Antibody-Mediated Delivery of Interleukin-2 to the Stroma of Breast Cancer Strongly Enhances the Potency of Chemotherapy

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Abstract

Purpose: There is an interest in the discovery of biopharmaceuticals, which are well tolerated and which potentiate the action of anthracyclines and taxanes in breast cancer therapy.

Experimental Design: We have produced a recombinant fusion protein, composed of the human antibody fragment scFv(F16) fused to human interleukin-2 (F16-IL2), and tested its therapeutic performance in the MDA-MB-231 xenograft model of human breast cancer. The F16 antibody is specific to the alternatively spliced A1 domain of tenascin-C, which is virtually undetectable in normal tissues but is strongly expressed in the neovasculature and stroma of breast cancer.

Results: When used as monotherapy, F16-IL2 displayed a strikingly superior therapeutic benefit compared with unconjugated recombinant IL-2. The administration of doxorubicin either before (8 days, 24 h, or 2 h) or simultaneously with the injection of F16-IL2 did not decrease the accumulation of immunocytokine in the tumor as measured by quantitative biodistribution analysis. Therapy experiments, featuring five once per week coadministrations of 20 µg F16-IL2 and doxorubicin, showed a statistically significant reduction of tumor growth rate and prolongation of survival at a 4 mg/kg doxorubicin dose but not at a 1 mg/kg dose. By contrast, combination of F16-IL2 with paclitaxel (5 and 1 mg/kg) exhibited a significant therapeutic benefit compared with paclitaxel alone at both dose levels. F16-IL2, alone or in combination with doxorubicin, was well tolerated in cynomolgus monkeys at doses equivalent to the ones now used in clinical studies.

Conclusions: F16-IL2 may represent a new useful biopharmaceutical for the treatment of breast cancer.

Breast cancer is a major cause of morbidity and mortality in women ages >45 years, especially in the United States, where every year >180,000 new cases are diagnosed and >45,000 women die of the disease (1). The survival of all patients with metastatic breast cancer varies according to certain prognostic factors: a long disease-free interval after primary therapy is more favorable than a short interval, nonvisceral sites of metastasis carry a better prognosis than visceral sites, and a single site of metastasis is more favorable than multiple sites. Whereas tamoxifen and aromatase inhibitors are frequently used for the treatment of estrogen receptor-positive patients, anthracyclines and taxanes represent standard elements in the adjuvant treatment of breast cancer and for the therapy of metastatic disease. For patients with metastases recurring after primary breast treatment, median survival is ~2 years. There is a clear need for better therapeutic options both in the adjuvant and in the metastatic breast cancer setting (2, 3).

The selective delivery of bioactive agents (e.g., cytotoxic drugs, radionuclides, or immunostimulatory cytokines) at the tumor site, while sparing normal tissues, represents one of the most promising avenues for the development of anticancer therapies with unprecedented efficacy and tolerability (2, 3). Markers of angiogenesis and of the tumor stroma represent particularly attractive targets for the antibody-based delivery of therapeutic agents in view of their selective, abundant, and accessible expression in aggressive solid tumors (4–7). In collaboration with the group of Luciano Zardi, our group has developed the human antibody L19, specific to the EDB domain of fibronectin, a marker of angiogenesis (8), whose ability to selectively localize at sites of neovasculature has been extensively documented (8–31). A large number of L19 derivatives have been produced and tested in animals for their therapeutic performance (for recent reviews, see refs. 28, 32); three of them are currently being investigated in multicenter clinical trials for the therapy of cancer. However, the EDB domain of fibronectin is only weakly expressed in breast cancer (33), and better targets are needed for the...
Translational Relevance
The development of more selective anticancer agents is one of the main goals of modern cancer research. One avenue toward the development of better drugs for the therapy of cancer consists in the antibody-mediated delivery of therapeutic agents (e.g., cytokines) to tumor sites, sparing normal tissues. In this context, certain antigens in the modified tumor stroma and neovascularature appear to be ideal targets in view of their abundance, stability, and specificity.

As described in this article, the immunocytokine F16-IL2, consisting of the human monoclonal antibody F16 specific to the extradomain A1 of tenascin-C fused to human IL-2, displays a potent anticancer therapeutic activity in murine models of human breast cancer. This activity is potentiated when the immunocytokine is administered in combination with chemotherapy. Based on the excellent safety profile observed in cynomolgus monkeys, F16-IL2 has recently entered two phase lb clinical trials: one in patients with breast and ovarian cancer in combination with doxorubicin and one in patients with breast and lung cancer in combination with paclitaxel.

antibody-based delivery of bioactive agents such as interleukin-2 (IL-2) to vascular tumor sites.

Tenascin-C is a glycoprotein of the extracellular matrix. It comprises several fibronectin type 3 homology repeats that can be either included or omitted in the primary transcript by alternative splicing, leading to small and large isoforms that have distinct biological functions (34, 35). Whereas the small isoform is expressed in several tissues, the large isoform of tenascin-C exhibits a restricted pattern of expression. It is virtually undetectable in healthy adult tissues but is extremely abundant in many cancer types, including virtually all forms of aggressive breast cancer (34). Radiolabeled derivatives of monoclonal antibodies to domains A1 and D of tenascin-C have been used for over a decade for imaging and radioimmunotherapy in patients with cancer (36, 37).

We have recently described the human monoclonal antibody fragment scFv(F16), which is specific to the alternatively spliced A1 domain of tenascin-C and which is able to selectively accumulate at neovascular tumor sites in animal models of cancer (38). The F16 antibody exhibits a virtually undetectable staining of most normal organs (including breast), whereas it strongly reacts with neovascular and stromal components of most human breast, lung, and head/neck cancers (38). The overexpression of large splice isoforms of tenascin-C in breast cancer has been well documented (34). Interestingly, the murine antibody BC2, recognizing both A1 and A4 domains of human tenascin-C (39), has been shown to selectively localize at tumor sites in animal models of breast cancer (40). It is generally believed that the extradomains A1 to D of tenascin-C are inserted or omitted “as a block” in the mRNA, except for domain C, which exhibits a more restricted pattern of expression (41–43).

In this article, we report the cloning, expression, and characterization of F16-IL2, a noncovalent homodimeric fusion protein consisting of the human scFv(F16) with a short 5-amino acid linker fused to human IL-2. F16-IL2 displayed an excellent ability to selectively localize to neovascular sites in mouse xenograft models of human breast cancer as evidenced by quantitative biodistribution studies done in the presence or absence of preadministration of doxorubicin. Furthermore, F16-IL2 was found to potentiate the therapeutic action of both doxorubicin and paclitaxel in mice bearing s.c. human MDA-MB-231 breast tumors, with weight loss below 10% for all therapy groups in the study. F16-IL2 has been found to be safe in cynomolgus monkeys, when used alone or in combination with doxorubicin at equivalent doses compared with ones planned for clinical studies in humans. The data presented in this article provide the scientific rationale for two phase lb studies of F16-IL2 in combination with paclitaxel and with doxorubicin for patients with metastastic breast cancer, which have recently started.

Materials and Methods

Cell lines and reagents. For the tumor experiments, the tumor cell line MDA-MB-231 human breast adenocarcinoma (American Type Culture Collection, LGC Promochem) was used. The cells were cultured in DMEM (Invitrogen) containing 10% FCS (Invitrogen) and incubated at 37°C and 5% CO2. The CTLL-2 cell line was obtained from the American Type Culture Collection. CTLL-2 cells were cultured in RPMI (Invitrogen) supplemented with 10% FCS, 2 mMol/L l-glutamine, 1 mMol/L sodium pyruvate, 50 μmol/L 2-mercaptoethanol (all purchased from Invitrogen), and 20 units/mL IL-2 (Roche). The human antibody fragment scFv(F16) has been described elsewhere (38). The CHO-K1 cells used for F16-IL2 expression were kindly provided by Luciano Zardi (Istituto Giannina Gaslini) and cultured in RPMI containing 10% FCS and 1 mMol/L l-glutamine or ProCHO-5 protein-free medium (Lonza) supplemented with 8 mMol/L l-glutamine. Recombinant human IL-2 (Proleukin) was purchased from Prorero Pharma, doxorubicin was from Sigma, and paclitaxel was from Bristol-Myers Squibb.

Cloning of F16-IL2. For cloning of F16-IL2, the huIL2 gene was amplified from a previously described vector directing the expression of L19-IL2 (44). To obtain the fusion protein in a noncovalent homodimeric format, the linker between heavy (VH) and light (VL) chains of the already existing scFv(F16) had to be shortened to 5 amino acids (GSSGG). Therefore, the VH gene for the F16-IL2 was PCR amplified priming on the region corresponding to the signal peptide using the primers NotI-VH-F16-for (5’-CAGTGGTGGAGTCTCAGGACTTGTCGTCGACTGGAGGTG-3’), and F16-IL2-for (5’-TTCGACCGACGTGACCGGTTTCC-3’). The VL gene was amplified with the primers NotI-VL-F16-back (5’-CTGCCTCAGCTGACCTGGAGCCTCT-3’) and NotI-VL-F16-for (5’-GACGTATTCTCGACCTGCGCGCGCTCAAGGCTCAGGTCGTTGCTCCCGCCGA-3’). Subsequently, the VH and VL genes were amplified with the primer pairs NotI-Sip-VL-F16-back and 5aa-VL-F16-forward (5’-AGAGACAGCCGCCCTCTGAGCACCGTGCAGCGGTTCCT-3’) or 5aa-VL-F16-back (5’-TCGAGAGCTCGAAGGTCGTTGCGGTTCCTGAGTCGTTGCGCCGA-3’). NotI-Sip-VL-F16-forward, followed by a PCR assembly with the oligos NotI-Sip-VL-F16-back and NotI-VL-F16-forward, leading to the formation of the complete scFv(F16) gene with a 5-amino acid linker subsequence. Subsequently, the antibody and cytokine gene portions were assembled by PCR and cloned into the mammalian cell expression vector pcDNA3.1(+)(+) using the HindIII and NotI sites, yielding vector pM1.

Expression and purification of F16-IL2. Adherent CHO-K1 cells grown in RPMI as described above were stably transfected with pM1 using Lipofectamine 2000 transfection reagent (Invitrogen). Selection was carried out in the presence of G418 (0.5 g/L). Clones of G418-resistant cells were screened for expression of the fusion protein by
ELISA using recombinant domain A1 of tenascin-C as antigen (38) and an anti-huIL2 antibody (eBioscience) for detection. Following generation of monoclonal cell lines, the best expressing clone was adapted to growth in ProCHO-5 protein-free medium for large-scale production of F16-IL2. The fusion protein could be purified from cell culture medium by protein A affinity chromatography, as there is a staphylococcal protein A binding site present on most VH3 domains (45–47). The size of the fusion protein was analyzed in reducing and nonreducing conditions on SDS-PAGE and in native conditions by fast protein liquid chromatography gel filtration on a Superdex S-200 size exclusion column (GE Healthcare).

**Bioactivity assay.** To determine the cytokine activity of F16-IL2, a CTLL-2 cell proliferation assay was done. CTLL-2 cells were seeded into 96-well plates at a concentration of $4 \times 10^4$ per well in 200 µL complete medium containing varying amounts of huIL2 standard (National Institute for Biological Standards and Controls) or the fusion protein at a maximum of 375 pmol/L IL-2 equivalents and serial dilutions. After 48 h at 37°C, 20 µL Cell Titer 96 Aqueous One Solution (Promega) was added and incubated for 2 h before reading at 490 nm. The cytokine activity was determined by comparing the absorbance reading of the samples to that of the standard.

**Fig. 1.** Cloning, expression, and characterization of F16-IL2. A, schematic representation of the cloning strategy and domain assembly of the scFv-huIL2 fusion protein F16-IL2 with a 5-amino acid (aa) linker between heavy and light chains. B, SDS-PAGE. C, gel-filtration analysis of affinity-purified F16-IL2 reveals complete noncovalent homodimer formation. Arrows, elution volumes of two markers: left arrow, IgG (150 kDa); right arrow, bovine serum albumin (67 kDa). D, BIAcore analysis done at 370 nmoL/L concentration of F16-IL2 revealed a slow kinetic dissociation from the antigen. E, F16-IL2 displayed biological activity comparable with the one of recombinant human IL-2 used as a standard in a CTLL-2 cell proliferation assay. **SP**, signal peptide; **N**, NH$_2$ terminus of the fusion protein; **MW**, molecular weight of the protein markers; **nr**, nonreducing; **r**, reducing.
added to each well. The plate was incubated for another 4 h and gently mixed and the absorbance was read at 490 nm. The experiment was done in triplicate for F16-IL2 and in duplicate for huIL2.

**Tumor mouse models.** Tumor-bearing mice were obtained by s.c. injection of \( 2 \times 10^6 \) MDA-MB-231 cells in 10- to 12-week-old female BALB/c nude mice (Charles River Laboratories). Normally, 9 days after tumor cell implantation, when tumors were clearly palpable and injected i.v. into the lateral tail vein with radioiodinated F16-IL2 either alone or following a single i.v. injection of doxorubicin (10 mg/kg; 8 days, 24 h, or 2 h). Antibody immunoreactivity after labeling was evaluated by loading a sample of radiolabeled F16-IL2 onto TNC-A1-Sepharose resin followed by radioactive counting of the flow-through and eluate fractions. Immunoreactivity, defined as the ratio between the counts of the eluted protein and the sum of the counts of the eluted and flow-through fractions, was 91%. Mice were sacrificed 24 h after injection of the fusion protein (12.5 ng, 3.3 μCi/mouse). Organs were weighed and radioactivity was counted with a Packard Cobra γ-counter. Radioactivity content of representative organs was expressed as %ID/g.

**Immunohistochemistry on frozen tissue sections.** MDA-MB-231 tumor sections of 8 to 10 μm thickness were treated with ice-cold acetone, rehydrated in TBS [50 mmol/L Tris, 100 mmol/L NaCl (pH 7.4)], and blocked with 20% FCS. The antibody F16 in small immunoprotein format (38) was added onto the sections in a final concentration of 5 μg/mL. The small immunoprotein format consists of a covalent homodimer, in which each monomeric unit comprises a scFv fused to a human sCH4 domain of the secretory isoform S2 of human IgE (17). This domain promotes the formation of homodimers that are further stabilized by disulfide bonds between the COOH-terminal cysteine residues, resulting in a 75 kDa homobivalent miniantibody. Bound small immunoprotein antibody was detected with rabbit anti-human IgE antibody (DAKO) followed by biotinylated goat anti-rabbit IgG antibody (Biopba) and streptavidin-alkaline phosphatase complex (Biopba). Fast red (Sigma) was used as phosphate substrate and sections were counterstained with Gill's hematoxylin no. 2 (Sigma).

**Immunofluorescence studies of tumor-infiltrating cells.** For immunofluorescence analysis of lymphocytic infiltration following therapy, female BALB/c nude mice bearing s.c. MDA-MB-231 tumors were injected weekly for 2 weeks i.v. with saline, 20 μg F16-IL2, 4 mg/kg doxorubicin and 20 μg F16-IL2, or 5 mg/kg paclitaxel and 20 μg F16-IL2. Twenty-four hours after the second injection, the mice were sacrificed and the tumors were excised and embedded in OCT compound (Micron International) and frozen in liquid nitrogen. Cryostat sections of 10 μm were cut of each tumor, transferred to glass slides (SuperFrost Plus; Menzel-Gläser), and stored at -80°C until needed. For immunofluorescent staining, slides were thawed and fixed by immersion in -20°C acetone for 10 min. The sections were then circled by a water-repellent pen and slides were washed in PBS for 5 min. Blocking was done by incubating the sections with 20% FCS in PBS for 1 h. Following washing with PBS for 2 × 5 min, sections were incubated with the primary antibodies rat anti-mouse F4/80 (anti-macrophage; Abcam), rat anti-mouse CD45 (BD Biosciences PharMingen), or rabbit anti-asialo-GM1 (anti-NK; Wako Pure Chemical Industries) in 12% bovine serum albumin in PBS for 1 h at room temperature or overnight at 4°C. Sections were washed 3 × 5 min with PBS at room temperature and then incubated with fluorescent Alexa 488- or 594-coupled secondary antibodies (BD Biosciences PharMingen) and Hoechst (4',6-diamidino-2-phenylindole) in 12% bovine serum albumin-PBS. Finally, sections were washed 3 × 5 min in PBS and mounted with Glycergel (DAKO) and a cover glass (VWR International). Images were obtained using the individual fluorescent channels using an Axioskop 2 mot plus (Carl Zeiss).

**Toxicology studies in cynomolgus monkey.** Toxicology studies were contracted to Nerviano Medical Sciences (Nerviano) and done in accordance with GLP guidelines. During the study, 9 male and 9 female cynomolgus monkeys (Macaca fascicularis) were assigned to 3 groups (3 males and 3 females per group) and administered i.v. over 1 h (once weekly for 7 weeks) with F16-IL2 at 0.57 mg/dose or at 0.1425 mg/dose in combination with 0.35 mg/kg doxorubicin.
A similarly constituted control group received the vehicle alone. Animals were assessed daily for reaction to treatment and the following investigations were done: body weight, food consumption, ophthalmoscopy, electrocardiography, blood pressure, hematology, and clinical chemistry. In addition, blood sampling for toxicokinetics, flow cytometry, and antibody determinations was done at time points defined in the Supplementary Table.


On completion of the study, all animals were sacrificed and underwent full necropsy and histopathology examination.

Immunogenicity tests were done by sandwich ELISA, coating F16-IL2 at 2 μg/ml on plastic plates and detecting monkey anti-fusion protein antibodies with suitable horseradish peroxidase-conjugated secondary antibodies (Nerviano Medical Sciences).

**Statistical analysis.** Data are expressed as mean ± SE. Differences in tumor volume between different mice populations were compared using Student’s t test, where P values < 0.05 were considered to be significant.

### Results

**Cloning, expression, purification, and characterization of F16-IL2.** The fusion protein F16-IL2, consisting of scFv(F16) with a 5-amino acid linker between VH and VL, sequentially fused to human IL-2, was cloned into the HindIII/NotI sites of the mammalian expression vector pcDNA3.1, yielding vector pJM1. The VH, VL, and IL-2 gene segments were sequentially assembled using PCR-based techniques (Fig. 1A; see also Materials and Methods). A short amino acid linker between VH and VL domain forces the formation of noncovalent homodimers (49). CHO-K1 cells were stably transfected with the pJM1 vector, yielding a monoclonal cell line capable of growing in suspension and directing the expression of F16-IL2 at >50 mg/L yields in fermenters (data not shown). Figure 1B shows an SDS-PAGE analysis of F16-IL2 following purification by protein A chromatography and ion exchange polishing, revealing a single band of the correct size in both reducing and nonreducing conditions. As expected, a single peak was observed in gel-filtration analysis on a Superdex S-200 column, corresponding to a noncovalent homodimer of F16-IL2 of apparent ~80 kDa (Fig. 1C). The fusion protein was found to display a slow kinetic dissociation from the antigen in a BIACore assay (Fig. 1D) and to exhibit biological activity comparable with the one of recombinant human IL-2 used as a standard in a Pharmacopoeia lymphocyte proliferation assay (Fig. 1E).

**Biodistribution studies with radioiodinated F16-IL2.** MDA-MB-231 tumors grafted s.c. into nude mice were chosen as a model to study the tumor-targeting properties of F16-IL2. This tumor is derived from an invasive ductal cell carcinoma and represents one of the most commonly used animal models of breast cancer (50). Figure 2 shows the immunohistochemical findings of a MDA-MB-231 section, stained with the F16 antibody, revealing a strong and uniform labeling of neovascular and stromal structures. To test whether the ability of F16-IL2 to selectively localize on breast cancer was affected by preadministration of a chemotherapeutic agent, we did quantitative biodistribution studies using an i.v. injection of radioiodinated F16-IL2 either alone or following a single i.v. injection of doxorubicin (10 mg/kg; 8 days, 24 h, or 2 h). Figure 3 shows that the tumor-targeting performance of F16-IL2 was not negatively affected by the preadministration of doxorubicin, with ~5% ID/g in the tumor and with excellent tumor/normal organ ratios 24 h after the injection of the radiolabeled immunocytokine (~15:1).

**Therapy studies in combination with doxorubicin and paclitaxel.** Both doxorubicin and paclitaxel are frequently administered to patients with once weekly schedules. We conducted therapy studies in nude mice bearing s.c. grafted MDA-MB-231 tumors. Previous studies with the L19-IL2 immunocytokine had revealed that the targeted delivery of IL-2 to solid tumors mediated an anticancer effect mainly through NK cells and that the therapeutic performance in nude mice was comparable with the one observed in immunocompetent mice bearing the same tumor (44). Using a three injections a week schedule, a dose of 20 μg in the...
mouse and of 3.75 mg in patients with cancer (22.5 million IU IL-2) were found to be well tolerated, in line with the conversion factor between mouse and human body surface (51).

Therapy was started 9 days following tumor cell implantation, when tumors had reached 50 to 100 mg in size. Five weekly i.v. administrations of doxorubicin (1 or 4 mg/kg) slowed down tumor growth but did not result in cures (Fig. 4A). We found higher doses of doxorubicin to give rise to excessive toxicity (data not shown). F16-IL2 (20 µg; a suboptimal dose at the once weekly schedule) exhibited a tumor growth inhibition, which was comparable with the one observed in the high-dose doxorubicin group. By contrast, equimolar amounts of recombinant IL-2 did not exhibit any therapeutic benefit, similar to what had been reported previously for other animal models of cancer (21, 22, 44), reinforcing the concept that the antibody-mediated targeted delivery of IL-2 may dramatically improve the antitumor
properties of this cytokine. The combination of doxorubicin (1 mg/kg) with F16-IL2 did not result in additional tumor growth retardation. By contrast, high-dose doxorubicin (4 mg/kg) combined with F16-IL2 resulted in substantially improved tumor growth retardation compared with both single doxorubicin \( (P = 0.04) \) and F16-IL2 \( (P = 0.01) \) treatment (Fig. 4A). Furthermore, single F16-IL2 was significantly better than recombinant IL-2 \( (P = 0.0098) \). Throughout the treatment, weight loss was <10% for all therapy groups, indicating that the regimens were well tolerated.

Using a similar administration schedule, we tested the therapeutic activity of paclitaxel at low-intermediate doses (1 and 5 mg/kg) either alone or in combination with F16-IL2 (20 \( \mu g \)). This time, coadministration of F16-IL2 dramatically improved the therapeutic performance of paclitaxel at both dose levels (Fig. 4B; F16-IL2 + paclitaxel 5 mg/kg versus paclitaxel 5 mg/kg: \( P = 0.0447 \); F16-IL2 + paclitaxel 1 mg/kg versus paclitaxel 1 mg/kg: \( P = 1.6 \times 10^{-6} \)). At the highest dose level, tumors remained smaller than 500 mg for over 80 days, with only five treatments. Also in this study, weight loss was <10% for all mice at any time point during the treatment.

Ex vivo microscopic analysis of cell infiltration. To assess the effect of F16-IL2 in terms of immunohistology of cells infiltrating tumors, we sacrificed groups of mice 24 h after the second therapeutic treatment (day 8 after the first injection) and analyzed tumor sections by immunofluorescence (3 mice per group). Figure 5 shows a comparative analysis of infiltrating cells from mice treated with saline, F16-IL2 alone, F16-IL2 plus doxorubicin, and F16-IL2 plus paclitaxel. Staining with an anti-CD45 antibody revealed that all three F16-IL2 treatment groups presented a higher level of infiltrating leukocytes compared with the saline treatment group. The highest number of tumor-infiltrating cells was detected in the F16-IL2 plus paclitaxel treatment group. A similar staining pattern was observed with an anti-asialo-GM1 antibody, which preferentially stains natural killer cells (52), and with the macrophage-specific antibody F4/80 (ref. 53; Fig. 5). This is consistent with our previous studies of tumor-infiltrating leukocytes in mice treated with immunocytokines (15, 18, 21, 44). In contrast to our experience with other mouse strains, detection of infiltrating natural killer cells using NK1.1 and NKp46 did not provide stainings of sufficient quality (data not shown).

Safety studies in cynomolgus monkey. The superior performance of doxorubicin- or paclitaxel-based chemotherapy when administered in combination with F16-IL2, compared with chemotherapy alone, motivated us to move forward to combination phase Ib clinical trials in patients with metastatic breast cancer. In preparation for these studies, we did a toxicity assessment of weekly administrations of F16-IL2 either alone or in combination with doxorubicin in cynomolgus monkeys.

Once weekly administrations of F16-IL2 for 7 weeks at a dosage of 80 million IU IL-2 human equivalents (0.57 mg F16-IL2/dose), or at a dosage of 20 million IU human equivalents (0.1425 mg F16-IL2/dose) in combination with doxorubicin, was very well tolerated with few side effects observed. A doxorubicin dose of 0.35 mg/kg/wk was chosen, in consideration of the fact that a single injection of 2 mg/kg doxorubicin leads to severe side effects, which require over 2 months for recovery (54), and that three weekly injections of 1.3 mg/kg
doxorubicin cause up to 90% reduction in reticulocytes and 80% reduction in neutrophils. Ophthalmoscopy, electrocardiography, blood pressure, coagulation, and clinical chemistry variables were all unaffected (Supplementary Table).

Overall, findings reported in the study related directly to the pharmacologic action of either F16-IL2 (the IL-2 moiety) or doxorubicin, with the hemolymphopoietic system mostly affected. Minor reversible changes in hematologic variables (e.g., 20% decrease in hematocrit and ~20% increase in WBC counts) were observed, which returned to baseline at the end of the 7-week treatment period (Supplementary Table). Minor microscopic changes were seen in the spleen, thymus, and lymph nodes.

In animals receiving F16-IL2 in combination with doxorubicin, moderate lymphocyte depletion was observed in the spleen, mesenteric lymph nodes, and thymus, which correlated with a slight reduction in organ weight recorded at necropsy. For animals receiving doxorubicin, these findings, together with the slower rebound effect for lymphocytes, were considered to be a consequence of the known myelosuppressive properties of this chemotherapeutic agent (55).

Toxicokinetic analysis revealed an essentially monophasic blood clearance profile for F16-IL2 when used as monotherapy or in combination with doxorubicin, in both cases with a $t_{1/2}$ ~ 2 h (Fig. 6). This pharmacokinetic profile was very similar to the one observed for L19-IL2 in cynomolgus monkeys. As expected with i.v. administration, peak serum concentrations were observed within 5 min of completion of dosing. Samples were collected at pre-dose, 5 and 30 min, and 1, 3 and 24 h after end of infusion, in a total of 6 monkeys per group.

F16-IL2 was found to be only weakly immunogenic in monkeys, based on a sandwich ELISA assay with a threshold set at 0.3 absorbance units, using plasma in a 1:10 dilution. ELISA values were below the threshold at the earlier time points tested (0, 8, and 15 days) and only slightly exceeded the threshold at days 22 and 43 in the F16-IL2 treatment groups (Supplementary Figure).

In conclusion, the weekly administration of F16-IL2 alone or in combination with doxorubicin was successfully administered to cynomolgus monkeys with few side effects observed. Pharmacologically relevant findings were observed, indicating that the combination of both these agents does not result in unexpected toxicity. This further supports the hypothesis that combining F16-IL2 and doxorubicin (or other existing chemotherapeutic agents) in the clinic will lead to superior benefits for cancer patients.

**Discussion**

The data presented in this study indicate that the immuno-cytokine F16-IL2 is able to substantially increase the therapeutic efficacy of anthracycline- and taxane-based chemotherapy in a mouse xenograft model of human breast cancer. Anthracyclines and taxanes represent standard elements in the adjuvant treatment of breast cancer and for the therapy of metastatic...
disease. The demonstration of a median survival increase on combination of doxorubicin or paclitaxel with the anti-HER-2/neu antibody trastuzumab (Herceptin; ref. 56) in a subpopulation of breast cancer patients positive for the antigen provides a rationale for the clinical development of combination therapeutic regimens.

In our preclinical studies, the tumor-targeting performance of F16-IL2 was found not to be influenced by the prednisone administration of chemotherapy. A dramatic increase of survival time was observed when F16-IL2 was combined with doxorubicin at a high dose (4 mg/kg) but not at a lower dose (1 mg/kg), indicating a rather narrow therapeutic window. By contrast, a strong synergistic effect of F16-IL2 and paclitaxel was observed at both doses tested (1 and 5 mg/kg). In all experiments, addition of the immunocytokine to the chemotherapeutic regimens did not result in additional toxicities as indicated by constant weight monitoring. It should be noted that suboptimal doses of both paclitaxel and F16-IL2 were used in this study. Our choice was motivated by the intention to mimic as closely as possible the doses and regimens, which will be used in clinical studies, rather than aiming for the best possible therapeutic results in the mouse setting. Using a once weekly schedule, doxorubicin and paclitaxel are generally administered at 20 and 80 mg/m² doses, respectively, in patients with breast cancer. Comforted by the excellent safety profiles observed in cynomolgus monkeys, F16-IL2 has recently entered two phase I clinical trials, in combination with doxorubicin (breast and lung, and ovarian cancer). In summary, we have shown that the immunocytokine F16-IL2 strongly increases the potency of taxane- and anthracycline-based chemotherapy without apparent additional toxicities in a mouse xenograft model of human breast cancer. Combination clinical trials in patients with metastatic breast, lung, and ovarian cancer, featuring weekly administrations of F16-IL2, have recently started.

Disclosures of Potential Conflicts of Interest

D. Neri is a founder and shareholder of Philogen S.p.A, the company that owns F16-IL2 and that has taken this product to the clinic.

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