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# Structure–Activity Relationship Studies on the Immune Stimulatory Effects of Base-Modified CpG Toll-Like Receptor 9 Agonists

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*Dedicated to Prof. Dr. Wojciech Stec on the occasion of his 65th birthday*

*Synthetic oligodeoxynucleotides containing unmethylated deoxycytidylyl-deoxyguanosine dinucleotide (CpG) motifs are able to stimulate potent immune responses through a signaling pathway involving Toll-like receptor 9 (TLR9). We have investigated the structure–activity relationship (SAR) of base-modified CpG oligonucleotides with TLR9 by measuring TLR9 activation by 20-mer oligonucleotides having just a single human recognition motif (5′-GTCGTT-3′) in functional cell-based TLR9 assays. Substitution of guanine by hypoxanthine and 6-thioguanine resulted in activity similar to the unmodified parent molecule, whereas purine, 2-aminopurine, 2,6-diaminopurine, and 8-oxo-7,8-dihydroguanine substitution resulted in approximately 40–60% reduction in activity, and 7-deazaguanine substitution led to the strongest (80%) reduction in TLR9 stimulation. Furthermore, none of the investigated modifications at C5 and N4 of cytosine were well tolerated with respect to human TLR9 stimulation. Our results are compatible with a SAR model in which guanine is recognized by the Hoogsteen site, and C5 is most critical for recognition of cytosine. In addition, we found significant species-specific differences between human and murine TLR9 recognition, which demonstrates the importance of choosing appropriate assay systems for SAR studies.*

## Introduction

Cells of the innate immune system recognize certain molecular structures of pathogens by a limited set of pattern recognition receptors (PRRs). The family of Toll-like receptors (TLRs) are probably the best understood class of PRRs, consisting of at least twelve different TLR subtypes (TLR1 to TLR12).[1] In general, activation of TLRs results in stimulation of the innate immune response, including secretion of proinflammatory cytokines, up-regulation of co-stimulatory molecules, and secretion of cytokines and chemokines. Among the TLRs, TLR9 plays an important role in that it specifically recognizes deoxycytidylyl-deoxyguanosine dinucleotide (CpG) motifs (Figure 1), which are present at relatively high frequency in bacterial DNA and many viruses.[2,3] In contrast to vertebrate DNA, CpG motifs of bacterial DNA are not methylated at C5 of the cytosine base. Furthermore, it has been found that relatively short oligodeoxynucleotides (ODNs) are capable of mimicking the immune stimulatory effects of bacterial DNA.[3,4]

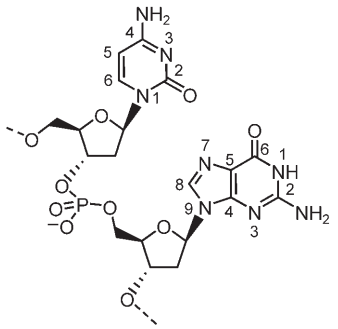

**Figure 1.** Chemical structure of the CpG recognition motif.

In recent years, various CpG ODNs have entered clinical trials to be developed as TLR9 agonists for the treatment of cancer, infectious diseases, asthma, and allergies.[5] The first generation of CpG ODNs are modified as phosphorothioates (PS) at every internucleotide linkage, thus making them more resistant to nucleases present in serum and in cells.[6] The ODNs in development usually have a length of 18 to 25 nucleotides and frequently contain more than one CpG motif. Thus, CPG 7909 (5′-TCGTCGTTTTGTCGTTTTGTCGTT-3′),[5,7] the gold standard of immune stimulatory ODN, contains four CpG dinucleotides. In theory, multiple motifs within one ODN can compete with each other for the same binding site on TLR9.[8] The optimal sequence context for these motifs to stimulate human immune cells is the hexanucleotide sequence 5′-GTCGTT-3′,

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which appears three times in CPG 7909, whereas for rodents, the optimal recognition motif has been reported to be 5′-GACGTT-3′.[9] In previous structure–activity relationship (SAR) studies,[10] ODNs were frequently based on the murine motif and tested in mouse or human cells,[11,12] leading to ambiguous results. Most of the SAR studies reported so far were based on measuring cytokine secretion or determining spleen enlargement in mice following administration of CpG oligonucleotides. On the basis of these results, conclusions were made regarding recognition of CpG ODN by human TLR9. The aim of the present study is to investigate the influence of base modifications at the CpG motif on the immune stimulation of TLR9 in a direct readout.

Another goal was to use a model CpG ODN in which the CpG motif was as close as possible structurally to the naturally occurring ligand for human TLR9. It has been reported that PS ODNs display higher immune stimulatory effects than partially PS-modified ODNs or phosphodiester (PO) ODNs.[13,14] Furthermore, PO ODNs lacked immune stimulatory activity in cultures of murine thymocytes in vitro, while PS ODNs showed significant proliferation of murine thymocytes.[15,16] The superior activity of PS ODNs has been attributed to their increased nuclease stability and better cellular uptake.[14,17] Therefore, we have chosen a 20-nucleotide sequence containing just a single human hexanucleotide motif designed for optimal recognition by human TLR9 (ODN **1** in Figure 2). In this CpG ODN all internucleotide residues are modified to phosphorothioate, except the linkage between C and G, which was maintained as a phosphodiester as it occurs in the natural CpG TLR9 ligand. In this study, ODN **1** served as a parent molecule to study SAR of ODNs, in which either cytosine or guanine was replaced by a modified base (Figure 2, Figure 3, and Figure 4). All modified ODNs were investigated for their potency to stimulate human or murine TLR9 using HEK293 cells expressing a luciferase gene controlled by a NF-κB promoter construct that constitutively expresses the genes for human TLR9 or murine TLR9, respectively. Our results show that modification of the guanine base of the CpG motif is generally better tolerated than modification of the cytosine base, and that there are significant differences between the recognition by human and murine TLR9. These findings have implications for the choice of appropriate assay systems for the optimization of TLR9 agonists. We also propose a SAR model in which guanine is recognized by N7 and O6 (Hoogsteen base pairing site) and cytosine by its N4 function and C5 spatial requirements.

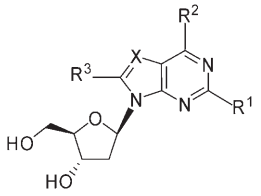
## Results

### Effect of guanine base substitution at CpG on TLR9 stimulation

Figure 2 shows the investigated ODNs with guanine base modifications at CpG of ODN **1**. For ODNs **2** to **4**, functional groups at the guanine base of the parent molecule were deleted. Removal of the 2-amino group in guanine leads to hypoxanthine and the corresponding nucleoside is called inosine (ODN **2**). Deletion of O6 results in a 2-aminopurine base (ODN

ODN **1**: T***G*T*C-G*T*T***T*T*T*T*T*T*T*T*T*T*T*T*T*T

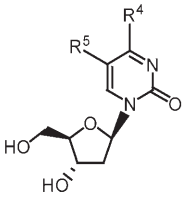
ODN **2-8**: T*G*T*C-Pu*T*T*T*T*T*T*T*T*T*T*T*T*T*T*T




| ODN | $R^1$ | $R^2$ | $R^3$ | X | Pu |
|---|---|---|---|---|---|
| **1** | $NH_2$ | OH | H | N | guanine |
| **2** | H | OH | H | N | hypoxanthine |
| **3** | $NH_2$ | H | H | N | 2-aminopurine |
| **4** | H | H | H | N | purine |
| **5** | $NH_2$ | SH | H | N | 6-thioguanine |
| **6** | $NH_2$ | $NH_2$ | H | N | 2,6-diaminopurine |
| **7** | $NH_2$ | OH | OH | N | 8-oxo-7,8-dihydroguanine |
| **8** | $NH_2$ | OH | H | CH | 7-deazaguanine |

**Figure 2.** Chemical modifications of guanine base (Pu). Phosphorothioate (PS) linkages are indicated as asterisks (*). The human hexanucleotide motif (GTCGTT) in ODN **1** is depicted in bold.

ODN **9-13**: T*G*T*Py-G*T*T*T*T*T*T*T*T*T*T*T*T*T*T*T

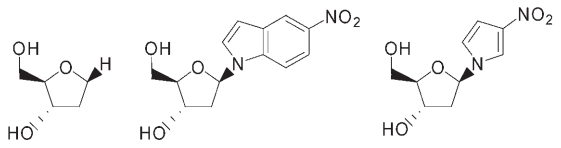



| ODN | $R^4$ | $R^5$ | Py |
|---|---|---|---|
| **1** | $NH_2$ | H | cytosine |
| **9** | $NH_2$ | $CH_3$ | 5-methylcytosine |
| **10** | $NH_2$ | OH | 5-hydroxycytosine |
| **11** | $NH_2$ | Br | 5-bromocytosine |
| **12** | $NH_2$ | $-C\equiv C-CH_2-NH_2$ | 5-aminopropargylcytosine |
| **13** | $NH-C_2H_5$ | H | N4-ethylcytosine |

**Figure 3.** Chemical modifications of cytosine base (Py).

ODN **14-19**: T*G*T*Py-Pu*T*T*T*T*T*T*T*T*T*T*T*T*T*T*T




D NI NP

| ODN | Py | Pu |
|---|---|---|
| **14** | D | G |
| **15** | C | D |
| **16** | NP | G |
| **17** | C | NP |
| **18** | NI | G |
| **19** | C | NI |

**Figure 4.** Substitution of deoxycytidine and deoxyguanosine by abasic residues (D, also called dSpacer) and universal base-containing nucleosides (NI = 5-nitroindole, NP = 3-nitropyrrole).

**3**), while simultaneous deletion of the 2-amino group and O6 results in a purine base (ODN **4**). In ODNs **5** to **8**, donor or acceptor functional groups of guanine were replaced by other functional groups. Thus, O6 was replaced by a sulfur or amino group, yielding 6-thioguanine (ODN **5**) and 2,6-diaminopurine (ODN **6**), respectively. Furthermore, 8-oxo-7,8-dihydroguanine (ODN **7**; replacement of H8 by OH for which the amide form is the preferred tautomer) and 7-deazaguanine (ODN **8**; replacement of the ring nitrogen atom by CH) were investigated for their ability to stimulate TLR9.

To gain a better insight into how modification of the heterocyclic nucleobase influences stimulation at the TLR9 level, the potency and efficacy of CpG ODNs **1** to **8** in inducing NF-κB signaling in stably transfected HEK293 cells expressing human or murine TLR9 and an NF-κB-luciferase readout construct was investigated. Human TLR9 (hTLR9) was stimulated with different concentrations of parent CpG ODN **1** or the base-modified analogues ODN **2** to **8**, and the level of NF-κB activity assessed at 16 h (Figure 5a and b). ODN **2**, with guanine replaced by hypoxanthine, shows maximal hTLR9 stimulation comparable to that of the unmodified ODN **1**, although a slightly higher concentration is required to obtain this effect. In contrast, replacement of guanine by 2-aminopurine (ODN **3**) or simply purine (ODN **4**) results in an approximate twofold decrease in efficacy (that is, maximal NF-κB activity at the highest concentration tested) and decreased potency to stimulate hTLR9. Stimulation of hTLR9 is fully compatible with the exchange of guanine with 6-thioguanine (ODN **5**; Figure 5b), whereas the exchange of O6 with an amino group (ODN **6**) leads to a strong reduction in activity, similar to introducing oxygen in position 8 of guanine (ODN **7**). Surprisingly, replacement of N7 by CH (ODN **8**) results in almost complete loss of activity in hTLR9 (Figure 5b). The effect of base modifications in murine TLR9 (mTLR9) turned out to be even more pronounced, with less tolerance for base modification relative to the effects on hTLR9 (Figure 5c and d). Thus, ODN **2**, with guanine replaced by hypoxanthine, results in an about threefold decrease in mTLR9 stimulation relative to unmodified ODN **1**, whereas only very little mTLR9 stimulation is preserved if guanine is replaced by either 2-aminopurine (ODN **3**) or purine (ODN **4**). In contrast to the effective stimulation of hTLR9 by ODN **5** with 6-thioguanine as base, ODN **5** stimulates mTLR9 with only about 20 percent of the efficacy of the parent molecule and has markedly reduced potency (Figure 5d), similar to ODN **8**, with 7-deazaguanine as base. Replacement of guanine in ODN **1** by 2,6-diaminopurine (ODN **6**) or 8-oxo-7,8-dihydroguanine (ODN **7**) results in almost complete loss of mTLR9 stimulation.

### Effect of modification of cytosine at CpG on TLR9 stimulation

In another set of ODN analogues, the cytosine base at CpG in ODN **1** was replaced by C5-substituted or N4-substituted cytosine derivatives (Figure 3). Substitution of cytosine at C5 is of particular interest, as it has been previously shown to be the major determinant by which vertebrate immune cells discriminate self (methylated C) from a danger signal evoked by invading pathogens (unmethylated C). To investigate the tolerance of TLR9 recognition to substitution at C5, we introduced methyl, hydroxy, bromo, and aminopropargyl substituents (ODN **9** to **12**). Finally, the 4-amino group of cytosine was alkylated to give N4-ethylcytosine (ODN **13**). Investigation of the ODNs with modified cytosine bases in hTLR9 stimulation shows significant but varying reductions in hTLR9 activation (Figure 6a). While the parent molecule shows a stimulation index of about 15 in the hTLR9 assay, substitution at C5 by methyl (ODN **9**) or bromo (ODN **11**) reduces the stimulation index to about 4 at the highest concentration. Replacement of cytosine by 5-hydroxycytosine (ODN **10**) leads to an even stronger decrease of hTLR9 stimulation, and the aminopropargyl analogue (ODN **12**) is virtually inactive. The loss in activity by N4-ethyl substitution (ODN **13**) is similar to that for 5-methyl and 5-bromo substitution of cytosine.

In the murine receptor assay, substitution at C5 by bromo is well tolerated, since ODN **11** shows activity similar to the parent molecule ODN **1** (Figure 6b). While 5-methylcytosine also retains about 30% of the immune stimulatory activity of the parent cytosine, all other modifications at cytosine (5-hydroxy, 5-aminopropargyl, and N4-ethyl) result in almost complete loss of the ability to stimulate mTLR9.

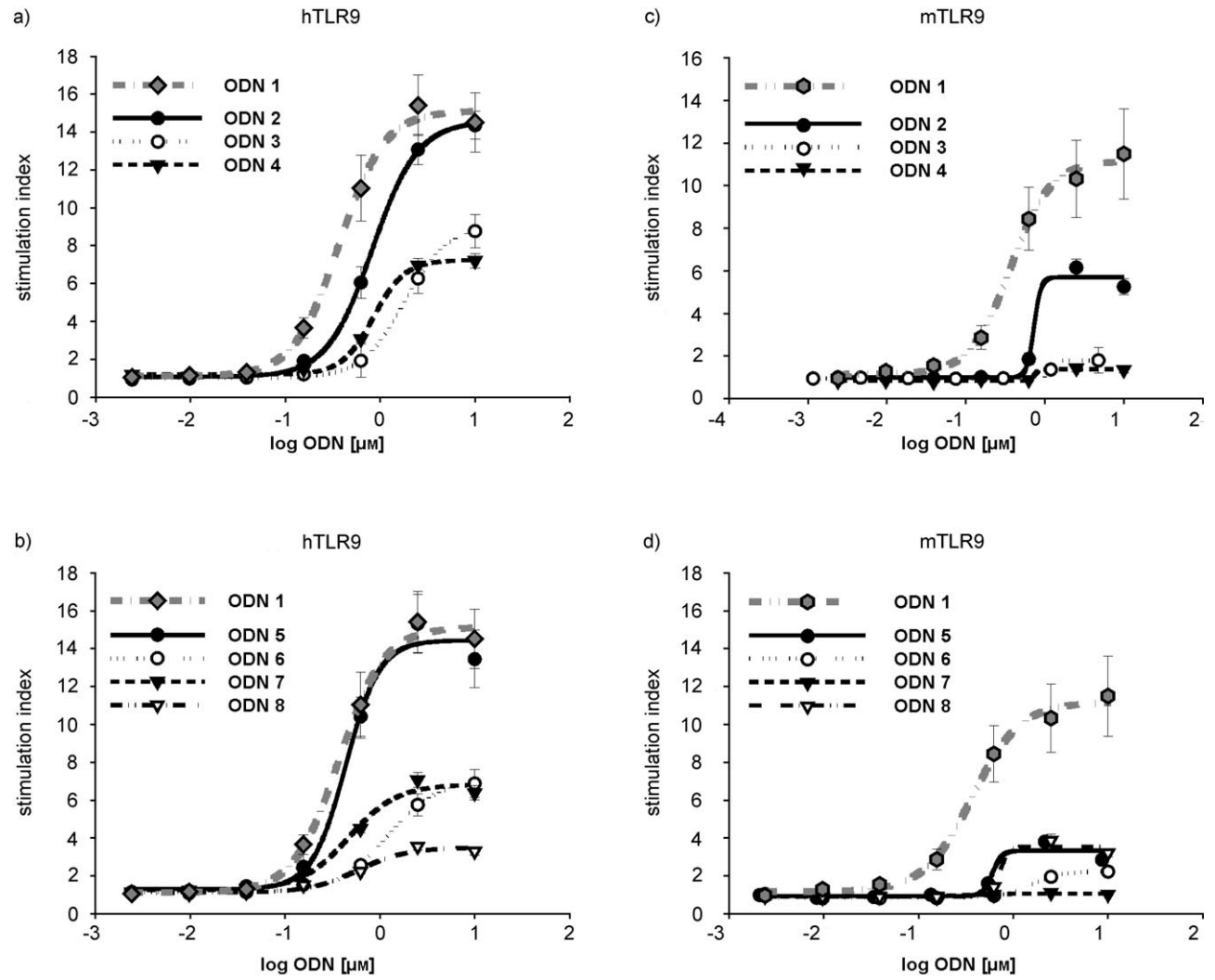



**Figure 5.** HEK293 cells stably transfected with a vector expressing either human TLR9 (a and b) or murine TLR9 (c and d) and an NF-κB-luciferase construct were incubated for 16 h with the ODN indicated. NF-κB stimulation was measured through luciferase activity. Stimulation indices (±SD) were calculated in reference to luciferase activity of medium alone. Shown is one representative out of at least two independent experiments.

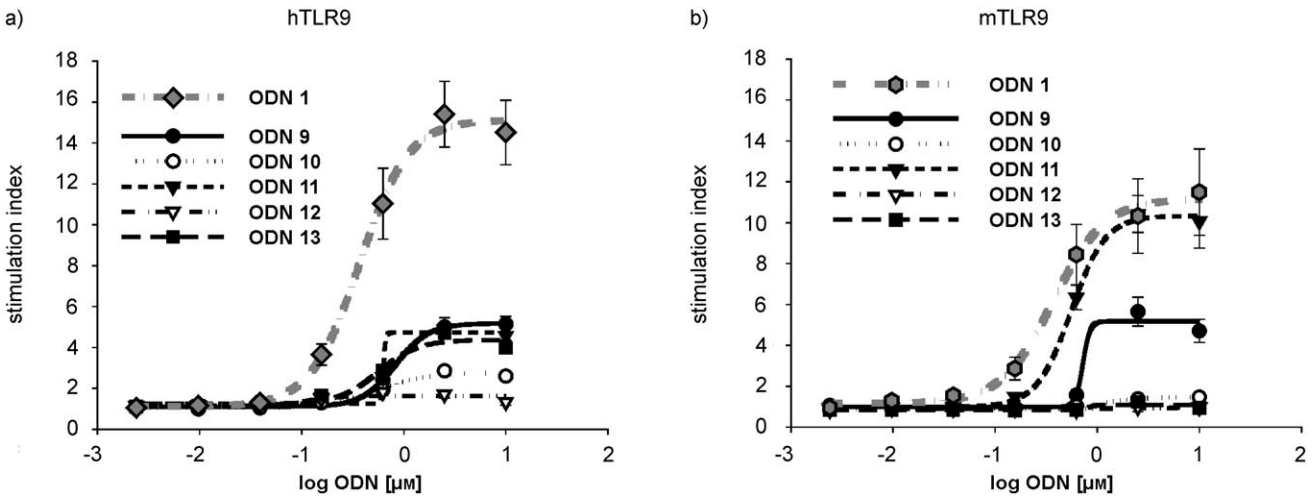



**Figure 6.** HEK293 cells stably transfected with a vector expressing either human TLR9 (a) or murine TLR9 (b) and an NF-κB-luciferase construct were incubated for 16 h with indicated ODN. NF-κB stimulation was measured through luciferase activity. Stimulation indices (±SD) were calculated in reference to luciferase activity of medium alone. Shown is one representative out of at least two independent experiments.

**Influence of universal bases or deletion of bases at CpG on TLR9 stimulation**

The substitution of naturally occurring heterocyclic bases by hydrophobic, non-hydrogen bonding analogues, also called universal bases, has been suggested previously,[18,19] whereby 5-nitroindole (NI) is expected to have steric bulk and shape similar to that of purines, and 3-nitropyrrole (NP) appears more appropriate to replace pyrimidines. While incorporation of a nitro-substituted aromatic system would be expected to increase base stacking interactions, deletion of the whole nucleobase (abasic residues, also called dSpacers (D)) would abrogate base stacking (Figure 4). Therefore, we incorporated D, NI, or NP instead of either C (ODNs **14**, **16**, and **18**) or G (ODNs **15**, **17**, and **19**) in ODN **1** and investigated their ability to stimulate TLR9. Replacement of cytosine by hydrogen, nitroindole, or nitropyrrole totally abolishes immune stimulatory activity both in human and murine TLR9 assay (Figure 7). In contrast, replacement of guanine by hydrogen, nitroindole, or nitropyrrole (ODNs **15**, **17**, and **19**) retains about one-third of the immune stimulatory potency in the hTLR9 assay, whereas those derivatives are incapable of stimulating mTLR9.

## Discussion

In recent years, CpG ODNs and analogues thereof have gained increasing interest as potential immune stimulatory drugs for the treatment of cancer, infectious diseases, asthma, and allergies. CpG ODNs act by stimulating a specific protein target, namely TLR9, found in immune system cells which direct the immune system to fight diseases. The TLR9-mediated stimulation of immune cells results in a multiplicity of effects, including secretion of Th1-promoting cytokines such as type I interferon, enhancement of natural killer cell responses, and activation of B cells. Recent Phase I and Phase II clinical trials with cancer patients suggest that first-generation phosphorothioate CpG ODNs show antitumor activity in these patients.[5]

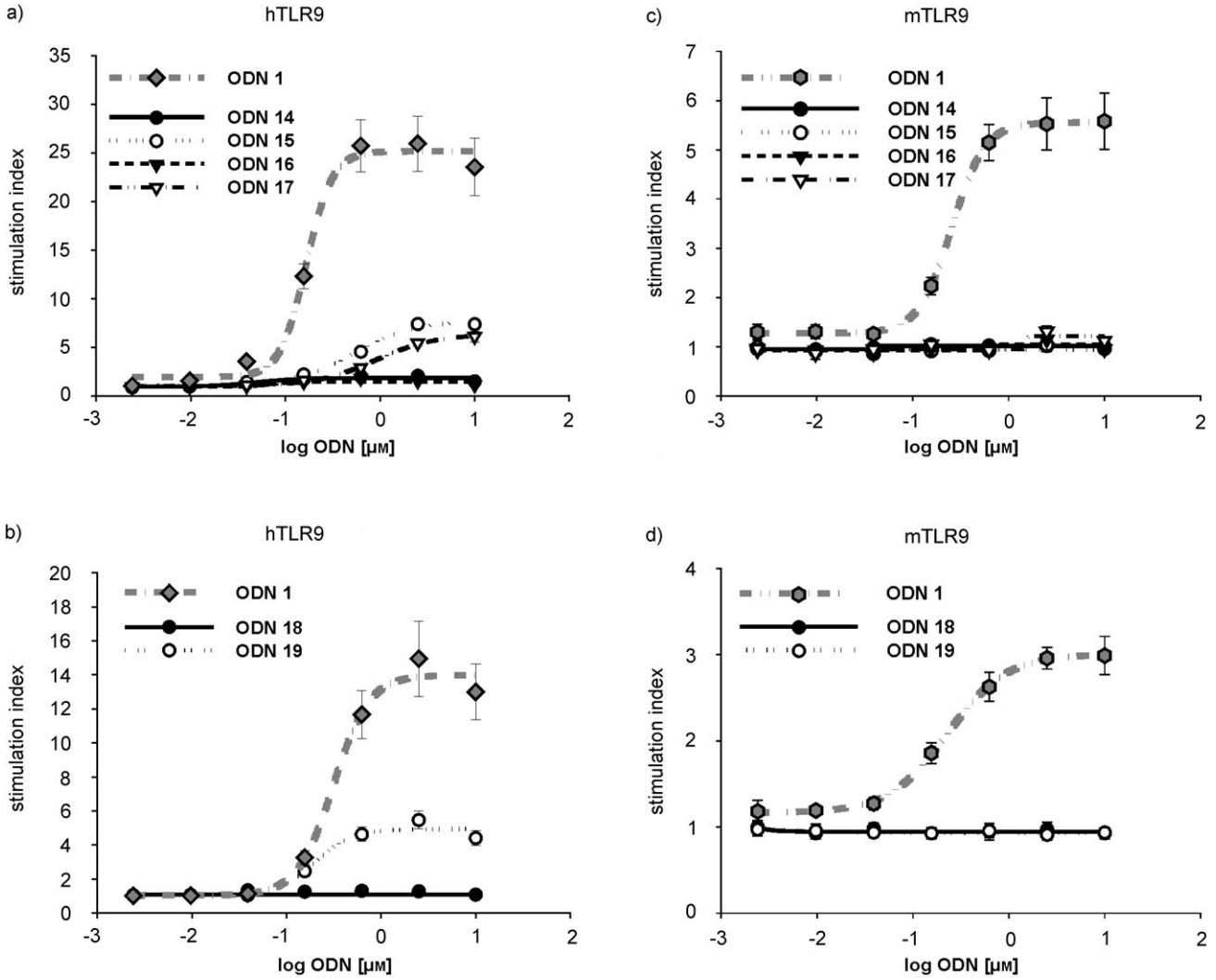



**Figure 7.** HEK293 cells stably transfected with a vector expressing either human TLR9 (a and b) or murine TLR9 (c and d) and an NF-κB-luciferase construct were incubated for 16 h with indicated ODN. NF-κB stimulation was measured through luciferase activity. Stimulation indices (± SD) were calculated in reference to luciferase activity of medium alone. Shown is one representative out of at least two independent experiments.

To investigate SAR of CpG ODNs with human TLR9, we selected ODN **1**, a 20-mer having just a single human hexanucleotide recognition motif (5′-GTCGTT-3′) embedded within a homothymidylate sequence. The presence of multiple CpG motifs has been avoided, as they may compete for the same binding site at the receptor, leading to results which are sometimes difficult to interpret. Since natural CpG TLR9 agonists such as bacterial or viral DNAs have a phosphodiester linkage at the unmethylated CpG dinucleotide, we also chose a phosphodiester linkage between C and G, but all other internucleotide bonds were modified to phosphorothioate, making the oligonucleotides relatively stable to nucleases present in serum and within cells. The phosphodiester linkage at the CpG also avoids the formation of diastereomers at the CpG motif, which have previously been shown to be recognized differently by TLR9, whereby the Rp diastereoisomer showed higher immune stimulatory activity.[20]

The aim of this study was to shed light on the molecular mechanism of recognition of CpG ODN by TLR9, in particular for the human receptor. We have investigated various modifications of the heterocyclic bases at the CpG motif. The importance of base recognition in receptor binding specificity is already highlighted by the much greater recognition of unmodified cytosine base relative to 5-methylcytosine; this is the basis of the vertebrate immune system's ability to distinguish a danger signal (bacterial DNA) from self-DNA (unmethylated cytosine).[3,4]

**Table 1.** Comparison of $EC_{50}$ values and maximal activity of modified ODN relative to ODN **1**.

| | hTLR9 | | mTLR9 | |
|---|---|---|---|---|
| ODN | $EC_{50}$ [nM][a] | Activity [%][b] | $EC_{50}$ [nM][a] | Activity [%] [b] |
| **1** | 320 ± 155 | 100 | 283 ± 76 | 100 |
| **2** | 670 ± 210 | 98 ± 2 | 680 ± 66 | 28 ± 12 |
| **3** | 930 ± 690 | 61 ± 4 | n.c.[c] | < 10 |
| **4** | 830 ± 14 | 45 ± 8 | n.c. | < 10 |
| **5** | 400 ± 57 | 102 ± 12 | 605 ± 7 | 17 ± 1 |
| **6** | 895 ± 430 | 44 ± 4 | 1550 ± 71 | 11 ± 2 |
| **7** | 415 ± 78 | 42 ± 3 | n.c. | < 10 |
| **8** | 500 ± 127 | 19 ± 6 | 720 ± 0 | 18 ± 5 |
| **9** | 817 ± 145 | 31 ± 4 | 637 ± 80 | 23 ± 12 |
| **10** | 583 ± 42 | 15 ± 2 | n.c. | < 10 |
| **11** | 660 ± 142 | 26 ± 7 | 530 ± 111 | 86 ± 6 |
| **12** | n.c. | < 10 | n.c. | < 10 |
| **13** | 600 ± 42 | 24 ± 5 | n.c. | < 10 |
| **14** | n.c. | < 10 | n.c. | < 10 |
| **15** | 575 ± 78 | 29 ± 8 | n.c. | < 10 |
| **16** | n.c. | < 10 | n.c. | < 10 |
| **17** | 1020 ± 255 | 26 ± 6 | n.c. | < 10 |
| **18** | n.c. | < 10 | n.c. | < 10 |
| **19** | 153 ± 67 | 19 ± 13 | n.c. | < 10 |

[a] $EC_{50}$ values are given ± SD from at least two independent experiments. [b] Percent activity ± SD was calculated in reference to activity of ODN **1** (set to 100%) at maximum concentration tested (10 μM). [c] $EC_{50}$ values of ODN with less than 10% of activity of ODN **1** were not calculated (n.c.).

To unambiguously assign distinct molecular interactions of CpG ODN to the human receptor, we used a human TLR9 reporter assay system and compared the results with those obtained from a murine TLR9 assay. We investigated two series of CpG derivatives, one in which the hydrogen donor and acceptor function at guanine were either deleted or exchanged by other functional groups, and another involving various modifications of the C5 of cytosine and N4-alkylated cytosine. Using a murine lymphocyte proliferation assay for investigation of immune stimulatory activity, it has been previously reported that 7-deazaguanine-modified CpG showed activity similar to unmodified CpG, whereas replacing guanine with hypoxanthine resulted in a 65% decrease in lymphocyte proliferation.[11,12] All other investigated modifications of guanine (such as purine, 2,6-diaminopurine, and 2-aminopurine) were previously reported to induce only insignificant immune stimulation (8–19%) in a mouse cell assay. These results are in sharp contrast to the results of the human TLR9 assay presented herein. Replacement of guanine by 7-deazaguanine resulted in an 81% reduction in maximal hTLR9 activation (Table 1). However, TLR9 activation was almost totally maintained when guanine was exchanged with hypoxanthine as base (deoxyinosine as nucleoside), which is in agreement with our previous findings with a different sequence and a fully phosphorothioate-modified ODN.[21] Both the $EC_{50}$ value and maximal hTLR stimulation were unchanged when the O6 of guanine was replaced by sulfur (6-thioguanine). Furthermore, replacing guanine by 2-aminopurine, purine, 2,6-diaminopurine, or 8-oxoguanine resulted in about 40–60% of the parent ODN efficacy in hTLR9 activation. These results strongly suggest that recognition of the guanine base in the CpG motif is primarily determined by the N7 and exocyclic O6 functions, which means that guanine is recognized from the "Hoogsteen base pairing site" by hTLR9. The decreased activity of 8-oxo-7,8-dihydroguanine could be caused by a shift in equilibrium from the *anti* to the *syn* orientation of the guanine base. This in turn might suggest that the guanine base in the *anti* conformation is preferable for recognition by TLR9. However, the observed effect could also be due to a change in function of N7 from a hydrogen acceptor to a hydrogen donor when guanine is modified to 8-oxo-7,8-dihydroguanine. Interestingly, only one modification at guanine, namely hypoxanthine, showed even modest activation (28%) of murine TLR9, whereas all other modifications resulted in a drop of more than 80% of maximal mTLR9 activation. Thus, the strongest difference between human and murine receptor was observed for 6-thioguanine. Since replacement of the O6 of guanine by sulfur results in an isoelectronic derivative, the sharp drop in activity at the murine receptor appears to be caused by a spatial constraint effect rather than an electronic effect.

Substitution of the hydrogen atom at C5 of cytosine with a methyl group resulted in the expected decrease in hTLR9 stimulation of about 70%. Surprisingly, substitution by a hydroxy group at the C5 of cytosine results in an even stronger decrease (85%), which is in contrast to the similar activity reported using the mouse lymphocyte assay.[11] However, the activity in the mouse system is unlikely to be a result of better recognition by the murine receptor, as the 5-hydroxycytosine derivative does not exhibit significant activity in the murine TLR9

assay. In this respect it is notable that the 5-bromocytosine derivative shows 86% and 26% maximal stimulation in the mTLR9 and hTLR9 assays, respectively. Combined with the results obtained for 6-thioguanine, these findings demonstrate that the spatial requirements at the murine receptor differ substantially from those of the human receptor. Substitution at the C5 of cytosine by a 5-aminopropargyl residue resulted in virtually complete loss of activity for both hTLR9 and mTLR9. Whereas alkylation of N4 by an ethyl substituent at cytosine preserved moderate activity (24% of maximal stimulation) for hTLR9, the same modification completely abolished recognition by mTLR9. Taken together, the results with the modifications at cytosine suggest that both the primary exocyclic amino group as well as the spatial requirements at C5 of cytosine are very important for the observed immune stimulatory effects of CpG ODNs. The need for correct space filling at the cytosine position is also underscored by the fact that deletion of cytosine in the CpG motif (ODN **14**) or substitution by a universal base, such as 3-nitropyrrole or 5-nitroindole, results in complete loss of activity. This is in contrast to deletion of the guanine base in the CpG motif, as the corresponding ODN **15** still elicits approximately 30% of the maximal hTLR9 activation of the parent molecule, though the potency is reduced as shown by the increase in the $EC_{50}$ value to about 575 nM. Surprisingly, substitution of guanine by 5-nitroindole results in a decreased $EC_{50}$ value of about 150 nM, compared with the $EC_{50}$ value of ODN **1** (approximately 320 nM). This suggests that 5-nitroindole may be capable of providing high-affinity binding to hTLR9, while the maximal stimulation (19%) of hTLR is strongly hampered. The smaller 3-nitropyrrole base shows significantly lower ability to stimulate hTLR9 ($EC_{50} = 1020$ nM), but reached maximal stimulation of 26% at 10 μM relative to the parent molecule. The observed residual activity of abasic CpD (ODN **15**) and replacements by universal bases (ODNs **17** and **19**) in the human TLR9 assay again contrasts with the results in the murine TLR9 assay, in which all three derivatives are devoid of any stimulatory effects. These results contrast with those of a previous report in which it was suggested, through less direct assays, that a nucleobase is absolutely required at both C and G positions in CpG ODN.[22]

By comparing the results from the human and murine TLR9 assays, it becomes clear that the previously reported results for 7-deazaguanine and 5-hydroxycytosine using the mouse lymphocyte proliferation assay[11] cannot be adequately explained by differences in species specificity, but might be explained by off-target effects other than the interaction with TLR9.

In conclusion, we have shown in our SAR studies that all investigated modifications at C5 and N4 of cytosine are only of limited compatibility with recognition by hTLR9, and the following ranking order (maximal stimulation of hTLR9) was observed: cytosine > 5-methylcytosine ~ 5-bromocytosine ~ N4-ethylcytosine > 5-hydroxycytosine > 5-aminopropargylcytosine. Furthermore, regarding guanine modifications, N7 is of utmost importance for recognition by hTLR9, while 2-amino, 6-amino, and 6-oxo are less critical for optimal activation. Only substitution of guanine by hypoxanthine and 6-thioguanine results in activity similar to the unmodified parent molecule. The ranking for maximal stimulation of hTLR9 is: guanine ~ 6-thioguanine ~ hypoxanthine > 2-aminopurine > purine ~ 2,6-diaminopurine ~ 8-oxoguanine > 7-deazaguanine. In view of the natural evolution of the CpG recognition motif, it may not come as a surprise that none of the investigated base modifications led to superior recognition by hTLR9, albeit two guanine modifications (6-thioguanine and hypoxanthine) show TLR9 activation similar to the natural CpG TLR9 agonist. To our knowledge, this is the first systematic SAR study of base modifications at phosphodiester-linked CpG dinucleotide motif in immune stimulatory ODN using human TLR9 activation as readout. The different results obtained from the human versus murine TLR9 in in vitro assay systems strongly underscore the importance of using human cell-based assay systems for optimization of potential drug candidates in CpG ODN drug development. Likewise, the use of in vivo mouse models for ranking CpG ODN in the selection of drug candidates for human use might also be of limited value.

## Experimental Section

Oligodeoxynucleotides: All ODN were purchased from Biospring or provided by Coley Pharmaceutical GmbH, controlled for identity (MALDI-TOF) and purity (capillary gel electrophoresis or ion-pair RP-HPLC) by Coley Pharmaceutical GmbH. ODN were suspended in sterile, endotoxin-free Tris-EDTA (Sigma), and stored and handled under aseptic conditions to prevent both microbial and endotoxin contamination. All dilutions were carried out using endotoxin-free Tris-EDTA.

TLR assays using stably transfected HEK293 cells expressing the human TLR9 or murine TLR9 were performed as previously described.[21,23] Briefly, HEK293 cells were transfected by electroporation with vectors expressing the respective TLR and a 6x NF-κB-luciferase reporter plasmid. Stable transfectants ($3 \times 10^4$ cells/well) were incubated with ODN for 16 h at 37 °C in a humidified incubator. Each data point was performed in triplicate. Cells were lysed and assayed for luciferase gene activity (using the BriteLite kit from Perkin–Elmer). Stimulation indices were calculated in reference to reporter gene activity of the medium without addition of ODN. $EC_{50}$ values were calculated using the Sigma Plot program (SSPS Inc.) using sigmoidal regression curves (4 parameters).

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