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Application of Molecular Mimicry to Target GD2 Ganglioside

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1. Introduction

GD2 ganglioside is an important example of tumour-associated carbohydrate antigens. Over expression of the GD2 ganglioside is a hallmark of neuroblastoma, while its expression on normal cells is restricted. The antigen is stably expressed on cells of the tumour, and retained during a therapy of the disease (Kramer et al., 2001). The facts allow to use its presence to diagnose neuroblastoma, monitor response to treatment, and target the tumour cells (Navid et al., 2010; Reuland et al., 2001; Swerts et al., 2005).

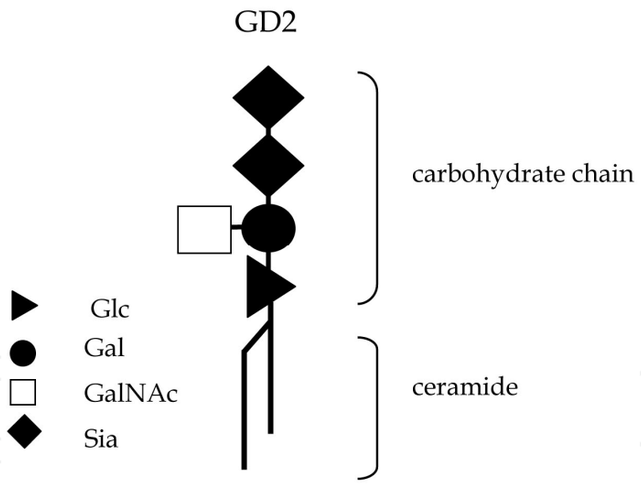

Fig. 1. A schematic representation of the GD2 ganglioside structure (abbreviations: Glc, glucose; Gal, galactose; GalNAc,, N-acetylgalactosamine; Sia, sialic acid).

The GD2 ganglioside is a member of glycolipids, compounds that are commonly present in outer plasma membrane of animal cells. The GD2 ganglioside is a polar molecule. The hydrophilic part is made from a carbohydrate chain, charged due to the presence of two sialic acid residues, and exposed to cell outside. The second part of the molecule is hydrophobic in nature, functions as a membrane anchor, and it is formed by a ceramide, fatty acid sphingosine (Fig. 1). Gangliosides were shown to be involved in many aspects of cancer biology. Changes in their expression may influence cell signalling pathways,

morphology, proliferation, invasiveness, and tumour progression, as shown in cell cultures and *in vivo* (Ruan et al., 1999; Yoshida et al., 2001). The gangliosides are shed from cell membranes, and are present in sera of cancer patients (Czaplicki et al., 2009). Immunomodulating activities of the GD2 ganglioside were also described (Ladish et al., 1994). The GD2 ganglioside allows for successful targeting of neuroblastoma with monoclonal antibodies binding the tumour-associated carbohydrate antigen (Kushner et al., 2011; Yu et al., 2011). Novel active approaches targeting the GD2 ganglioside are under development to improve weak-immunogenicity of the self antigen, including GD2 ganglioside-KLH conjugates, anti-idiotypes, and peptide mimetics (Foon et al., 2000; Chapman et al., 2000,). In the chapter we review approaches to improve immunogencity of the GD2 ganglioside, with emphasis on application of peptide mimetics.

2. Molecular mimicry phenomenon

A molecular mimicry phenomenon relies on similarities between chemically diverse molecules, *i.e.*, a surrogate and an original antigen (epitope) in the context of their ability to bind to a given target. Numerous data highlight that peptides, which are small molecules, consisting of amino acids linked with the peptide bond in a linear order, can be carbohydrate mimetics, inhibitors of carbohydrate-protein interactions, but also immunogens. Such peptides can be isolated from phage-displayed peptide libraries, synthetic libraries constructed with combinatorial chemistry, or rationally designed (Aina et al., 2007; Johnson & Pinto, 2008; Pande et al., 2010).

Antigenicity of peptide mimetics relies on their ability to bind to receptors, such as antibodies, enzymes, or lectins. Their reactivity can be measured with several assays (ELISA, Western blot). Additional competition tests for binding between mimicking peptides and the natural ligand can prove, if the peptides can bind at or near the antigen binding site. Further studies often include evaluation of residues critical for binding, their replacement, shortening or extension of the peptide length. All of these can help to characterise the binding. Moreover, structures of free and the receptor-bound mimetics can be compared experimentally, or with application of computational docking (Huang et al., 2011). As a result, lead compounds can be identified, and further developed to replace natural antigens in diagnostic assays, and in therapy (Deroo & Muller, 2001).

The nature of the peptide-carbohydrate mimicry is extensively studied, as more examples are reported (Jonson & Pinto, 2008). On the molecular level, structural mimicry can be distinguished, if a peptide can replicate three-dimensional (3D) interactions made by the carbohydrate with the target binding site. The contacts are mediated due to a certain arrangement of functional groups. The interaction does not require for such a peptide to have a similar 3D shape, as the mimicked carbohydrate. Instead, it may be facilitated by a high degree of flexibility that characterises peptides. This can allow for an arrangement of peptide functional groups within the target binding site that is critical for the structural mimicry to occur. Another type of interactions, namely functional mimicry, takes place, when a peptide does not interact with the binding site of the target in the same way, as the mimicked carbohydrate. Here, the peptide binds engaging different set of residues at the binding site of the target. A peptide can also made contacts at a distinct site, as compared to the carbohydrate. Based on the extensive data published, one can conclude that there is no consistent or preferred mechanism by which peptides mimic carbohydrates. Moreover,

mixed types of the above described binding modes are often observed (Agostino et al., 2011, see also examples in the paragraph 4.3).

Immunological mimicry may be desired, if the use of an original antigen in vaccine formulation is not the best suited. Immunologically functional mimetics (mimotopes) induce antibodies that cross react with the original antigen. Mimotopes can be useful, if a natural antigen is unknown, hard to purify, or synthesise, or weakly immunogenic (Popkov et al., 2000; Tai et al., 1985). This also applies in general to carbohydrates that due to chirality are costly, and uneasy in their synthesis. Here, application of carbohydrate mimicking peptides as surrogates is justified, and often possible (Fleuridor et al., 2001; Kieber-Emmons et al., 2000). However, discrepancies between antigenicity and immunogenicity of mimicking peptides are often observed (El Kasmi et al., 1999; Willers et al., 1999). Many antigenic peptides isolated with antibodies are not immunogenic, because they do not bind the same paratope, as the natural epitope. Based on the published data, it is still unclear to what extent antibody bound conformation of a peptide, or conformation of the peptide in solution is related to immunogenicity (Theillet et al., 2009). It is known, that biologically functional mimicry can be affected for example by the sequence, flexibility of free peptides, a phage environment, conjugation to carrier protein, expression on a protein scaffold, a fit induced during interactions between the selecting antibody and the mimic.

Many features of peptides can justify their use in place of carbohydrates. The methodology of their synthesis is well established, and can be automatic. The peptide structure can be modified in order to delay their degradation *in vivo*. Although, peptides in general are highly flexible, there are approaches to restrain their conformation through introduction of structure constrains, or their presentation as a part of a larger protein scaffold. More importantly, peptides are thymus dependent antigens, and therefore attractive surrogates of generally weakly immunogenic carbohydrates. Furthermore, peptides can be conjugated to carrier proteins, and even transferred to a DNA sequence, depending on the delivery schedule, and types of the immune response desired. The molecular mimicry lays a basis for application of anti-idiotypic antibodies, and peptide mimetics of the GD2 ganglioside isolated from phage displayed-libraries, as novel candidates to induce immune responses against neuroblastoma.

3. Anti-idiotypic antibodies to target the GD2 ganglioside

Monoclonal antibodies are effective means to fight cancer. The molecules can affect signalling pathways critical for malignancy, trigger, or enhance anti-tumour immune responses (Weiner et al., 2009). Monoclonal antibodies binding the GD2 ganglioside, *e.g.*, the 14.G2a (mouse monoclonal antibody, IgG2a), the 3F8 (mouse monoclonal antibody, IgG3), the ch14.18 (chimeric antibody consisting of variable regions of the murine monoclonal antibody 14.18, and the constant regions of human IgG-K) were tested in clinical trials (Modak & Cheung, 2010). Recently, treatment with the ch14.18 was shown to significantly improve event-free survival, and overall survival in a phase III clinical trial in patients with high risk neuroblastoma (Yu et al., 2011). Moreover, approaches to reduce the treatment-associated pain are being developed (Kushner et al., 2011; Sorkin et al., 2010). On the one side, HAMA (human anti-mouse antibody), and HACA (human anti-chimeric antibody) responses can be induced in patients after administration of antibodies, and if acute unable the further treatment. On the other side, induction of idiotypic network measured by

presence of anti-anti-idiotypic antibodies and binding the GD2 ganglioside antibodies, 6 and 11 months after the 3F8 administration, was associated with long-term survival of patients with stage 4 neuroblastoma (Cheung et al., 2000).

Three major types of anti-Ig molecules can be induced, based on which part of the antibody they will recognize, *i.e.*, anti-isotypic, anti-allotypic, anti-idiotypic antibodies. Isotypic determinants consist of epitopes specific to one of five heavy chains (H), or one of two light chains (L) of antibodies. Allotypic determinants reflect allelic polymorphism existing in various regions of Ig constant genes. Thus, a particular isotype of an antibody common to members of a species may exist in several allogenic forms. Additionally, each Ig molecule has a unique (not-shared by any other member of the species) relatively minor differences that are defined by the hypervariable sequences in the variable region (*i.e.*, antigen binding region). The collection of such unique determinants on a given Ig defines its idiotypes. It is suggested that network of anti-idiotypic antibodies may have a physiological function by regulating immune responses (Mak & Saunders, 2006).

Anti-idiotypic antibodies binding specific paratopes can be induced with anti-GD2 ganglioside antibodies (termed Ab1). Some anti-idiotypic antibodies can be used as surrogate antigens in immunizations to target the GD2 ganglioside. Variable regions of such anti-idiotypic antibodies (Ab2β) may "immunologically resemble" the GD2 ganglioside, yet are not identical with the glycolipid. They react with the Ab1 antibody, and upon administration induce anti-anti-idiotypic antibodies (Ab3), also specific toward the GD2 ganglioside (Ab1') (Bhattacharya-Chatterjee et al., 2000; Saleh et al., 1993).

The first anti-idiotypic antibody for neuroblastoma, used in clinical trials, was the monoclonal anti-idiotypic antibody for the 3F8, A1G4. In the phase I study in relapsed or high risk patients, administration of the anti-idiotype intravenously (i.v.) at 0.1, 0.3, 1 mg/kg for a total of 10 doses, induced anti-GD2 ganglioside antibody responses at all doses tested. No dose limiting toxicities (DLTs) were observed (Modak & Cheung, 2007 as cited in Modak & Cheung, 2010). 1A7 is yet another anti-idiotypic antibody. It was raised against the 14.G2a antibody. The 1A7 was tested in melanoma patients. 47 advanced melanoma patients received 1, 2, 4, 8-mg doses of the 1A7 mixed with QS-21 adjuvant (100 μg), subcutaneously (s.c.), weekly for 4 weeks and then monthly until disease progression. In 40 out of 47 patients that continued on study beyond 3 months, anti-GD2 ganglioside antibody responses were detected. In 20 patients the antibody concentrations ranged from 34-240 μg/ml. The responses were predominately IgG. There was no additional toxicity, such as abdominal pain. Authors concluded that although objective responses had been minimal, the approach should be tested in prospective randomised trials to further investigate possible favourable impact on disease progression (Foon et al., 2000).

Zeytin et al. constructed DNA vaccine (pc1A7VHLnVL) encoding secreted form of the single chain variable fragment of the 1A7 (1A7scFV). Intramuscular injection of the DNA vaccine to mice induced both antibodies against the 1A7 antibody, and against the GD2 ganglioside. However, no cellular immune responses were observed (Zeytin et al., 2000). On the contrary, GD2 ganglioside specific delayed-type hypersensitivity reactions (DTH) to the challenge with syngeneic GD2-positive melanoma cells D142.34 were induced by s.c. injection of mouse anti–idiotypic antibody TA412G Cl1 with complete Freund adjuvant in C57BL/6 mice, but not in in C57BL/6 $CD4^{-/-}$ mice. This suggested involvement of $CD4^{+}$ T cells in the observed DTH reactions. Additionally, 3 out of 6 of anti-idiotypic antibodies (TA17A Cl2, TA412G Cl1, TB310B Cl1), precipitated by alum and administered to rabbits, induced IgG antibodies binding GD2 ganglioside positive melanoma WM115, and glioma 251 MG cells (Basak at al., 2003).

4. Generation of peptide mimetics of the GD2 ganglioside

Peptide displayed on bacteriophages, plasmids, polysomes, and various types of synthetic peptide libraries are invaluable sources of new ligands. They can be further tested as therapeutic leads, and therefore have a potential commercial value. In the following chapters, the technology of screening of peptide libraries based on filamentous bacteriophage display will be characterised. This will be followed by review of currently undertaken approaches to generate peptides mimicking the GD2 ganglioside.

4.1 The filamentous bacteriophage life cycle

The phage display is a powerful and challenging method to study interactions of proteins with other molecules including peptides, nucleic acids, and carbohydrates. Generally, the technique applies filamentous bacteriophages of the genus *Inovirus* (M13, fd, f1) infecting *Escherichia coli*, although other viral species, *i.e.*, T4, λ could be employed for peptide display.

The wild filamentous viruses are rod-like structures, 930 nm in length, and 6.5 nm in diameter. Their protein capsid is mainly build of a small protein, the major coat protein p8, which is present in 2700 copies, and covers like a cylinder a circular single-stranded DNA genome. The DNA is build of 6400 bases, and encodes for eleven genes. At one end of the virion, there are five copies of each of p3 and p6 proteins, and the other end contains five copies of each of p7 and p9 proteins. The filamentous phages infect male *E. coli* bacteria that have F pilus, made of subunits of pilin protein. The structure interacts with the p3 protein in the first step of infection. The further steps of the life cycle involve amplification of viral ssDNA, which is mediated by viral proteins p2, p5, p10 and host bacterium proteins. A double stranded circular DNA called a replicative form (RF) arises as an intermediate in the process. The minus strand of the RF form is used for transcription. Numerous copies of the viral ssDNA, covered with dimers of the p5 protein are produced as a result of the replication. This induces collapse of the DNA to a rod, leaving only the packaging signal exposed.

The viral proteins that build the capsid, as well as proteins p4, p1, p11 are synthesised, and then embedded in bacterial membranes. The p4, p1, p11 proteins form sites (channels) in the bacterial envelope which bring cytoplasmic and outer membranes in a close contact. Here, a process of assembly of new viral particles takes place. The filamentous phages do not kill the infected bacteria, but slow the ratio of bacterial growth by half. The assembly process allows packing of all the capsid protein, integrated to the cytoplasmic membrane, around the DNA extruding from the cytoplasm. The end that leaves first the bacterium contains the p7 and the p9 protein, interacting with the packaging signal. The process of exchange of the p5 to the p8 continues till the end of the DNA is reached. Then, the p3 and the p6 are added, and a newly formed bacteriophage particle is released (Rakonjac et al., 2011).

4.2 Phage display technology

Because of the nature of the membrane-associated assembly, there is virtually no constrains on the size of DNA to be packed in the capsid. Therefore, the bacteriophage vectors are useful for construction of genomic, cDNA libraries, and display of peptides or even whole proteins on the surface of phage particles. The most prominent example of the protein display is construction of libraries of antibody fragments that are proved sources of human fragments of monoclonal antibodies (Rakonjac et al., 2011).

A large number of libraries consisting of variants of peptides are available. They differ by number of clones, length of peptide sequences that can be random, or constrained due to the

presence of cysteine residues forming a disulfide bond. Although all protein building the capsid can be used for display, the most common are libraries utilizing the p3, and the p8 proteins for presentation of foreign sequences. The pivotal concept is that the peptides displayed on the bacteriophage surface, and the DNA sequence coding for that peptide are physically linked in one viral particle. This enables easy verification of sequences of peptides isolated from the libraries. The libraries are constructed by cloning of oligonucleotides encoding for peptides in frame with the gene of the protein used for display. Oligonucleotides encoding for peptides can be inserted between sequences encoding the leader sequence and the mature p8 (Fig. 2). Peptides are encoded by NNK or NNS (where N is A, T, C, G; K is G and T; S is G and C). The cloning can result in all copies of the p3 and the p8 expressed as fusion proteins, or only some of the proteins carrying peptides. The later can be achieved by introduction into the viral genome of an additional copy of the recombinant gene, besides the wild-type gene. The recombinant gene can also be delivered to bacteria on a separate genome (a phagemid). Moreover, peptide libraries can be expressed as a part of a larger protein scaffold (Uchiyama et al., 2005).

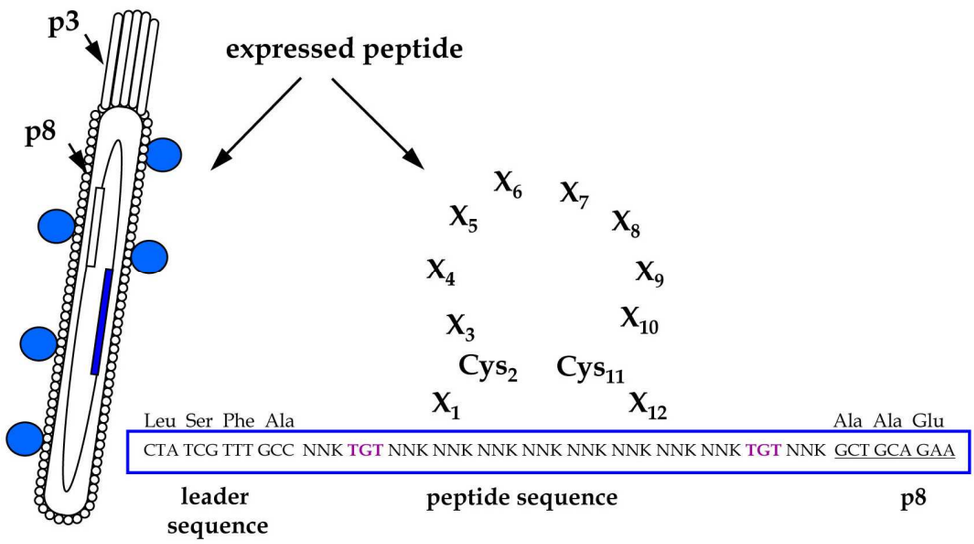

Fig. 2. A schematic representation of the constrained peptide library LX-8. Peptides are expressed as fusion with the p8 protein. Two genes encoding p8 proteins are present in the bacteriophage genome, a wild type (white), and a recombinant one (colour filled). The leader sequence is removed during processing of the p8 (Bonnycastle et al., 1996).

The process of screening of phage-displayed peptide libraries is called a panning. During the purification process a peptide library is allowed to interact with screening molecules such as for examples antibodies, enzymes, receptors, lectins, or hormones. The target receptor can be coated to plastic surfaces, membranes, or used in a solution phase. Affinity interactions are basis for most of the screening protocols. Therefore pre-adsorption steps are necessary to reduce the numbers of phage clones that bind to other than screening molecules, *e.g.*, blocking agents, washing buffer ingredients, and surfaces used for target coating. Phages captured via their displayed peptides by the target screening molecule can be retained during washing steps. Then, they are amplified in bacteria, and the panning is repeated. A resulting pool of phages is enriched in peptides that bind the target molecule used for the screening. From such pools single clones can be isolated. Next, the peptides are

decoded through DNA sequencing, and then characterised in the phage-displayed environment, as well as in a free form (Fig. 3). The screening can also be designed and

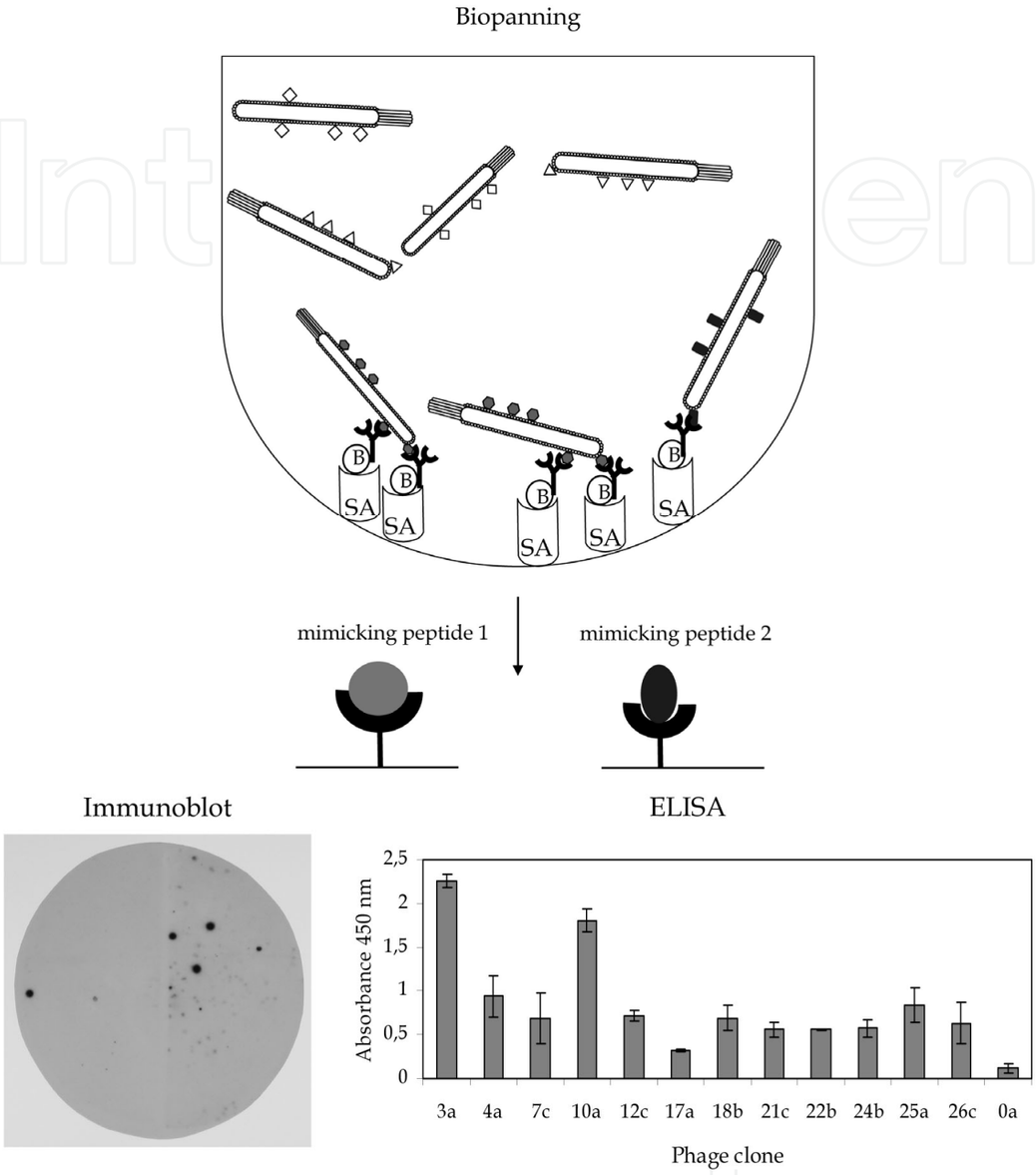

Fig. 3. A schematic representation of a biopanning (top). Biotinylated antibodies are immobilised in streptavidin-coated wells. The pre-adsorbed library of phage-displayed peptides can interact with the immobilised target molecules (see text for more details). Immunoblotting and ELISA can be used to analyse phage clones isolated from peptide libraries (bottom). The examples from the screening of the LX-8 library with the 14.G2a were included (see Horwacik et al., 2007 for more details). Replicas of bacteria infected with phages on nitrocellulose membranes were incubated with the 14.G2a antibody and the secondary HRP-conjugated antibody, and then developed (bottom left). ELISA with immobilised 14.G2a antibody was used to capture specific phage clones. Phage binding was detected with anti-p8 antibodies. The 0a phage is a negative control (bottom right).

conducted on target molecules expressed on the surface of whole cells, or even in mice. Applicability of the technique is enormous, and includes new ligand identification, quest for agonist and antagonists of receptors, elucidation of binding motifs, epitope mapping, analyses of enzymatic activity, new drug and vaccine design (Dudak et al., 2011, Smith & Petrenko, 1997).

4.3 Isolation and optimisation of peptide mimetics of the GD2 ganglioside isolated from phage-displayed libraries

It is a common finding that in addition to binding to original epitopes, antibodies can cross-react with several antigens (Van Regenmortel, 2001). Phage display techniques were used to exploit the molecular mimicry phenomenon to isolate peptide surrogates to tumour-associated carbohydrate antigens, *e.g.*, Lewis Y (Kieber-Emmons et al., 1999), sialylated Lewis a/x (O et al., 1999), and GD3 (Willers et al., 1999). The examples of the peptides mimicking the GD2 ganglioside, the antibodies used for their identification, as well as references were for convenience collected in the Table 1. Following is the description of their antigenic and immunogenic properties, analysed with experimental and computational tools, as well as strategies that were used to improve their mimicry.

Antibody	Peptide name	Peptide sequence	Reference
ME36.1	P9	LDVVLAWRDGLSGAS	Qiu et al., 1999
	P10	GVVWRYTAPVHLGDG	Monzavi-Karbassi et al., 2007
	P10s	WRYTAPVHLGDG	
Ch14.18	MD	CDGGWLSKGSWC	Förster-Waldl et al., 2005
	MA	CGRLKMVPDLEC	Bleeke et al., 2009
	C3	CGRL**HL**VPDLEC	
14.G2a	47	EDPSHSLGLDVALFM	Bolesta et al., 2005
	47-LDA	EDPSHSLGLD**A**ALFM	
14.G2a	94	RCNPNMEPPRCF	Horwacik et al., 2007
	94-12-F/W-AAEGD	RCNPNMEPPRC**WAAEGD**	Horwacik et al., 2011

Table 1. Examples of mimicking peptides isolated with the anti-GD2 ganglioside monoclonal antibodies.

4.3.1 Peptides isolated with the ME36.1 antibody

The ME36.1 monoclonal antibody displays reactivity with the gangliosides GD2 and GD3. The antibody was used to screen the 15-mer library build with the fUSE5 vector. Qiu et al. reported in 1999 results of a panning of the library with the ME36.1 antibody. 16 peptides were isolated. Sequence analysis revealed presence of mainly unique sequences, which indicated that many peptides could mimic the GD2 ganglioside binding to the ME36.1. Interestingly, some similarities between the peptides isolated with the ME36.1 and the BR55-2 (binding Lewis-Y) were found, *i.e.*, presence of WRY and AP sequences in the peptide P10 (GVVWRYTAPVHLGDG), and WRDG in the peptide P9 (LDVVLAWRDGLSGAS) that is similar to YRGD found in a peptide isolated with the BR55-2.

ELISA was applied to compare ganglioside reactivity of IgM in sera samples of non-immunised animals, and animals immunized with the P10. For vaccinations the P10 was presented as multiple antigenic peptides of 8 peptide clusters (MAP), injected intraperitoneally (i.p.) with QS-21 adjuvant. The obtained results suggested that the P10 might induce serum IgM antibodies reactive with multiple gangliosides. The analysis of data comparing dilutions yielding half-maximal binding to different gangliosides showed that the peptide could functionally mimic GD1b, GD1a, GD3, and only to a lesser extent GD2 and GM2. The finding was supported with molecular modelling approach revealing that the P10 interacts with the ME36.1 with only two hydrogen bonds (with Tyr33 and Ser100 from the heavy chain) in common, as compared to the GD2 ganglioside binding network to the antibody (*i.e.*, with Tyr33, His35, Asp50, Asn52, Asn59, and Ser100 from the heavy chain, and Tyr93 from the light chain).
In efforts to improve the immunological mimicry of the GD2 ganglioside, a shorter peptide missing the N-terminal GVV sequence was designed (P10s). It showed a rise in numbers of bonds involved in the ME36.1 interactions (Tyr33, Asp50, Asn59, and Ser100 from the heavy chain, and Tyr93 from the light chain). Additionally, immunisation with the P10s induced IgM in sera with augmented reactivity toward GD2, GD3, GM2 (as compared to the P10). Moreover, the sera collected from mice immunised with the P10s showed higher binding to the GD2 ganglioside positive MCF7 human breast cancer cells, and WM793 melanoma cells, as compared to sera from mice immunised with the P10 (Monzavi-Karbassi et al., 2007).

4.3.2 Peptides isolated with the ch14.18 antibody

Förster-Waldl et al. published in 2005 results from their panning experiments (Förster-Waldl et al., 2005). The group screened a peptide phage-displayed library expressed in the context of the p3, and constrained by presence of additional cysteines at N and C termini of the peptides. The authors reported isolation of 13 different phage-displayed peptides that specifically recognized the ch14.18 antibody, in contrast to wild type phages. An isotype control antibody (cetuximab) was included in the binding assays. In most of the clones R is present in the position 2 of the decamers flanked by cysteines, hydrophobic V, L, or M are in the positions 5 or 6, and acidic D or E occupies the position 10. The antigenicity of the phage-expressed peptides was positively verified with application of a competitive ELISA, to measure their specific binding to the ch14.18 against simultaneously added GD2 ganglioside. Two peptide sequences were chosen for further studies, *i.e.*, the MD peptide (CDGGWLSKGSWC) and the MA peptide (CGRLKMVPDLEC).
In the next step, a computer model of interactions between the ch14.18 and the GD2 ganglioside, or the two peptides was built. The peptide MD was shown not to block the binding site as effectively as the peptide MA. In 2006, additional *in silico* data were published by Fest et al. Eight residues of the ch14.18 were found to interact with both the two peptides and the GD2 ganglioside, *i.e.*, Tyr37, His54, Ser96, Val99, Pro100, Leu102 from the light chain, as well as Gly98 and Gly100 from the heavy chain. Also, dissociation constants were determined with surface plasmion resonance (Fest et al., 2006). Immunogenicity of the both peptides was investigated after their conjugation to KLH. In the sera of mice immunised with both peptide conjugates antibodies of IgM and IgG isotypes recognising the GD2 ganglioside were raised, as detected with ELISA. The sera samples also bound specifically to the GD2 positive M21 melanoma cells (Reimer et al., 2006).
The MA sequence was recently improved, as reported by Bleeke and co-authors. This was achieved after another two rounds of screenings with the ch14.18 of synthetic sub-libraries,

produced on a solid support, and containing systematic alterations based on the MA sequence (Bleeke et al., 2009). The experiments yielded a new mutated sequence C3 CGRL**HL**VPDLEC with significantly improved binding to the ch14.18, as determined with dot blot and surface plasmion resonance measurements using BIACORE.

4.3.3 Peptides isolated with the 14.G2a antibody

Bolesta and co-authors reported screening with the 14.G2a of 15-mer library of linear peptides, fused with the p8 protein (in the f88-4 vector). 6 peptides were isolated, and shown to bind to the 14.G2a antibody by ELISA. Moreover, all the peptides in the free form competed for binding to the 14.G2a with the GD2 ganglioside present on a human neuroblastoma cell line IMR-32 (Bolesta et al., 2005). Importantly, three peptides (47, 9, and 51) cross-reacted with GD2 ganglioside binding antibodies from a serum sample of a neuroblastoma patient. Competition experiments between peptides 47, 9, and 51 showed that they mimic an overlapping epitope of the GD2 ganglioside.

The peptide 47 (EDPSHSLGLDVALFM) with highest score in the competition tests was chosen for further optimisation. Also here, molecular modelling was conducted to build a model of interactions between the 47 peptide, and the antigen binding site of the 14.G2a. Additionally, alanine scanning enabled identification of amino acid residues pivotal for the observed binding. As a result, the LGLDVALFM sequence was found essential for the interactions with the 14.G2a. More importantly, substitution of V with A yielded the 47-LDA (EDPSHSLGLD**A**ALFM) peptide that was 2-fold more effective than the original 47, in the competition assays against the IMR-32 cells.

Based on the findings, the 47-LDA peptide, and two universal T helper epitopes (PADRE, and $P18_{MN}$ epitope from envelope protein of HIV-1 isolate MN) were encoded in a DNA vaccine, constructed in the pNGVL-7 vector that contained the tissue plasminogen activator secretory signal sequence. The vaccine induced anti-GD2 ganglioside IgG antibodies in BALB/c mice. The level of GD2 ganglioside cross-reactive antibodies was further increased with prime-boost strategy that utilised first the 47-LDA construct, and then the GD2 antigen administered in incomplete Freund's adjuvant. The sera samples obtained from immunisations with the DNA construct alone, as well as in combination with the GD2 ganglioside boost, mediated complement dependent lysis of the GD2 positive IMR-32 neuroblastoma, and HT-144 melanoma cells (Bolesta et al., 2005).

Yet another 5 sequences binding to the 14G2a antibodies were isolated, but this time from the constrained library LX-8 (Horwacik et al., 2007). The library displays 12-amino acid peptides. Their structure is constrained by the presence of two cysteines at the positions 2 and 11. Binding motifs were identified through sequence comparisons. LTG or LSG motifs are present in the centre in 3 of 5 isolated peptides. D or N is present in the position 3 of 4 peptides, while P occupies the position 4 in 2 peptides, and L is present in the positions 9 (in 2 peptides), and 10 (in 3 peptides). Finally, S occupies the position 12 in 2 peptides. All five peptides were shown to mimic the GD2 ganglioside, as analysed by ELISA. The peptides presented in the phage-bound form, as well as the free synthetic form competed for binding to the 14.G2a with the GD2 ganglioside present on the IMR-32 cells (Fig. 4). More importantly, the presence of the disulfide bond, formed by the cysteines 2 and 11, is pivotal for the observed mimicry of the GD2 ganglioside for all 5 constrained peptides.

To further characterise antigenic features of the peptides, we analysed reactivity of 3 peptides (65, 85, 94) with other than the 14.G2a mouse monoclonal antibodies that had been raised against gangliosides, *i.e.*, the anti-GD2 antibody 126 (IgM), anti-GD2 antibody ME361-

S2A (IgG2a), and the anti-GD3 antibody ME3.6 (IgG3). But, the peptides did not bind to them, which highlights the fact that peptide-carbohydrate mimicry is observed in the context of a given receptor (the 14.G2a in this case). Additionally, the peptides were shown to occupy overlapping binding sites on the 14.G2a. This finding was supported by *in silico* models of interactions of the peptides with the Fab fragment of the 14.G2a (Horwacik et al., 2011).

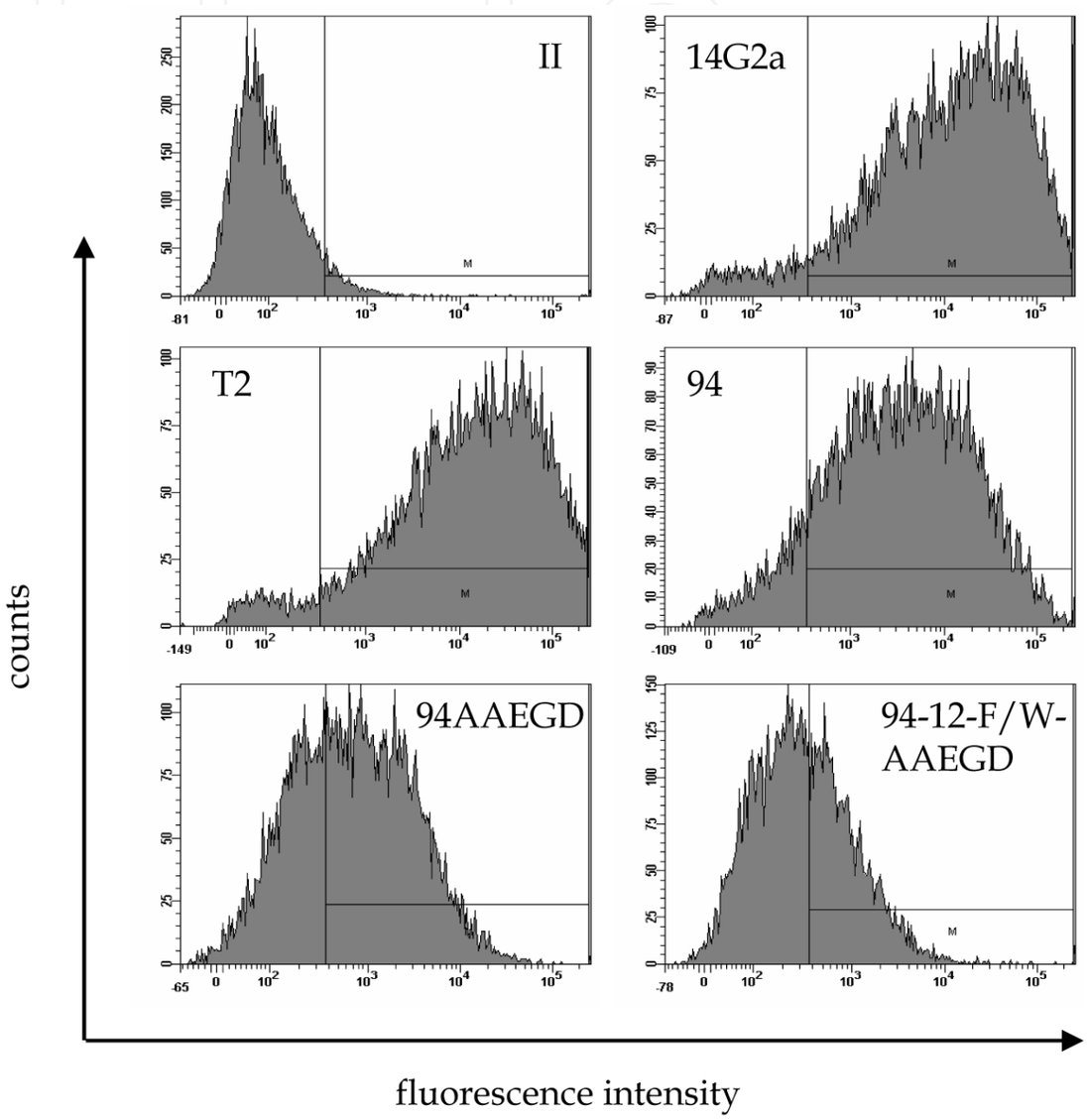

Fig. 4. The peptide 94 can compete with the GD2 ganglioside present on IMR-32 neuroblastoma for biding to the 14.G2a (as analysed by flow cytometry). The inhibitory effect is significantly improved for modified peptides 94-AAEGD and 94-12-F/W-AAEGD. No inhibition was detected with a control peptide T2. The pool of GD2-positive IMR-32 cells was determined by comparison of cells stained with the 14.G2a and the secondary FITC-conjugated $(Fab')_2$ fragments specific to mouse immunoglobulins (14.G2a), and cells stained only with the secondary antibody fragments (II).

The peptide 94 (RCNPNMEPPRCF) was chosen for further analysis and optimisation of the binding to the 14.G2a. Alanine scanning, replacement analysis, and truncation experiments allowed to elucidate structure-activity relationships that are the basis for the observed mimicry of the GD2 ganglioside by the peptide 94. The experiments showed that C2, N3, M6, E7, C11 are indispensable for the observed binding, as their substitution by alanine resulted in a complete loss of the 14.G2a binding. A new peptide with better binding to the antibody was identified by replacement of F at the position 12 with W. The antigenicity was even further improved with extension of the C terminus with the AAEGD sequence, taken from the N terminus of the viral coat protein p8 (Horwacik et al., 2011). The peptide 94-12-F/W-AAEGD (RCNPNMEPPRC**WAAEGD**) was the best in the competition assay against the IMR-32 cells, and showed 18-fold decrease in the measured IC50 values (half maximal inhibition concentration), as compared to the original 94 sequence (Fig. 4). The obtained peptides are currently being further analysed to verify relationships between their antigenicity and immunogenicity.

4.4 Vaccination using the GD2 mimetics in preclinical settings

Many of the isolated peptides mimicking the GD2 ganglioside were tested in pre-clinical experiments, using several modes of delivery (*e.g.*, DNA vaccines, dendritic cell-based vaccines, KLH-conjugated peptides) with application of syngeneic (Bleeke et al., 2009; Gil et al., 2009; Kowalczyk et al., 2007; Wierzbicki et al., 2008; Wondimu et al., 2008), and heterotransplant mouse models (Bolesta et al., 2005).

4.4.1 Tests of GD2 ganglioside mimics in melanoma models

Sera were collected from animals immunised with the 47-LDA DNA vaccine/GD2 ganglioside boost. Therapeutic efficacy of purified fractions of mouse IgG antibodies containing the GD2 ganglioside cross-reactive antibodies was tested in a heterotransplant model, based on s.c. injection of a human MV3 melanoma (10^6 cells). Multiple i.v. injection of the antibodies were conducted, starting 3 days after the tumour challenge. They lead to significant decrease in tumour growth in the group of animals treated with the GD2 antibodies, as compared to control mice treated with murine IgG. However, none of the mice on therapy with the GD2 ganglioside specific antibodies showed a complete resolution of the melanoma tumours (Bolesta et al., 2005).

Similar indications of anti-melanoma efficacy of vaccines based on the GD2 mimicking peptides were gathered on a syngeneic model with s.c. injections of GD2 ganglioside positive melanoma cells D142.34 to C56BL/6 mice. Peptide vaccines containing the P9, or the P10 conjugated to KLH, or in the form of MAP were delivered s.c. with QS-21 adjuvant. GD2 ganglioside specific antibodies were raised by the immunisations with both peptides, as compared to control group injected with an unrelated peptide. In animals that received vaccines with the peptides, DTH reactions were detected 48, and 72 h after administration of irradiated D142.34 cells, in contrast to injections with GD2 ganglioside negative B78.H1 melanoma cells. In a prophylactic setting, the vaccines with the P10 were more effectively inhibiting s.c. growth of the D142.34 cells than the vaccines with the P9, as evidenced by tumour volume and weight measurements. Moreover, P10-KLH vaccinations in a therapeutic setting, starting 10 days after challenge with the D142.34 cells, delayed growth of tumours, as compared to control peptide vaccination. Although, the statistical

significance could not be achieved in the therapeutic setting, the data emphasise that induction of protective responses targeting the melanoma cells with active immunisation with the GD2 mimicking peptides was possible (Wondimu et al., 2008).

4.4.2 Tests of GD2 ganglioside mimics in syngenic neuroblastoma models

A syngeneic mouse model based on injections of NXS2 cells that show heterogeneous expression of the GD2 ganglioside to mice of A strain was reported in 1997 (Lode et al., 1997). The model was applied to test DNA constructs bearing Ig κ leader sequence, T helper epitope for HIV-1 envelope protein, and MA, or MD epitopes separated with flexible glycine-serine linkers in the pSA vector. The construct was used for oral mice immunisation after transfection to attenuated *Salmonella typhimurium* SL7207. The regiment used in a prophylactic setting was shown to significantly reduce spontaneous liver metastases, as determined about a month after removal of s.c. NXS2 tumors. This was correlated with induction of anti-GD2 antibodies responses in sera of mice vaccinated with MA or MD, as well as with significant increase in activity of spleen derived NK cells from pSA-MD immunised mice. The later was evidenced by ability of the NK cells to lyse YAC-1 cells, but not NXS2 cells. However, the effect was only observed with the effector cells co-cultured with irradiated NXS2. Additionally, orally delivered *Salmonella typhimurium* SL7207, carrying the empty vector pSA, was shown to be an effective adjuvant to establish GD2 ganglioside specific antibodies, and NXS2 protective immune responses in A mice, with protein vaccines containing the peptides MD and C3 conjugated to KLH, and absorbed on alum. No $CD8^+$ T cell immune responses were observed with the DNA vaccines. No experiments in therapeutic setting were described (Bleeke et al., 2009; Fest et al., 2006).

On the contrary, sequence of the peptide 47-LDA delivered in the form of DNA construct effectively induced antibody and cell mediated immune responses that inhibited growth of s.c. NXS2 tumour in A mice in therapeutic setting. Kowalczyk and co-authors showed that protection against tumour challenge with NXS2 cells required both innate (NK cells), and adoptive cell-mediated immunity ($CD4^+$ and $CD8^+$ T cells). Furthermore, administration of plasmids encoding IL-15, and IL-21 cytokines together with the 47-LD construct markedly enhanced the tumour protective immunity. In the group of animals that were treated with the combined immunisation regiment, delivered from 1 day after s.c. NXS2 challenge, 6 of 8 animals remained tumour-free more than 90 days. The inclusion of the plasmids encoding IL-15, and IL-21 in the vaccination protocol, significantly increased levels of GD2 ganglioside cross-reactive IgG in sera of mice, as well as CDC and ADCC against NXS2 cells. More importantly, re-challenge experiment in the group of 8 animals that remained tumour free, showed that all the animals survived additional 90 days, in contrast to control mice that developed tumours by day 30. Finally, adoptive transfer of $CD8^+$ T cells from 47-LDA vaccinated and cured mice to NXS2-challenged mice, significantly influenced tumour control, as most of the mice survived tumour-free for more than 90 days (Kowalczyk et al., 2007).

The $CD8^+$ T cell responses induced with the 47-LDA construct were further investigated. Interestingly, they were shown to be MHC class I restricted to syngeneic neuroblastoma cells (NXS2, Neuro2a), but independent of the GD2 ganglioside expression. This suggested that than GD2 vaccine induced responses cross-reactive with other the GD2 antigen present on neuroblastoma cells. The hypothesis was confirmed with experiments showing that the 14G2a cross reacts with a 105 kDa protein, ALCAM/CD166 (activated leukocyte cell

adhesion molecule). When the expression of CD166 was silenced using specific shRNA in Neuro2a, the cells were no longer killed by $CD8^+$ T cells from mice immunised with the 47-LDA construct. Additionally, such cells induced progressive growth of s.c. tumours in mice, despite of the combined immunisation with the 47-LDA, IL-15, IL-21 constructs that preceded the challenge. This finding shed new light on application of peptide mimicking the GD2 ganglioside. Here the 47-LDA was shown to induce responses to conformation-dependent epitope of CD166 protein (Wierzbicki et al., 2008).
In the following report in 2009, the 47-LDA mimotope was expressed as a fusion protein with the mouse IgG2a Fc region, and used for vaccination based on dendritic cells (DC). Targeting the mimotope vaccine to activating Fcγ receptors of DC allowed for selective expansion of adoptively transferred $CD8^+$ T in NXS2-tumour bearing syngeneic mice. Anti-tumour responses were also observed with virotherapy, after delivery of recombinant oncolytic vaccinia virus expressing the 47-LDA- Fcγ2a fusion protein (Gil et al., 2009).

5. Conclusion

Tumour associated-carbohydrate antigens are highly over expressed in tumour cells, and therefore are attractive targets for both passive and active immunization approaches. Both, mimicking peptides and anti-idiotypic antibodies can functionally mimic carbohydrates, including the GD2 ganglioside. Furthermore, such surrogate antigens often induce humoral and cellular immune responses, which the carbohydrate itself is usually unable to induce. Peptides are easier to manufacture and modify than carbohydrates. Additionally, they can be used to manipulate immune responses, *i.e.*, to present cryptic epitopes, and to break tolerance by focusing the response on critical epitopes delivered in a different molecular environment. The data on application of the molecular mimicry to target the GD2 ganglioside adds knowledge on structural-activity relationships of such mimicking peptides, and anti-idiotypic antibodies, and correlates of their immunogenicity. This extends our understanding of vaccine design in cancer. Hopefully, this will boost research on future vaccines targeting neuroblastoma.

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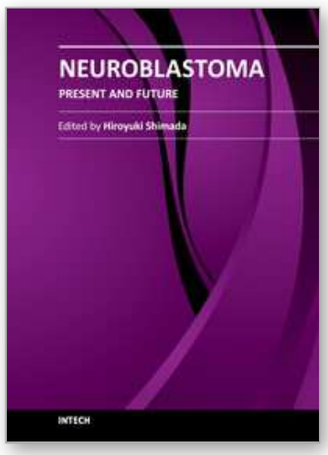

Neuroblastoma - Present and Future

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Neuroblastoma, once called "enigmatic", due to "unpredictable" clinical behaviors, is composed of biologically diverse tumors. Molecular/genomic properties unique to the individual tumors closely link to the clinical outcomes of patients. Establishing risk stratification models after analyzing biologic characteristics of each case has made a great success in patient management. However, the trend of improving survival rates in neuroblastoma over the last 30 years has started to level off, and currently available treatment modalities have almost reached to their maximized intensity. Furthermore, aggressive treatment causes significant long-term morbidities to the survivors. We really need to make the next step to the level of personalized medicine with more precise understanding of neuroblastoma biology. This book includes useful data and insights from the world's experts in this field. I believe this book can make an excellent contribution to all the investigators working hard and fighting for the children stricken by this disease.

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