Preclinical pharmacokinetics and safety of Sym004: A synergistic antibody mixture directed against epidermal growth factor receptor

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ADA, anti-drug antibodies; AG ratio, albumin:globulin ratio; CIEX, cation-exchange; CL, clearance; CRC, colorectal cancer; DRF, dose range finding; EGFR, epidermal growth factor receptor; ELISA, enzyme-linked immunosorbent assay; FDA, Food and Drug Administration; GLP, good laboratory practice; mAb, monoclonal antibody; SCCHN, squamous cell carcinoma head and neck
STATEMENT OF TRANSLATIONAL RELEVANCE

The therapeutic efficacy of currently approved anti-EGFR monoclonal antibodies (cetuximab and panitumumab) for cancer-therapy still leaves room for further improvement. We have previously shown that Sym004, a mixture of two novel noncompetitive anti-EGFR monoclonal antibodies, displayed superior inhibition of cancer cells \textit{in vitro} and tumor growth \textit{in vivo} compared to cetuximab.

The present preclinical safety studies show that co-administration to monkeys of the two monoclonal antibodies as one drug product, i.e. Sym004, results in a rapid target mediated clearance and an early onset of pronounced anti-EGFR mediated activities. Compared with monoclonal anti-EGFR antibodies, Sym004 do not induce any distinct or novel adverse findings in the monkeys.

The Sym004 development program is unique in that we investigate a fixed combination of two unmarketed investigational monoclonal antibodies and the preclinical safety studies enabled initiation of the ongoing clinical Phase I/II development of Sym004 as a fixed combination therapy.
Abstract

Purpose: Sym004 is a novel therapeutic antibody mixture product comprising two unmarketed monoclonal antibodies (mAbs) targeting the epidermal growth factor receptor (EGFR). In previous preclinical proof-of-concept studies, Sym004 was shown to elicit superior cancer cell growth inhibition activities compared with marketed anti-EGFR mAbs. This paper describes the design and results of the preclinical safety program conducted to support early clinical development of Sym004.

Experimental design: Tissue cryosections from various species were stained with Sym004 to evaluate tissue cross-reactivity. The pharmacokinetics of Sym004 were evaluated in a mouse xenograft model and in Cynomolgus monkeys. Monkeys received once-weekly intravenous infusions of Sym004 in the range 2-24 mg/kg for 6-8 weeks. Cetuximab (a marketed anti-EGFR mAb) and the individual antibodies comprising Sym004 were included in the repeat dose toxicity studies at single dose level.

Results: Sym004 had a staining pattern similar to cetuximab in tissue panels from both human and non-human primates. Once-weekly dosing of Sym004 to Cynomolgus monkeys did not cause accumulation, whereas administration of the individual antibodies resulted in prolonged half-life and accumulation. In direct comparisons with cetuximab, Sym004 did not induce any distinct or novel adverse findings in the animals. However, an early onset of pronounced, reversible and anticipated anti-EGFR-mediated pharmacological effects, such as skin rash, dehydration, and liquid feces, was observed. Only minor adverse effects were recorded in animals treated with the individual antibodies comprising Sym004.
Conclusion: Sym004 was well tolerated and did not induce any unexpected toxicities. The preclinical safety data enabled initiation of the ongoing clinical development.
Introduction

Dysfunctions in the EGF receptor (EGFR) tyrosine kinase pathway are associated with increased cellular growth, angiogenesis, metastasis, and reduced apoptosis of cancer cells and thus play a key role in malignant tumor growth. Upon ligand binding, the EGFR dimerizes, which leads to autophosphorylation and activation of intracellular signaling. Overexpression and excessive activation of the receptor results in hyperactivation of the signaling pathway, poor response to treatment, disease progression and poor survival. Blocking EGFR-transmitted signaling by direct targeting of the receptor is thus an attractive therapeutic strategy (1). The FDA has currently approved two mAbs targeting the EGFR for the treatment of colorectal cancer (CRC) and/or squamous cell carcinoma head and neck (SCCHN), namely cetuximab (Erbitux®) and panitumumab (Vectibix®), (2,3). Both mAbs bind to the EGFR extracellular domain III, competing with ligand and thereby intervening with dimerization and subsequent signaling (4,5). However, the therapeutic efficacy of these mAbs (6) still leaves room for further improvement of EGFR targeted therapies, for example by employing mixtures of noncompetitive anti-EGFR mAbs, which may offer more complete target inhibition.

Sym004 is a 1:1 mixture of the two chimeric anti-EGFR IgG1 antibodies mAb992 and mAb1024, which bind distinct non-overlapping epitopes on domain III of EGFR. The epitopes of mAb992 and mAb1024 both overlap with the epitopes of cetuximab and panitumumab. Functional characterization of Sym004 has demonstrated that the two individual antibodies act synergistically in inhibiting the proliferation of cancer cells \textit{in vitro}. The superior anti-proliferative effect is caused by efficient induction of EGFR internalization and subsequent

\footnote{K. Koefoed et al. Rational identification of an optimal antibody mixture for targeting the epidermal growth factor receptor, submitted for publication.}
degradation of the EGFR. Compared with marketed anti-EGFR mAbs, Sym004 elicits superior cancer cell growth inhibition in preclinical models (7).

We evaluated the preclinical pharmacokinetics and safety of Sym004 preceding the initiation of clinical development. In GLP-compliant tissue cross-reactivity studies, Sym004 showed a staining pattern similar to the marketed anti-EGFR mAb cetuximab in a full tissue panel from human and non-human primates. Subsequent repeat dose toxicity studies in Cynomolgus monkeys were designed to evaluate the pharmacokinetics and toxicity of Sym004 and the two individual antibodies constituting Sym004. Sym004 was well tolerated and did not accumulate upon once-weekly i.v. administration for eight weeks. In contrast, the half-life was prolonged and accumulation was observed when the individual antibodies were administered. In direct comparisons with cetuximab, once-weekly administration of Sym004 did not induce any distinct or novel adverse findings, although the extent and severity of expected anti-EGFR related effects, such as skin rash, dehydration, and liquid feces, were accelerated.
Materials and methods

EGFR antibodies

The two antibodies constituting Sym004, mAb992 and mAb1024, were produced separately in Chinese hamster ovarian cells and are chimeric, containing murine variable regions and human IgG1 constant regions. Production of mAb992, mAb1024, and Sym004 was carried out using procedures comparable to those used for manufacturing clinical trial materials, and subsequent characterization met specifications for identity, purity, potency, sterility and endotoxin. The test articles were supplied as sterile liquid formulations containing 5.4 mg/mL Sym004, 5.0 mg/mL mAb992, or 5.7 mg/mL mAb1024 in 10 mM sodium citrate, 150 mM NaCl, pH 6.0, and stored refrigerated (2-8 °C) in the dark until use. The marketed anti-EGFR chimeric IgG1 mAb, Erbitux® (cetuximab), 5.0 mg/mL, was stored refrigerated (2-8 °C) in the dark until use.

Tissue cross-reactivity studies

To identify potential models for toxicology studies, tissue cross-reactivity of Sym004 was evaluated in cryosections from a selected panel of Cynomolgus monkey, Rhesus monkey, rabbit, rat, mouse and guinea pig tissues (i.e. cerebellum, fallopian tube, heart, prostate, skin and spleen). Subsequently, the cross-reactivity of Sym004 was evaluated in cryosections from a full panel of normal human and Cynomolgus monkey tissues. In brief, mAb992 and mAb1024 were individually labeled using the Immunoprobe™ biotinylation kit (Sigma-Aldrich, Dorset, UK) and then mixed in a ratio of 1:1 to generate biotinylated Sym004. The biotinylated test articles displayed fully retained binding to immobilized EGFR (data not shown). Tissue sections were fixed in acetone and tissues were evaluated with different concentrations (ranging from 0.1 to 10 µg/mL) of biotinylated Sym004 or with 10 µg/mL of biotinylated chimeric IgG1 (anti-tetanus) as
negative control antibody. Biotinylated cetuximab (10 µg/mL) was included as a positive control. Binding was visualized with streptavidin-conjugated peroxidase and diaminobenzidine.

In vivo study designs

The EGFR-expressing cancer cell line A431NS was obtained from the American Type Culture Collection and maintained according to the supplier’s instructions. Female nude BALB/c mice (6-8 weeks old) were inoculated subcutaneously into the right flank with 10⁶ A431NS cancer cells as described in (7). When tumors reached a volume of ~200 mm³, mice with or without the EGFR-expressing A431NS tumor xenografts were administered a single i.p. injection of 50 mg/kg Sym004. To investigate target and non-target mediated clearance (CL) of Sym004, blood samples for pharmacokinetic analysis were drawn at the time points indicated in the results. Plasma levels of Sym004 were analyzed using a standard anti-human IgG enzyme-linked immunosorbent assay (ELISA).

Two safety studies in Cynomolgus monkeys (Macaca fascicularis) were conducted in accordance with the requirements of European Directive 2003/63/EC and all subsequent amendments, together with any relevant International Conference on Harmonization guidelines. Experimentally naïve male and female purpose-bred Cynomolgus monkeys were obtained from Bioculture Ltd (Mauritius). After an acclimation period, animals were approximately 2.2 to 3.7 years of age and ranged in weight from 2.3 to 4.3 kg at start of dosing. As a part of the daily clinical observations, special attention was paid to signs of dehydration and superficial infections of the areas of the skin affected by rashes. Appropriate treatment was provided as needed at the discretion of the responsible veterinary.
Investigations performed during the two repeat-dose toxicity studies described below included clinical observations, neurological assessments, body weight, food consumption, ophthalmoscopy, electrocardiography, blood pressure, hematology, clinical chemistry, immunogenicity, toxicokinetics, cytokine release, organ weight, and, at necropsy, macroscopic observations and microscopic histopathology. Unless otherwise stated, the results are reported as group mean values ± standard deviation (SD).

Six-week repeat-dose range finding study

For the 6-week repeat-dose range finding (DRF) study, female Cynomolgus monkeys were administered Sym004 once-weekly at doses of 0 (vehicle control) 12/8, 24/16, or 36/24 mg/kg by intravenous (i.v.) infusions for six consecutive weeks (first number indicates loading dose on study day 1; second number indicates maintenance dose levels administered from day 8 onwards). As a reference, cetuximab was administered once-weekly to animals at a single dose level of 24/16 mg/kg. All animals were dosed on day 1, day 8, day 15, day 22, day 29 and day 36, followed by necropsy on day 43. The number of animals in each group is indicated in Table 2. Serum samples for analysis of cytokines IL-2, IL-4, IL-5, IL-6, TNFα and IFNγ were subjected to flow cytometric analysis in 96-well filter plates using a commercially available kit (BD™ cytometric bead array non-human primate Th1/Th2 cytokine kit, Becton & Dickinson, UK) according to the manufacturer’s instructions. The pharmacokinetic profile of Sym004 and cetuximab after a single infusion (12 mg/kg) was evaluated in two satellite groups, each consisting of two male and two female animals.

Eight-week repeat-dose toxicity study with 4-week recovery
In the GLP-compliant 8-week repeat-dose toxicity study, monkeys were assigned to six groups (five animals/sex/group). Animals were administered eight once-weekly doses of 0 (control), 2, 7, or 14 mg/kg of the Sym004 antibody mixture. To mimic the concentration of the individual antibodies in the highest Sym004 dose group, eight once-weekly doses of 7 mg/kg mAb992 or 7 mg/kg mAb1024 were administered to separate groups of animals. Animals were dosed on day 1, day 8, day 15, day 22, day 29, day 36, day 43 and day 50. The test articles were given by short *i.v.* infusions of undiluted solutions following a 0.22 µm filtration step (Durapore® PVDF membrane). The control group was infused with the vehicle (10 mM sodium citrate, 150 mM NaCl) at a rate and volume corresponding to the highest dose level of Sym004. All main study animals (three animals/sex/group) were terminated on day 57 (one week after the 8th and last infusion). To assess the reversibility of any potential treatment-related effects, recovery animals (two animals/sex/group) were terminated at the completion of the 4-week recovery period (day 85), *i.e.* 35 days after the 8th and last infusion.

**Assay methods**

**Pharmacokinetics**

In the 6-week DRF study, blood samples for assessment of systemic exposure of Sym004 and cetuximab were collected from repeat dose animals by venipuncture. For each animal, peak serum levels were measured in samples collected 1 hour after 1st, 4th and 6th dosing, and through serum levels in samples collected 168 hours after 1st, 3rd and 6th dosing. In the single dose group animals (12 mg/kg of Sym004 or cetuximab), blood samples for pharmacokinetics were collected at 0 (pre-dose) 1, 2, 4, 8, 24, 48, 72, 120 and 168 hours post-dose, and days 10, 13, 16, 19 and 25 post-dose.
In the 8-week repeat-dose toxicity study, blood samples for pharmacokinetics were collected from all animals before dosing and 0.25, 4, 24, 72, 120 and 168 hours after the 1st (day 1), 4th (day 22) and 8th (day 50) dose occasion. In addition, pharmacokinetic samples were obtained during the treatment-free period from recovery animals on days 10, 13, 16, 19, 25 and 35 post-dose.

The concentration of the test articles Sym004, cetuximab, mAb992 and mAb1024 in serum samples from the Cynomolgus monkeys was measured using generic quantitative enzyme-linked immunosorbent assay (ELISA). Test article specific calibrators were used to generate Sym004, cetuximab, mAb992 and mAb1024 standard curves that were regressed according to an asymmetric 5-parameter model. The four different ELISAs were validated for their intended purpose according to current recommendations for validation of ligand binding assays for quantification of macromolecules in a biological matrix (8-11). In brief, serum samples, standards and quality controls were diluted within the calibration range 7.5 to 80 ng/mL (75 to 800 ng/mL in 100% serum) and captured on microplate wells pre-coated with monkey IgG-adsorbed polyclonal goat anti-human IgG (Bethyl Laboratories Inc. Montgomery, TX, US). After incubation and washing, the analytes captured were detected with horseradish-peroxidase conjugated monkey IgG-adsorbed, polyclonal goat anti-human IgG.

Non-compartmental pharmacokinetic parameters were estimated from the serum concentration-time profiles of the test articles derived from the individual monkeys in all dose groups using WinNonlin software (version 5, Pharsight Corporation, Mountain View, CA, US). Pharmacokinetic parameters in mice with or without xenografts were estimated from group mean plasma concentration-time profiles.
The relative distribution ratio of mAb992 and mAb1024 in serum after infusion of Sym004 to monkeys was determined by qualified cation-exchange (CIEX) analysis of selected toxicokinetic samples from the 8-week repeat-dose toxicity study. In brief, total IgG from the pharmacokinetic samples (4, 24, 72, 120 and 168 hours after infusion of Sym004) was purified on a protein-A matrix. Subsequently, the purified total serum IgG was analyzed on a weak CIEX column, where the two antibodies in Sym004 were separated into two distinct peaks according to differences in charge. The relative distribution (%) of mAb992 and mAb1024 in serum samples from animals infused with Sym004 was determined by integration of the two individual peak areas. Individual peak areas could not be evaluated in animals with serum levels of Sym004 <35 µg/mL.

Anti-drug antibodies

A double-antigen ELISA format was used to measure generation of anti-drug antibodies (ADA) in serum samples from the 8-week repeat-dose toxicity study. In brief, serum samples were diluted 10-fold in buffer and added to microtiter plates containing covalently attached Sym004 and biotinylated Sym004 in solution. ADA in serum samples, forming a bridge between Sym004 and biotinylated Sym004, was detected with horseradish-peroxidase conjugated streptavidin. Polyclonal rabbit anti-mAb992 and rabbit anti-mAb1024 mixed 1:1 (w/w) were used as positive controls. The method detected anti-Sym004, anti-mAb992 and anti-mAb1024, and was validated according to recent recommendations for the validation of immunoassays used for ADA detection (12). Blood samples for assessment of ADA were taken before dosing and 168 hours after the 3rd, 6th and 8th infusion of Sym004, mAb992, or mAb1024. In addition, blood samples were taken before the terminal sacrifice from all animals in the recovery group.
Results

*Tissue cross-reactivity studies*

Tissue cross-reactivity studies in different species (Table 1) showed similar staining patterns in tissues from human, Cynomolgus monkey and Rhesus monkey. Some staining was also observed in rabbit cerebellum, prostate and skin, whereas tissue panels from rat, mouse and guinea pig tested negative. Sym004 tissue cross-reactivity was subsequently assessed in 35 different tissues from three individual humans and three individual Cynomolgus monkeys (data not shown). In summary, Sym004 bound to tissue panels from humans and Cynomolgus monkeys with similar distribution patterns, frequency and intensity. Specific positive staining was observed in tissues of epithelial origin, endothelial tissues, neurological tissues, stromal tissues and interstitial cells, photoreceptor cells, muscle fibers, and smooth muscle and cells within lymphoid tissues. The reference mAb cetuximab showed a similar pattern of staining to Sym004 in both human and Cynomolgus tissues, suggesting that the staining detected is specific for EGFR. These results support the choice of Cynomolgus monkeys as single species for the nonclinical safety program for Sym004.

*Pharmacokinetics and anti-drug antibodies*

Pharmacokinetics after a single intraperitoneal injection of Sym004 at 50 mg/kg was investigated in nude BALB/c mice with and without EGFR-expressing A431NS tumor xenografts. In mice with EGFR-expressing xenografts, the terminal half-life ($T_{1/2}$) of Sym004 was shorter, the clearance (CL) was faster and area under curve ($\text{AUC}_{0-\infty}$) was smaller compared with non-tumor bearing mice (Figure 1A).
Single dose pharmacokinetics were further investigated in Cynomolgus monkeys, where groups consisting of two male and two female monkeys received a single i.v. infusion of 12 mg/kg Sym004 or cetuximab. The serum concentration versus time curves of Sym004 and cetuximab both displayed multi-phase elimination profiles with no apparent gender differences (Figure 1B). Following a rapid distribution phase lasting about 24 hours, the serum concentration profiles were characterized by three elimination phases, named phase 2, 3 and 4 (Figure 1B, table insert). Phase 3 had a steeper elimination slope than phase 2 and phase 4, indicating that clearance of Sym004 and cetuximab accelerated when serum concentrations fell below ~50 µg/mL. The onset of the accelerated clearance phase (phase 3) occurred earlier with Sym004 than with cetuximab. This observation could be explained by a faster EGFR internalization rate following Sym004 administration, as previously described for in vitro experiments (5). Maximum serum concentration (C\text{max}), AUC\text{0-600h} and CL were similar for Sym004 and cetuximab.

To evaluate the systemic exposure of Sym004 after repeated dosing, we conducted a 6-week DRF study measuring peak (1 hour after dosing) and trough serum levels (168 hours after dosing) after once-weekly administration of Sym004 or cetuximab. All animals had measurable serum concentrations of Sym004 or cetuximab throughout the study. The exposure to Sym004 increased almost proportionally to dose, with animals receiving an equal dose of Sym004 or cetuximab (24/16 mg/kg) having almost identical peak and trough serum levels (Figure 1C and Figure 1D). Serum trough levels did not increase in any of the dose groups during the course of the study, indicating that at the dose levels tested, Sym004 and cetuximab do not accumulate after once-weekly dosing for up to 6 weeks.
We investigated the toxicokinetics of Sym004 and the individual antibodies mAb992 and mAb1024 as a part of the 8-week GLP-compliant repeat-dose study. Monkeys received once-weekly *i.v.* infusions of the individual antibodies at 7 mg/kg or three different doses (2, 7 or 14 mg/kg) of Sym004.

Sym004 binding antibodies (ADA) were detected from day 22 onwards. During the course of the study, ADA were detected in 10/10, 6/10 and 4/10 of the animals dosed with Sym004 at 2, 7 and 14 mg/kg, respectively. In each of the groups administered mAb992 or mAb1024 individually, ADA were detected in 1/10 animals. The majority of the ADA-positive animals showed reduced exposure in the terminal elimination phases, which is likely to be attributable to increased clearance of Sym004. Despite the increased clearance, animals in the 7 and 14 mg/kg Sym004 dose groups were still exposed to pharmacologically active drug levels throughout the entire study. In the low (2 mg/kg) dose group, however, exposure was clearly demonstrated up to at least day 22, but was substantially reduced on day 50.

The pharmacokinetic parameters in ADA-negative animals were determined by non-compartmental analysis of serum concentration-time data obtained after the 1st (day 1), 4th (day 22) and 8th (day 50) infusion of the test articles, and during the recovery period (Figure 2). In accordance with the single dose pharmacokinetic profile of Sym004 described above, the serum elimination curves were multi-phase, showing increased inclination when serum levels of Sym004 fell below concentrations of ~50 µg/mL. This observation was evident for the 2 and 7 mg/kg Sym004 dose groups within the sampling frame of 0.25 to 168 hours after the 1st, 4th and 8th infusion. After infusion of Sym004 at 14 mg/kg, the serum concentrations declined in a monophasic manner, which could indicate saturation of EGFR-mediated clearance. In the treatment-free recovery period, serum levels of Sym004 rapidly declined below 10 µg/mL in the
7 or 14 mg/kg dose groups. In the 2 mg/kg dose group, Sym004 was not detectable from 240 hours post-dose onwards (Figure 2D). Serum concentrations of the individual antibodies both declined in a monophasic manner during the 8-week dosing period (Figure 2 A-C). However, an increased clearance rate was observed for mAb1024 during the recovery period (Figure 2D).

C_max of Sym004 increased in a dose-proportional manner, while AUC_0-168h of Sym004 increased more than proportionally to dose. Within the Sym004 dose groups, the mean elimination half-life (T_1/2) was 19.0 ±1.3, 18.0 ±2.4, and 65.7 ±11.1 hours in the 2, 7 and 14 mg/kg dose groups, respectively. During the course of the study, there was no tendency towards an increase in T_1/2, C_max and AUC_0-168h, confirming that repeated dosing did not cause Sym004 to accumulate in the monkeys. In contrast, repeated dosing with 7 mg/kg of either of the individual antibodies, mAb992 or mAb1024, resulted in a 2-3 fold increase in AUC_0-168h at day 50 compared with day 1. The increase in AUC_0-168h was most pronounced for mAb992, consistent with a 3-fold prolonged elimination half-life at day 50 (236 ±40 hours) compared with day 1 (83 ±12 hours).

Since Sym004 comprises a 1:1 mixture of two different mAbs, we investigated whether the individual antibodies displayed different pharmacokinetic profiles after administration of Sym004 to monkeys. The relative distribution ratio in serum after the 8th dosage of Sym004 (7 or 14 mg/kg) was determined by CIEX chromatography in order to qualitatively resolve the presence of both antibodies. After the 8th weekly infusion of Sym004, mAb992 and mAb1024 were both present in serum at close to a 1:1 ratio (Figure 3), and there was no evidence of selective interdependent clearance or accumulation of either of these individual antibodies.

**Safety evaluation in Cynomolgus monkeys**
Clinical in-life observations

There were no decedents, moribund behavior or prescheduled necropsies during the entire course of the *in vivo* studies. None of the test articles induced effects on local tolerance as evaluated by enhanced clinical observation of sites of injection. The most pronounced treatment-related clinical signs (rash, dehydration and liquid feces) are summarized in Table 2. Skin rashes, in the inguinal areas, lower abdomen and axillar areas, were a main feature of treatment and generally presented after 1-3 weekly doses of Sym004. The onset and severity of the rashes followed a dose-related trend and, in the case of the highest Sym004 dose (36/24 mg/kg), blistering rash on the head was observed following the 6th dose occasion. In many instances, the initial ‘dry’ rash became moist or wet and more reddened during the course of the studies, a condition described as ‘severe rash’ in Table 2. The most likely cause was the occurrence of superficial infection on the affected skin, which was effectively treated with topical application of antibiotic/steroid cream (Fuciderm®). In animals dosed with Sym004, the initial signs of rash generally persisted throughout the remaining dose occasions. In contrast, rash in the cetuximab group was of minor severity, transient, and only recorded in one animal (Table 2A).

In the 8-week repeat-dose study, rash was recorded in 8/10 animals dosed with Sym004 at 14 mg/kg, and four of these animals required treatment for severe rash (Table 2B). Three animals developed rash after dosing with 7 mg/kg Sym004 or with 7 mg/kg mAb992, while only one animal developed rash in the mAb1024 group. Compared with the rash in animals treated with Sym004 at 7 or 14 mg/kg, the rash in animals treated with the individual antibodies was less severe and was only transiently observed during the course of the study. The higher incidence of rash noted in animals treated with mAb992 than in animals treated with mAb1024 is in
agreement with previous in vitro pharmacological studies, in which mAb992 was observed to be more efficient in inhibiting tumor cell growth than mAb1024 (7). All but one case of rash disappeared after cessation of dosing, as did all other treatment-related clinical signs, such as dehydration and liquid feces.

**Clinical pathology parameters**

Food consumption, urine analysis, ophthalmology, cytokines and hematology parameters were generally unaffected by treatment. One exception was that all three animals given 36/24 mg/kg Sym004 in the DRF study had increased neutrophil counts on study day 22, which coincided with superficial infections of rashes. These infections were treated with Fuciderm® and the neutrophil counts reverted to the pretreatment range by study day 43.

Globulin levels increased and albumin levels decreased in a time- and dose-dependent manner, causing a lowering of the albumin:globulin (AG) ratio in the animals treated with Sym004 or cetuximab. Sym004 had a more pronounced effect on the AG ratio than cetuximab, whereas the individual antibodies were without effect. After cessation of dosing, the AG ratio returned to baseline levels at day 85. Although not statistically significant and subject to high individual variation, serum levels of gamma glutamyl transferase (γGT) on study day 43 (in DRF study) and study day 57 (in 8-week repeat-dose study) were increased (approximately 2-fold compared with the control and pretreatment baseline) in animals infused with 36/24, 24/16 or 14 mg/kg Sym004. The increase in γGT returned to pretreatment levels at the end of the recovery period. There were no other obvious treatment-related changes in liver parameters or other clinical chemistry data.

**Anatomic pathology**
Weight loss was recorded in monkeys given Sym004 or cetuximab, whereas control animals and those given the individual antibodies gained weight during the study (Figure 4A and B). During the treatment-free recovery period, all animals gained weight (Figure 4C), indicating that the weight loss is reversible. After euthanasia by exsanguination, all animals had a complete necropsy evaluation including full \textit{in situ} examination of tissues and organs, as well as recording of organ weight. There were no macroscopic findings due to local or systemic effects of Sym004, cetuximab, mAb992 or mAb1024. Absolute (data not shown) and relative kidney weights in Sym004 treated animals were higher than in the control, cetuximab, mAb992 or mAb1024 treated animals (Figure 4D and E). At the main necropsy, relative kidney weight was increased by up to 43% and 33% (36/24 and 14 mg/kg groups, respectively) compared with the control. In recovery animals, no increase in absolute or relative kidney weight was observed compared with the control, indicating reversibility of the effect of Sym004 on kidneys (Figure 4F). In the 6-week DRF study, tubular nephropathy and dilation were observed in animals given 24/16 and 36/24 mg/kg Sym004 or 24/16 mg/kg cetuximab (Table 2A). This constituted dose-limiting clinical signs guiding the choice of the reduced Sym004 doses used in the 8-week repeat-dose study. Furthermore, a loading dose was not included, since this was not scheduled for the planned clinical trial. In the 8-week repeat-dose study, repeat dosing of Sym004 at 2-14 mg/kg did not result in any pathological findings in kidneys (Table 2B). There were no other obvious changes in organ weight or microscopic findings suggesting local or systemic effects of Sym004, mAb992 or mAb1024.

\textit{Safety pharmacology}
No appreciable treatment-related effects were observed for evaluated cardiovascular (ECG, blood pressure) or CNS parameters (neurological assessments). Behavioral, autonomic and neurological parameters were normal in all animals. However, in animals given 24/16 mg/kg Sym004, 24/16 mg/kg cetuximab or 14 mg/kg Sym004, intermittent and transient tremors were noted, along with itchy/scratching behavior, 4-5 hours after dosing on a number of occasions. No indication of increased cytokine levels was observed and a potential reason for the observed tremors remains unclear.
Discussion

EGFR is a validated target for cancer therapy and currently two anti-EGFR mAbs, panitumunab and cetuximab, are being marketed for the treatment of CRC and/or SCCHN. The first-in-class anti-EGFR antibody 1:1 mixture Sym004 comprising the two chimeric antibodies mAb992 and mAb1024 as one drug product may offer more complete target inhibition, thereby improving the effectiveness of anti-cancer treatment and addressing refractory tumors.

The present nonclinical safety program was designed to support first in man trials with Sym004 and assess the pharmacokinetic and safety profile in non-human primates. In addition, we tested mAb992 and mAb1024 at a single dose level to address any putative differential toxicity or pharmacokinetics of the individual antibodies when administered separately.

Sym004 has been shown to efficiently induce EGFR internalization and subsequent degradation in a range of cancer cell lines of different tissue origins (7). When we administered Sym004 to mice with a human tumor xenograft expressing high levels of EGFR, systemic clearance of Sym004 was significantly increased, suggesting the presence of a target mediated elimination pathway in the tumor bearing mice. Although a hypermetabolic state in the xenografted mice may have contributed to the increased serum clearance, we speculate that the EGFR expressing tumor acts as an antigen sink, effectively removing Sym004 from circulation by EGFR mediated binding, internalization and degradation. This hypothesis is supported by previous reported ex vivo studies of A431NS xenograft tumors, where it was found that tumors in the mice were completely devoid of EGFR after 3 weeks of twice weekly injections with Sym004 (7).
The Sym004 mAbs both bind to Cynomolgus EGFR and the staining pattern of Sym004 in Cynomolgus monkey tissues resembled that of human tissue. Therefore, this species was selected for the repeat dose toxicity studies. Repeat dosing of Sym004 to Cynomolgus monkeys resulted in generation of ADA. In the majority of the ADA-positive animals, increased clearance of Sym004 was observed, but all animals in the intermediate (7 mg/kg) and high (14 mg/kg) dose groups were still exposed to pharmacologically active drug levels throughout the entire study. High frequency of ADA has also been reported in monkey studies with the fully human mAb panitumumab (13). However, preclinical immunogenicity is not predictive of immunogenicity in humans (14). The inverse dose relationship in frequency of detected ADA responses may be due to drug interference in the ADA assay. This could also explain the apparent low frequency of detectable ADA in animals dosed with the individual antibodies because, when administered separately, the individual antibodies displayed a long T$_{1/2}$. Consequently, high trough levels were present in the corresponding ADA samples.

In Cynomolgus monkeys, Sym004 clearance accelerated when serum concentrations fell below $\sim$50 µg/mL, a finding of anti-EGFR antibody pharmacokinetics that has been described previously (15). It is assumed that elimination of anti-EGFR antibodies in Cynomolgus monkeys and humans involves a specific, saturable elimination process, i.e. target-mediated clearance via EGFR internalization in parallel to a non-specific, and at therapeutic antibody concentrations non-saturable elimination process (16, 17). At non-saturating Sym004 serum concentrations, rapid clearance via EGFR internalization predominated, resulting in an elimination phase characterized by a steep slope in the serum concentration versus time curve. In direct comparisons with cetuximab, this phase occurred earlier for Sym004, which may be due to a more efficient EGFR internalization process for Sym004 than for cetuximab, as demonstrated in
Efficient EGFR-mediated clearance of Sym004 from the circulation may explain why Sym004 did not accumulate following repeat dosing. In accordance with the less efficient EGFR internalization mediated by the individual antibodies (7), systemic accumulation of mAb992 and mAb1024 was observed when these were administered separately to Cynomolgus monkeys. Importantly, no evidence of selective interdependent clearance or accumulation of mAb992 or mAb1024 was noted following repeated dosing with Sym004.

We speculate that the efficient clearance of Sym004 compared to the individual antibodies is mainly due to rapid EGFR mediated internalization. Antibody-mediated receptor internalization is a key mechanism for abrogating receptor activation (18), which may explain the increased frequency and severity of rash in animals treated with Sym004. EGFR is primarily expressed in cells of epithelial origin, such as skin and gastrointestinal tract, and is crucial for normal development of these tissues (19, 20). The most commonly observed toxic effects from inhibition of EGFR activity by monoclonal antibodies is rash and diarrhea (19), and an association of rash with the therapeutic efficacy of cetuximab in cancer patients has previously been reported (21, 22). Therefore, Sym004 may present an attractive and efficient therapeutic option for patients with EGFR-expressing cancers.

In the 6-week DRF study, cetuximab was included as a reference compound at a single dose level to assess whether the more potent and efficacious *in vitro* and *in vivo* pharmacological activity of Sym004 (7) would translate into a distinct safety profile in monkeys. Compared with cetuximab at equal dose levels, Sym004 did not induce any distinct or novel adverse findings but, importantly, there was an early onset of rash. The first observed incidences of rash were recorded after the first once-weekly dosing with Sym004 at 12/8 and 24/16 mg/kg. This is in
contrast to reports for cetuximab, where the first observed skin toxicities are reported to occur on study day 64 (12/7.5 mg/kg) and study day 22 (38/24 mg/kg) according to data from a 39-week repeat dose study described in the FDA pharmacological review of cetuximab (16).

The dose-limiting clinical signs of tubular nephropathy and tubular dilation in the kidneys observed in the 6-week DRF study after administration of high doses of cetuximab or Sym004 (Table 2A) were not unexpected, since EGFR is expressed in the kidney tubules, mesangial cells and parietal epithelial cells, and is involved in maintaining tubular integrity (23). An anti-EGFR mediated effect on kidneys has previously been reported for cetuximab (16).

The unique mechanism of action of Sym004, and the resulting potent pharmacological activity, is dependent on the presence of both antibodies, as shown in both in vitro and in vivo studies (7). We therefore hypothesized that the full toxicity of Sym004 in monkeys would only manifest itself when both mAb992 and mAb1024 were present. This was confirmed in the 8-week repeat dose study. In spite of accumulation of both individual antibodies, when administered separately, no or only minimal effects were observed after eight once-weekly dose occasions with the individual antibodies. This effect appeared to be higher for mAb992 than for mAb1024, which is in accordance with in vitro pharmacological findings, where it was shown that mAb992 is superior to mAb1024 in ability of blocking ligand binding (7). The present study showed that co-administration of mAb992 and mAb1024, as one drug product, i.e. Sym004, resulted in a strongly enhanced anti-EGFR mediated activity. Treating cancer with two antibodies targeting different epitopes on the same receptor is also being developed by others. A strongly enhanced antitumor activity was observed in xenograft models by combining the two independently developed anti-HER-2 mAbs, trastuzumab and pertuzumab (24). Combined
administration of trastuzumab and pertuzumab, are currently being tested in late stage clinic
studies in metastatic breast cancer patients (25).

In summary, the safety studies on Sym004 and the individual antibodies constituting Sym004 reported here showed that Sym004 possesses an anticipated and enhanced anti-EGFR mediated activity. The safety studies, in conjunction with the previously reported \textit{in vitro} and \textit{in vivo} pharmacological characterization (7), enabled initiation of the ongoing clinical Phase I/II development of Sym004 as a fixed combination therapy. This strategy was found to be in agreement with the subsequently issued draft FDA guidance for industry regarding codevelopment of two or more unmarketed investigational drugs for use in combination (26).
FIGURE LEGENDS

Figure 1. Pharmacokinetics of Sym004 and cetuximab. A) Plasma levels (mean+SD, N=10) after a single i.p. injection of Sym004 at 50 mg/kg in female nude BALB/c mice with (○) and without (●) EGFR-expressing A431NS tumor xenografts. Insert: Pharmacokinetic parameters estimated from group mean plasma concentration-time profiles. B) Single dose pharmacokinetics in Cynomolgus monkeys. Mean+SD serum levels of Sym004 (filled symbols) or cetuximab (open symbols) as a function of time (0-600 hours) after single i.v. doses at 12 mg/kg in female (●, ○) or male (■, □) monkeys. Insert: PK parameters, mean values with SD in brackets. C) Peak serum levels 1 hour after 1st, 4th and 6th once-weekly dosing; and D) trough serum levels 168 hours after 1st, 3rd and 6th once-weekly dosing with Sym004 or cetuximab. Bars represent mean serum levels+SD after dosing with the indicated dose levels. Note: Trough serum levels were determined to be 0.4 µg/mL 168 hours after the 3rd and 6th once-weekly dose occasion of Sym004 at 8 mg/kg (not seen on scale).

Figure 2. Repeat dose pharmacokinetics following once-weekly repeated dosing with Sym004 or the individual antibodies. Serum concentration-time course of Sym004, mAb992 and mAb1024 as a function of time (0-168 hours) after the 1st (A), 4th (B), and 8th (C) once-weekly i.v. dosing of Sym004 at 2 (●), 7 (▲), and 14 (■) mg/kg, or after dosing with the individual antibodies mAb992 (∇) or mAb1024 (Δ) at 7 mg/kg. D) Serum concentration-time course of Sym004, mAb992 and mAb1024 as a function of time (240-840 hours) during the 4-week recovery period. All values represent mean+SD.

Figure 3. Relative distribution in serum of mAb992 and mAb1024 after infusion of Sym004. Chromatographic analysis of pharmacokinetic samples was performed after the 8th (day 50) once-weekly infusion of Sym004 at A) 7 mg/kg or B) 14 mg/kg. All values represent mean±SD of the relative distribution (%) of the individual antibodies in serum from the indicated number (N) of animals with Sym004 serum levels above 35 µg/mL.
Figure 4. Body weight gain and kidney weight relative to body weight at necropsy. Left panel: 6-week DRF study, group mean body weight gain (A) and relative kidney weights (D) at necropsy (study day 43) after six once-weekly infusions of vehicle (control), Sym004 or cetuximab at the indicated dose levels. Centre panel: 8-week repeat-dose toxicity study, group mean body weight gain (B) and relative kidney weight (E) at necropsy of main animals (study day 57) after eight once-weekly infusions of vehicle (control), Sym004, mAb992 or mAb1024 at the indicated dose levels. Right panel: Recovery animals in 8-week repeat-dose toxicity study, group mean body weight gain (C) and relative kidney weight (F) 35 days after the 8th once-weekly infusion of vehicle (control), Sym004, mAb992 or Mab1024 at the indicated dose levels.*= p<0.05, ** = p<0.01, *** = p<0.001 compared with the control group (one-way ANOVA with Dunnet’s multiple comparison test).
References


13. FDA/Center for Drug Evaluation and Research: Vectibix Pharmacological Reviews. URL: http://www.accessdata.fda.gov/drugsatfda_docs/nda/2006/125147s0000TOC.


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<th>Heart</th>
<th>Prostate</th>
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1) The reference monoclonal antibody cetuximab displayed a similar staining pattern to Sym004 in the tissues listed.
Table 2. Summary of clinical observations.

A) Clinical observations in the 6-week, once-weekly repeat-dose DRF study

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<td>0</td>
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<tr>
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<tr>
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<tr>
<td>Tubular nephropathy</td>
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<td>3</td>
</tr>
<tr>
<td>Tubular dilation</td>
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1) First number indicates loading dose on Day 1; second number indicates maintenance dose level administered from Day 8 onwards

B) Clinical observations in the 8-week, once-weekly repeat-dose toxicity study

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<tr>
<th>mg/kg/dose</th>
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<th>mAb992 7</th>
<th>mAb1024 7</th>
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Figure 1

A

A431NS Xenograft: No Yes
Cmax (µg/mL) 494 409
AUC0-600 (h·µg/mL) 175748 56272
CL (mL/h/kg) 0.28 0.89
T½ (h) 339 67

B

Test article: Sym004 Cetuximab
Cmax (µg/mL) 309 (29) 273 (36)
AUC0-600 (h·µg/mL) 13727 (878) 14051 (1603)
CL (mL/h/kg) 0.88 (0.05) 0.86 (0.10)
T½ (h) Phase 2 49 (6) 61 (10)
T½ (h) Phase 3 16 (2) 19 (1)
T½ (h) Phase 4 299 (193) 121 (10)
Figure 2

(A) Day 1: 1st dosing
(B) Day 22: 4th dosing
(C) Day 50: 8th dosing
(D) Day 57-85: Recovery

Serum levels (µg/mL) vs. Time (hours)
Figure 3

A

N=8  N=7  N=5

Relative distribution (%)

Hours post infusion

B

N=6  N=6  N=6  N=4

Relative distribution (%)

Hours post infusion

N=6  N=6  N=6  N=4

N=5

N=7

N=8

mAb992

mAb1024
Figure 4
Preclinical pharmacokinetics and safety of Sym004: A synergistic antibody mixture directed against epidermal growth factor receptor


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