Journal of Pharmaceutical and Biomedical Analysis 75 (2013) 33–40

Contents lists available at SciVerse ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Development of generic immunoassay for the detection of a series of aminoglycosides with 6′-OH group for the treatment of genetic diseases in biological samples

Moran Shalev[a], Jeyakumar Kandasamy[a], Nir Skalka[b], Valery Belakhov[a], Rina Rosin-Arbesfeld[b], Timor Baasov[a,*]

[a] The Edith and Joseph Fischer Enzyme Inhibitors Laboratory, Schulich Faculty of Chemistry, Technion – Israel Institute of Technology, Haifa 32000, Israel
[b] Department of Anatomy and Anthropology, Sackler School of Medicine, Tel Aviv University, Tel Aviv, Israel

ARTICLE INFO

Article history:
Received 14 August 2012
Received in revised form 1 November 2012
Accepted 9 November 2012
Available online 20 November 2012

Keywords:
Aminoglycosides
Genetic diseases
Nonsense mutations
Enzyme linked immunosorbent assay
Generic antibody
Residue monitoring

ABSTRACT

Over the last two decades, a growing number of scientific evidences highlighted the potential therapeutic value of several structures of aminoglycoside antibiotics (including gentamicin and G418) for the treatment of various genetic diseases caused by nonsense mutations. These findings resulted in a fast evolvement of synthetic derivatives of aminoglycosides which were shown to be more target specific and less toxic than the clinically used antibiotics. The emerging progress in drug design and development has necessitated the urge to develop a fast, easy and accurate procedure for the determination of these potential therapeutic agents in various biologically derived matrices. Here we describe the preparation of a generic polyclonal antibody that was used for the development of homologous and heterologous immunoassays for the detection of a wide range of natural and synthetic aminoglycoside derivatives, highlighted today as potential therapeutic agents for the treatment of various genetic diseases. A common two-ring scaffold, NB82, present in the majority of compounds exhibiting potent biological activity, was used as a generic immunization hapten for the immunization of two rabbits. By using a series of chemical steps, NB82 was selectively conjugated *via* the N-1 position through glutaric acid linker to a carrier protein. Sensitivity (I_{50}) values for the recognition of three representative compounds NB82, NB84 and NB124 were determined to be 10 ± 3 ng mL^{-1}, 0.5 ± 0.04 μg mL^{-1} and 1 ± 0.12 μg mL^{-1}, respectively. Limits of detection were determined to be 1 ± 0.3 ng mL^{-1} for NB82, 20 ± 7 ng mL^{-1} for NB84 and 15 ± 8 ng mL^{-1} for NB124. The developed assays were further exploited for the *in vivo* monitoring of the therapeutic compounds in mice serum. Serum experimentations exhibited similar detection limits as observed for the PBS calibration experiments, demonstrating no interference with assays sensitivity, with rather high recovery ratios ranging from 92 to 107% in whole blood samples.

1. Introduction

Aminoglycosides are highly potent antibacterial agents, which are known to exert their deleterious effects on bacterial cells by interfering with the translation process [1,2]. Over the last 20 years, a few natural aminoglycosides have been highlighted as promising therapeutic agents for the treatment of several genetic disorders caused by nonsense mutations [3–5]. These mutations generally lead to the production of truncated, non-functional proteins. In human, these mutations have been linked to nearly 2000 genetic disorders such as cystic fibrosis (CF), Duchenne muscular dystrophy (DMD), ataxia-telangiectasia, Hurler syndrome, hemophilia A, hemophilia B and Tay-Sachs [6,7]. For many of these diseases there is no effective treatment. Aminoglycoside antibiotics including gentamicin, paromomycin and G418 (Fig. 1) have been shown to suppress premature termination codons and partially restore

Abbreviations: 2-DOS, 2-deoxy-streptamine; AHB, (S)-4-amino-2-hydroxybutanoyl; CB, carbonate buffer; CF, cystic fibrosis; CE, capillary electrophoresis; DCC, N′,N′-dicyclohexylcarbodiimide; DDW, double distilled water; DMD, Duchenne muscular dystrophy; DMF, dimethylformamide; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; FIA, fluoroimmunoassays; GAG, glycosaminoglycan; GC, gas chromatography; GTA, glutaraldehyde; HPLC, high-performance liquid chromatography; HRP, horseradish peroxidase; TLC, thin layer chromatography; KLH, Keyhole Limpet Hemocyanin; MPS I-H, mucopolysaccharidosis type-I-Hurler; NHS, Nhydroxysuccinimide; OVA, ovalbumin; PBS, phosphate-buffered saline; PBST, PBS containing Tween-20; PMe_3, trimethylphosphine; RIA, radioimmunochemical assays; S.E.M., standard error mean; THF, tetrahydrofuran; TMB, 3,3′,5,5′-tetramethyl benzidine.

* Corresponding author. Tel.: +972 4 829 2590; fax: +972 4 829 5703.
E-mail address: chtimor@techunix.technion.ac.il (T. Baasov).

0731-7085/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.jpba.2012.11.014

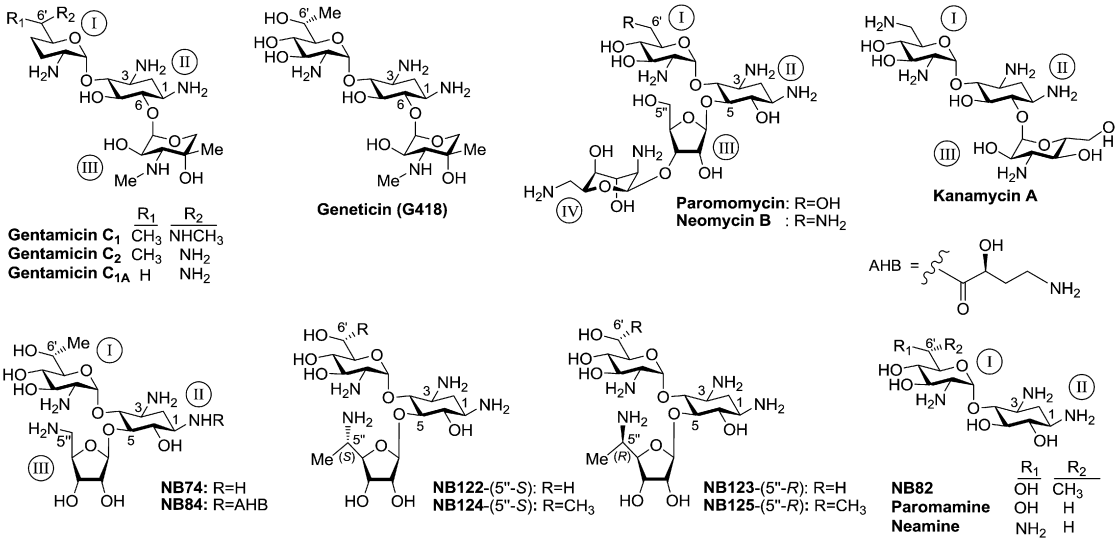

Fig. 1. Chemical structures of standard and semi-synthetic aminoglycoside derivatives used in this study.

functional protein production for more than twenty genetic diseases [8]. However, the potential toxicity associated with aminoglycosides has limited their clinical applications in suppression therapy. Over the last few years several synthetic derivatives of aminoglycosides were developed to overcome some of the enhanced toxicity issues. Many of these derivatives have been designed and synthesized in our lab [9–12]. Our most recent lead compounds of NB-series, including NB74, NB84, and NB122–NB125 (Fig. 1) demonstrated a remarkable biological activity altogether with reduced toxicity values in various *in vitro*, *ex vivo* and *in vivo* systems [10–14].

Further progress in drug development necessitated a fast and effective methodology for the detection and quantification of the developed lead NB-compounds in various biological derived matrices. Over the last few decades many analytical and bioanalytical assays have been suggested for the qualitative and quantitative analysis of aminoglycosides that are in clinical, veterinary and agricultural use to treat bacterial infections. These methods were applied for the detection of drug residues in a wide range of biological derived matrices such as cell tissues [15], serum [16], milk [17], eggs [15,18], and honey [18]. Chemical methodologies included the application of gas chromatography (GC) [19], thin layer chromatography (TLC) [20], high-performance liquid chromatography (HPLC) [21] and capillary electrophoresis (CE) [22]. Biological methodologies included the development of microbiological assays [23], radiochemical and radioimmunochemical assays (RIA) [24], enzyme linked- and fluoro-immunoassays (ELISA, FIA) [16,25,26], nano-sensors and nano-particle based immunoassays [27,28].

Immuno-based methodologies such as ELISA, FIA and RIA have shown to be as sensitive and accurate as the chemical methods [29]. These methods could be easily applied on a wide variety of biologically derived matrices, are simple to perform and analyze, and do not require the use of high cost instrumentations. Immuno-based methodologies are commercially available for the detection of some widely used natural aminoglycosides such as gentamicin, streptomycin, dihydrostreptomycin, neomycin and kanamycins (*e.g.* MaxSignal® gentamicin ELISA Test Kit, Aminoglycosides enzyme immune-assay-EIA kit for the detection of gentamicin, neomycin, streptomycin and dihydrostreptomycin – Europroxima, and kanamycins ELISA kit – Wanger). These commercially available kits can be exploited for highly sensitive detection of aminoglycosides in various matrices, and some of them are used in hospitals to monitor the serum levels of clinically used aminoglycosides such as gentamicin and amikacin. However, these assays are mostly used for the detection of aminoglycoside antibiotics that are based on a neamine core, containing an amino group at their 6′ position (ring I, Fig. 1). The use of the derived antibodies for the detection of therapeutic derivatives containing other substituents on the neamine core is quite limited due to low cross-reactivity values. Recent documentations demonstrated the superiority of 6′-hydroxyl containing aminoglycosides such as paromomycin and G418 over the 6′-amino derivatives in the treatment of various genetic disorders [4]. These derivatives are mostly based on either a paromamine or a 6′-(R)-methyl-paromamine (NB82) 2-ring core (Fig. 1) and are extensively explored for the treatment of various genetic diseases [3,9–14]. To our knowledge, no immunoassays exist for the detection of aminoglycosides containing a 6′-hydroxyl group.

The aim of the present study was to generate a generic antibody that will be able to recognize a wide range of potential therapeutic members containing a 6′-hydroxyl group. For this purpose, the 2-ring scaffold NB82 designed in our lab, was chosen to serve as an immunogen for the development of a generic antibody. The resulting antibody was shown to cross-react with a series of standard and synthetic derivatives of aminoglycosides that shared structural similarity with the immunization scaffold using a homologous ELISA. The antibody was further used for the development of 3 highly sensitive heterologous ELISAs for the detection of selected potential therapeutic agents. The assays were shown to have high recovery from spiked blood and serum samples and have been applied for *in vivo* monitoring of the synthetic derivatives in mice serum. The approach of a generic immunoassay developed in this study can be applied for the development of similar assays for the detection of other classes of drug candidates.

2. Materials and methods

2.1. Materials

Paromomycin, neomycin, geneticin (G418), gentamicin and kanamycin were purchased from Sigma as sulfate salts. All the synthetic derivatives including neamine, paromamine, NB82, NB74, NB84, NB122, NB123, NB124 and NB125 were prepared as described previously by Baasov and coworkers [9–12] and were used as their sulfate salts. All other chemicals and biochemicals, unless otherwise stated, were obtained from Merck or Sigma.

Rabbits immunization has been performed in Sigma–Aldrich (Rehovot, Israel) following the standard immunization protocols.

2.2. General techniques

^{1}H NMR spectra was recorded on a Bruker Avance™ 500 spectrometer at 500 MHz. Chemical shifts reported (in ppm) were relative to the internal standard Me_4Si ($\delta = 0.0$) with $CDCl_3$ as the solvent. ^{13}C NMR spectra was recorded on a Bruker Avance™ 500 spectrometer at 125.8 MHz, and the chemical shifts reported (in ppm) were relative to the residual solvent signal for $CDCl_3$ ($\delta = 77.00$). Mass spectra analysis was obtained on a Bruker Daltonix Apex 3 mass spectrometer under electrospray ionization (ESI). Reactions were monitored by TLC on Silica Gel 60 F254 (0.25 mm, Merck), and spots were visualized by charring with a yellow solution containing $(NH_4)Mo_7O_{24}{\cdot}4H_2O$ (120 g) and $(NH_4)_2Ce(NO_3)_6$ (5 g) in 10% H_2SO_4 (800 mL). Flash column chromatography was performed on Silica Gel 60 (70–230 mesh).

Data analysis. The sensitivity (I_{50}) and limit of detection (I_{20}) values were obtained from fitting concentration *vs* % binding curves to a log–logit plot using the Origin software, version 8.5 (Mirocal Software, North Hampton, MA, USA). Correlation coefficient between the assays linear range (I_{20}–I_{80}) was ≥0.98 in all standard curves. Data precision was calculated according to the National Committee of Clinical Laboratory Services guideline [30].

2.3. Preparation of 6′-(R)-methyl-2′,3-diazido-1-N-[5-oxopentanoic acid]-paromamine (**compound B**, Fig. 2)

A mixture of **compound A** [10] (500 mg, 1.2 mmol) and glutaric anhydride (0.29 mg, 2.5 mmol) in 20 mL anhydrous methanol was stirred under argon for 6 h at room temperature. The reaction was monitored by TLC (EtOAc/MeOH 60:40) which indicated completion after 6 h. The reaction mixture was evaporated under vacuum to dryness and purified by flash chromatography, resulting in 400 mg (62% yield) of pure **compound B** (Fig. 2). The purity of the product was determined by NMR and MALDI-TOF mass spectra analysis. ^{1}H NMR ($CDCl_3$, 500 MHz): 'Ring I' δ H 1.26 (d, 3H, $J = 6.0$ Hz, CH3), 3.10 (dd, 1H, $J1 = 4.2$ Hz, $J2 = 10.5$ Hz, H-2′), 3.38 (t, 1H, $J = 9.7$ Hz, H-4′), 3.92–3.98 (m, 2H, H-5′ and H3′), 4.04 (m, 1H, H-6′), 5.74 (d, 1H, $J = 3.1$ Hz, H-1′); 'Ring II' δ H 1.43 (ddd, 1H, $J1 = J2 = J3 = 12.5$ Hz, H-2ax), 2.20 (td, 1H, $J1 = 4.5$, $J2 = 12.5$ Hz, H-2eq), 3.26 (t, 1H, $J = 9.3$ Hz, H-4), 3.52 (m, 3H, H-3, H-5, H-6), 3.78 (m, 1H, H-1). Linker peaks appeared in the spectrum as follows: δ H 1.92 (t, 2H, $J = 7.2$ Hz), 2.29 (m, 2H), 2.36 (t, 2H, $J = 7.5$ Hz). ^{13}C NMR ($CDCl_3$, 125.8 MHz): δ C 18.0, 22.2, 33.9, 34.2, 36.2, 50.4, 61.4, 64.7, 69.3, 72.3, 74.2, 75.2, 76.2, 78.7, 80.3, 98.7 (C-1′), 175.5 (C═O), 177.1 (C═O). MS: calculated mass for $C_{18}H_{30}N_7O_{10}$ ($[M+H]^+$) m/z: 504.20; measured m/z: 504.20.

2.4. Preparation of immunization conjugate (**compound E**, Fig. 2)

A mixture of **compound B** (100 mg, 0.2 mmol), N-hydroxysuccinimide (NHS) (23 mg, 0.2 mmol) and N′,N′-dicyclohexylcarbodiimide (DCC) (41.2 mg, 0.2 mmol) in 10 mL dimethylformamide (DMF) was stirred for 4 h at room temperature followed by a 12 h incubation at 4 °C. The mixture was then centrifuged for 15 min at 2500 × *g* at room temperature. 1 mL of the supernatant containing 10 mg of the **compound C** were added to 10 mg KLH dissolved in 4 mL of 0.13 M $NaHCO_3$ at pH 9.2. The reaction was incubated 12 h at 4 °C, followed by size exclusion product purification for 25 min at 3000 × *g* at room temperature using Centricon 30 (Amicon, Millipore, Billerica, MA) to afford **compound D**. The concentrated **product D** (Fig. 2) was washed 3 times with 4 mL of $NaHCO_3$ solution. The final volume was adjusted to 4 mL by adding $NaHCO_3$ solution. The reduction of azido-groups was performed by adding 100 μL trimethylphosphine (PMe_3) solution (1 M solution in tetrahydrofuran – THF) and the mixture was allowed to react 12 h at 4 °C. The final product E (referred as a **compound E**, Fig. 2) was further purified using size exclusion purification as described above, followed by 2 washes with 5 mL $NaHCO_3$ solution. The final volume was adjusted to 5 mL by adding double distilled water (DDW) and was stored at −20 °C pending to use.

2.5. Immunization and antiserum production

The immunization conjugate (**compound E** from the above section, 0.5 mL) were emulsified with either Complete Freund's adjuvant (1st immunization), or incomplete adjuvant (2nd to 4th boosts) prior to immunization. The emulsion was injected subcutaneously to two rabbits 8244 and 8245 (∼1 mg conjugate were injected per rabbit at each time point). Bleeds were collected after each boost and were tested for anti-antigen activity using the checkerboard assay (Fig. S1). The 3rd bleed from Rabbit 8245 has been shown to have the highest anti-antigen activity, and was therefore used for further experimentations (Fig. S1).

2.6. Preparation of NB82–OVA coating antigen

NB82–OVA conjugates were prepared similarly to immunization conjugate, as described above, except that 5 mg ovalbumin (OVA), that were dissolved in 2.5 mL $NaHCO_3$ solution, were conjugated to **compound B** at molar ratios of 1:1, 1:2, 1:5 and 1:10. The final volume of the resulted NB82–OVA conjugate was adjusted to 1 mL by adding 0.13 M $NaHCO_3$, and the pH of the solution was adjusted to 9.6 by adding dilute solution (0.01 M) of NaOH. Protein content has been determined to be 5 mg mL^{-1} using the Bradford assay (BioRad protein assay, BioRad, Hercules, CA) and the resulting conjugates antigen activity was evaluated toward 3rd bleed antiserum from Rabbits 8244 and 8245 using the checkerboard assay (data not shown). The 1:1 NB82–OVA conjugate crossed with 3rd bleed antiserum derived from Rabbit 8245 has been shown to have the highest sensitivity, and was therefore used for further experimentations (Fig. S2). The conjugates were stored in aliquots at −20 °C.

2.7. Preparation of NB82/84/124–GTA–OVA coating antigens

To prepare coating antigens for heterologous-cross-reactivity assays we used glutaraldehyde (GTA) coupling method as described by Chen at al. [31]. In brief, 20 mg OVA was dissolved in 10 mL of 0.01 M phosphate-buffered saline (PBS) at pH 6.5 (the pH adjustment was performed prior to the addition of OVA by adding dilute solution, 0.01 M, of HCl). 20 mg of each compound (NB82/84/124 sulfate) was added to the solutions and stirred for a few min at room temperature 1.5 mL of freshly prepared 1% GTA solution was added in a drop-wise manner using a 1 mL syringe equipped with a 0.5 mm × 16 mm needle. The reaction mixture was allowed to be stirred at room temperature for 15 min, of which yellow color indicated the imine formation. Reduction of imines to amines has been performed by adding 115 mg sodium borohydride, followed by 12 h incubation at 4 °C. The final product has been purified using Centricon 30 for 30 min at 3000 × *g*, followed by 2 washes with 1 mL 0.01 M PBS pH 7.2. The final volume of conjugates has been adjusted to 1 mL using PBS pH 7.2, and their protein content has been determined to be 17.5–19 mg mL^{-1} using the Bradford methodology. Conjugates antigen reactivity was tested against 3rd bleed antiserum from Rabbits 8244 and 8245 using the checkerboard assay

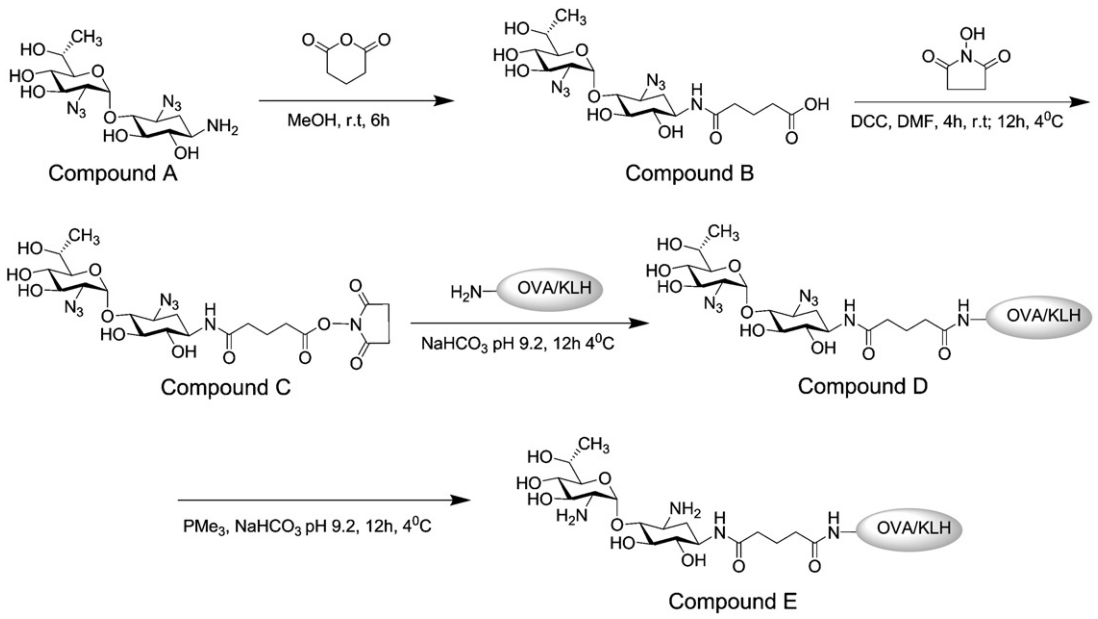

Fig. 2. Synthetic scheme for the preparation of immunization/coating conjugates.

(data not shown). The conjugates were stored in aliquots at −20 °C pending to use.

2.8. NB82 indirect competitive ELISA

The assay developed was an indirect competitive ELISA in which tested samples or standard solutions were allowed to compete with an immobilized coating antigen for antiserum binding. The assay has developed by using microtiter 96 well plates (F96 Maxisorp, Nunc Immuno Plate, Roskilde, Denmark). Wells were coated with 100 μL NB82–OVA (1:1) conjugate, diluted 1:8000 (62.5 ng conjugate per well) in 0.5 M carbonate buffer, pH 9.6 (CB). After an overnight incubation at 4 °C, wells were washed 3 times with PBST (defined as PBS pH 7.2 containing 0.3% [v/v] Tween-20). 50 μL of unknown samples or standard solutions (in PBS, mice serum or fetal bovine serum – FBS, Biological Industries, Israel) were added to the wells, followed by the addition of 50 μL of antiserum (Rabbit 8245) diluted 1:80,000 in PBST. The standard samples were comprised of 12 serial dilutions of NB82, ranging from 10 to 0.0049 μg mL^{-1} dissolved in PBS or serum. Plates were incubated overnight at 4 °C, and washed 3 times with PBST. 100 μL secondary antibody (2nd Ab) conjugated to horseradish peroxidase (HRP) (anti-rabbit HRP conjugated, Sigma), diluted 1:20,000 in PBST were added to each well. Plates were incubated for 2 h at room temperature, rinsed with PBST, and tested for HRP activity by the addition of 100 μL substrate solution – 3,3′,5,5′-tetramethyl benzidine (TMB substrate chromagen, Dako, Glostrup, Denmark). The reaction was stopped after 20 min by adding 50 μL of 4 N sulfuric acid, and the absorbance was measured with ELISA reader (Spectra Max M2, Molecular Devices, Sunnyvale, CA) at 450 nm. In all experiments a 5 mg mL^{-1} OVA solution in PBS has been used as a negative control to evaluate and normalize the unspecific binding of antisera. Unspecific binding values obtained were almost negligible in all experiments. Wells coated with coating antigen that were not introduced with a free antigen sample were used as a positive control and determined the 100% binding of antisera.

2.9. Cross-reactivity experiments

Cross-reactivity values of antisera with various aminoglycosides were determined using the heterologous indirect ELISA as described above by adding the subjected compounds (instead of NB82) at 24 serial dilutions ranging from 10 mg mL^{-1} to 1.19 ng mL^{-1}, except for NB84, of which serial dilution ranged from 1 mg mL^{-1} to 0.119 ng mL^{-1}. All aminoglycosides tested were at their sulfated form and were dissolved and diluted in PBS or serum. The standard solutions of the compounds NB82, NB84 and NB124 were shown to be stable in PBS and standard serum (FBS) solutions that were frozen (−20 °C) for a few months and demonstrated similar values using at least 5 repeated freezing/thawing cycles.

2.10. NB82/84/124 indirect competitive ELISA

In order to enhance antiserum sensitivity toward heterocompounds that share similar epitopes with the derivate of which antiserum Antibodies has been produced against, an indirect heterologous-assay has been developed. The assay has been performed similarly to the indirect assay for NB82 as described in Section 2.8, except for the coating antigens of which for the NB82 heterologous-test, NB82–GTA–OVA conjugate diluted 1:128,000 in CB was used as the coating antigen; for the NB84 heterologous-test, NB84–GTA–OVA conjugate diluted 1:64,000 in CB was used as the coating antigen; and for the NB124 heterologous-test, NB124–GTA–OVA conjugate diluted 1:16,000 in CB was used as the coating antigen. Solutions that contained 17.5–19 mg mL^{-1} OVA in PBS were used for coating negative control wells. Cross-reactivity experiments have been performed as indicated in Section 2.9.

2.11. Determination of NB compounds level in mice serum

Determination of NB compounds serum levels included an intraperitoneal (IP) injection of NB84/NB124 to mice and collection of blood samples at various time points. The protocol included the IP injection of 28.5 μg/g body weight of the relevant NB compound to 7 male mice (C57/BL, 2.5–3.5 months old), followed by collection of fresh blood samples (∼200 μL each) at 7 time points (5, 10, 20, 30, 40, 50, 60 min). Blood was collected to sterile tubes containing 20 μL of 50 mM EDTA pH 8.0. Serum was further extracted by centrifugation at 2000 × *g* at 4 °C for 10 min. NB compounds content in serum samples was determined by using the pre-developed microtiter assay, as described above, when compared to a linearized calibration curve transformed to a log–logit plot using the Origin

software, version 8.5. Each sample was tested in duplicates at five dilutions. All *in vivo* samples were tested immediately after serum preparation. Serum recovery ratios were determined by spiking experiments of which whole blood samples derived from untreated mice were spiked with known amounts of the relevant aminoglycosidic derivatives. Blood samples were incubated for 1 h at 4 °C followed by serum preparation by centrifugation as described above.

3. Results and discussion

3.1. Preparation of immunization conjugate

The main goal of the present study was to develop an immunochemical assay for monitoring lead compounds of NB-series in biologically derived samples. The specific aims included the production of a generic polyclonal antiserum that will be able to recognize a wide variety of NB compounds (Fig. 1) that have previously shown to exhibit a great potential for the treatment of various nonsense mutations related genetic diseases [9–14].

NB82, a semi-synthetic 2-ring fragment derived from G418, is a common scaffold, appearing in nearly all compounds of NB-series (Fig. 1). We assumed that by choosing NB82 as hapten for immunogen preparation we could obtain polyclonal antibodies that will be able to recognize a diverse selection of these compounds. Similar methodology was previously described by Van Amerongen and coworkers [32] by using neamine as an immunogenic hapten for the generation of polyclonal antiserum capable of recognizing some natural aminoglycosides such as neomycin, kanamycin A and gentamicin.

Our next challenge was to choose a proper conjugation strategy for the preparation of immunization conjugate. Some of our lead compounds, like NB84, contain an (S)-4-amino-2-hydroxybutanoyl (AHB) moiety at their N-1 position (Fig. 1). Our earlier studies have demonstrated that the addition of AHB group selectively at N-1 position significantly enhances the readthrough activity of the resulted derivatives in comparison to that of the parent structure without the AHB, while its toxicity profile was not significantly affected [9–12]. Taking into account that the antibody raised against a hapten linked to the carrier protein through N-1 position would have similar affinity to both compounds with and without AHB, further substantiated our strategy for linking NB82 to a carrier protein selectively at N-1 position.

In order to selectively conjugate the NB82 scaffold to a carrier protein *via* the N-1 position, we first performed a selective azidation of NB82 at 2′ and 3 positions to yield 2′,3-diazido-NB82 (**compound A**, Fig. 2). Treatment of **compound A** with glutaric anhydride afforded **compound B** in 62% isolated yield. The carboxylic acid function of **compound B** at its N-1 side-chain was activated by reaction with N-hydroxysuccinimide in the presence of DCC to yield **compound C**. Conjugation of the NHS-activated **compound C** with the carrier protein KLH was followed by the conversion of two azido groups to the corresponding amines using Staudinger reaction (Me_3P, pH 9.2) to yield the immunization conjugate NB82–KLH (**compound E**, Fig. 2). The resulting conjugate was used as an immunization agent and was injected to two rabbits.

3.2. Development of an indirect competitive ELISA

The two rabbits immunized with the NB82–KLH conjugate showed high antibody serum titers, as indicated in checkerboard homologous-assays performed by using the NB82–OVA conjugate as a coating antigen (Figs. S1 and S2). These experiments were further used to determine the optimal concentrations of coating antigen (NB82–OVA, 1:1), rabbit antiserum (8245) and secondary antibody to be 1:8000, 1:80,000 and 1:20,000, respectively. These conditions were used to determine the I_{50} value and assay's detection limit (I_{20}) of NB82 to be 50 ± 6 ng mL^{-1} and 8 ± 1 ng mL^{-1} ($n = 10$), respectively (Fig. 3, Table 1).

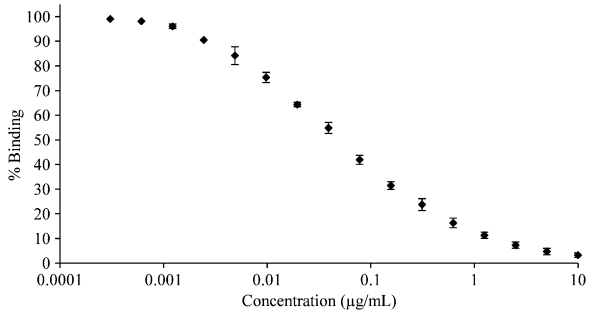

Fig. 3. Standard curve of NB82 (homologous assay). NB82–OVA conjugate dilution 1:8000; antisera dilution (Rabbit 8245) 1:80,000. I_{50} 50 ± 6 ng mL^{-1}; limits of detection: 8 ± 1 ng mL^{-1}. Each value represents the mean ± S.E.M. (standard error mean) of 10 independent repeats performed in duplicates.

In order to evaluate the efficiency of the assay for the detection of various aminoglycosides, 13 representative members were chosen to be detected for antiserum cross-reactivity *via* homologous-cross-reactivity assays, using the NB82–OVA conjugate as a coating antigen. Five of them including G418, gentamicin, paromomycin, neomycin and kanamycin A are commercial aminoglycosides (Fig. 1). Six compounds including NB74, NB84, NB122, NB123, NB124 and NB125, are semi-synthetic agents systematically developed in our laboratory for the treatment of genetic diseases caused by premature stop codon mutations [10–12]. Three additional compounds selected in this study included 2-ring structures as common scaffolds: neamine, a common scaffold of neomycin B and kanamycin A; paromamine, a common scaffold of paromomycin, NB122 and NB123; NB82, a common scaffold of G418, NB74, NB84, NB124 and NB125. As can be seen from the data in Fig. 4, antiserum antibodies were able to recognize all tested members at various sensitivity ranges, where NB82, as expected, demonstrated the lowest I_{50} value, thus indicating highest antibody affinity (Table 1).

The affinities of serum antibodies toward the structurally related aminoglycosides containing a secondary (R)-6′-OH on ring I (NB82, G418, NB74, NB84, NB124, NB125) were higher than those obtained for similar compounds containing either a primary 6′-OH (NB122, NB123, paromomycin, paromamine) or a 6′-NH_2 (neomycin, neamine, gentamicin, kanamycin A). These results correlate well with our expectations that the resulting antibodies

Table 1
Concentrations at 50% inhibition (I_{50}) and limits of detection (I_{20}) of various aminoglycosides using the homologous ELISA with NB82–OVA as a coating antigen.[a]

Compound	I_{50} (μg mL^{-1})	Detection limit (I_{20}) (μg mL^{-1})
NB82	0.05 ± 0.006	0.008 ± 0.001
G418	0.4 ± 0.04	0.06 ± 0.02
NB125	1.25 ± 0.14	0.3 ± 0.03
Paromamine	3.0 ± 0.34	0.6 ± 0.1
NB84	3.1 ± 0.75	0.7 ± 0.1
NB74	3.5 ± 0.15	0.7 ± 0.1
NB124	12 ± 1.1	3 ± 0.7
Gentamicin	130 ± 8.99	20 ± 3
NB123	250 ± 22.6	40 ± 1
Paromomycin	325 ± 56.6	50 ± 2
NB122	400 ± 34.2	80 ± 4
Neomycin	700 ± 71.7	90 ± 4
Kanamycin A	1000 ± 144	120 ± 11
Neamine	2000 ± 251	150 ± 14

[a] NB82–OVA conjugate dilution 1:8000 was used as coating antigen; antisera dilution (Rabbit 8245) 1:80,000. Each value represents the mean ± S.E.M. of at least 3 experiments performed in duplicates.

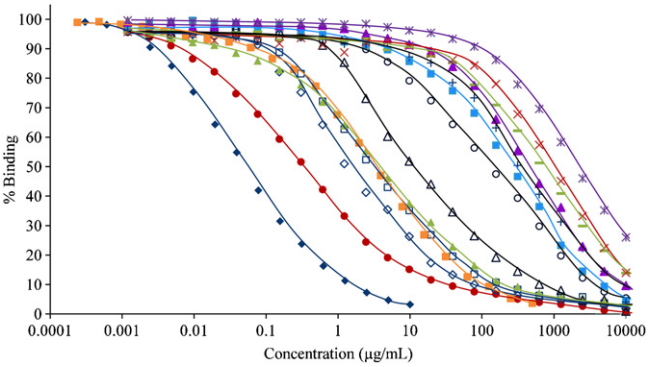

Fig. 4. Homologous assay cross-reactivity. Antiserum cross-reactivity of natural and synthetic representative aminoglycoside derivatives: NB82 (♦), geneticin (G418) (●), NB125 (◊), paromamine (□), NB84 (■), NB74 (▲), NB124 (△), gentamicin (○), NB123 (■), paromomycin (+), NB122 (▲), neomycin (–), kanamycin A (×), and neamine (★). NB82–OVA was used as coating antigen (dilution 1:8000); antiserum dilution (Rabbit 8245) 1:80,000. Each value represents the mean of at least 3 experiments performed in duplicates. Mean I_{50} values ±S.E.M. are summarized in Table 1.

Table 2
Concentrations at 50% inhibition (I_{50}) and limits of detection (I_{20}) of 3 representative members (NB82, NB84 and NB124) using the heterologous ELISAs.[a]

Compound	Coating antigen	I_{50} (μg mL^{-1})	Detection limit (I_{20}) (μg mL^{-1})
NB82	NB82–OVA	0.05 ± 0.006	0.008 ± 0.001
	NB82–GTA–OVA	0.05 ± 0.009	0.007 ± 0.001
	NB84–GTA–OVA	0.04 ± 0.006	0.007 ± 0.001
	NB124–GTA–OVA	0.01 ± 0.003	0.001 ± 0.0003
NB84	NB82–OVA	3.1 ± 0.75	0.7 ± 0.1
	NB82–GTA–OVA	6 ± 0.3	0.5 ± 0.1
	NB84–GTA–OVA	0.5 ± 0.04	0.02 ± 0.007
	NB124–GTA–OVA	0.8 ± 0.03	0.03 ± 0.009
NB124	NB82–OVA	12 ± 1.1	3 ± 0.7
	NB82–GTA–OVA	7 ± 0.5	0.5 ± 0.06
	NB84–GTA–OVA	2 ± 0.1	0.03 ± 0.009
	NB124–GTA–OVA	1 ± 0.12	0.015 ± 0.008

[a] NB82–OVA conjugate dilution was 1:8000; NB82–GTA–OVA conjugate dilution was 1:128,000; NB84–GTA–OVA conjugate dilution was 1:64,000; NB124–GTA–OVA conjugate dilution was 1:16,000; antisera dilution (Rabbit 8245) 1:80,000. Each value represents the mean ± S.E.M. of at least 3 experiments performed in duplicates.

will show higher specificity toward compounds that share greater similarity with the parent compound used for immunization. Using NB82–OVA as the coating agent the antiserum indicated a rather high cross-reactivity value of 12.5% for G418, with I_{50} of 400 ng mL^{-1} (Fig. 1, Table 1). These values can be attributed to the high number of similar epitopes G418 shares with NB82, as can be seen in Fig. 1.

Cross-reactivity values of the structurally related synthetic derivatives NB74, NB84, NB124 and NB125 where in the range of 0.4–4%, with I_{50} values of 1.25–12 μg mL^{-1} (Table 1). These compounds are derived from G418; therefore they all contain the NB82 scaffold. The observed 1–2 orders of magnitude gaps in cross-reactivity values (when compared to G418) can be explained by the difference in their substitution pattern regarding the 2-deoxy-streptamine (2-DOS) ring (ring II, Fig. 1). NB compounds are 4,5-di-substituted 2-DOS derivatives, whereas G418 is a 4,6-di-substituted 2-DOS member. Since immunization conjugate has been linked *via* the N-1 position, it is reasonable to hypothesize that the epitopes detected by the various antibody populations are located in the 1st sugar ring (ring I) and the glycosidic bond relative positions of the 2-DOS ring (ring II); as far as possible from the N-1 position. Following this line of thought, compounds of NB-series substituted with ring III at C5 position will enhance greater steric interference with the antibody than that of G418 in which ring III is substituted at C6 position. According to this rationale, compounds that differ only in the substitution pattern at N-1 position should have similar affinities to the antibody. Indeed, as can be seen from the data in Table 1, NB74 and NB84 that differ at their N-1 position demonstrated similar cross-reactivity values of 3.5 ± 0.15 μg mL^{-1} and 3.1 ± 0.75 μg mL^{-1}, respectively. Cross-reactivity values for compounds that did not contain NB82 as a main scaffold were shown to be less than 0.04%, with I_{50} values of 130–2000 μg mL^{-1} (NB122, NB123, paromomycin, neomycin, gentamicin, kanamycin A and neamine, Fig. 4, Table 1).

Interestingly, the antibody populations generated were able to differentiate between the diastereomeric compounds that differ only by the configuration at a single chiral carbon center. Among the series of compounds tested in this study we had two such pairs of compounds: NB122 (5″-S) *vs* NB123 (5″-R), and NB124 (5″-S) *vs* NB125 (5″-R). The I_{50} values of NB125 (5″-R) were 1 order of magnitude lower than those obtained for its 5″-diastereomer, NB124 (5″-S) (Table 1). Similarly, the values obtained for NB123 (5″-R) were twice lower than those obtained for NB122 (5″-S). These results demonstrate a stereo-selective preference toward the 5″-R isomers. An opposite tendency was observed for these compounds regarding their readthrough activity and cytotoxicity values [11]. These observations could be attributed to the different pattern of interactions between the 5″-groups and the antibody binding pocket.

3.3. Development of heterologous-assays

The cross-reactivity values obtained in the homologous assay demonstrated the antibodies ability to bind a wide range of synthetic and natural derivatives. However, the enhanced affinity toward the homologous coating conjugate (NB82–OVA) used in these assays resulted in a 1–5 orders of magnitude gap between the affinities to NB82 and the other tested compounds. Previous studies have demonstrated that using heterologous conjugates as coating antigens can significantly enhance the sensitivity of such assays toward different antigens [32]. In this case, a heterologous coating conjugate will have a lower affinity to the antibody, and consequently, the free hapten and or similar structures will be able to displace the antibodies more sensitively. Therefore, in attempts to enhance the sensitivity of our assay, we developed heterologous ELISAs toward 2 leading NB compounds, NB84 and NB124, which have shown the greatest activity in *ex vivo* and *in vivo* experimentations [10–14]. Three heterologous-coating conjugates have been prepared using the GTA conjugation method [31]: NB82–GTA–OVA (control), NB84–GTA–OVA and NB124–GTA–OVA. Conjugate preparation was followed by the development of 3 indirect heterologous-assays to test cross-reactivity values of 3 representative semi-synthetic members. The obtained results indicated a major improvement in heterologous-antigen recognition, where I_{50} values for NB82, NB84 and NB124 were reduced from 50 ± 6 ng mL^{-1} to 10 ± 3 ng mL^{-1}, from 3.1 ± 0.75 μg mL^{-1} to 0.5 ± 0.04 μg mL^{-1} and from 12 ± 1 μg mL^{-1} to 1 ± 0.12 μg mL^{-1}, respectively (Table 2). Detection limits, while using the heterologous-assays were 0.001 ± 0.0003 μg mL^{-1} for NB82, 0.02 ± 0.007 μg mL^{-1} for NB84 and 0.015 ± 0.008 μg mL^{-1} for NB124 (Table 2).

3.4. Determination of selected NB compounds serum levels in mice

Our next challenge was to use the pre-developed assay to monitor aminoglycosides content in mice serum *in vivo*. For this purpose, we initially determined the antibodies affinities and detection

Table 3
Concentrations at 50% inhibition (I_{50}) and limits of detection (I_{20}) of 3 representative members (NB82, NB84 and NB124) in mice serum.[a]

Compound	Matrix	I_{50} ($\mu g\,mL^{-1}$)	Detection limit (I_{20}) ($\mu g\,mL^{-1}$)
NB82	PBS	0.01 ± 0.003	0.001 ± 0.0003
	Serum	0.008 ± 0.001	0.001 ± 0.0002
NB84	PBS	0.5 ± 0.04	0.02 ± 0.007
	Serum	0.4 ± 0.1	0.02 ± 0.009
NB124	PBS	1 ± 0.12	0.015 ± 0.008
	Serum	0.8 ± 0.06	0.01 ± 0.005

[a] For NB82 and NB124 detection: NB124–GTA–OVA conjugate dilution 1:16,000 was used as coating antigen; for NB84 detection: NB84–GTA–OVA conjugate dilution 1:64,000 was used as coating antigen; antisera dilution for all experiments was 1:80,000 (Rabbit 8245). Each value represents the mean ± S.E.M. of at least 3 experiments performed in duplicates.

limits to aminoglycosides in serum. We prepared 24 serial dilutions of 3 representative members NB82, NB84 and NB124 in PBS, FBS and mice serum and quantified them by using our pre-developed ELISA. In these experiments, NB82 was used as a positive control to test serum interference with ELISA protocol, whereas NB84 and NB124 were chosen due to their high potential as suppression therapy candidates resulting from previous results [10–14] and preliminary data in various *in vitro*, *ex vivo* and *in vivo* models. The measured I_{50} and I_{20} values for all three compounds tested (NB82, NB84 and NB124) in mice serum were similar to those observed in PBS solutions (Table 3). Next, we determined the recovery of these derivatives from whole mice blood samples by spiking 250 μL blood samples with 10, 25 or 50 $\mu g\,mL^{-1}$ of the relevant compound, followed by serum extraction. Recovery ratios were determined to be 97–102% for NB82, 92–105% for NB84 and 100–107% for NB124 (Table 4). Assays imprecision (coefficients of variation percentage) was determined lower than 6% for all tested samples.

To monitor serum levels of NB84 and NB124 *in vivo*, we carried out IP injections at therapeutic doses (28.5 mg/kg body weight) to mice, and collected blood samples at various time points post injection. Similar doses of NB84 (30.0 mg/kg body weight) were previously shown to enhance therapeutic effect *in vivo* in Idua-W392X model mice for MPS I-H disease without causing any detectable side effects [13]. ELISA measurements of aminoglycosides content in serum samples indicated that the serum concentrations of NB84 and NB124 reached the peak levels of 121.6 $\mu g\,mL^{-1}$ and 140 $\mu g\,mL^{-1}$, respectively, within 10 min post injection, and then declined to ~10 $\mu g\,mL^{-1}$ and 28 $\mu g\,mL^{-1}$ by 60 min after injection (Fig. 5). Remarkably similar kinetics were previously reported for the aminoglycosides gentamicin and amikacin detected in mice serum [25,26]. The obtained results also correspond to the alpha phase in the 3-compartment pharmacokinetic model of aminoglycosides serum availability, of which, a fast deterioration is observed in the first 60 min after injection [33].

Table 4
Recovery of NB82, NB84 and NB124 from whole blood samples.[a]

Compound	Spiked concentration ($\mu g\,mL^{-1}$)	Detected concentration ($\mu g\,mL^{-1}$)	% precision	% recovery
NB82	10	10.0 ± 0.0	100	100
	25	24.2 ± 0.5	96	97
	50	50.8 ± 0.8	97	102
NB84	10	9.9 ± 0.2	96	99
	25	26.3 ± 0.3	98	105
	50	46.1 ± 1.5	94	92
NB124	10	10.3 ± 0.1	98	103
	25	26.7 ± 0.7	95	107
	50	50.0 ± 0.1	99	100

[a] Each value represents the mean ± S.E.M. of at least 3 experiments performed in duplicates.

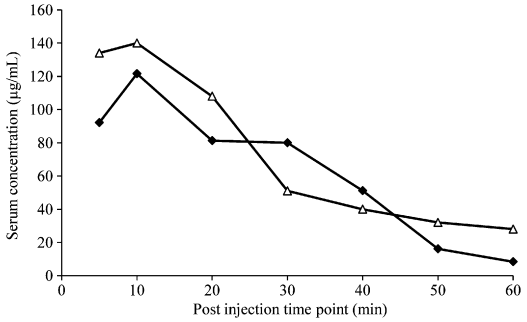

Fig. 5. Serum levels of NB84 and NB124 following a single dose IP injection in male mice (C57/BL, 2.5–3.5 months old), Each point represents the aminoglycoside concentration (NB84 – black parallelogram, NB124 – white triangle) in a single blood sample taken from different mice at various time points following the injection of 28 μg/g body weight NB compound. The concentrations were determined using the pre-developed heterologous-immunoassays. Each sample was prepared at 5 different concentrations and tested in duplicates.

Due to the relatively high toxicity of aminoglycosides, monitoring the serum levels of these compounds in treated patients is highly important. According to the Physicians Desk Reference (PDR) [34], the recommended intravenous (IV) doses of gentamicin and amikacin for antibacterial treatment in patients are 6–7.5 mg/kg/day and 15 mg/kg/day, respectively. The maximum peak serum levels allowed for these compounds are 12 $\mu g\,mL^{-1}$ and 35 $\mu g\,mL^{-1}$. However, in a recent clinical trial, performed in France, testing the potential use of gentamicin for the treatment of CF patients carrying various nonsense mutations, higher gentamicin peak serum levels of 20–40 $\mu g\,mL^{-1}$ were allowed [35]. These values are 2–4 folds higher than those approved for antibacterial treatment. No toxic effects were reported using the declared doses during the 15 days trial, of which gentamicin was administrated once daily at 10 mg/kg body weight. Our previous studies have indicated that all the compounds of NB-series used in this study lack antibacterial activity and exhibit significantly lower toxicity than that of gentamicin [9–14]. However, whether the observed peak levels of 121.6 $\mu g\,mL^{-1}$ and 140 $\mu g\,mL^{-1}$, upon the administration of ~30 mg/kg body weight of NB 84 and NB 124, are within the safety doses or not for the administration in humans, is not clear yet and requires further investigation. Nevertheless, the sensitivity and versatility of the generic immunoassay developed in this study are well adequate for the detection of NB84 and NB124; lowest detection limits of 20 ng mL^{-1} and 15 ng mL^{-1}, respectively that are far below the peak levels of these compounds observed in mice serum.

4. Summary and conclusions

Using a common two-ring scaffold, NB82, as an immunization hapten, we were able to develop a generic antibody that was further used for the development of highly sensitive ELISAs that were used for the detection of a wide range of natural and synthetic derivatives. The unique conjugation method used in this study enabled us to selectively conjugate the NB82 scaffold to a carrier protein *via* the N-1 position; therefore enabling to partially control the epitopes selection, directing the recognition toward a wide variety of therapeutic agents. Using heterologous-coating antigens we were able to monitor the assays sensitivity toward 3 selected representative members: NB82, NB84 and NB124 (Fig. 1). Sensitivity (I_{50}) values for the recognition of these representatives were determined to be 10 ± 3 ng mL^{-1}, 0.5 ± 0.04 $\mu g\,mL^{-1}$ and

$1 \pm 0.12\ \mu g\ mL^{-1}$, respectively (Table 2). Limits of detection were determined to be $1 \pm 0.3\ ng\ mL^{-1}$ for NB82, $20 \pm 7\ ng\ mL^{-1}$ for NB84 and $15 \pm 8\ ng\ mL^{-1}$ for NB124 (Table 2). These values were similar to the previously reported sensitivities for the detection of natural aminoglycosides in various analytical and bioanalytical manners [29].

The developed methodology has been successfully applied for monitoring selected aminoglycosides serum levels *in vivo*. Spiking experimentations demonstrated relatively high and consistent recovery ratios for all tested compounds. *In vivo* serum kinetics were shown to be similar for the 2 representative compounds tested and correlated well with pre-existing information regarding the serum levels of standard aminoglycosides *in vivo* [25,26]. The assays developed in this study can further be exploited for the detection of NB compounds and other aminoglycosides containing 6′-OH group at ring I in various biological derived samples. Similar methods can be applied for the generation of new and improved scaffolds for the development of a wide variety of bioassays.

Acknowledgments

We thank Dr. Aart Van Amerogen (Wageningen University & Research Centre, The Netherlands) for generous supply of antineamine antibodies, enabled us to perform an initial screen of our NB-compounds. This work was supported by the NIH/NIGMS grant (1 R01 GM094792-01 A1) and by the German-Israeli Foundation (GIF) for Scientific Research and Development (G-1048-95.5/2009). M.S. acknowledges the Schulich Fellowship for PhD students; J.K. acknowledges the Schulich Postdoctoral Fellowship; V.B. acknowledges the financial support by the Center of Absorption in Science, the Ministry of Immigration Absorption and the Ministry of Science and Technology, Israel (Kamea Program).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jpba.2012.11.014.

References

[1] B.D. Davis, Mechanism of bactericidal action of aminoglycosides, Microbiol. Rev. 51 (1987) 341–350.
[2] S. Jana, J.K. Deb, Molecular understanding of aminoglycoside action and resistance, Appl. Microbiol. Biotechnol. 70 (2006) 140–150.
[3] J.F. Burke, A.E. Mogg, Suppression of a nonsense mutation in mammalian cells *in vivo* by the aminoglycoside antibiotics G-418 and paromomycin, Nucleic Acids Res. 13 (1985) 6265–6272.
[4] M. Manuvakhova, K. Keeling, D.M. Bedwell, Aminoglycoside antibiotics mediate context-dependent suppression of termination codons in a mammalian translation system, RNA 6 (2000) 1044–1055.
[5] E. Kerem, Pharmacologic therapy for stop mutations: how much CFTR activity is enough? Curr. Opin. Pulm. Med. 10 (2004) 547–552.
[6] Online Mendelian Inheritance in Man, OMIM®, McKusick-Nathans Institute of Genetic Medicine, Johns Hopkins University, Baltimore, MD, 2012, World Wide Web URL: http://omim.org/OMIM
[7] J. Atkinson, R. Martin, Mutations to nonsense codons in human genetic disease: implications for gene therapy by nonsense suppressor tRNAs, Nucleic Acids Res. 22 (1994) 1327–1334.
[8] K.M. Keeling, D.M. Bedwell, Suppression of nonsense mutations as a therapeutic approach to treat genetic diseases, Wiley Interdiscip. Rev. RNA 2 (2011) 837–852.
[9] I. Nudelman, A. Rebibo-Sabbah, M. Cherniavsky, V. Belakhov, M. Hainrichson, F. Chen, J. Schacht, D.S. Pilch, T. Ben-Yosef, T. Baasov, Development of novel aminoglycoside (NB54) with reduced toxicity and enhanced suppression of disease-causing premature stop mutations, J. Med. Chem. 52 (2009) 2836–2845.
[10] I. Nudelman, D. Glikin, B. Smolkin, M. Hainrichson, V. Belakhov, T. Baasov, Repairing faulty genes by aminoglycosides: development of new derivatives of geneticin (G418) with enhanced suppression of diseases-causing nonsense mutations, Bioorg. Med. Chem. 18 (2010) 3735–3746.
[11] J. Kandasamy, D. Atia-Glikin, V. Belakhov, T. Baasov, Repairing faulty genes by aminoglycosides: identification of new pharmacophore with enhanced suppression of disease-causing nonsense mutations, Med. Chem. Commun. 2 (2011) 165–171.
[12] T. Baasov, D. Atia-Glikin, J. Kandasamy, V. Belakhov, Aminoglycosides and uses thereof in targeting genetic disorders, PTC/IL2011/000889 [WO 2012/066546 A1], Israel, 2011.
[13] D. Wang, V. Belakhov, J. Kandasamy, T. Baasov, S.C. Li, Y.T. Li, D.M. Bedwell, K.M. Keeling, The designer aminoglycoside NB84 significantly reduces glycosaminoglycan accumulation associated with MPS I-H in the Idua-W392X mouse, Mol. Genet. Metab. 105 (2012) 116–125.
[14] C. Brendel, V. Belakhov, H. Werner, E. Wegener, J. Gartner, I. Nudelman, T. Baasov, P. Huppke, Readthrough of nonsense mutations in Rett syndrome: evaluation of novel aminoglycosides and generation of a new mouse model, J. Mol. Med. (Berl.) 89 (2011) 389–398.
[15] B. Solomun, N. Bilandzic, I. Varenina, G. Scortichini, Validation of an enzyme-linked immunosorbent assay for qualitative screening of neomycin in muscle, liver, kidney, eggs and milk, Food Addit. Contam. Part A: Chem. Anal. Control Expo. Risk Assess. 28 (2011) 11–18.
[16] Y. Jin, J.W. Jang, M.H. Lee, C.H. Han, Development of competitive direct enzyme-linked immunosorbent assay for the detection of gentamicin residues in the plasma of live animals, Asian-Australas. J. Anim. Sci. 18 (2005) 1498–1504.
[17] N.F. Xu, C.L. Qu, W. Ma, L.G. Xu, L.Q. Liu, H. Kuang, C.L. Xu, Development and application of one-step ELISA for the detection of neomycin in milk, Food Agric. Immunol. 22 (2011) 259–269.
[18] I.A. Galvidis, M.A. Burkin, Monoclonal antibody-based enzyme-linked immunosorbent assay for the aminoglycoside antibiotic kanamycin in foodstuffs, Russ. J. Bioorg. Chem. 36 (2010) 722–729.
[19] M. Preu, D. Guyot, M. Petz, Development of a gas chromatography mass spectrometry method for the analysis of aminoglycoside antibiotics using experimental design for the optimisation of the derivatisation reactions, J. Chromatogr. A 818 (1998) 95–108.
[20] U. Hubicka, J. Krzek, H. Woltynska, B. Stachacz, Simultaneous identification and quantitative determination of selected aminoglycoside antibiotics by thin-layer chromatography and densitometry, J. AOAC Int. 92 (2009) 1068–1075.
[21] A. Kaufmann, P. Butcher, K. Maden, Determination of aminoglycoside residues by liquid chromatography and tandem mass spectrometry in a variety of matrices, Anal. Chim. Acta 711 (2012) 46–53.
[22] M.N. El-Attug, J. Hoogmartens, E. Adams, A. Van Schepdael, Optimization of capillary electrophoresis method with contactless conductivity detection for the analysis of tobramycin and its related substances, J. Pharm. Biomed. Anal. 58 (2012) 49–57.
[23] E. Adams, L. Liu, K. Dierick, S. Guyomard, P. Nabet, S. Rico, P. Louis, E. Roets, J. Hoogmartens, Neomycin: microbiological assay or liquid chromatography? J. Pharm. Biomed. Anal. 17 (1998) 757–766.
[24] P.R. Oeltgen, S.R. Hamann, R.A. Blouin, Comparison of radioimmunoassay (RIA) and enzyme-immunoassay (EMIT) for the determination of serum gentamicin, Clin. Chem. 26 (1980) 970.
[25] M. Du, J.R. Jones, J. Lanier, K.M. Keeling, J.R. Lindsey, A. Tousson, Z. Bebok, J.A. Whitsett, C.R. Dey, W.H. Colledge, M.J. Evans, E.J. Sorscher, D.M. Bedwell, Aminoglycoside suppression of a premature stop mutation in a Cftr−/− mouse carrying a human CFTR-G542X transgene, J. Mol. Med. (Berl.) 80 (2002) 595–604.
[26] M. Du, K.M. Keeling, L. Fan, X. Liu, T. Kovacs, E. Sorscher, D.M. Bedwell, Clinical doses of amikacin provide more effective suppression of the human CFTR-G542X stop mutation than gentamicin in a transgenic CF mouse model, J. Mol. Med. (Berl.) 84 (2006) 573–582.
[27] S.R. Raz, M.G.E.G. Bremer, W. Haasnoot, W. Norde, Label-free and multiplex detection of antibiotic residues in milk using imaging surface plasmon resonance-based immunosensor, Anal. Chem. 81 (2009) 7743–7749.
[28] Y. Zhu, J.I. Son, Y.B. Shim, Amplification strategy based on gold nanoparticle-decorated carbon nanotubes for neomycin immunosensors, Biosens. Bioelectron. 26 (2010) 1002–1008.
[29] D.A. Stead, Current methodologies for the analysis of aminoglycosides, J. Chromatogr. B 747 (2000) 69–93.
[30] National Committee of Clinical Laboratory Services, EP5A2, Evaluation of Precision Performance of Quantitative Measurement Methods, Approved Guideline, 2nd ed., NCCLS, Wayne, PA, 2004.
[31] Y. Chen, Z. Wang, Z. Wang, S. Tang, Y. Zhu, X. Xiao, Rapid enzyme-linked immunosorbent assay and colloidal gold immunoassay for kanamycin and tobramycin in swine tissues, J. Agric. Food Chem. 56 (2008) 2944–2952.
[32] E.E. Loomans, W.J. Van, M. Koets, A. Van Amerongen, Neamin as an immunogen for the development of a generic ELISA detecting gentamicin, kanamycin, and neomycin in milk, J. Agric. Food Chem. 51 (2003) 587–593.
[33] J. Turnidge, Pharmacodynamics and dosing of aminoglycosides, Infect. Dis. Clin. North Am. 17 (2003) 503–528.
[34] M. Mehta, PDR provides latest Food and Drug Administration – approved dosage guidelines, Arch. Intern. Med. 161 (2001) 2622.
[35] I. Sermet-Gaudelus, M. Renouil, A. Fajac, L. Bidou, B. Parbaille, S. Pierrot, N. Davy, E. Bismuth, P. Reinert, G. Lenoir, J.F. Lesure, J.P. Rousset, A. Edelman, In vitro prediction of stop-codon suppression by intravenous gentamicin in patients with cystic fibrosis: a pilot study, BMC Med. 5 (2007) 5.