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In Vivo Kinetics and Biodistribution of a HIV-1 DNA Vaccine after Administration in Mice

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In this study we have investigated the pharmacokinetics and tissue distribution of GX-12, a multiple plasmid DNA vaccine for the treatment of HIV-1 infection. Plasmid DNA was rapidly degraded in blood with a half-life of 1.34 min and was no longer detectable at 90 min after intravenous injection in mice. After intramuscular injection, plasmid DNA concentration in the injection site rapidly declined to less than 1% of the initial concentration by 90 min post-injection. However, sub-picogram levels (per mg tissue) were occasionally detected for several days after injection. The relative proportions of the individual plasmids of GX-12 remained relatively constant at the injection site until 90 min post-injection. The concentration of plasmid DNA in tissues other than the injection site peaked at 90 min post-injection and decreased to undetectable levels at 8 h post-injection. The rapid *in vivo* degradation of GX-12 and absence of persistence in non-target tissues suggest that the risk of potential gene-related toxicities by GX-12 administration, such as expression in non-target tissues, insertional mutagenesis and germline transmission, is minimal.

Key words: GX-12, Plasmid DNA vaccine, HIV-1, Pharmacokinetics, Biodistribution

INTRODUCTION

Two decades after of the first clinical reports of acquired immunodeficiency syndrome (AIDS) were published, the cumulative number of worldwide HIV infections has exceeded 60 million with the estimated number of mortalities due to AIDS reaching 13 million (UNAIDS, 2001). Unfortunately, a cure of the viral disease has yet to be realized despite the active researches currently on-going in the field.

Combination drug regimens using antiretroviral agents such as protease inhibitors and reverse transcriptase inhibitors are currently adopted for the treatment of HIV infection and AIDS. Highly active antiretroviral therapy (HAART) has enabled the reduction of plasma HIV titer to below detection limit, and its availability has led to a substantial decrease in AIDS-related mortalities in developed countries (Palella *et al.*, 1998). However, antiretroviral therapies are subject to several concerns such as acute and chronic toxicities (Yeni *et al.*, 2002), potential generation of resistant strains (Little *et al.*, 2002), inability of complete virus eradication (Finzi *et al.*, 1999) and a high cost which limits its applications in developing countries where the impacts of the AIDS epidemic are the most devastating (UNAIDS, 2001). In view of the clinical and economical limitations of antiretroviral therapy, it is generally perceived that the development of an effective and economical vaccine could be an attractive alternative for slowing down the AIDS epidemic (Gottlieb, 2001). Conventional vaccination approaches such as inactivated whole virus, attenuated virus or subunit vaccines have proven to be ineffective or pose an unacceptable risk to the person receiving the vaccine. A more recent and promising approach is DNA vaccination, which has several advantages such as the ability to induce both humoral and cellular immune responses, flexibility of design, safety, ease of manufacture, and suitability for prime-boost strategies in combination with viral vectors (Fomsgaard, 1999).

Genexine Co. and Dong-A Pharmaceutical Co. have

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jointly developed GX-12, a novel candidate vaccine for the treatment of HIV-1 infection and AIDS. GX-12 is a DNA vaccine that consists of four plasmid vectors encoding major (*env*, *pol*, *gag*) and accessory genes (*rev*, *vif*, *nef*, *tat*, *vpu*) of HIV-1 subtype B as well as a human interleukin-12 mutant (hIL-12m) gene. Due to the inclusion of multiple genes, the vaccine stimulates a broad immune response against various humoral and cellular epitopes, which is potentially advantageous in controlling viral immune escape. Expression of the IL-12 gene enhances the proliferation of peripheral T lymphocytes and HIV-1 specific CTL responses to increase the therapeutic efficacy of the vaccine (Ha *et al.*, 2002). Previously, a SIV vaccine analogue of GX-12 elicited protective immunity against SIV infection in monkey experiments, thus demonstrating the potential efficacy of the HIV vaccine candidate (Hunsmann, 2001).

A number of theoretical safety concerns that are not encountered in conventional pharmaceutical agents exists with DNA vaccines, including toxicity associated with the expression of the encoded genes, autoimmune diseases and the potential for chromosomal integration. To address these safety issues, the *in vivo* fate of the DNA compound has to be characterized in preclinical studies, usually by polymerase chain reaction (PCR) or related gene amplification techniques. In this study, we have investigated the *in vivo* kinetics and biodistribution of GX-12 using PCR. The study was conducted as part of the preclinical evaluation of GX-12 to support regulatory submission.

MATERIALS AND METHODS

Test substance

GX-12 consists of a mixture of four plasmids: pGX10-GE hx, encoding HIV-1 *gag* and *env*; pGX10-dpol jr encoding HIV-1 *pol*; pGX10-VN/TV jr encoding HIV-1 *vif*, *nef*, *tat* and *vpu*; and pGX10-hIL-12m encoding a human IL-12 mutant gene. The plasmids are formulated in 150 mM phosphate buffer, pH 7.0, each at a concentration of 0.5 mg/mL. The plasmid vectors were constructed by cloning the respective genes into the eukaryotic expression vector pGX10. The pGX10 backbone carries a prokaryotic origin of replication (ColE1), a bacterial kanamycin resistance gene and a eukaryotic expression cassette consisting of a human CMV promoter, adenovirus tripartite leader sequence, SV40 late polyA and SV40 enhancer. The vaccine was produced to clinical grade according to a proprietary process established at Dong-A Pharm. Co. Briefly, *E. coli* DH5α cell lines carrying the plasmids were grown in kanamycin-containing medium in a 15 L fermentor. The fermentation broth was subjected to a series of purification steps including alkaline lysis, PEG precipitation, anion exchange chromatography and gel filtration chromatography. The purified plasmids were mixed to the final composition and dialyzed against formulation buffer.

Intravenous administration of GX-12

GX-12 was administered to male ICR mice (Charles River Laboratories), 6 weeks of age, as a single injection of 50 μL volume (100 μL total plasmid DNA) via the tail vein. Control mice received a 50 μL injection of vehicle control (150 mM phosphate buffer, pH 7.0). Five mice were used per time point. Blood samples were drawn from the retro-orbital plexus at 1, 5, 15, 30, 45, 60, 90, 120 min and 8 h post-administration. The samples were immediately frozen by immersion in liquid nitrogen after addition of EDTA. The blood samples were stored at -75 °C until further analysis.

Intramuscular administration of GX-12

Single 50 μL doses of GX-12 (100 μL total plasmid DNA) were injected into the left femoral muscles of male ICR mice. Control mice received a 50 μL injection of vehicle control. Five mice were used per time point. At 5 min, 15 min, 30 min, 90 min, 120 min, 8 h, day 1, day 3, day 7, day 14 and day 30 mice were sacrificed and tissue samples (brain, heart, stomach, intestines, liver, lung, spleen, kidney and testis) were taken for subsequent analysis. The tissue samples were frozen immediately by immersion in liquid nitrogen and stored at -75°C until analysis. Blood samples were drawn from the retro-orbital plexus and rapidly frozen by immersion in liquid nitrogen after the addition of EDTA. The blood samples were stored at -75°C until further analysis.

Isolation of GX-12 from blood samples

Fifty μL of blood was diluted 20-fold in TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) and extracted with equal volumes of phenol/chloroform. After addition of 1 μL of salmon sperm DNA as a co-precipitation agent, the DNA was precipitated by the addition of 1 volume of 3 M sodium acetate and 2 volumes of ethanol. The precipitated DNA was separated by centrifuging at 15,000 rpm for 10 min and dissolved in nuclease-free water.

Isolation of GX-12 from tissue samples

About 50~200 mg of tissue was minced and suspended in 600 μL of resuspension buffer (50 mM Tris-HCl, pH 7.5, 10 mM EDTA, 100 μL/mL RNase A) and homogenized using a tissue homogenizer (Bellco). After addition of 600 μL of lysis buffer (0.2 M NaOH, 1% SDS) and 600 μL of neutralization buffer (1.32 M potassium acetate, pH 4.8) the cell debris was removed by centrifugation at 15,000 rpm for 10 min. The supernatant was extracted with equal volumes of phenol/chloroform. DNA was precipitated by adding 2 volumes of ethanol. After separation by centrifug-

ing at 15,000 rpm for 10 min, the DNA was dissolved in nuclease-free water.

Analysis of GX-12 by Polymerase chain reaction (PCR)

The DNA isolated from tissue and blood samples was subjected to PCR amplification of the kanamycin resistance gene, which is present in all four plasmids of GX-12. The primer sequence was 5'-GAAGGCGATGCGCTGCGAAT-3' and 5'-GTCAAGACCGACCT GTCCGG-3'. Reaction mixture containing Taq polymerase was prepared using the PCR Core System II kit (Promega, USA). PCR was carried out using a RTC-100 thermocycler (MJ Research Co., USA), with 35 cycles of denaturation at 95°C for 1 min, annealing at 52°C for 1 min and extension at 74°C for 1 min. Various dilutions of GX-12 were used as positive standards. The 624 bp amplicon was verified by 1% agarose gel electrophoresis and ethidium bromide staining. For quantification, densitometric analysis was carried out using BIO-PROFIL system and BIO-1D Image Analysis software (Vilber Lourmat, France). For each batch of DNA isolation and PCR analysis, a negative control using DNA isolated from control animal tissue and a positive control using DNA isolated from control animal tissue spiked with GX-12 was processed in parallel. Experimental results were considered valid only if the negative control gave a negative result and the positive control yielded the expected 624 bp PCR product.

The PCR analysis had a detection limit of 0.1~0.5 pg, with a log linear range of 0.005~10 ng. To determine the quantification range of the combined procedure of DNA isolation, PCR and densitometric analysis, tissue samples were spiked with 0.001~100 ng of GX-12. A recovery rate of 70~130% and an intra- and inter-assay CV of less than or equal to 30% was set as the criterion for the effective quantification range. The quantification limits of GX-12 in muscle and blood samples were determined to be 0.05 pg/mg tissue and 0.1 pg/mL respectively.

To determine the *in vivo* kinetics of the individual plasmids constituting GX-12, PCR using primers specific for the inserted genes was carried out. The primer sequences were 5'-AATCCTGGCCTGTTAGAAAC-3' and 5'-TATGTCACTTCCCCTTGGTT-3' for the amplification of pGX10-GE hx, 5'-TGGCCATTGACAGAAGAAAA-3' and 5'-TCAGGATGGAGTTCATAACC-3' for the amplification of pGX10-dpol jr, 5'-GGGGATGCTAGATTGGTAAT-3' and 5'-TCAGTGGAAATCTGACCCCT-3' for the amplification of pGX10-VN/TV jr, and 5'-GGCCATATGGGAACTGAAGA-3' and 5'-GAAGAAGCTGCTGGTGTAGA-3' for the amplification of pGX10-hIL-12m. PCR cycling conditions were 95°C, 1 min, 53°C, 1 min and 72°C, 1 min with 35 cycles total. The sizes of the PCR products were 570 bp for pGX10-GE hx, 641 bp for pGX10-dpol jr, 821 bp for pGX10-VN/TV jr and 503 bp for pGX10-hIL-12m. Agarose gel electrophoresis and densitometric analysis was carried out as described above.

Statistical analysis

Statistical analysis of data was performed using Student's t-test for comparison of differences between group mean values. A p value of less than 0.05 was considered significant.

RESULTS

In vivo kinetics of GX-12 after intravenous administration

GX-12 was rapidly degraded in blood after intravenous administration (Fig. 1). Analysis of plasma concentrations of GX-12 after administration of 50 μL of GX-12 via the tail vein showed that less than 1% of the initial concentration remained at 30 min post-administration, and no GX-12 was detected at 60 min post-administration. The half-life of GX-12 in blood was 1.34 min and the area under the curve (AUC) was 102 $\mu g \cdot min \cdot mL^{-1}$. PCR analysis of the individual plasmids of GX-12 showed that the plasmids were degraded at similar rates, with the relative proportions of the plasmids ranging from 17 to 31% until 30 min post-administration (Fig. 2).

In vivo kinetics of GX-12 after intramuscular administration

GX-12 at the site of administration decreased rapidly, presumably due to degradation and mass transfer (Fig. 3). However, trace quantities of GX-12 remained at the administration site for a considerable time period. PCR analysis of GX-12 after intramuscular administration showed that at 30 min post-administration approximately 33% of the initial concentration was present, while less than 1%

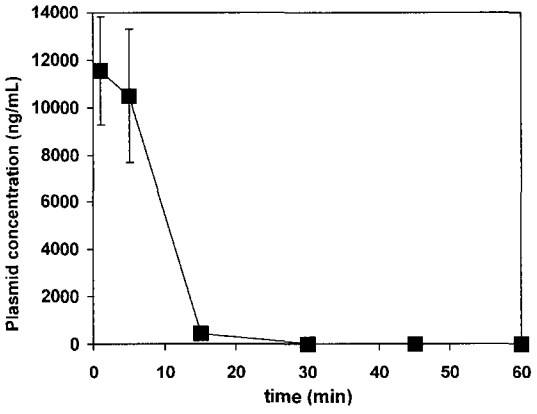

Fig. 1. Plasmid levels in blood following intravenous administration of 50 μL of GX-12 (100 μg of plasmid DNA) to mice. The data represent average levels ± SD from five mice per time-point.

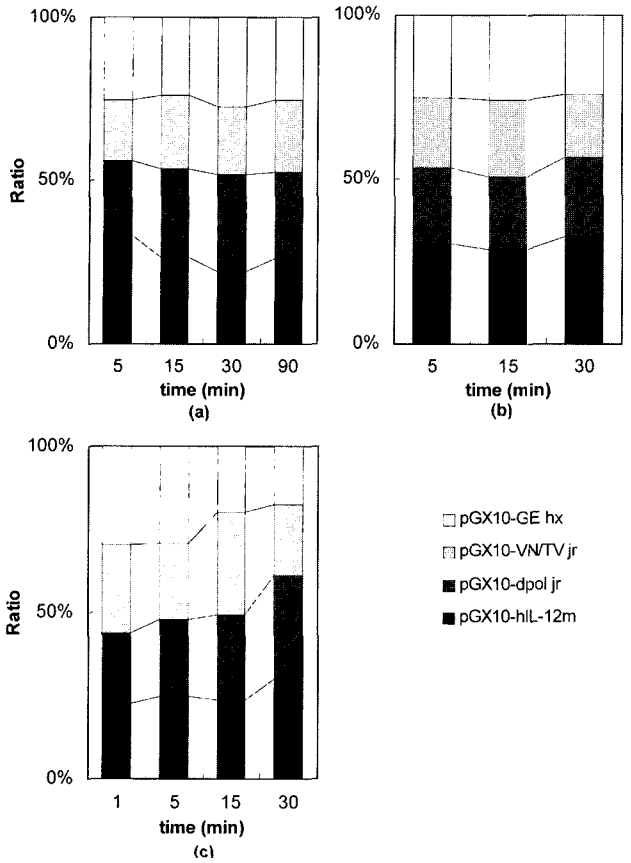

Fig. 2. Relative proportions of the four plasmids of GX-12 following administration of 50 μL of GX-12 (100 μg of plasmid DNA) to mice. (a) In muscle (injection site), after intramuscular injection; (b) in blood, after intramuscular injection; (c) in blood, after intravenous injection.

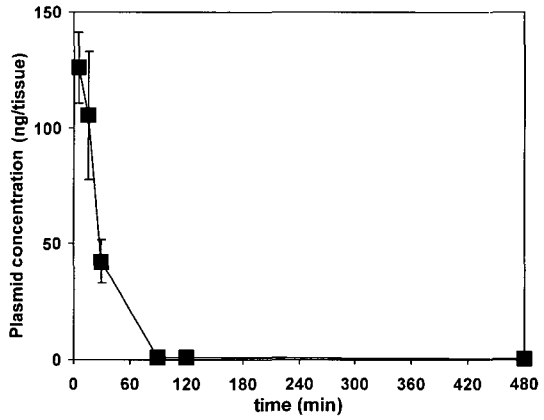

Fig. 3. Plasmid levels in muscle (injection site) following intramuscular administration of 50 μL of GX-12 (100 μg of plasmid DNA) to mice. The data represent average levels ± SD from five mice per time-point.

was remaining at 90 min. Sub-picogram (per mg tissue) quantities of GX-12 were occasionally detectable for several days after administration (Table I). In the extreme case, plasmid DNA below quantification limit was observed at day 14 in one animal. PCR analysis of the individual plasmids

Table I. Tissue distribution of plasmid DNA at 90 min following intramuscular administration of 50 μL of GX-12 (100 μg of plasmid DNA)[a]

Tissue	90 m	8 h	1 d	7 d	14 d	30 d
Liver	5/5	0/5	0/5	0/5	0/5	0/5
Brain	5/5	2/5	0/5	0/5	0/5	0/5
Kidney	5/5	0/5	0/4	0/5	0/5	0/5
Spleen	3/5	0/5	0/5	0/5	0/5	0/5
Heart	4/5	0/5	0/5	0/5	0/5	0/5
Testis	4/5	0/5	0/5	0/5	0/5	0/5
Lung	5/5	2/5	0/5	0/5	0/5	0/5
Muscle (injection site)	5/5	5/5	2/5	1/5	1/5	0/5

[a]Mice received 100 μg of GX-12 in a 50 μL volume as a single intramuscular injection. The mice were sacrificed at the indicated time points and total DNA was extracted from the tissues and analyzed by PCR as described in Materials and Methods. The data are presented as number of positive tissues/number of tissues analyzed.

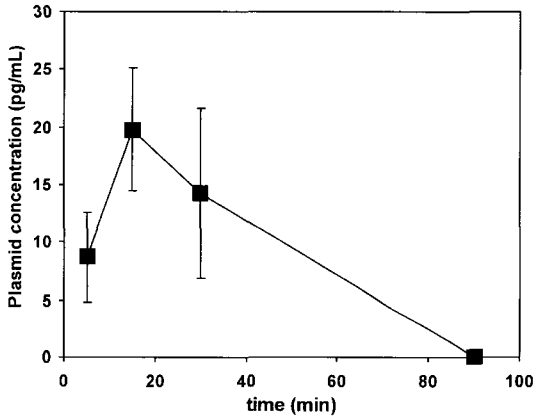

Fig. 4. Plasmid levels in blood following intramuscular administration of 50 μL of GX-12 (100 μg of plasmid DNA) to mice. The data represent average levels ± SD from five mice per time-point.

of GX-12 showed that the plasmids were present at relative proportions of 19-28% until 90 min post-administration (Fig. 2).

The plasma concentration of GX-12 after intramuscular administration showed a maximum of 19.8±5.4 pg/mL at 15 min and decreased thereafter (Fig. 4). At 90 min, plasmid was not detectable in the blood. The relative proportions of the four plasmids were in the range of 19-32% until 30 min post-administration (Fig. 2). The AUC of GX-12 was 825 pg·min·mL^{-1}.

Tissue distribution of GX-12 after intramuscular administration

GX-12 concentration in tissues other than the administration site peaked at 90 min post-administration, with picogram (per mg tissue) levels of plasmid DNA detected in the majority of the tissues investigated. However, plasmid

Table I. Plasmid DNA levels in various tissues at 90 min following intramuscular administration of 50 μL of GX-12 (100 μg of plasmid DNA)[a]

Tissue	Concentration (pg/mg tissue)
Liver	17.1 ± 3.9
Brain	0.4 ± 0.2
Kidney	1.9 ± 0.2
Spleen	0.8 ± 0.7
Heart	2.0 ± 1.6
Testis	1.9 ± 1.2
Lung	2.9 ± 0.6
Muscle (injection site)	1251.1 ± 1080.4

[a] Mice received 100 μg of GX-12 in a 50 μL volume as a single intramuscular injection. Total DNA was extracted from the tissues and analyzed by PCR as described in Materials and Methods. The data are presented as group average level standard deviation. For each data point, tissue samples from five animals were analyzed (n=5).

DNA in tissues decreased rapidly and was undetectable at 24 h post-administration (Table I). The highest concentration of plasmid DNA was detected in the liver at 90 min (17.1±3.9 pg/mg tissue), while concentrations in other organs were less than 3 pg/mg tissue (Table II). The plasmid DNA concentration in testis, which is of particular interest due to its implications in potential germline transmission, showed a maximum of 1.9±1.2 pg/mg tissue at 90 min but was undetectable thereafter.

DISCUSSION

As delineated in several guidelines concerning the safety aspects of gene transfer products (Food and Drug Administration, 1996; World Health Organization, 1998), investigation of the *in vivo* fate in animal models is regarded a mandatory requirement in the preclinical evaluation of gene therapy vectors and DNA vaccines. Thus, the present study was conducted to characterize the pharmacokinetics and tissue distribution of GX-12, a novel HIV/AIDS DNA vaccine candidate, after administration in mice using gene amplification by polymerase chain reaction (PCR).

GX-12 was rapidly degraded after intravenous administration with a half life of 1.34 min. In the case of intramuscular administration, less than 1% of the initial concentration remained at 90 min post-administration. The rapid degradation observed in this study is in accordance with previous reports on the pharmacokinetics of plasmid vectors. For example, Kawabata *et al.* (1995) observed over 99% degradation of naked DNA after 90 min of an intravenous administration, while Lew *et al.* (1995) reported a blood half life of less than 5 min and no detection after 60 min. For intramuscular administration, Manthorpe *et al* (1993) observed a >95% degradation of plasmid in the muscle at 90 min post-administration. Such a rapid degradation of plasmid *in vivo* is generally thought to be due to the action of extracellular nuclease. In spite of the instability of GX-12 *in vivo*, the short residence time seems to be sufficient for effective cellular uptake and expression. This assumption is supported by efficacy studies conducted in mice and macaques in which the administration of GX-12 analogues induced immunological responses (Hunsmann, 2001; Lee *et al.*, 1999).

Although the reported values for the *in vivo* half lifes of plasmid DNA vary widely in different studies, the concentrations of the four plasmids of GX-12 were observed to exist at comparable proportions during the time course of *in vivo* degradation until most of the plasmid DNA was degraded (Fig. 2) in the current study. The fairly constant proportions of the four different vectors may reflect similar degradation rates of the structurally related plasmids being subjected to identical experimental conditions (i.e. animal species, administration route, formulation etc.). An implication of these results is that all four plasmids should be effectively available during the critical time frame for cellular uptake and biological action after clinical administration.

GX-12 was observed to be distributed in all organs investigated after intramuscular administration. However, at the highest levels the concentrations of plasmid DNA in these tissues were still 2-3 orders of magnitude lower than in the injection site. Furthermore, plasmid transferred from the injection site to organs was rapidly degraded. No plasmid DNA was detected in non-target tissues after 8 h post-administration, in contrast to the injection site where plasmid DNA was detected for up to two weeks. The short persistence in non-target tissues observed in this study is consistent with other biodistribution studies in which plasmid DNA was detected in most tissues until 24 h post-administration but long-term persistence (up to several months) was observed only at the injection site (Lew *et al.*, 1995; Parker *et al.*, 1999; Winegar *et al.*, 1996; Manam *et al.*, 2000). Considering the high degradation rate of plasmid DNA in blood and organs and the fact that the administration dose of 100 μg DNA used in this study corresponds to an approximately 50-fold higher dose (per bodyweight) than expected in clinical applications (4 mg DNA/60 kg), it is thought to be highly unlikely that significant amounts of plasmid DNA will reach non-target organs via blood circulation or persist in these organs in actual clinical situations. Thus, the risk of adverse events such as insertional mutagenesis or germline transmission is considered to be negligible.

The conclusions of this study are complementary by two separate studies in which genomic integration and mRNA production in the administration site or gonads

were not detectable by PCR or RT-PCR (Dong-A Pharm., published results). The combined results of these studies and the present work advocate the genetic safety of GX-12 required for the initiation of human clinical trials.

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