FY25 Generic Drug Science and Research Initiatives Public Workshop,
Assessment Challenges with Complex Active Ingredients: Peptides & Oligonucleotides
June 3, 2025

Immunogenicity of Oligonucleotides

Sudhir Agrawal, D.Phil.
Founder and President, ARNAY Sciences

sagrawal@arnaysciences.com

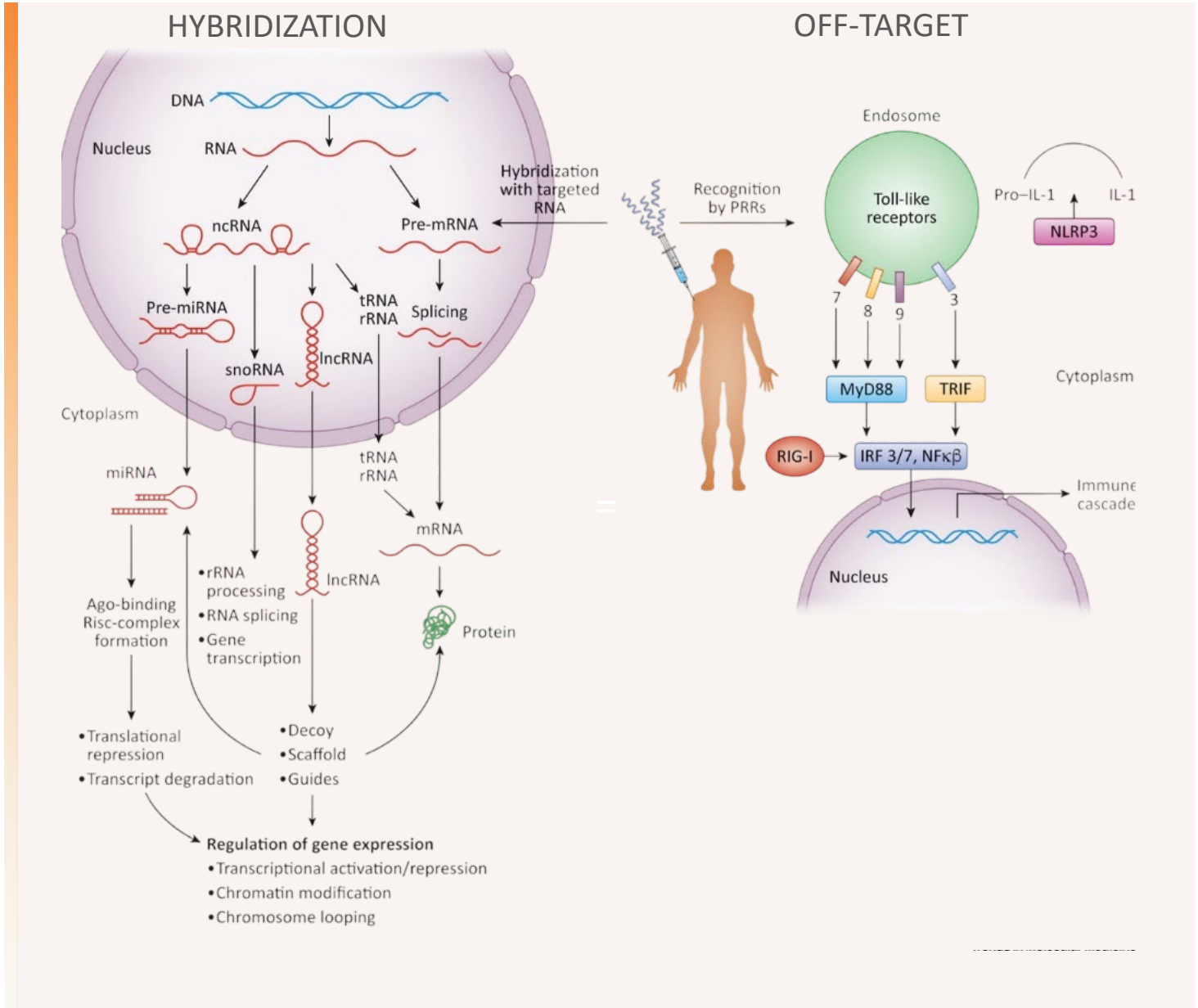
Disclosures:

Founder and President, Arnay Sciences

Advisor/SAB member of Quralis, Dyne Therapeutics, Haya Therapeutics, Alloy Therapeutics, Quiver Bio, ProGenis, and SynGenis

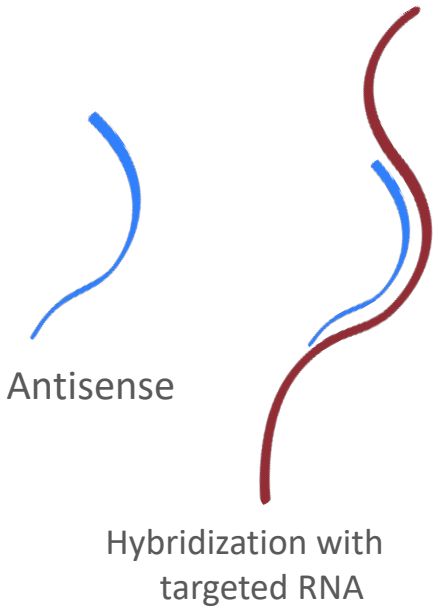
Affiliate Professor in the Department of Medicine, UMass Chan Medical School; Business advisor, Harvard's Initiative on RNA Medicine; SAB member, Dan Lewis Foundation for Brain Research

RNA therapeutics



RNA Therapeutics Are Stepping Out of the Maze
Sudhir Agrawal
Trends Mol. Med. 2020 Dec;26(12): 1061-1064

Antisense oligonucleotide: Mechanisms of action



Modulation of protein expression

- RNase H mediated excision > Degradation of targeted RNA
- Splicing > Expression of the desired protein
- 5’UTR > Increased Expression of the desired protein
- ncRNA/lncRNA > Increased or decreased translation
- miRNA > Gene regulation
- siRNA>Degradation of targeted RNA
- ADAR mediated editing > A-I editing in mRNA
- CRISPR -Editing of DNA

Chemistry of antisense therapeutics

Late eighties

Chemistry of antisense oligonucleotides

Recent

Chemical engineering of oligonucleotides

Oligonucleotides act as pathogen-associated molecular patterns (PAMPs)

Immunogenicity of Oligonucleotides

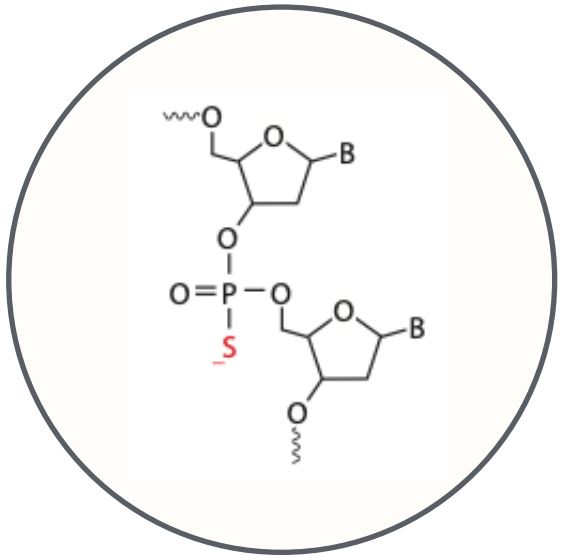
Considerations for creating the next generation RNA therapeutics: Oligonucleotide chemistry and Innate immune responses to nucleic acids

S Agrawal.

Nucleic Acid Therapeutics, 2024,, 37-51

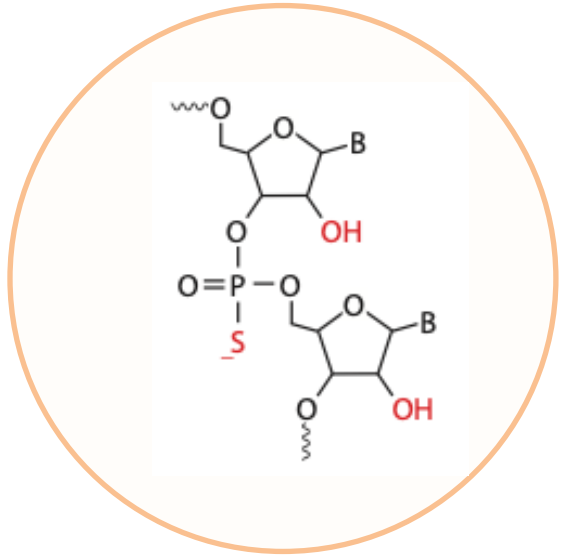
Phosphorothioate Oligonucleotides

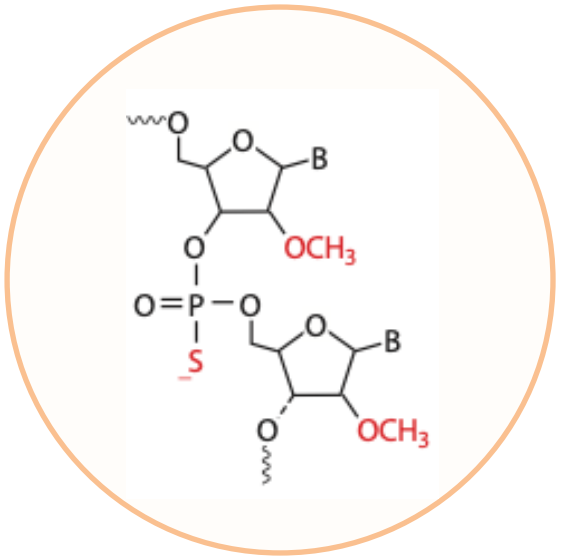
- Nuclease stability
- Affinity with target RNA
- Aqueous Solubility



Phosphorothioate DNA

RNase H substrate

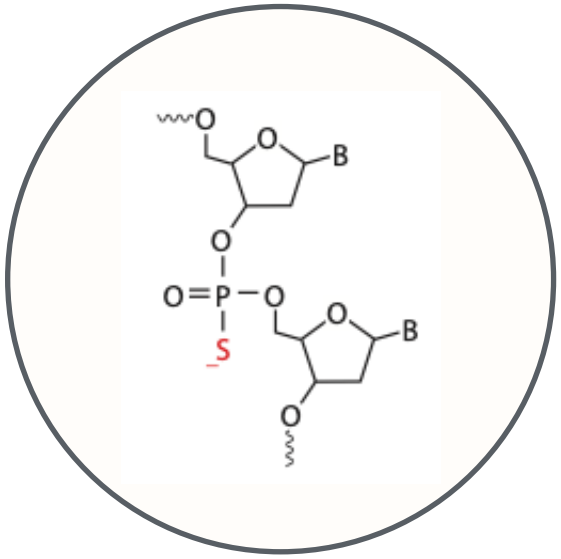




Modified RNA and 2’-Substituted RNA

Lack of RNase H activation

Phosphorothioate Oligonucleotide: Observation of off-target activity



Phosphorothioate DNA

- First-Generation Antisense Candidates
- RNase H-mediated degradation of targeted RNA
- Over twenty antisense candidates advanced to clinical development
  - Antiviral
  - Anticancer
- Immune and polyanionic characteristics
- Mechanism of action of antisense questioned
- Development discontinued

Oligodeoxynucleotide phosphoramidates and phosphorothioates as inhibitors of human immunodeficiency virus

S Agrawal, J Goodchild, MP Civeira, AH Thornton, PS Sarin, PC Zamecnik

PNAS USA. 1988, 85(19)7079-7083

1992-1994

PS-DNA: First observation of complement activation in primates

5’-CTCTCG**CACCCATCTCTCTCCTTCT-3’**, PS –DNA, IV administration

Serum Complement CH50

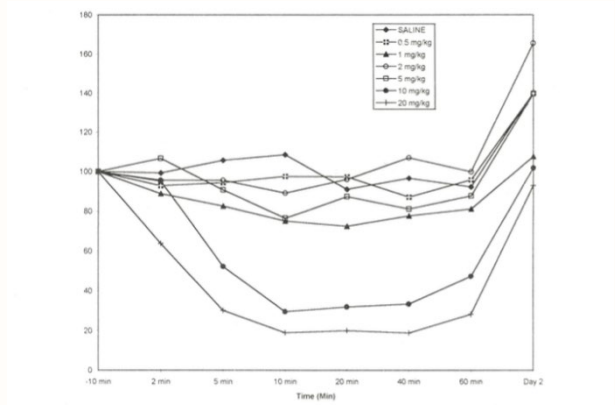


FIG. 4. Level of serum complement (CH50) in monkeys (average of two animals per group), as percent individual baseline following infusion of various doses of GEM 91 intravenously over a 10-min period. The blood samples were drawn 10 min prior to dosing, and at 2 min, 5 min, 10 min, 20 min, 40 min, 60 min, and 24 hr postdosing, and analyzed for level of C50 complement. Day 2 represents a time point between 24 and 30

Serum Complement C5a

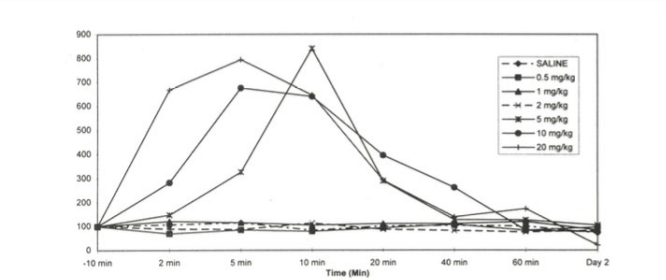


FIG. 5. Level of serum complement (C5a) in monkeys (average of two animals per group), as percent individual baseline following intravenous infusion of various doses of GEM 91 over a 10-min period. Day 2 represents a time point between 24 and 30 hr.

Prolongation of aPTT

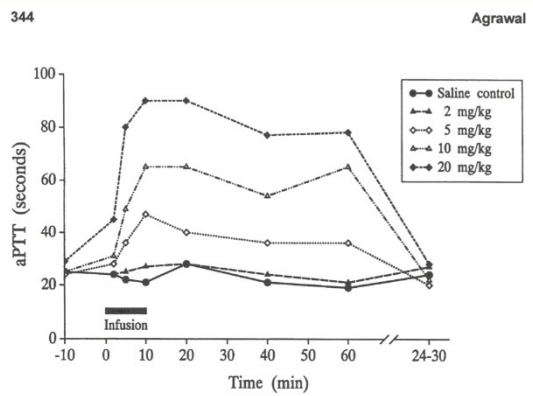


Figure 8. Activated partial thromboplastin tissue in monkeys following infusion of various doses of GEM®91 i.v. over a 10 min. period. The blood samples were drawn at times indicated and analyzed for aPTT. *(reprinted, with permission, from Galbraith et al., 1994).*

Complement activation and hemodynamic changes following intravenous administration of phosphorothioate oligonucleotides in the monkey

WM Galbraith, WC Hobson, PC Giclas, PJ Schechter, S Agrawal

Antisense Res Dev. 1994 Fall;4(3):201-6

1992-1994

PS-DNA: First observation of thrombocytopenia

5’-CTCTCG**CACCCATCTCTCTCCTTCT-3’**, PS –DNA, IV administration

Thrombocytopenia

TABLE 1. HEMATOLOGICAL PARAMETERS

Test	Time from start of infusion (Minutes) –10 min	2 min	5 min	10 min	20 min	40 min	60 min	24 hr
Saline Control (10 min infusion)								
Platelet Count (Thsd/mm³)	489	475	444	484	452	432	442	389
Hematocrit (%)	34.2	34.5	35.6	33.2	33.4	33.7	32.7	34.7
WBC (Thsd/mm³)	8.4	7.9	8.0	7.6	7.8	8.0	8.7	16.1
Neutrophil (%)	57	51	49	56	51	57	63	73
Dose 5 mg/kg (10 min infusion)								
Platelet Count (Thsd/mm³)	445	429	363	242	338	329	326	440
Hematocrit (%)	43.1	42.1	42.4	41.8	46.0	47.6	47.7	40.8
WBC (Thsd/mm³)	5.8	5.8	6.1	2.1	2.0	9.6	16.0	13.7
Neutrophil (%)	63	61	40	3	8	65	74	79
Dose 10 mg/kg (10 min infusion)								
Platelet Count (Thsd/mm³)	391	397	267	192	226	270	282	353
Hematocrit (%)	39.6	39.0	37.7	36.4	38.6	43.2	40.4	33.1
WBC (Thsd/mm³)	10.1	10.0	9.7	3.7	2.5	11.9	16.3	8.7
Neutrophil (%)	40	34	38	3	6	51	47	76
Dose 20 mg/kg (10 min infusion)								
Platelet Count (Thsd/mm³)	346	330	165	179	175	225	245	331
Hematocrit (%)	40.5	39.0	39.4	37.6	46.0	48.1	47.8	41.2
WBC (Thsd/mm³)	10.8	10.0	8.4	4.0	5.0	26.0	33.7	28.7
Neutrophil (%)	37	40	34	2	2	46	51	88

- Plasma concentration dependent
- Sequence Independent
- Length-dependent/ polyanionic
- Avoided by slow infusion

Complement activation and hemodynamic changes following intravenous administration of phosphorothioate oligonucleotides in the monkey

WM Galbraith, WC Hobson, PC Giclas, PJ Schechter, S Agrawal

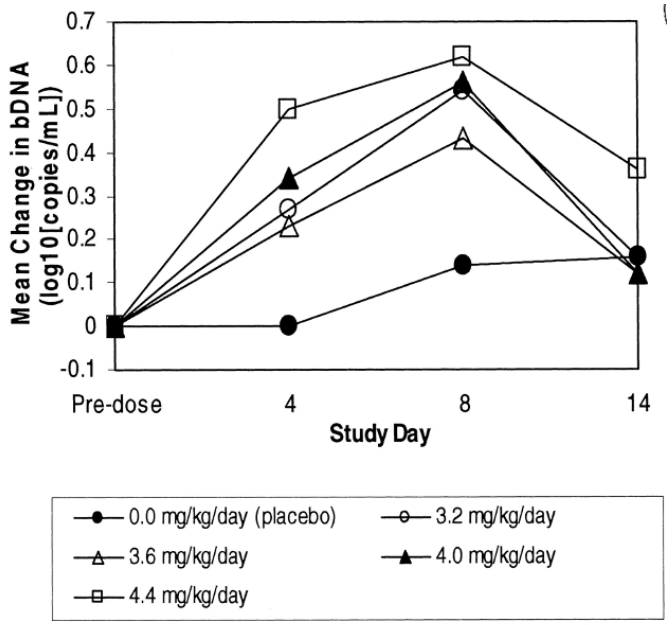
Antisense Res Dev. 1994 Fall;4(3):201-6

1993-1996

First observation of immune activation with PS-DNA antisense

5’-CTCTCGCACCCATCTCTCTCCTTCT-3’ (PS-DNA)

- IV infusion or SC administration in HIV-1-infected patients
- Chills/Flu-like symptoms, induration at the site of injection
- SC administration was more severe than with IV infusion
- Thrombocytopenia in a few subjects
- Increase of HIV-1 RNA in the systemic circulation?



Was induction of HIV-1 Through TLR9?

Sudhir Agrawal; R. Russell Martin

J Immunol (2003) 171 (4): 1621-1622

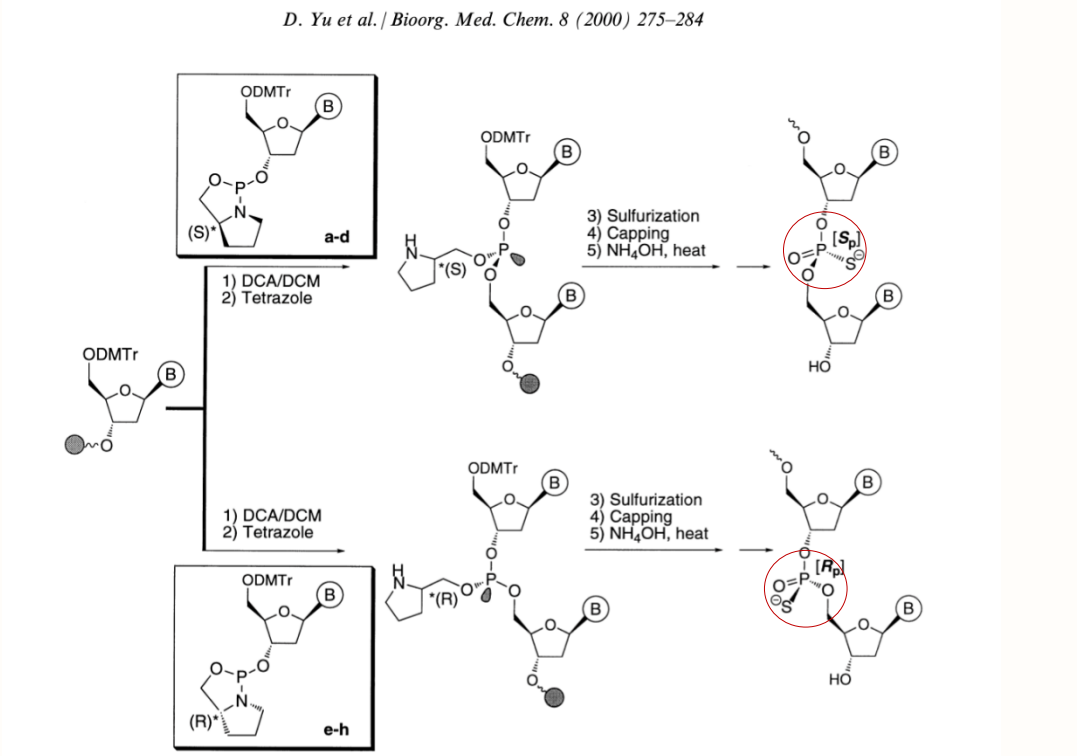
PS oligonucleotides: Impact of stereoisomers on off-target activity?

Rp isomer

- Less stable against nucleases
- Better RNase H substrate
- Increased CpG-mediated immune activity

Sp isomer

- More stable against nucleases
- Poor RNase H substrate
- Increased protein binding
- Increased Polyanionic characteristics
- Decreased CpG immune activity



Enzymatic Synthesis of Stereoregular (All Rp) Oligonucleotide Phosphorothioate and It's Properties

Jinyan Tang, Allysen Roskey, Ying Li & Sudhir Agrawal

Nucleosides and Nucleotides, 1995, 14:3-5, 985-990,

Solid-phase stereoselective synthesis of oligonucleotide phosphorothioates: the nucleoside bicyclic oxazaphospholidines as novel synthons

Radhakrishnan P Iyer, Mao-Jun Guo, Dong YU, Sudhir Agrawal

Tetrachedron Letters. Volume 39, Issue 17, 23 April 1998, Pages 2491-2494

Stereo-enriched phosphorothioate oligodeoxynucleotides: synthesis, biophysical and biological properties

D Yu, ER Kandimalla, A Roskey, Q Zhao, L Chen, J Chen, S Agrawal

Bioorg Med Chem. 2000 Jan;8(1):275-84

Characteristics of phosphorothioate DNA and RNA

Phosphorothioate DNA

- Nuclease stability ++
- Affinity with RNA +
- RNase-H substrate – yes
- Antisense potency ++
- Complement activation ++
- Prolongation of aPTT++
- Immune/Inflammatory responses ++

Phosphorothioate 2’-substituted RNA

- Nuclease stability +++
- Affinity with RNA +++
- RNase-H substrate – no
- Antisense potency +
- Complement activation –
- Prolongation of aPPT –
- Immune/Inflammatory responses -

Complement activation and hemodynamic changes following intravenous administration of phosphorothioate oligonucleotides in the monkey

WM Galbraith, WC Hobson, PC Giclas, PJ Schechter, S Agrawal

Antisense Res Dev. 1994 Fall;4(3):201-6

Novel enzymatic and immunological responses to oligonucleotides

S Agrawal, O K Rustagi, D R Shaw

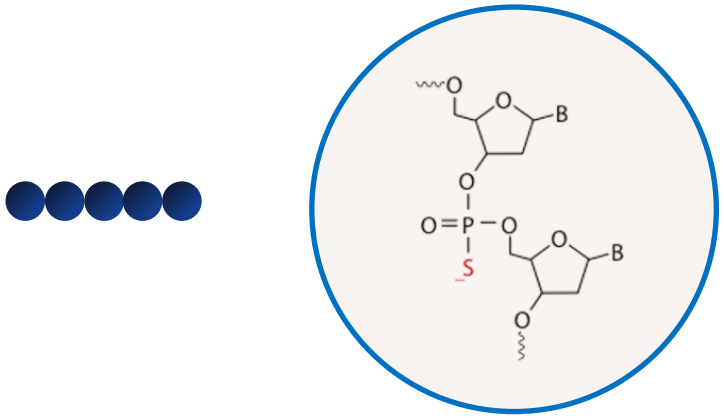
Toxico Lett. 1995 Dec; 82083:431-4

Effect of different chemically modified oligodeoxynucleotides on immune stimulation

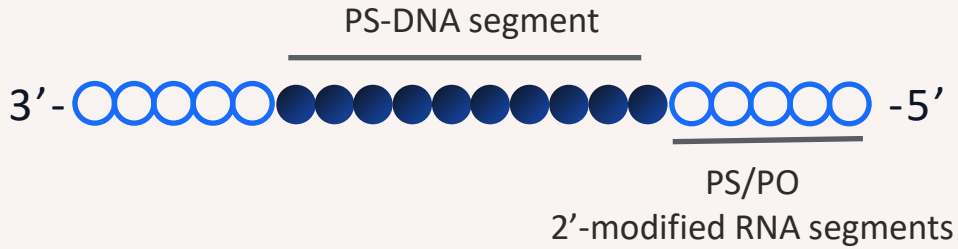
Q Zhao, J Temsamani, P L Iadarola, Z Jiang, S Agrawal

Biochem Pharmacol. 1996 Jan 26;51(2): 173-82

Foundational Chemistry in antisense: Enabling development and approval



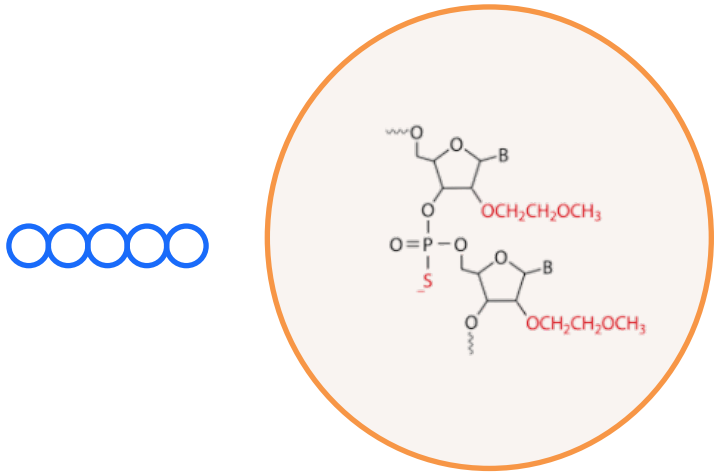
Gapmer



Hybrid oligonucleotide Phosphorothioates

Valeri Metelev, and Sudhir Agrawal

Patent numbers: 5,652,355: 6,143,881; 6,346,614; 7,045,609; filed in 1992



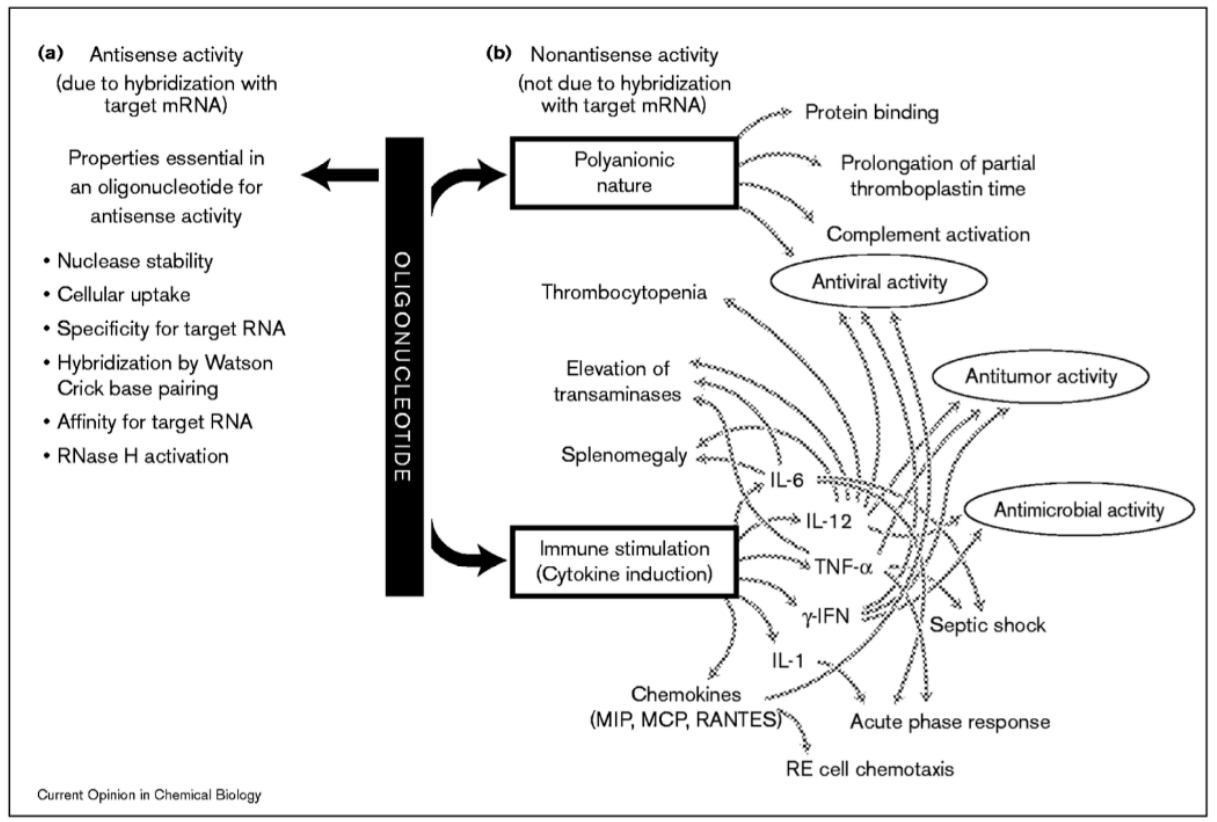
Splice modulator

Repair of thalassemic human beta-globin mRNA in mammalian cells by antisense oligonucleotides

H Sierakowska MJ Sambade, S Agrawal, R Kole

PNAS USA. 1996 Nov 12:93(23):12840-4

Oligonucleotide therapeutics: Off-target activity



Antisense Therapeutics
Sudhir Agrawal* and Quiyan Zhao
Current Opinion in Chemical Biology 1998, 2:519-528

Immune activity of PS DNA: Sequence and modification dependent

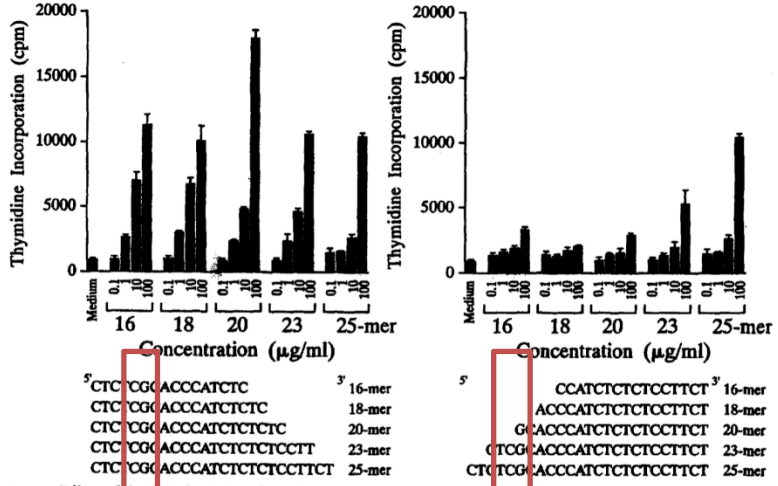


FIG. 2. Effect of the length of phosphorothioate oligonucleotides on murine splenocyte proliferation. GEM 91 length was reduced either from the 5′- or the 3′-end. Cells were incubated with these different oligomers and pulse-labeled with [^{3}H]thymidine. The final concentration of 10 μg/mL corresponds to 1.9 to 1.2 μM for a 16- to 25-mer. Bars represent the SD of triplicate cultures.

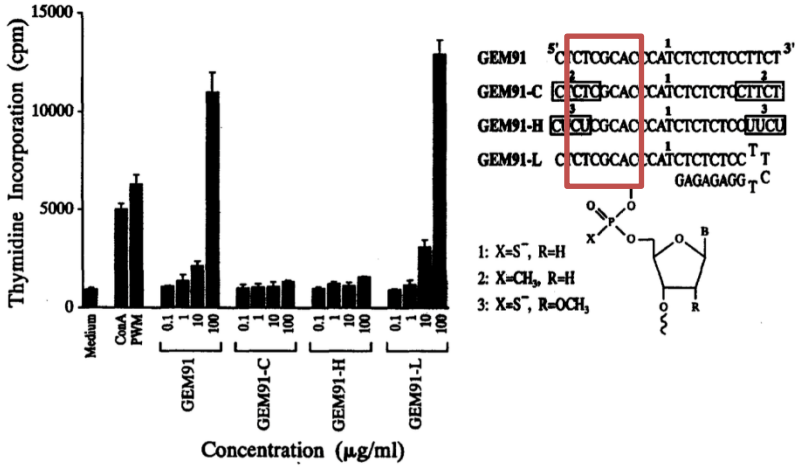


FIG. 3. Effects of GEM 91 and its analogs on murine splenocyte proliferation. The chemical modifications of GEM 91 are indicated. The numbers 1, 2, and 3 represent the different linkages. Bars represent the SD of triplicate cultures.

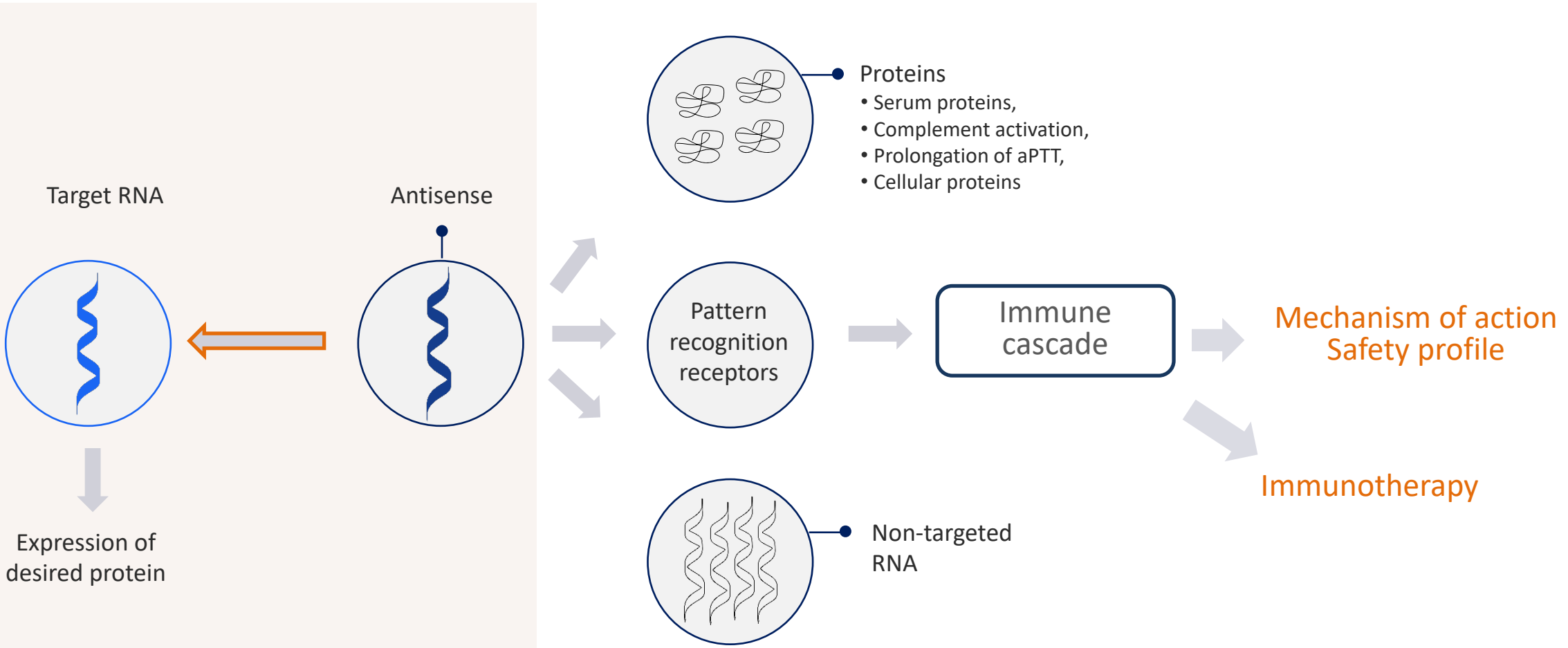
HIV-1 antisense 5’-CTCTCGCACCCATCTCTCTCCTTCT-3’ (PS-DNA)

“It is possible that lymphocyte activation could arise from a discrete protein interaction on the cell membrane or following the entry of the oligonucleotide into the cell, since oligonucleotide can bind to some proteins in a nonspecific manner and modifications of the oligonucleotide backbone may affect their protein binding properties”.

Effects of different chemically modified oligodeoxynucleotides on immune stimulation
Q Zhao, J Temsamani, PL Iadarola, Z Jiang, and S Agrawal
Biochem. Pharm. 1996, 51, 173-182

SEQUENCE AND MODIFICATIONS DEPENDENT

Off–target activity



Chemistry of antisense therapeutics

Late eighties

Chemistry of antisense oligonucleotides

Recent

Chemical engineering of oligonucleotides

Oligonucleotides act as pathogen-associated molecular patterns (PAMPs)

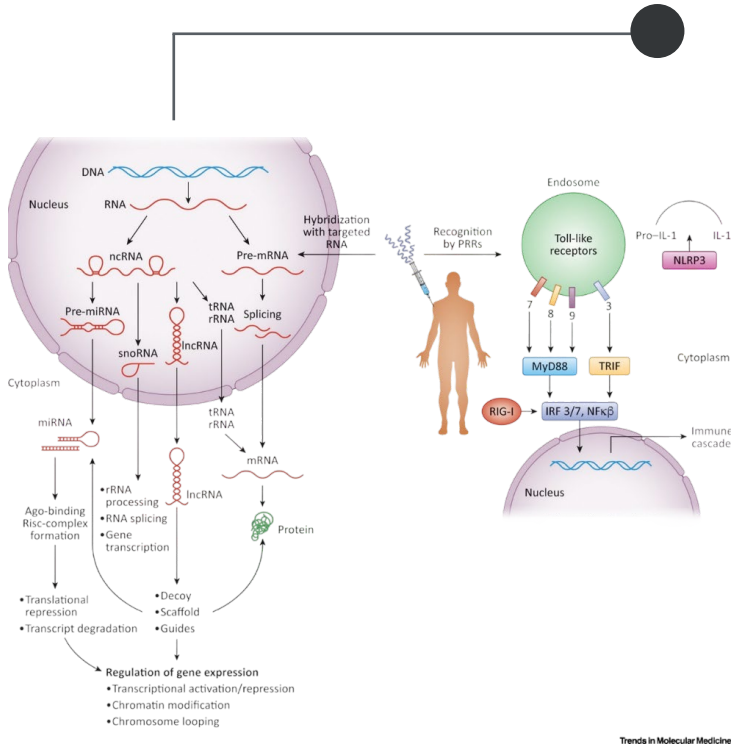
Immunogenicity of Oligonucleotides

Considerations for creating the next generation RNA therapeutics: Oligonucleotide chemistry and Innate immune responses to nucleic acids

S Agrawal.

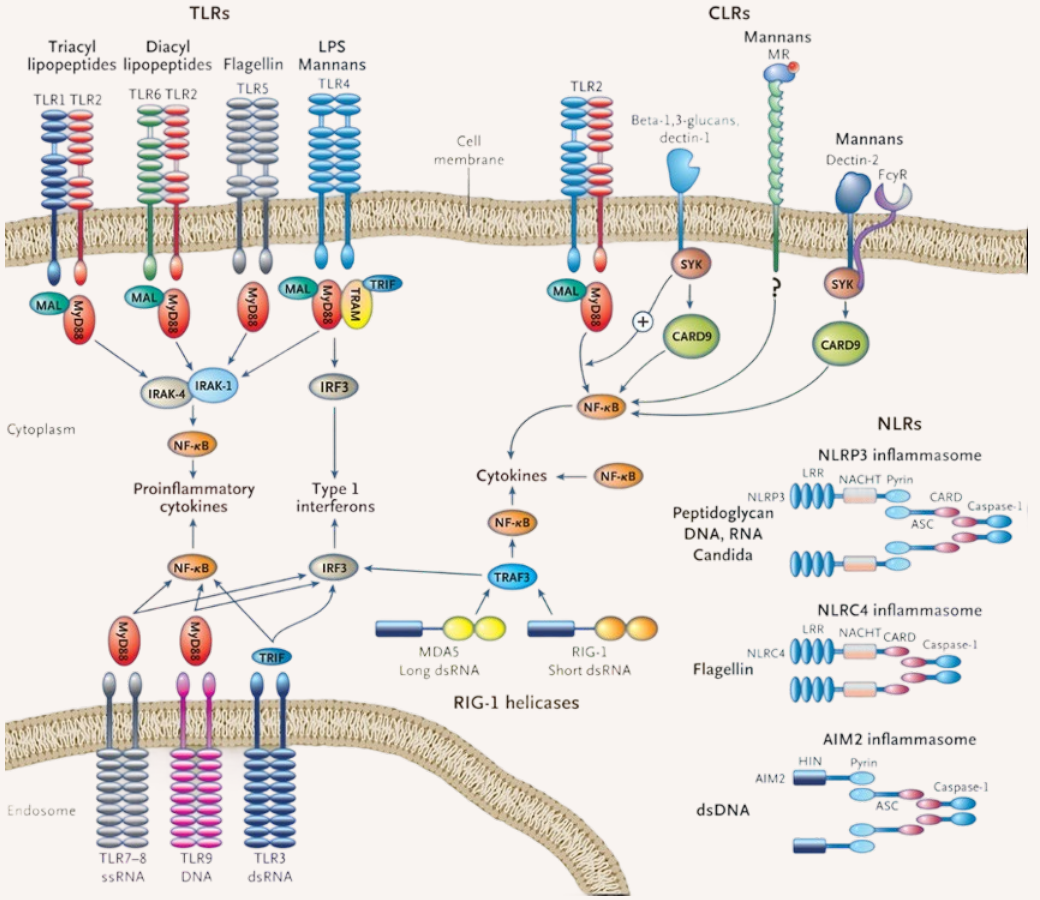
Nucleic Acid Therapeutics, 2024,, 37-51

Oligonucleotides as pathogen-associated molecular patterns (PAMPs)



Was Induction of HIV-1 Through TLR9?
Sudhir Agrawal, Russell Martin
J Immunol (2003) 171(4):1621-1622

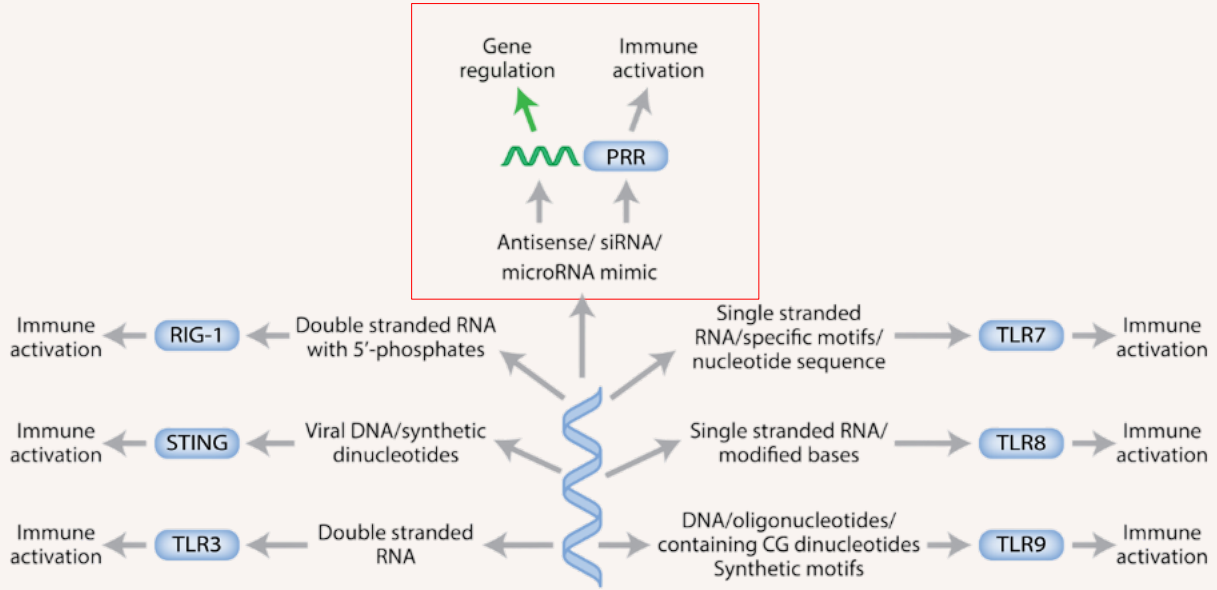
Pattern Recognition Receptors (PRRs)
Expression varies in rodents/primates and humans



Nucleic acids and oligonucleotides: TOLLS to be paid

- Expression of PRRs varies in species
- DNA or RNA as PAMPS
- Single-stranded or double-stranded
- Sequence/motif preference
- Immune cells

Sequence and modifications affect the interactions with PRRs

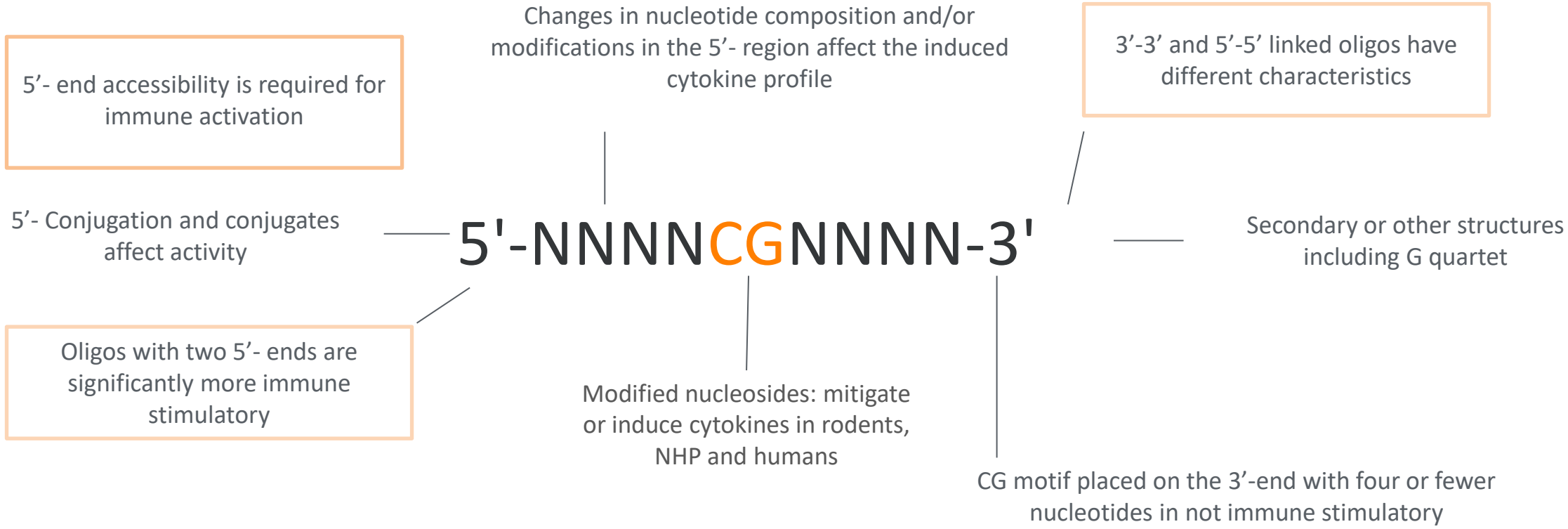


Intratumoral immunotherapy: activation of nucleic acid sensing pattern recognition receptors

Sudhir Agrawal and Ekambar R. Kandimalla

Immunooncology Technol. 2019 Oct; 3: 15-23.

Oligonucleotide sequence and chemical modifications



Synthetic agonists of Toll-like receptors and therapeutics applications.

Agrawal, Sudhir, and Ekambar R. Kandimalla

Advances in Nucleic Acid Therapeutics. 2019. 306-338

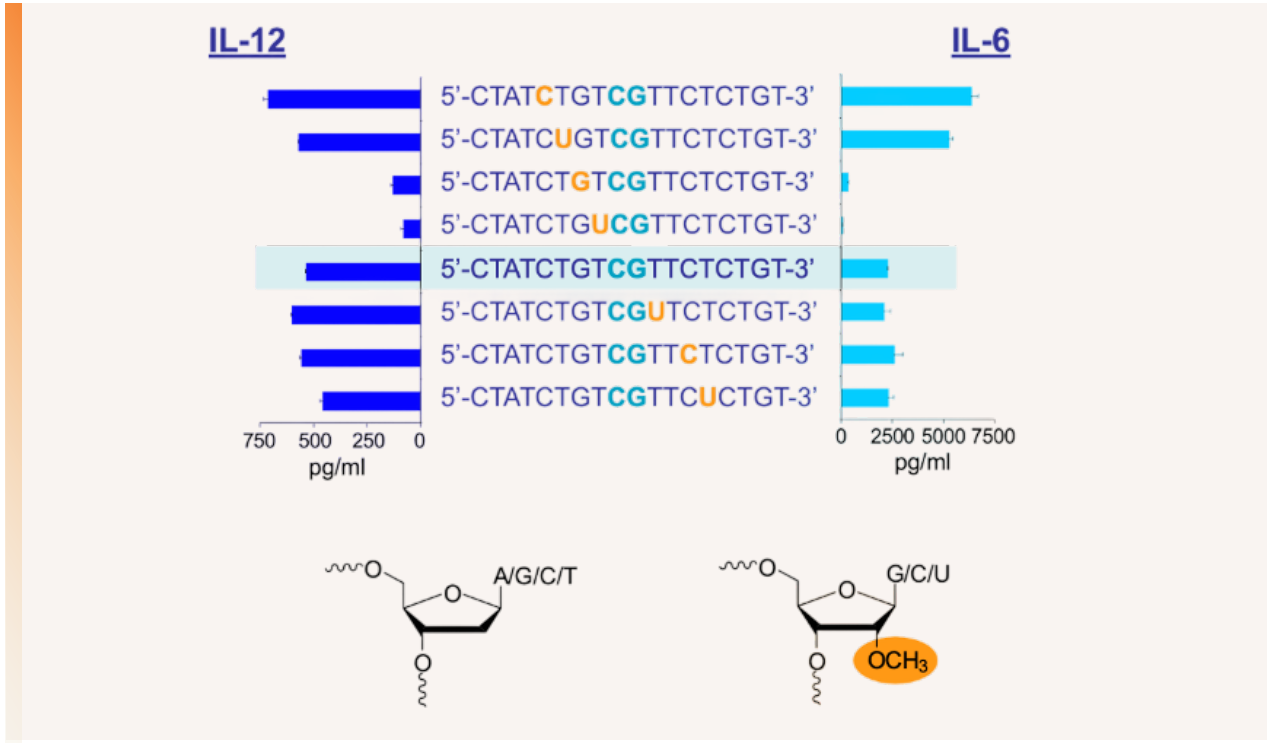
Oligonucleotide-based Toll-like Receptor antagonists and therapeutic applications.

Kandimalla, Ekambar R, and Sudhir Agrawal

Advances in Nucleic Acid Therapeutics. 2019. 80-102

1995-1997

Site of modification in oligonucleotide affects the outcome as PAMPs



Mouse spleen cells (BALB c), 1 microgram/ml 24-hour treatment

Importance of nucleotide sequence and chemical modification of antisense oligonucleotides

Sudhir Agrawal

Biochimica et Biopysica Acta.,1999,1489,53-68

Synthetic agonists of Toll-like receptors 7,8, and 9

Sudhir Agrawal, E R Kandimalla

Biochem Soc Trans. 2007 Dec; 35(Pt.6):1461-7

Design synthesis and biological evaluation of novel antagonist compounds of Toll-like receptors 7, 8 and 9

Ekambar R Kandimalla, Lakshmi Bhagat, Daqing Wang, Dong Yu, Tim Sullivan, Nicola La Monica, Sudhir Agrawal

Nucleic Acids Res. 2013 Apr; 41(6): 3947-3961

Immune responses in human PBMCs: TLR9 agonists

Immune responses are sequence, structure, and modification dependent

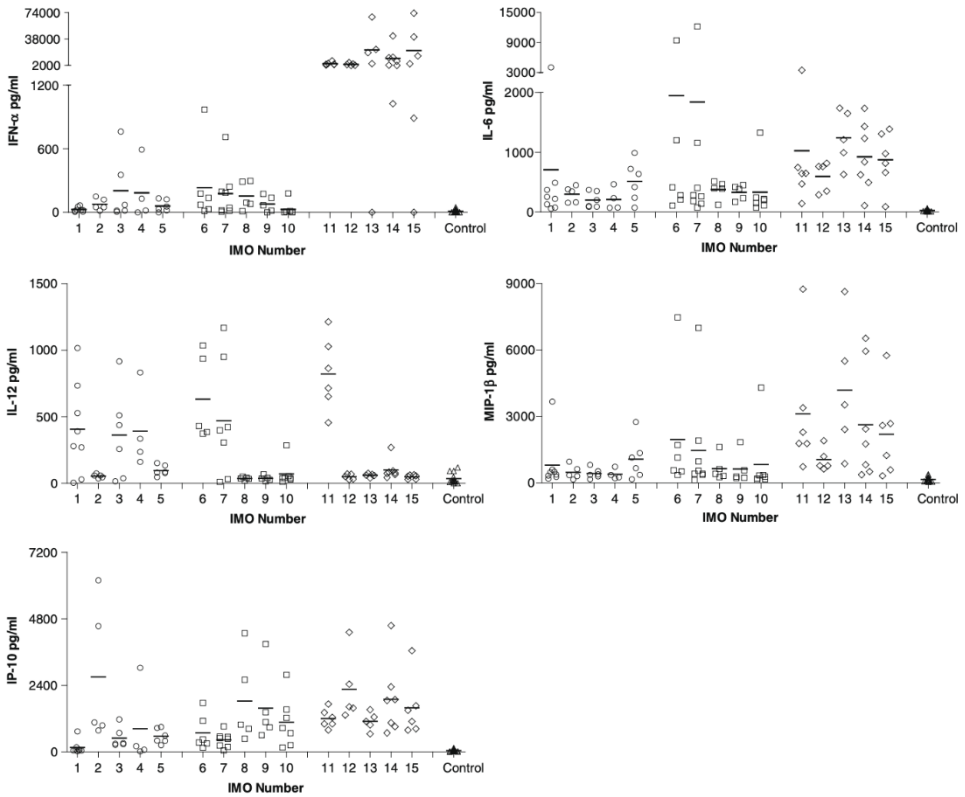


Fig. 2. IMOs induce cytokines and chemokines in human PBMCs. Supernatants from human PBMCs cultured for 24 h with 10 μg/ml IMOs were assayed by luminex/multiplex for cytokine induction. Each data point represents an individual donor (mean of 2 or 3 wells), and bars represent the mean of all donors.

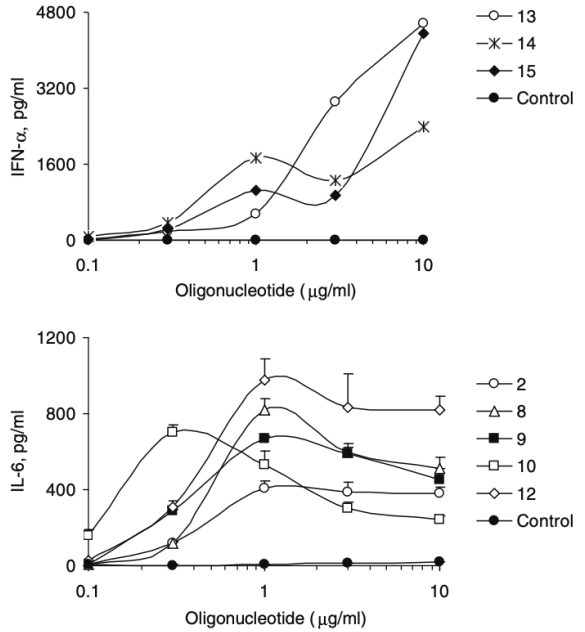


Fig. 3. IMOs induce dose-dependent cytokine production in human PBMCs. Human PBMCs were cultured with multiple doses of IMOs, and supernatants were assayed as in Fig. 2. Shown are representative dose–response curves of two separate donors for IL-6 and IFN-α.

Synthesis and immunological activities of novel agonists of toll-like receptor 9
M Struthers et al.
Cellular Immunology, 263 (2010), 105-113

Immune responses in human PBMCs and pDCs: TLR7 and 8 agonists

Immune responses are sequence, structure, and modification dependent

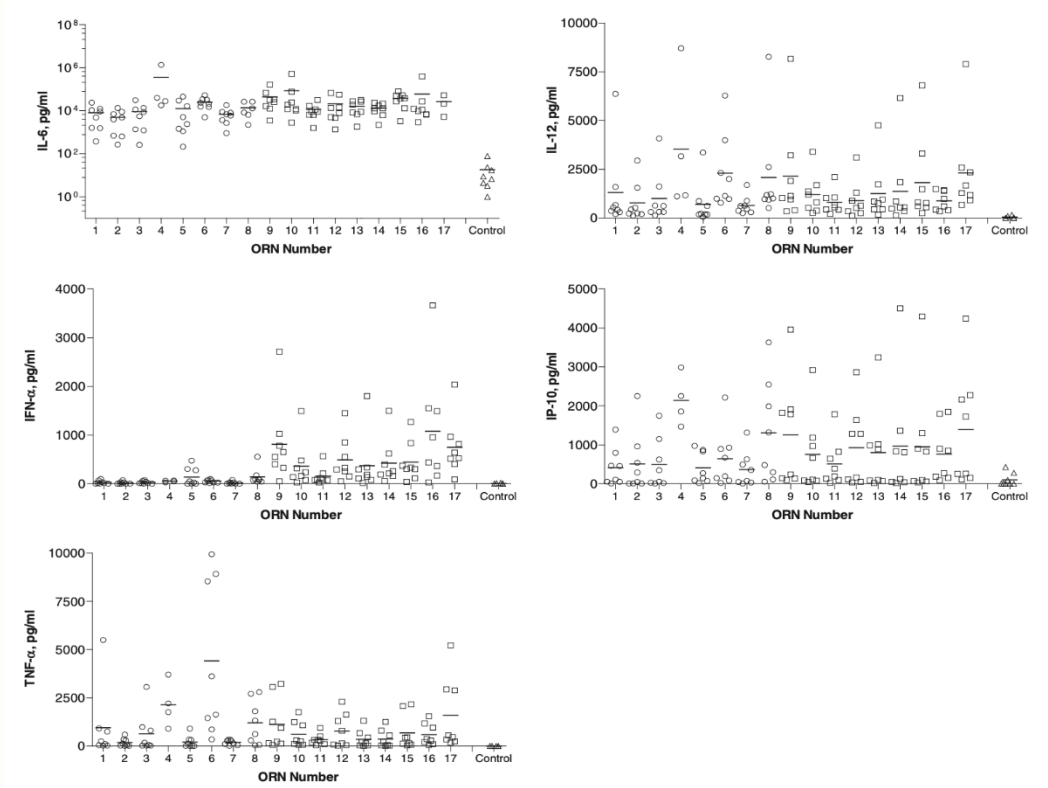


Fig. 2. Cytokine induction by ORNs in human PBMC. Supernatants from human PBMC cultured for 24 h with 100 μg/ml ORNs were assayed by luminex/multiplex for cytokine induction. Each data point represents the response of an individual donor, and bars represent the mean of all donors.

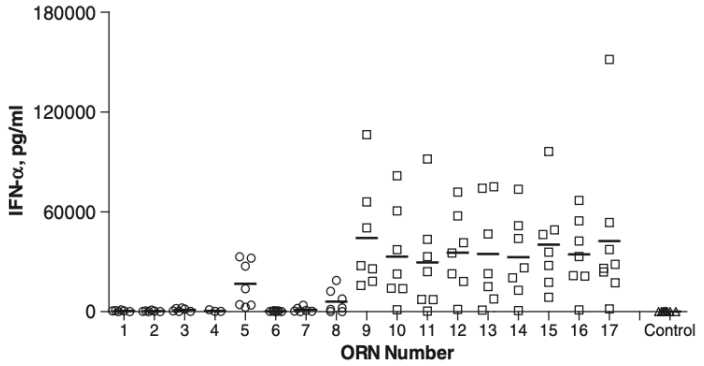
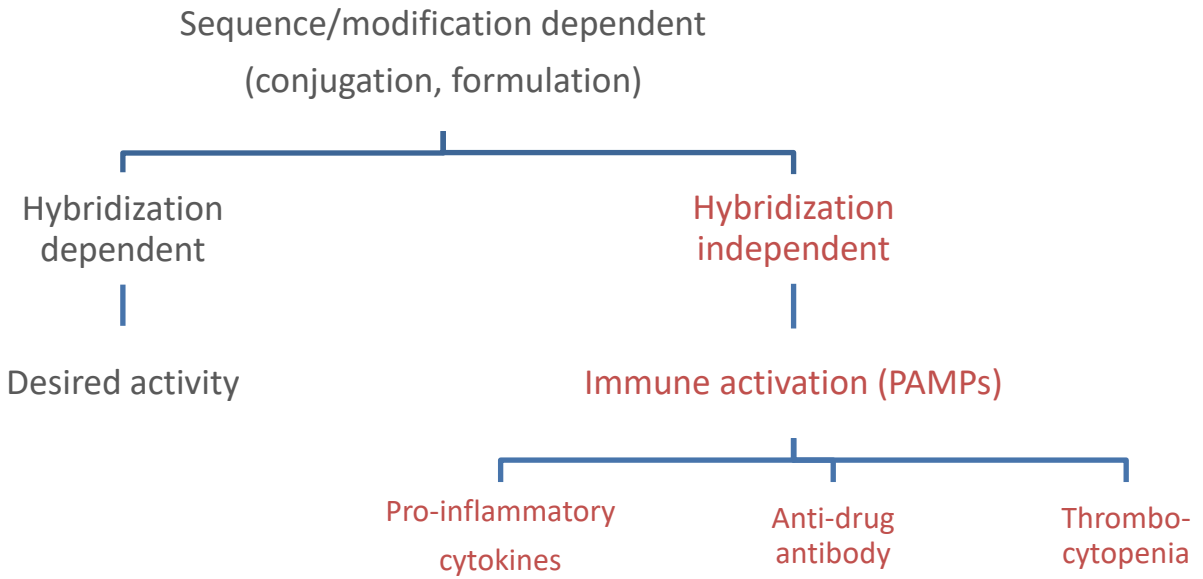
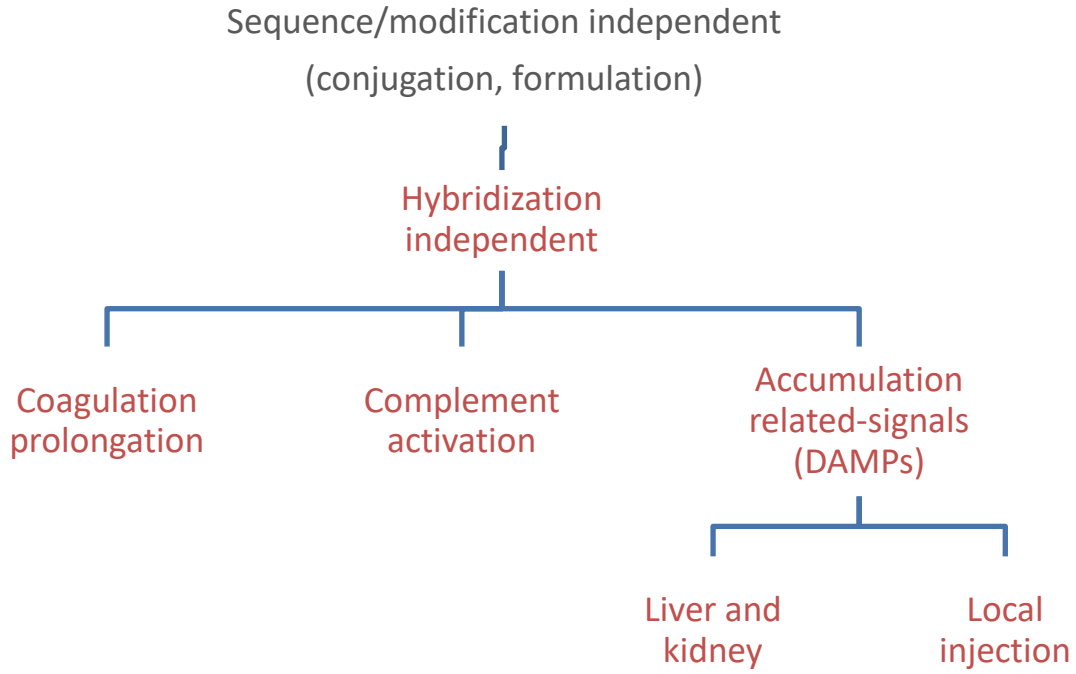


Fig. 4. IFN-α induction in human pDC. Freshly isolated human pDC were cultured with 50 μg/ml ORNs for 24 h, and supernatants were assayed for IFN-α as in **Fig. 2**. Each data point represents the response of an individual donor, and bars represent the mean of all donors.

Synthesis and immunological activities of novel Toll-like receptor 7 and 8 agonists
Kandimalla R et al.
Cellular Immunology, 263 (2010), 105-113

Oligonucleotide-mediated immune modulation: An overview





Modified figure from

Preclinical and clinical drug metabolism, pharmacokinetics, and safety of therapeutic oligonucleotides

P Andersson and C den Besten

In Advances in Nucleic Acid Therapeutics. 2019. 474-531 ed. S Agrawal and MJ Gait

Immunogenicity of oligonucleotides: Ex-vivo assays for evaluations

- Reporter cells: mice and human PAMPs/ligands
- Mouse: PBMCs and splenocytes
- Primates: PBMCs, pDCs, mDCs, B-cells
- Humans*: PBMCs, pDCs, mDCs, B-cells, Monocytes

**Gender, age, and autoimmune disorder, medications, etc.*

Synthetic agonists of Toll-like receptors and therapeutics applications.

Agrawal, Sudhir, and Ekambar R. Kandimalla

Advances in Nucleic Acid Therapeutics. 2019. 306-338

Oligonucleotide-based Toll-like Receptor antagonists and therapeutic applications.

Kandimalla, Ekambar R, and Sudhir Agrawal

Advances in Nucleic Acid Therapeutics. 2019. 80-102

Chemistry of antisense therapeutics

Late eighties

Chemistry of antisense oligonucleotides

Recent

Chemical engineering of oligonucleotides

Oligonucleotides act as pathogen-associated molecular patterns (PAMPs)

Immunogenicity of Oligonucleotides

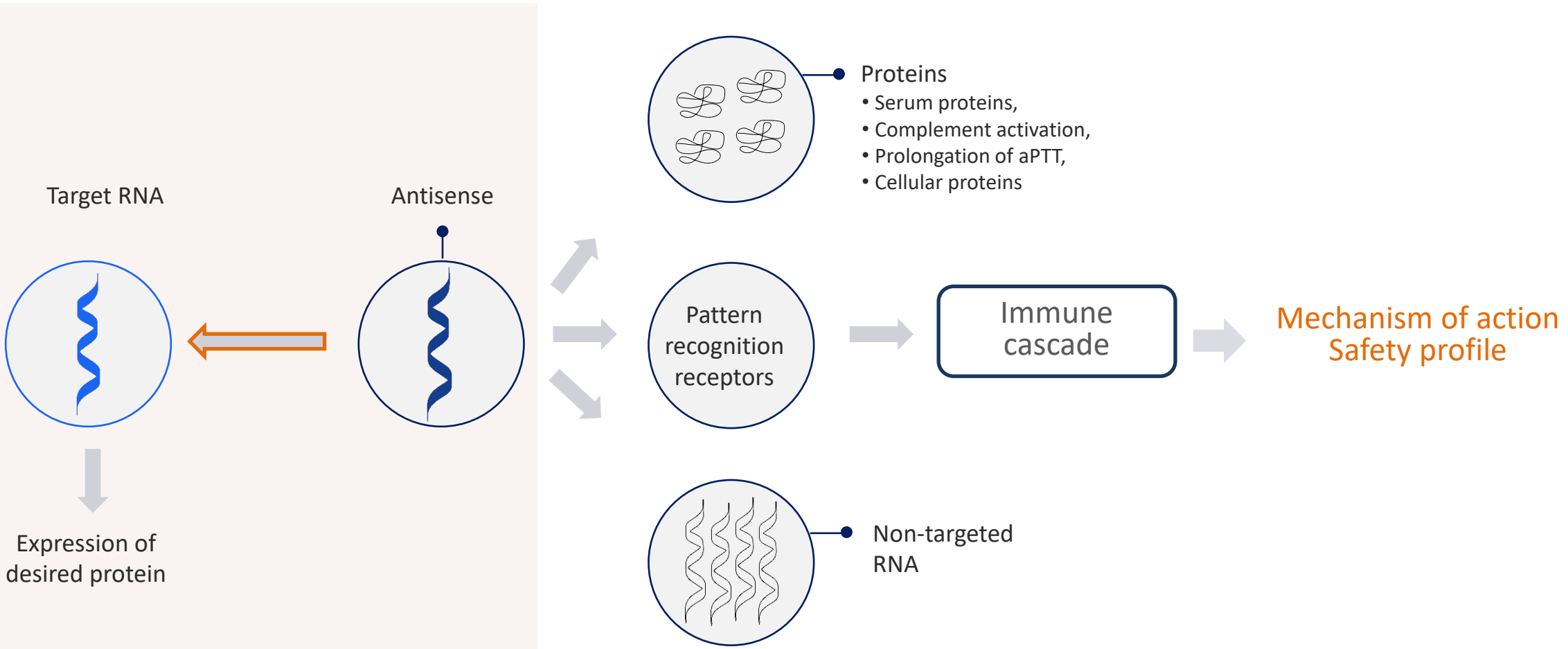
Considerations for creating the next generation RNA therapeutics: Oligonucleotide chemistry and Innate immune responses to nucleic acids

S Agrawal.

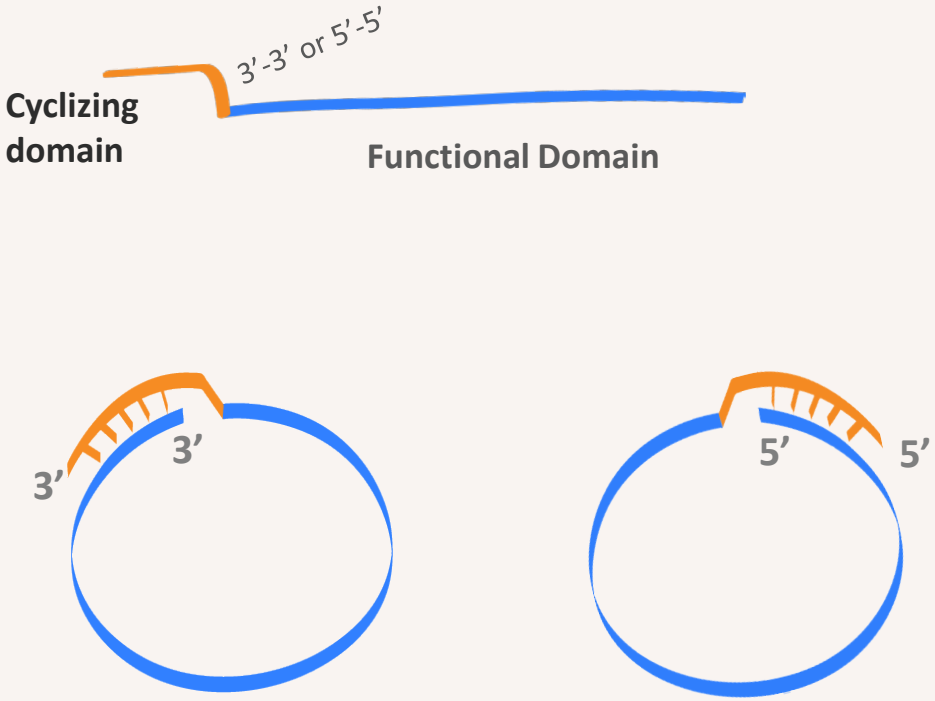
Nucleic Acid Therapeutics, 2024,, 37-51

SEQUENCE AND MODIFICATIONS DEPENDENT

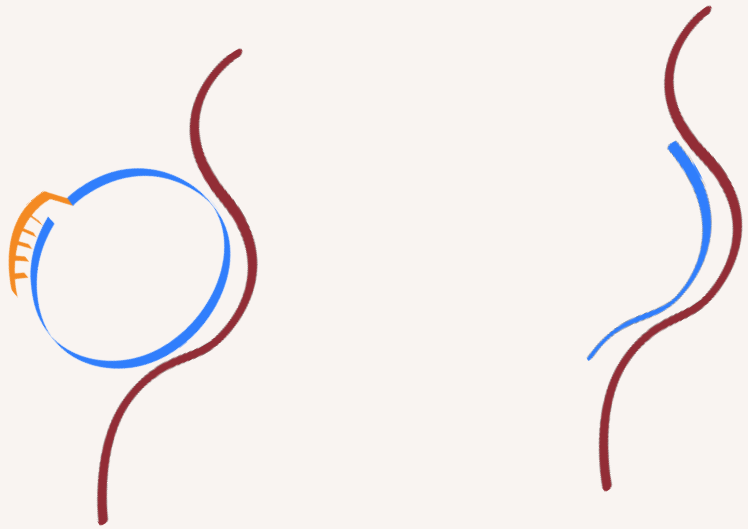
Off–target responses: Mitigation strategy in creating optimized therapeutics



Transient cyclic structures



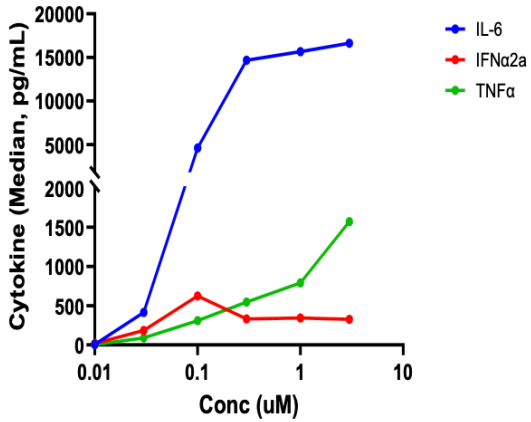
Single-molecule

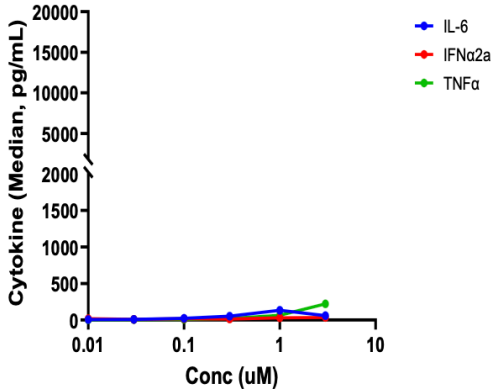


Hybridization with target RNA leads to the opening of cyclic structure

Agrawal, WO 2023/049275

5’-5’- linked oligonucleotide mitigates immune responses





5’-TCGGCTAACGTATGCGAGGAC-3’-3’-AGCCGA-5’

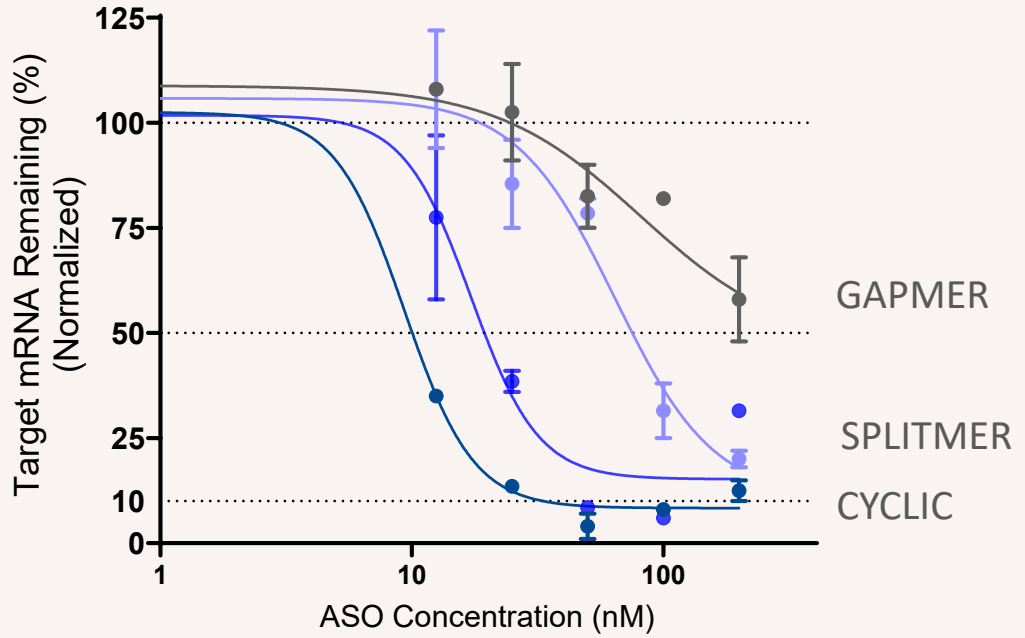
3’-CTCCTG–5’-5’-TCGGCTAACGTATGCGAGGAC-3’

N-PO DNA; N-PS DNA, N-CG motif

Mouse splenocytes; oligo conc. 3.0, 1.0, 0.3,0.1, 0.03uM; 24 hr. treatment, Multiplexed MSD-based readout

Agrawal, WO 2023/049275

Cyclic antisense: Increased potency



Target: PNPLA3,I148M

3’-GACGAAGTACGGGAGATG-5’

3’-GACGAAGTACGGGAAGATG-5’

3’-GACGAAGTACGGGAAGATG-5’-5’-**CTGCTT-3’**

3’-GACGAAGTACGGGAAGATG-5’-5’-**CTGCT-3’**

Agrawal, WO 2023/049275

HepG2 Cell line; RNAiMax, transfection agent; conc 200nM, 100nM, 50nM, 25nM and 12.5nM; duration of treatment- 24 hours; qPCR; host gene POLR2A

In summary

- Chemistry has provided drug-like properties to oligonucleotides
- Gapmers and 2’-modified oligonucleotides have emerged as foundational platforms
- Immunogenicity of oligonucleotides is sequence and modification-dependent
- Chemical engineering of oligonucleotides enhances potency and specificity
  - Transient cyclic structured oligonucleotides mitigate immune responses
  - Broad applicability in addressing multiple mechanisms of action

OVER THREE DECADES

Acknowledgments

Late eighties

Colleagues and collaborators
of over three decades

Financial Support:
Academic institutions and companies *

*Names included in publications

Recent

ARNAY

In collaboration with:

Alloy Therapeutics®

Vinod Vathipadiekal and team