucial parameters such as hemoglobin levels, erythrocyte and reticulocyte count, and hematocrit to acceptable levels. This can be achieved with only one dose per week. EPO, with multiple linkage to PEG (MIX), is also effective, but to a lesser degree.

The safety profile of the novel EPO derivative Ro 50-3821 seems to be superior to that of rhEPO. No significant antibody formation or increase in blood pressure was found during treatment with Ro 50-3821, whereas rhEPO induced significant antibody production in a large percentage of animals.

Therefore, Ro 50-3821 may offer important clinical advantages compared to currently used agents, since it could be administered less frequently, may have a favorable safety profile, and/or entails a lower risk of unfavorable immunological responses.

MATERIALS AND METHODS

Before commencement, the study was approved by the Regional Commission, Karlsruhe, Germany. All experiments were performed in accordance with federal and local laws, as well as institutional regulations.

Male Sprague–Dawley rats, 14 weeks old (Janvier, France), weighing between 300 and 400 g, were fed standard rat chow (Ssniff, Soest, Germany) *ad libitum* with free access to tap water for drinking. All rats were two-thirds nephrectomized unilaterally by resection of both kidney poles, followed by a complete contralateral nephrectomy 2 weeks later[45] under intraperitoneal ketamine (Hostaket®, Germany; 75 mg/kg bw) and xylazine (Rompun® 2%, Bayer, Germany; 5 mg/kg bw) anesthesia.

The animals were allowed to recover from surgery for about 2 weeks. Thereafter, rats were trained for 1 week for systolic blood pressure measurements by tail cuff manometry using a commercially available tail sphygmomanometer (Hugo Sachs Elektronik, March, Germany).

Baseline blood sampling and premedication-phase blood pressure recordings were begun in the following week. Blood samples were obtained from the retrobulbar region under ether anesthesia. Hematocrit, hemoglobin, and erythrocyte, leucocyte, and thrombocyte counts were measured using a Cell Dyn 3500 automated hematology analyzer (Abbott, Wiesbaden, Germany). Reticulocytes were measured with an EPICS XL-MCL flow cytometer (Coulter Electronics, Krefeld, Germany, staining with thiazole orange). Creatinine, urea, aspartate aminotransferase, alanine aminotransferase, and alkaline phosphatase were determined with a Hitachi Automatic Analyzer.

For antibody determination, the enzyme-linked immunosorbant assay (ELISA) used in the present study as reference method is described fully elsewhere.[46] Briefly, 96-well polystyrol plates coated with streptavidin (Roche, Basel, Switzerland) were incubated with 125 μl biotinylated rhEPO in sodium phosphate 40 mM, pH 7.4 (Na_2HPO_4 and $NaH_2PO_4 \times 1\ H_2O$ containing 0.1% (v/v) Tween 20 (phosphate buffer (PB))). After washing three times with PB, the serum samples were added in duplicate 1:25 diluted in PB (without Tween 20) containing 0.5% bovine serum albumin (BSA), 2% sheep immunoglobulin (IgG), and 150 mM NaCl. After 3 h of incubation at room temperature (RT), the plates were washed three times with PB and subsequently incubated with sheep anti-rat Fab fragments conjugated with horseradish peroxidase 15 mU/ml final concentration, 1 h, RT). The substrate 2,2′-acino-di(3-ethylbenz-thiazoline-sulfonic) acid was added and, after 30 min of incubation at RT, the reaction was read at 405 and 492 nm using SLT microplate Reader SLT (SLT-Labinstruments GmbH, Grödig/Salzburg, Austria). To evaluate the specificity of the ELISA, the test was performed in parallel using BSA or rhEPO (100 μg/ml).

To test the reliability of each run, a dose–response curve of the control material was used. The control material is a conjugate of polyclonal anti-erythropoietin antibody from rabbit and human IgG. Three concentrations (5, 10, and 15 μg/ml) and zero control material were used to test the reliability of the reaction. The cutoff point for positive samples was previously evaluated as an absorbance decrease of 50%. The intra-assay coefficient of variation was 4.2% ($n = 20$) for absorbance and 5.4% ($n = 10$) for absorbance decrease (displacement).

The rats were randomly allocated to one of the three study protocols designed to investigate the pharmacodynamic properties of the different study drugs. The protocols consecutively examined the efficacy, frequency of administration, and dose–response characteristics. After allocation, the rats were given the first dose of medication according to the respective protocols.

Drugs

The drugs tested in these experiments were rhEPO (epoetin, working standard, Roche Diagnostics GmbH, Mannheim, Germany), Ro 50-3821, and an alternative compound (MIX). Ro 50-3821 and MIX were synthesized at Roche, Nutley, NJ, USA, and are chemically synthesized continuous EPO receptor activators.

These differ from EPO through integration of amide bonds between amino groups and methoxy PEG-succinimidyl butanoic acid.[41]

Scheme of Ro 50-3821 and alternative compound MIX:
$[CH_3\ O(CH_2\ CH_2O)_n\ CH_2\ CH_2\ CH_2CO]_m$-NH-EPO

Ro 50-3821 and MIX differ among themselves in the content or proportion of methoxy PEG-succinimidyl butanoic acid (m).

Physiologic NaCl solution was used as control. To prevent unspecific binding to surfaces, 0.125 ml freshly prepared blood serum of Sprague–Dawley rats was added per mg protein of each EPO medication. All study treatments were administered by s.c. injection.

Table 3 | Dose and frequency of study drug administration

Drug dose (μg/kg body weight)	Frequency of administration
Protocol 1	
Ro 50-3821 0.25 (*n*=11)	Once per week
Ro 50-3821 2.5 (*n*=11)	Once per week
MIX 0.25 (*n*=13)	Once per week
MIX 2.5 (*n*=11)	Once per week
rhEPO 0.25 (*n*=12)	Three times per week
NaCl (*n*=10)	Once per week
Protocol 2	
Ro 50-3821 0.25 (*n*=12)	Three times per week
Ro 50-3821 0.75 (*n*=12)	Once per week
rhEPO 0.25 (*n*=12)	Three times per week
rhEPO 0.75 (*n*=12)	Once per week
NaCl (*n*=11)	Once per week
Protocol 3	
Ro 50-3821 0.75 (*n*=13)	Once per week
Ro 50-3821 2.5 (*n*=13)	Once per week
Ro 50-3821 7.5 (*n*=13)	Once per week
rhEPO 0.75 (*n*=14)	Once per week
NaCl (*n*=12)	Once per week

n=number of animals in each dosing group.

Protocol 1

Protocol 1 was performed to investigate the erythropoietic efficacy of Ro 50-3821 and MIX in comparison to rhEPO and NaCl control. Rats were randomly allocated to one of six groups. All animals received the respective study medication for 4 weeks. Group 1 ($n=11$) received Ro 50-3821 (0.25 μg/kg bw) once per week, group 2 ($n=11$) received Ro 50-3821 (2.5 μg/kg bw) once per week, group 3 ($n=13$) received MIX (0.25 μg/kg bw) once per week, group 4 ($n=11$) received MIX (2.5 μg/kg bw) once per week, group 5 ($n=11$) received rhEPO (0.25 μg/kg bw) three times per week, and group 6 ($n=10$) received NaCl once per week. Blood samples obtained at the end of each week were analyzed as described above. After the last set of procedures, following 4 weeks of treatment, rats were killed under combined ketamin and xylazin anesthesia.

Protocol 2

During protocol 2, Ro 50-3821, the more effective of the two newly synthesized agents from protocol 1, was tested to characterize the most suitable frequency of application in comparison to rhEPO and NaCl (Table 3). Rats were randomly allocated to one of five groups. Group 1 ($n=12$) received a low dose of Ro 50-3821 (0.25 μg/kg bw) three times per week. Group 2 ($n=12$) received 0.75 μg/kg bw of Ro 50-3821 once weekly. Analogously, groups 3 and 4 received respective doses of rhEPO ($n=12$ for both), and group 5 ($n=11$) was administered isotonic saline solution once weekly.

Blood samples were obtained at the end of each week of treatment and animals were killed under combined ketamin and xylazin anesthesia after 4 weeks of treatment.

Protocol 3

The long-term effects of Ro 50-3821 in regular doses (0.75 μg/kg bw once weekly; $n=13$) as well as two high doses (2.5 and 7.5 μg/kg bw once weekly; $n=13$ for both) were compared to rhEPO (0.75 μg/kg bw once weekly; $n=14$) and NaCl in protocol 3. Blood samples for the determination of hematological parameters were obtained after 1, 2, 4, 6, 8, 10, and 12 weeks of treatment. Antibody levels against the study drugs were measured after 1, 8, 10, and 12 weeks of treatment. Rats receiving Ro 50-3821 2.5 and 7.5 μg/kg bw were killed after 8 weeks of treatment, whereas the Ro 50-3821 0.75 μg/kg

Table 4 | Timeline of protocols 1–3

Study day	Protocol 1	Protocol 2	Protocol 3
−42	2/3 nephrectomy (left)	2/3 nephrectomy (left)	2/3 nephrectomy (left)
−35	Total nephrectomy (right)	Total nephrectomy (right)	Total nephrectomy (right)
−14	Blood sampling (week 0)	Blood sampling (week −1)	
− 7	First dose of study drug	Blood sampling (week 0)	Blood sampling (week 0)
0		First dose of study drug	First dose of study drug
		Action taken	
Weeks after initiation of study drug	Protocol 1	Protocol 2	Protocol 3
1	Blood sampling	Blood sampling	Blood sampling, antibody levels
2 and 3	Blood sampling	Blood sampling	
4	Blood sampling, sacrifice	Blood sampling, sacrifice	Blood sampling Blood sampling
8			Blood sampling, antibody levels Killing of rats receiving Ro 50-3821 2.5 and 7.5 μg/kg
10			Blood sampling, antibody levels
12			Blood sampling, antibody levels, killing

as well as the control groups were continued until 12 weeks after initiation of study medication.

Table 4 shows the timeline of the protocols, and Table 3 summarizes the doses used during their various stages.

Statistical methods

The results of the three protocols were analyzed independently from one another. Primary evaluation parameters were blood hemoglobin levels after the respective periods of treatment (4 weeks in protocols 1 and 2, 12 weeks in protocol 3). Values are presented as means $\pm$ s.d. Initial analyses compared the means using general linear models. In case of $P<0.05$, least-square differences were calculated, correcting for multiple comparisons, to analyze the differences between the groups. Statistical analyses were performed using SAS® for Windows version 8.1 (Cary, NC). In secondary analyses, additional variables characterizing erythropoiesis (RBC, reticulocyte count, hematocrit) were analyzed using the same statistical approach. In addition, all parameters were measured and analyzed after each week of treatment.

In protocol 3, antibody production was analyzed as a dichotomous variable (antibodies against the respective medication *present/not present*). Differences between groups were analyzed using the χ^2 test with *post hoc* decomposition. In case of cell counts smaller than five, Fisher's exact test was used.

Animals that died during the treatment phase of protocol 1 were excluded from analyses (three in the rhEPO and one in the MIX (0.25 μg/kg bw) groups). During protocol 2, no animal was lost. During protocol 3, nine animals had to be killed and one died (four in Ro 50-3821 (2.5 μg/kg bw), four in Ro 50-3821 (7.5 μg/kg bw), one in rhEPO (0.75 μg/kg bw), and one in the control group). Animals were used for analysis until the date of death. In addition to the above-outlined analyses, a worst-case scenario analysis was performed for antibody status. For this purpose, animals that died were added to the group of animals in which antibodies were detected.

All safety parameters were evaluated descriptively.

The statistician was blinded with respect to medication while performing the statistical analyses.

REFERENCES

1. Hsu CY, McCulloch CE, Curhan GC. Epidemiology of anemia associated with chronic renal insufficiency among adults in the United States: results from the Third National Health and Nutrition Examination Survey. *J Am Soc Nephrol* 2002; **13**: 504–510.
2. Kazmi WH, Kausz AT, Khan S *et al.* Anemia: an early complication of chronic renal insufficiency. *Am J Kidney Dis* 2001; **38**: 803–812.
3. Gretz N, Unger R, Grittman S *et al.* Renal function, anemia and blood pressure in patients with chronic renal failure. *Scand J Urol Nephrol* 1988; **108**(Suppl): 57–60.
4. Lundin AP. Quality of life: subjective and objective improvements with recombinant human erythropoietin therapy. *Semin Nephrol* 1989; **9**: 22–29.
5. Murphy ST, Parfrey PS. The impact of anemia correction on cardiovascular disease in end-stage renal disease. *Semin Nephrol* 2000; **20**: 350–355.
6. Jensen JD, Madsen JK, Jensen LW *et al.* Reduced production, absorption, and elimination of erythropoietin in uremia compared with healthy volunteers. *J Am Soc Nephrol* 1994; **5**: 177–185.
7. Lin FK, Suggs S, Lin CH *et al.* Cloning and expression of the human erythropoietin gene. *Proc Natl Acad Sci USA* 1985; **82**: 7580–7584.
8. Winearls CG, Oliver DO, Pippard MJ *et al.* Effect of human erythropoietin derived from recombinant DNA on the anaemia of patients maintained by chronic haemodialysis. *Lancet* 1986; **2**: 1175–1178.
9. Eschbach JW, Egrie JC, Downing MR *et al.* Correction of the anemia of end-stage renal disease with recombinant human erythropoietin. Results of a combined phase I and II clinical trial. *N Engl J Med* 1987; **316**: 73–78.
10. Erslev AJ, Besarab A. Erythropoietin in the pathogenesis and treatment of the anemia of chronic renal failure. *Kidney Int* 1997; **51**: 622–630.
11. Marchetti M, Barosi G. Clinical and economic impact of epoetins in cancer care. *Pharmacoeconomics* 2004; **22**: 1029–1045.
12. Tsakiris D. Morbidity and mortality reduction associated with the use of erythropoietin. *Nephron* 2000; **85**(Suppl 1): 2–8.
13. Dikow R, Schwenger V, Schomig M *et al.* How should we manage anaemia in patients with diabetes? *Nephrol Dial Transplant* 2002; **17**(Suppl 1): 67–72.
14. Cody J, Daly C, Campbell M *et al.* Recombinant human erythropoietin for chronic renal failure anaemia in pre-dialysis patients. *Cochrane Database Syst Rev* 2001, Issue 3, Art. No.: CD003266.
15. Gouva C, Nikolopoulos P, Ioannidis JP *et al.* Treating anemia early in renal failure patients slows the decline of renal function: a randomized controlled trial. *Kidney Int* 2004; **66**: 753–760.
16. MacDougall IC. Hyporesponsiveness to anemia therapy – what are we doing wrong? *Periton Dial Int* 2001; **21**: S225–S230.
17. Macdougall IC, Cooper AC. Erythropoietin resistance: the role of inflammation and pro-inflammatory cytokines. *Nephrol Dial Transplant* 2002; **17**(Suppl 11): 39–43.
18. Sunder-Plassmann G, Horl WH. Novel aspects of erythropoietin response in renal failure patients. *Nephrol Dial Transplant* 2001; **16**: 40–44.
19. Frankenfield D, Johnson CA, Wish JB *et al.* Anemia management of adult hemodialysis patients in the US results: from the 1997 ESRD Core Indicators Project. *Kidney Int* 2000; **57**: 578–589.
20. Peces R, de la Torre M, Alcazar R *et al.* Antibodies against recombinant human erythropoietin in a patient with erythropoietin-resistant anemia. *N Engl J Med* 1996; **335**: 523–524.
21. Prabhakar SS, Muhlfelder T. Antibodies to recombinant human erythropoietin causing pure red cell aplasia. *Clin Nephrol* 1997; **47**: 331–335.
22. Casadevall N. Antibodies against rHuEPO: native and recombinant. *Nephrol Dial Transplant* 2002; **17**(Suppl 5): 42–47.
23. Sunder-Plassmann G, Horl WH. The clinical potential of novel erythropoiesis stimulating protein. *Expert Opin Biol Ther* 2001; **1**: 733–739.
24. Macdougall IC. Darbepoetin alfa: a new therapeutic agent for renal anemia. *Kidney Int* 2002; 61 (Suppl 80): 55–61.
25. Egrie JC, Dwyer E, Browne JK *et al.* Darbepoetin alfa has a longer circulating half-life and greater *in vivo* potency than recombinant human erythropoietin. *Exp Hematol* 2003; **31**: 290–299.
26. Bukowski RM, Tendler C, Cutler D *et al.* Treating cancer with PEG Intron: pharmacokinetic profile and dosing guidelines for an improved interferon-alpha-2b formulation. *Cancer* 2002; **95**: 389–396.
27. Gupta SK, Pittenger AL, Swan SK *et al.* Single-dose pharmacokinetics and safety of pegylated interferon-alpha2b in patients with chronic renal dysfunction. *J Clin Pharmacol* 2002; **42**: 1109–1115.
28. Abuchowski A, Kazo GM, Verhoest Jr CR *et al.* Cancer therapy with chemically modified enzymes. I. Antitumor properties of polyethylene glycol–asparaginase conjugates. *Cancer Biochem Biophys* 1984; **7**: 175–186.
29. Nucci ML, Olejarczyk J, Abuchowski A. Immunogenicity of polyethylene glycol-modified superoxide dismutase and catalase. *J Free Radic Biol Med* 1986; **2**: 321–325.
30. Valderrabano F. Erythropoietin in chronic renal failure. *Kidney Int* 1996; **50**: 1373–1391.
31. Gretz N, Lasserre J, Kraft K *et al.* Efficacy and side effects of erythropoietin used in the treatment of anemia of uremic rats. *Contrib Nephrol* 1988; **60**: 236–244.
32. Cowgill LD, James KM, Levy JK *et al.* Use of recombinant human erythropoietin for management of anemia in dogs and cats with renal failure. *J Am Vet Med Assoc* 1998; **212**: 521–528.
33. Bleuel H, Hoffmann R, Kaufmann B *et al.* Kinetics of subcutaneous versus intravenous epoetin-beta in dogs, rats and mice. *Pharmacology* 1996; **52**: 329–338.
34. Cramer JA. Effect of partial compliance on cardiovascular medication effectiveness. *Heart* 2002; **88**: 203–206.
35. Andersson PO, Wikby A, Hornquist JO. Influence of insulin pen injection frequency on quality of life. *Diabetes Care* 1990; **13**: 1135–1136.
36. Bor-Kucukatay M, Yalcin O, Meiselman HJ *et al.* Erythropoietin-induced rheological changes of rat erythrocytes. *Br J Haematol* 2000; **110**: 82–88.
37. Maeda N, Kon K, Tateishi N *et al.* Rheological properties of erythrocytes in recombinant human erythropoietin-administered normal rat. *Br J Haematol* 1989; **73**: 105–111.

38. Yang J, Joo KW, Kim YS *et al.* Two cases of pure red-cell aplasia due to anti-erythropoietin antibodies. *J Nephrol* 2005; **18**: 102–105.
39. Cournoyer D, Toffelmire EB, Wells GA *et al.* Anti-erythropoietin antibody-mediated pure red cell aplasia after treatment with recombinant erythropoietin products: recommendations for minimization of risk. *J Am Soc Nephrol* 2004; **15**: 2728–2734.
40. Bennett CL, Luminari S, Nissenson AR *et al.* Pure red-cell aplasia and epoetin therapy. *N Engl J Med* 2004; **351**: 1403–1408.
41. Bailon P, Pahlke W, Brandt M *et al.* CERA (continuous erythropoiesis receptor activator) for the treatment of renal anemia: a new agent with an innovative mechanism of action. *Nephrol Dial Transplant* 2003; **18**: 166.
42. Tare N, Pill J, Haselbeck A. Preclinical pharmacodynamics and pharmacokinetics of Ro 50-3821 (continuous erythropoiesis receptor activator): a new erythropoietic agent for anemia management in patients with kidney disease. *Nephrol Dial Transplant* 2003; **18**: 166.
43. Reigner B, Jordan P, Pannier A *et al.* Phase I studies of the new erythropoietic agent, RO 50-3821 (continuous erythropoiesis receptor activator): demonstration of a dose-dependent response. *Nephrol Dial Transplant* 2003; **18**: 166.
44. Joy MS. Darbepoetin alfa: a novel erythropoiesis-stimulating protein. *Ann Pharmacother* 2002; **36**: 1183–1192.
45. Gretz N, Meisinger E, Strauch M. Partial nephrectomy and chronic renal failure: the 'mature' rat model. *Contrib Nephrol* 1988; **60**: 46–55.
46. Kientsch-Engel R, Hallermayer K, Dessauer A. Methods for measuring erythropoietin and erythropoietin antibodies using ELISA technique. *Contrib Nephrol* 1989; **76**: 100–105.