A STAT3-inhibitory hairpin decoy oligodeoxynucleotide discriminates between STAT1 and STAT3 and induces death in a human colon carcinoma cell line.

Inès Souissi, Patrick Ladam, Jean Cognet, Stéphanie Le Coquil, Nadine Varin-Blank, Fanny Baran-Marszak, Valeri Metelev, Remi Fagard

To cite this version:
Inès Souissi, Patrick Ladam, Jean Cognet, Stéphanie Le Coquil, Nadine Varin-Blank, et al.. A STAT3-inhibitory hairpin decoy oligodeoxynucleotide discriminates between STAT1 and STAT3 and induces death in a human colon carcinoma cell line.. Molecular Cancer, BioMed Central, 2012, 11 (1), pp.12. 10.1186/1476-4598-11-12. inserm-00687513

HAL Id: inserm-00687513
https://www.hal.inserm.fr/inserm-00687513
Submitted on 13 Apr 2012

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L’archive ouverte pluridisciplinaire HAL, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d’enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.
A STAT3-inhibitory hairpin decoy oligodeoxynucleotide discriminates between STAT1 and STAT3 and induces death in a human colon carcinoma cell line

Inès Souissi1,2, Patrick Ladam2,6, Jean AH Cognet7, Stéphanie Le Coquil1,2, Nadine Varin-Blank1,2, Fanny Baran-Marszak1,2,4, Valeri Metelev5 and Remi Fagard1,2,3*

Abstract

Background: The Signal Transducer and Activator of Transcription 3 (STAT3) is activated in tumor cells, and STAT3-inhibitors are able to induce the death of those cells. Decoy oligodeoxynucleotides (dODNs), which bind to the DNA Binding Domain (DBD) of STAT3, are efficient inhibitors. However, they also inhibit STAT1, whose activity is essential not only to resistance to pathogens, but also to cell growth inhibition and programmed cell death processes. The aim of this study was to design STAT3-specific dODNs which do not affect STAT1-mediated processes.

Results: New dODNs with a hairpin (hpdODNs) were designed. Modifications were introduced, based on the comparison of STAT3- and STAT1-DBD interactions with DNA using 3D structural analyses. The designed hpdODNs were tested for their ability to inhibit STAT3 but not STAT1 by determining: i) cell death in the active STAT3-dependent SW480 colon carcinoma cell line, ii) absence of inhibition of interferon (IFN) γ-dependent cell death, iii) expression of STAT1 targets, and iv) nuclear location of STAT3 and STAT1. One hpdODN was found to efficiently induce the death of SW480 cells without interfering with IFNγ-activated STAT1. This hpdODN was found in a complex with STAT3 but not with STAT1 using an original in-cell pull-down assay; this hpdODN also did not inhibit IFNγ-induced STAT1 phosphorylation, nor did it inhibit the expression of the STAT1-target IRF1. Furthermore, it prevented the nuclear transfer of STAT3 but not that of IFNγ-activated STAT1.

Conclusions: Comparative analyses at the atomic level revealed slight differences in STAT3 and STAT1 DBDs’ interaction with their DNA target. These were sufficient to design a new discriminating hpdODN that inhibits STAT3 and not STAT1, thereby inducing tumor cell death without interfering with STAT1-dependent processes. Preferential interaction with STAT3 depends on oligodeoxynucleotide sequence modifications but might also result from DNA shape changes, known to modulate protein/DNA interactions. The finding of a STAT3-specific hpdODN establishes the first rational basis for designing STAT3 DBD-specific inhibitors.

Keywords: Hairpin decoy oligodeoxynucleotide (hpdODN), STAT3, STAT1, Colon carcinoma cell line

Background

STAT3 belongs to the signal transducers and activators of transcription (STATs) family of transcription factors (TFs) [1]. STAT3 is activated in response to several cytokines and growth factors, including IL-6, IL-10, the epidermal growth factor (EGF), and interferon (IFN) α and is also weakly activated in response to other cytokines, including IFNγ in some cellular contexts [2]. Activation of STAT3 involves phosphorylation of tyrosine 705 by cytokine receptor-associated Janus Kinase (JAK); the involvement of the Src and Abl tyrosine kinases as well as EGFR have also been reported [3]. Tyrosine phosphorylation of STAT3 is followed by dimerization

* Correspondence: remi.fagard@avc.aphp.fr
1 INSERM, Unité 978, Bobigny 93017, France
Full list of author information is available at the end of the article

© 2012 Souissi et al; licensee BioMed Central Ltd. This is an Open Access article distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/2.0), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.
through phosphotyrosine-SH2 domain interaction; activated STAT3 enters the nucleus where it stimulates the transcription of its targets, including Cyclin-D1, Survivin, VEGF, C-Myc, Bcl-xL, and Bcl2 [4,5].

STAT3 is a key regulator of cell survival and proliferation [6]. Its constitutive activation has been observed in many human tumors, including colon, breast, lung, pancreas and prostate cancers, melanoma, head and neck squamous carcinoma, multiple myeloma, mantle cell lymphoma, and glioma [7,8]. However, in certain cell types such as PTEN-deficient glioblastoma, STAT3 can become a tumor suppressor [9,10].

STAT1 is another member of the STAT family. It is activated mainly by IFNs α and γ, and plays a major role as a pro-inflammatory, anti-pathogen and anti-proliferative factor [11,12]. Its biological function is thus mostly antagonistic to that of STAT3. Despite their 50% amino acid sequence homology [13], STAT1 and STAT3 are structurally very similar; yet some important differences have been noted in their DBD sequences [14]. Despite its major role as a tumor antagonist, STAT1 can also have functions in cancer cells, as documented in mouse leukemia [15].

Inhibition of STAT3 in tumor cells in which it is constitutively activated leads to cell death [16-18]. This is achieved using either non-specific inhibitors such as curcumin, which also inhibits other transcription factors (NF-κB), or inhibitors specifically designed to inhibit STAT3 through non-covalent binding to the SH2 domain, such as Stattic [19] or STA-21 [20]. Interestingly, these compounds have little effect in cells in which STAT3 is not activated, pointing to STAT3 as a highly valid target to focus on for the design of anti-cancer compounds. However, such compounds are still poorly developed.

TFs activate transcription of their target genes by binding to distinct short DNA consensus motifs. Decoy oligonucleotides (dODNs) containing these consensus motifs can bind the DNA binding domains (DBD) of the TFs and block their activity. dODNs [21] and hairpin dODNs (hpDODNs) [22] have been shown to induce the death of cells in which STAT3 is activated, suggesting that the DBD is another potential target for specific inhibitory compounds. Similarly to double-stranded oligonucleotides that are used to detect active dimers in electrophoretic migration shift assays, STAT3 hpDODNs interact with activated, dimeric STAT3. This interaction impairs the binding of the dimer to importins, resulting in the sequestration of STAT3 in the cytoplasm [23].

Yet, because of the high degree of similarity between STAT3 and STAT1 consensus DNA binding sites, STAT1 competes with activated STAT3 for dODN binding in interferon (IFN) γ-treated cells [22,23], thereby preventing inhibition of active STAT3. Under such conditions the dODN loses its ability to block cell proliferation. In addition, since STAT1 plays a key role in cell death processes [24,25], including caspases expression [26,27] and cooperation with p53 function [28,29], its inhibition by the dODN prevents cell death. Finally, IFNγ being a cell death inducer in several cell types [22,30-32], it is important to design reagents that do not interfere with STAT1, one of its key effectors. Thus, in order to elaborate target-specific anti-cancer compounds, the specificity of hpDODNs to STAT3 needs to be enhanced. It should be noted, however, that in certain cellular contexts STAT1 has been found to be a tumor promoter [15].

The difficulty in designing dODNs recognized by STAT3 but not STAT1 lies in the striking similarity of the consensus DNA sequences of the two TFs [33], in spite of their different cellular functions. Nevertheless, early studies on STAT3/STAT1-discriminating DNA motifs established some sequence preferences that differentiate these TFs [34-39], suggesting possibilities for designing STAT3/STAT1-discriminating dODNs. The notion that discrete nucleotide modifications in target DNA sequences might alter their recognition by closely related TFs is supported by the observation that a single nucleotide change in the κB consensus motif modified NF-κB subunit specificity [40]. Furthermore, DNA recognition by proteins relies in part on DNA shape, known to deviate from the ideal B-conformation. The nature of the nucleotides in the sequence influences conformation and dynamics: for instance, dG:dC stretches confer rigidity [41], pyrimidine/purine steps (particularly T/dA steps) confer flexibility and may also introduce kinks [42,43], and dA:T stretches can have complex configurations [41]. The coordinates from available crystal structures of both STAT1 [44] and STAT3 [45] (in complex with their DNA consensus sequence) were used to analyze their 3D structure using the UCSF Chimera program [46]. Based on the differences found, new hpDODNs were designed and tested for their STAT3/STAT1 discrimination ability by measuring SW480 colon carcinoma cell death and absence of inhibition of STAT1-dependent IFNγ-induced cell death. SW480 cells offer a relevant model since these cells show constitutive activation of STAT3, which is essential for their survival, and they are susceptible to IFNγ-induced cell death, which is a STAT1-dependent process. The newly designed hpDODNs were also compared for their relative binding capacity to STAT1 and STAT3 by performing in-cell pull-downs, and for their ability to prevent nuclear transfer using immunofluorescence.

Results
Striking similarities in the interactions of STAT1 and STAT3 with their consensus DNA sequence

Comparison of the 3D structures of STAT1 and STAT3 in complex with their oligonucleotide duplexes featuring
a consensus DNA sequence using the Chimera program showed that they are highly similar (Figure 1A), with an overall root mean square deviation of 0.63 Å between 317 atom pairs of the backbone. To focus our study on the interaction of the STAT1 and STAT3 DBDs with their consensus DNA sequence, only the amino acids in close contact with the DNA strands were examined. This revealed the striking similarity of STAT1 and STAT3 DNA-interacting amino acids (Figures 1B and 1C). Several differences were noted, however, including: 

![Image](http://www.molecular-cancer.com/content/11/1/12)

**Figure 1 3D comparison of STAT1 and STAT3 and of their DNA binding region.** A: View of the superimposed 3D structure of STAT1 and STAT3 monomers created with the Chimera program, showing the DNA binding region. B and C: close-up views of the DNA binding regions of STAT1 (B) and STAT3 (C). Amino acids shown are those within a 5 Å distance from DNA; note that Thr 327 did not appear using this selection and was added. Arrows point to amino acids of STAT1 and STAT3 interacting differently with DNA.
i) Glu 421, unique to STAT1, and located within direct H-bond distance from G 1017 [44], G 2002 and C 1018 (the latter possibly mediated by water) (Figures 1B and 1C; detailed view in Figure 2; see Table 1 for a list of H-bonds and Additional file 1: Table S1 for a list of the interactions of STAT1 and STAT3 with DNA); ii) the peptide backbone of a polar residue of STAT1, Thr 327, and of a hydrophobic residue of STAT3, Met 331, establish H bonds with C 1009 and C 1010 (Figure 1B); iii) a polar amino acid, Thr 419 for STAT1, and a charged amino acid, Arg 423 for STAT3, are identically positioned, facing the backbone of nucleotide 1018 (Figure 1B). To obtain STAT3/STAT1 discriminating sequences, we chose to design hpdODNs, by modifying the original consensus sequences and hpdODNs are depicted in Figure 3: for convenience we use the same numbering as in reference [45]).

**Nucleotide substitutions provide a hairpin decoy oligonucleotide which can discriminate between STAT1 and STAT3, inhibiting STAT3 in IFNγ-treated cells**

As previously shown [22,23], the consensus-carrying hpdODN A can efficiently induce the death of cells of the SW480 line (Figure 4A and 4B); but it also inhibits STAT1, thus blocking the STAT1-dependent IFNγ-induced mortality of these cells (Figure 4A, B and 4C) as previously shown [22,23]. hpdODN B was designed by replacing three base pairs in hpdODN A. T replaced dC in position 1003, dC replaced dG in 1011, and dG replaced dC in position 1017 (Figure 3). In the same assay, hpdODN B was found to efficiently induce SW480 cell death but was devoid of any action on IFNγ-induced cell death (Figure 4A and 4B), indicating a preference for STAT3 over STAT1. Features of hpdODN B consist in a stretch of pyrimidines spanning nucleotides 1005 to 1012, a d(TA) step (1003/1004) and a d(TG) step (1016/1017). To analyze the possible effect of only one change in the sequence of hpdODN A, hpdODN C was designed by replacing dG with dC in position 1011 (Figure 3). The killing efficiency of HpdODN C was lower than those of hpdODN A [22,23] and hpdODN B, but in contrast with the latter, it showed a capacity to compete with IFNγ-induced mortality, suggesting that it interacts with STAT1 (Figure 4A and 4B). Next, by placing dG in 1003, dC in 1004, dC in 1011 and dG in 1017 we obtained hpdODN D (Figure 3), which corresponded with a sequence with a marked preference for STAT1 as previously shown by others using a reporter assay [47]. hpdODN D did not induce SW480 cell mortality, but prevented IFNγ-induced killing (Figure 4A and 4B). Finally, hpdODN E, containing a mutated STAT3 binding site (Figure 3) did not induce cell death and did not compete with IFNγ-induced cell death (Figure 4A and 4B). A comparison of the different hpdODNs’ IFNγ-independent cell killing efficiency (taking the killing efficiency of hpdODN A as the 100% standard) showed that hpdODN B was twice as efficient as hpdODN A (Figure 4C) and that the control mutated hpdODN E had no effect on cell death (Figure 4C), as previously published [22,23].

**The new STAT3-specific hpdODN B inhibits STAT3 but not STAT1 phosphorylation and inhibits cyclin D1 but not IRF1 expression**

To detect the effect of the hpdODNs on STAT3 phosphorylation, IL-6-treated (50 ng/ml) SW480 cells were used. In cells treated with hpdODN B and hpdODN A for 16 h, STAT3 phosphorylation was suppressed (Figure 4E, lanes 2 and 3); the expression of cyclin D1 and of STAT3 itself (STAT3 being its own target) were considerably diminished (Figure 4E, lanes 2 and 3), in agreement with previous observations [22,23]. When cells were treated for 4 h with hpdODNs A and B, phospho-STAT3 was reduced without effect on STAT3 (Figure 4F); the control mutated hpdODN E had no effect (Figures 4E and 4F, lane 4). To confirm that hpdODN B was preferentially inhibiting STAT3 in SW480 cells, the induction of the STAT1-dependent IFNγ target IRF1 was studied. In cells treated with IFNγ (50 ng/ml for 16 h), both phosphorylation of STAT1 and expression of IRF1 increased (Figure 4D, lanes 1 and 2). Treatment with hpdODN A, but not hpdODN B, strongly reduced IRF1 expression (Figure 4D, lanes 3 and 4). In IFNγ-treated cells, the addition of hpdODN A reduced IFNγ-induced IRF1 expression (lane 5) whereas the addition of hpdODN B did not (lane 6). Interestingly, STAT1
phosphorylation on tyrosine was inhibited following treatment with hpdODN A (lane 5) but not with hpdODN B (lane 6). These data indicate that under these experimental conditions hpdODN B does not inhibit STAT1.

**Biotinylated hpdODN B interacts preferentially with STAT3**

Binding of STAT3 and STAT1 to hpdODNs has previously been analyzed directly within cells using biotinylated versions of the different hpdODNs [23]. To compare hpdODNs A and B, cells were treated, or not, with IFNγ, transfected with biotinylated hpdODNs, and pull-downs were performed. The pull-down efficiencies of hpdODN A (Figure 5, lanes 1 and 4) and B (Figure 5, lanes 3 and 6) for STAT1 and STAT3 were very different. Indeed, compared with hpdODN A, hpdODN B brought down STAT3 very efficiently, but not STAT1 (Figure 5, compare lanes 4 and 6). Furthermore, compared

| Table 1 Hydrogen bonds involved in the interaction of STAT1 and STAT3 with DNA |
|-------------------------------|----------------------------------|-----------------|-----------------|-----------------|
| **Nucleotide involved**  | **Residue(s) involved**  | **Nucleotide involved**  | **Residue(s) involved**  |
|-------------------------------|----------------------------------|-----------------|-----------------|-----------------|
| T 1005                        | Thr 427 (BB/Phos.)               | T 1005          | Val 432 (BB/Phos.) |
| T 1006                        | Lys 413 (BB/Phos.)               | T 1006          | Ser 465 (SC/Phos.) |
|                               | Ser 459 (BB/Phos.)               | T 1006          | Arg 382 (SC/Phos.) |
|                               | Arg 378 (BB/Phos.)               | T 1006          | Gln 469 (SC/Phos.) |
|                               | Ser 459 (BB/Phos.)               | T 1006          | Ser 465 (SC/Phos.) |
|                               | Gln 463 (BB/Phos.)               | T 1007          | Asn 466 ND2 (SC/Base) |
|                               |                                 | T 1007          | Arg 417 NH1 (SC/Phos.) |
|                               |                                 | C 1008          | Gln 344 NE2 (SC/Phos.) |
|                               |                                 | C 1009          | Gln 344 N (BB/Phos.) |
| C 1010                        | His 328 (SC/Phos.)               | C 1010          | Lys 340 (SC/Phos.) |
|                               |                                 | C 1010          | His 332 (SC/Phos.) |
| G 1011                        | Lys 567 (SC/Phos.)               | T 1012          | Asn 466 (SC/Phos.) |
| T 1012                        | Glu 421 (SC/Phos.)               | T 1012          | Asn 466 (SC/Phos.) |
| G 1017                        |                                 | C 1008          | Glu 421 (SC/Phos.) |
| C 1018                        | Thr 419 (BB/Phos.)               | T 2005          | Val 432 (BB/Phos.) |
|                               | Thr 419 (BB/Phos.)               | T 2005          | Val 432 (BB/Phos.) |
| G 2002                        | Glu 421 (SC/Phos.)               | T 2005          | Val 432 (BB/Phos.) |
|                               |                                 | T 2005          | Val 432 (BB/Phos.) |
| T 2006                        | Lys 413 (SC/Phos.)               | T 2006          | Ser 465 (SC/Phos.) |
|                               | Arg 378 (SC/Phos.)               | T 2006          | Arg 382 (SC/Phos.) |
|                               | Ser 459 (SC/Phos.)               | T 2006          | Ser 465 (SC/Phos.) |
|                               | Arg 378 (SC/Phos.)               | T 2006          | Gln 469 (SC/Phos.) |
| T 2007                        | Asn 460 (SC/BB)                  | T 2007          | Asn 466 (SC/Phos.) |
|                               | Lys 413 (SC/BB)                  | T 2007          | Asn 466 (SC/Phos.) |
| A 2008                        | Asn 460 (SC/Phos.)               | A 2008          | Glu 421 (SC/Phos.) |
|                               |                                 | C 2009          | Glu 421 (SC/Phos.) |
| G 2010                        | His 328 (SC/Phos.)               | G 2010          | Lys 340 (SC/Phos.) |
|                               |                                 | G 2010          | Lys 340 (SC/Phos.) |
|                               |                                 | G 2010          | Lys 340 (SC/Phos.) |
| G 2011                        | Lys 567 (BB/Phos.)               | A 2013          | Asn 466 (SC/Phos.) |
|                               |                                 | A 2013          | Asn 466 (SC/Phos.) |
| G 2012                        | Asn 460 (SC/Phos.)               | A 2013          | Asn 466 (SC/Phos.) |
|                               |                                 | A 2013          | Asn 466 (SC/Phos.) |
| G 2018                        | Glu 421 (SC/Phos.)               | G 2018          | Glu 421 (SC/Phos.) |

The nucleotides that are involved in binding with STAT1 or STAT3 are listed. For clarity, the numbering used is the same as in reference [45] (see Figure 3). The following abbreviations were used for the atoms involved in hydrogen bonding: phospho-diester (Phos.) or base moiety (Base) for the nucleotides. BB stands for backbone and SC for side-chain for the proteins.
with hpdODN A, hpdODN D, shown to interact preferentially with STAT1, was more efficient in pulling down STAT1 than STAT3 (compare lanes 8 and 9 with lanes 1 and 4). Finally, hpdODN E, a control hpdODN with mutations in the binding consensus, did not bring down either STAT3 or STAT1, which both remained nuclear (Figure 6, columns 2 and 5).

The new hpdODN B prevents the constitutive nuclear location of STAT3 in SW480 cells, but not that of IFNγ-activated STAT1

HpdODNs A and B were further compared for their ability to prevent the nuclear translocation of STAT3 and STAT1 in SW480 cells (treated or not with IFNγ) using immunofluorescence. Treatment of the cells with hpdODN A prevented the nuclear translocation of both STAT3 and STAT1 (for STAT1 nuclear translocation, IFNγ-treated cells were used) (Figure 6), as previously shown [22,23]. Treatment with hpdODN B prevented the nuclear translocation of STAT3 only, and not that of IFNγ-activated STAT1 (Figure 6, columns 1 and 4, see arrows), confirming its discriminative capacity. Notably, the control mutated hpdODN E had no effect on the subcellular location of either STAT3 or STAT1, which both remained nuclear (Figure 6, columns 2 and 5).

Discussion

A new hairpin decoy oligonucleotide (hpdODN) carrying STAT3’s DNA binding consensus sequence was designed following 3D analysis of protein/DNA interaction and shown to induce the death of STAT3-dependent tumor cells without interfering with STAT1, a key effector of cell death. In this paper, 3D structural analyses of the protein/DNA interaction of STAT1 and STAT3 demonstrated their high similarity, confirming previous reports [13]. These 3D analyses served as a basis for the design of new sequences with base substitutions. The new sequences were tested for their ability to induce cell death in an IFNγ-sensitive [22,23,48-51], active-STAT3-dependent colon carcinoma cell line [22]. This enabled the design of the STAT3-specific hpdODN labeled here as hpdODN B. The ability of hpdODN B to discriminate between STAT1 and STAT3 was assessed by: i) its ability to kill cells without interfering with IFNγ-induced cell death; ii) its ability to inhibit STAT3 targets, including cyclin D1, iii) the absence of inhibition of IFNγ-induced STAT1 phosphorylation and IRF1 expression, iv) its lack of interaction with STAT1 in pull-down assays and iv) its inability to inhibit IFNγ-induced STAT1 nuclear location. Indeed, hpdODN A treatment, but not hpdODN B treatment, reduced STAT1 phosphorylation, probably by impairing nucleocytoplasmic shuttling as previously suggested [22,23]. Nevertheless, despite its ability to discriminate between STAT1 and STAT3, hpdODN B probably has a residual affinity for STAT1, as shown by low detection of STAT1 in pull-down assays and the fact that cell death induction by hpdODN B and IFNγ are not additive.

The STAT3/STAT1-discriminating hpdODN was obtained by replacing key nucleotides that 3D analyses had shown to be in the vicinity of amino acids of the DBD that distinguish the two STATs; the similarity of their DNA consensus sequences, despite their different functions, has been recognized for some time [33]. Examination of the nucleotide modifications that led to STAT1/STAT3-discriminating hpdODN B showed that they are compatible with previous in vitro DNA-binding studies, such as the preference for T at 1003 and 1005, dC at 1010 and dA at 1015 of STAT3 [36-38]. The fact that T at 1003 does not favor STAT1 binding is also in agreement with the earlier suggestion that “selection for a dG:dC base pair at position 7 (here: 1017) is likely to involve Glu 421 (of STAT1) which can accept hydrogen bonds from guanine in the minor groove” [44]. This has also been noted by others [39]. Finally, altered recognition by a TF following single nucleotide changes has been previously shown, for instance with NF-κB subunit recognition of κB [40]. One notable property of the hpdODN B is its dissymmetry. A symmetric version (with dA replacing dG in 1017) was tested and is apparently not different from hpdODN B (not shown). Intriguingly, although the preference of hpdODN D for STAT1 was expected from previous data showing its
Figure 4 Comparative cell death induction efficiency of the different hpdODNs in the presence or not of IFNγ in the SW480 cell line

The efficiencies of hpdODN A, B, C, D and E in inducing cell death were compared, in the presence (+) or not (-) of IFNγ. A. Cell death, measured by determining propidium iodide (PI) labeling, induced by hpdODNs A to E (2 μg/ml) alone (-) or in the presence of IFNγ (50 ng/ml 16 h) (+). B. Cell death, measured with flow cytometry for annexin V, induced by hpdODNs A to E (2 μg/ml) alone (-), or in the presence of IFNγ (50 ng/ml 16 h) (+). In A and B, FITC denotes the detection of cells having incorporated the fluorescein-labelled hpdODNs. Note that experimental conditions were such that cell death was not maximal, to allow detection of differences between experiments. Experiments were repeated at least three times, a typical result is shown. C: Histograms from several experiments (at least three per condition) using hpdODNs A to E, obtained by computing PI incorporation and annexin V labelling data (such as those shown in Figures 4A and B). To facilitate comparison, data are presented as% of the control. D: Western blotting showing the phosphorylation of STAT1 on tyrosine (‘P-STAT1’) and expression level of...
STAT1-specific binding [47], its basis is not clear and may rest upon properties beyond nucleotide sequence such as DNA shape. The shape and flexibility of DNA strands are known to be influenced by their nucleotide content; here the 8-pyrimidine stretch in hpdODN B may confer a higher flexibility than hpdODN A and may account for a differential interaction with STAT3 Arg 423 and STAT1 Glu 421. In fact, the molecular dynamics studies which describe a scissor-like molecular movement upon DNA binding for STAT3, but not for STAT1 [13] suggest that the flexibility of the DNA target may play a role in binding and therefore underly the preference of hpdODN B for STAT3. It may also account for the greater sensitivity of STAT3 to an intact palindromic structure compared to STAT1, as previously stated [38]. Protein binding itself can affect DNA bending, as shown with the high-affinity target of the papillomavirus E2 [52]. Nevertheless, despite its efficiency, the precise mechanism whereby the hpdODN B discriminates between STAT1 and STAT3 in cells is not understood. Changes in DNA shape may play a role in the preferential recognition of hpdODN B by STAT3; co-factors may also be involved in DNA recognition by STAT3, and might associate more efficiently when hpdODN B is used. The process might also be more complex than mere differential DNA binding: STAT1 and STAT3 are reciprocally regulated [2,53-55] and the relative abundance of their active forms may itself play a key role in biological responses, as previously discussed [56]. Another level of complexity arises from the fact that in cells in which STAT3 has been suppressed, IFNγ-activated STAT1 induces the expression of mitogenic STAT3-targets [57,58]. Furthermore, STAT1 and STAT3 form heterodimers, whose function has not been elucidated to date. In this respect, quantification of the relative amounts of STAT1 and STAT3 bound to the hpdODNs A and B may help understand the complex interaction of these TFs. Preliminary experiments that aimed under different conditions. Therefore, it is possible that hpdODN B functions in cells by tilting the active STAT1/active STAT3 balance toward STAT1, thereby inducing cell death.

Conclusions
By combining 3D molecular interaction analysis and direct screening in cells, this work allowed the design of an hpdODN that can selectively inhibit STAT3 but not STAT1. The efficacy and potential of this approach resides in the direct testing of modified hpdODNs in cells, analyzing processes that depend on STAT3 (cell

![Figure 5](image_url)
Figure 5 Specific binding of hpdODN B to STAT3 detected by in-cell pull-down. Western-blotting of STAT1 and STAT3 bound to biotinylated hpdODNs after transfection and in-cell pull-down with avidine-coated beads. Cells were either not treated (−) (lanes 1, 2, 3 and 7, 8) or treated (+) (lanes 4, 5, 6 and 9) with IFNγ (50 ng/ml, 16 h), and hpdODN A (lanes 1 and 4), hpdODN B (lanes 3 and 6), hpdODN C (lanes 2 and 5) hpdODN D (lanes 8 and 9) and hpdODN E (lane 7) (hpdODNs were at 1 μg/ml for 16 h). Following cell lysis, protein concentration was measured, identical amounts of protein per sample (250 μg) were added, anti-tubulin western blotting from aliquots of the extracts was performed to show identical loading. Binding experiments were repeated at least three times. All lanes are part of the same blot (identical exposure time) and have been separated for clarity.
survival) or STAT1 (IFNγ-mediated death). These hpdODNs represent a basis for elaborating STAT3 DBD-specific low molecular weight compounds with anti-cancer properties.

Material and methods

Computer analysis of STAT3 and STAT1

The PDB (Protein Data Base) files for STAT1 (1BF5) [44] and STAT3 (1BG1) [45] were downloaded and analyzed using Chimera [46]. The STAT1 and STAT3 crystals used in the X-ray diffraction studies were proteins complexed with oligonucleotide duplexes featuring a consensus DNA sequence [34,35,59] (see Figure 3). To compare the STAT1 and STAT3 DBDs in a complex with their DNA consensus sequences, the missing complementary strand of the STAT3-bound oligonucleotide [45] was reconstructed through crystal symmetry operations.

Decoy oligonucleotides

The STAT3 decoy ODNs used were

RHN-(CH$_2$)$_6$-CATTTCCCCCTAAATGGAACATT-TAGGGGAATA-(CH$_2$)$_6$-NHR (hpdODN B); RHN-(CH$_2$)$_6$-CATTTCCCCCTAAATCGAAGATT-TAGGG-GAAATG-(CH$_2$)$_6$-NHR (hpdODN C); RHN-(CH$_2$)$_6$-GCTTTCCCCTAAATGGAACATTTAGGGGAAAGC-(CH$_2$)$_6$-NHR (hpdODN D); and the following mutated hpdODN as a negative control:

RHN-(CH$_2$)$_6$-CGATTGCCACACATGGGAAGATTG TGGCAATCG-(CH$_2$)$_6$-NHR (hpdODN E) (where R was either: hydrogen, fluorescein or biotin). The addition of fluorescein or biotin, followed by high-performance liquid chromatography, were carried out by the manufacturer (Eurogentec, Seraing, Belgium) using in-house protocols. The hairpin sequence GAA, previously shown to confer stability and nuclease resistance [60,61], was included in the dODNs. In the hpdODNs, the hairpin motif was built and incorporated in the X-ray structure using the BCE (Biomolecular Chain Elasticity) approach [62]; this showed that the hairpin did not interfere with the DBD-DNA interaction (not shown).

Cell culture and reagents

SW480 (colon adenocarcinoma) cells were grown in DMEM (Invitrogen, Lifetecnologies, Cergy-Pontoise, France), supplemented with 10% FCS (Lonza, Levallois-Perret, France), 100 U/ml penicillin, 10 μg/ml streptomycin (GibcoBRL), 1 mM sodium pyruvate (GibcoBRL), MEM vitamins (100x, Invitrogen) and 5 μg/ml plasmocin (Casla InvivoGen, Toulouse, France). Sodium orthovanadate (100 μM) was from Fischer (Ilkirkirch, France). Interferon γ (50 ng/ml) was from Promocell (GmbH, Heidelberg, Germany) or Sigma-Aldrich (Montigny-le-Bretonneux, France).

Transfections

Cells were grown in 4-well plates to a density of 0.25 × 10$^6$ cells/ml. When the cells reached 50-60% confluence, they were transfected with the different STAT3 hpdODNs or the control hpdODN (2 μg/ml, corresponding to 400 nM) into 150 μl of DMEM medium (without SVF) combined with polyethyleneimine (PEI, reference 408727, average MW: 25000, Sigma-Aldrich), to confer stability and nuclease resistance [60,61], was included in the dODNs. In the hpdODNs, the hairpin motif was built and incorporated in the X-ray structure using the BCE (Biomolecular Chain Elasticity) approach [62]; this showed that the hairpin did not interfere with the DBD-DNA interaction (not shown).

Flow cytometry and cell viability

To measure cell death, cells were resuspended in annexin V-binding buffer, incubated with 5 μl of propidium iodide (PI, BD Pharmingen, Morangis, France) and subjected to flow cytometry analysis, using a FACS
Canto II Flow Cytometer (BD). To enable selective analysis of the cells that had incorporated the various hpdODNs, fluorescein-labelled hpdODNs were used. Fluorescein-labelled cells were analyzed for PI incorporation (red channel) or annexin V labelling (APC, red channel). A cell death index was established through computation of averages.

Gel electrophoresis, western blotting
Cells were washed in Phosphate-Buffered Saline (PBS), lysed in sodium dodecyl sulfate (SDS) sample buffer (50 mM Tris-Cl pH 6.8 (Bio-Rad, Marnes-la-Coquette, France), 2% SDS (Sigma-Aldrich), 20% glycerol (Prolabo, Fontenay-sous-Bois, France), 1 mM sodium vanadate (Na3VO4, Labosi, Elancourt, France), 1 mM dithiothreitol (DTT) (Merck, Fontenay-sous-Bois, France) and 0.01% bromophenol blue (Sigma-Aldrich)), sonicated and stored at -70°C. Proteins (50 µg) were separated on SDS polyacrylamide gels (PAGE) (10%) and transferred onto nitrocellulose membranes; membranes blocked with dry skimmed milk (5%) in Tris Buffered Saline (TBS) were incubated with antibody overnight at 4°C. Anti-phospho-STAT1, anti-STAT1 and anti-STAT3 (1:1000) (Cell Signaling, Ozyme, Montigny-le-Bretonneux, France), anti-cyclin D1 and anti-IRF1 (1:1000) (Santa Cruz, Tebu-bio, Le Perray en Yvelines, France) were used. Blots were washed in TBS with Tween (0.05%) (TBS-T), incubated with peroxidase-coupled goat anti-mouse (Santa Cruz) or goat anti-rabbit (Upstate, Ozyme) secondary antibody (1:20,000), washed in TBS-T and revealed by chemiluminescence (Lumin-GLO reagent and peroxide; Cell Signaling) and autoradiography (X-Omat R film; Kodak). When necessary, membranes were stripped with BLOT Restore Kit (Chemicon International, Millipore, Saint-Quentin-en-Yvelines, France) and reprobed with anti-tubulin (Cell Signaling) or anti-actin (Santa Cruz) antibody to ensure equal loading of the gels. Prestained molecular weight standards (Fermentas, Saint-Rémy-lès-Chevreuse, France) were used.

Oligodeoxynucleotide pull-down
For in-cell hpdODN pull-down assays, cells were transfected with the biotinylated hpdODNs (1 µg/ml, 6 h), as described under oligonucleotide transfection, and then lysed in cell lysis buffer (1% NP40, 50 mM Hepes, pH 7.6, 140 mM NaCl) containing salmon sperm DNA (1 µg/assay). Protein concentration was measured in the samples. Extracts (250 µg of protein per tube) were recovered on avidin-sepharose beads (50 µL) (NeutrAvidin, Pierce); beads were incubated for 30 min at 4°C in binding buffer (1% NP40, 50 mM Hepes, pH 7.6, 140 mM NaCl). After washing with binding buffer, complexes were eluted in SDS sample buffer, separated on SDS-PAGE (8%), and subjected to immunoblotting using anti-STAT1 or anti-STAT3 antibodies (Cell Signaling) and processed as above.

Immunocytochemistry
Cells were grown at 50-60% confluence in 8-well plates (Lab-Tek, Nunc, Rochester, USA) to a density of 105 cells/ml. Cells were transfected with fluorescein-labelled hpdODNs, incubated (6 to 16 h), washed in PBS, fixed with 3.7% formaldehyde for 15 min, permeabilized in 0.1% Triton X-100 for 15 min and incubated in 5% FCS-0.1% Tween- PBS for 1 h. Cells were stained with anti-STAT3 or anti-STAT1 antibody (Cell Signaling) (1:100) for 2 h, then stained with an Alexa fluor 546-labeled secondary antibody (Invitrogen) (1:200) for 90 min. Cells, counterstained with 4',6-diamidino-2-phenylindole (DAPI), were mounted onto glass slides with Vectashield (Vectorlabs, Clinsciences, Montrouge, France). Fluorescence images were acquired using a Zeiss Axioplan 2 Deconvolution microscope (Carl Zeiss, Le Pecq, France) and analyzed with Metafer4 (Metasystems, Altussheim, Germany).

Additional material

Acknowledgements
We thank Martin Zacharias (Technical University Munich, Germany) for critical advice and fruitful discussions at the onset of this study. IS was supported by a one-year (2nd PhD year) grant from the Association pour la Recherche sur le Cancer (ARC). This work was funded in part by grants from OSEO (France), the Ligue nationale contre le cancer (France) and the Ligue départementale contre le cancer (comité du Val d’Oise).

Author details
1INSERM, Unité 978, Bobigny 93017, France. 2Université Paris 13, UFR SMBH, Bobigny 93017, France. 3AP-HP, Hôpital Avicenne, Service de biochimie, Bobigny 93009, France. 4AP-HP, Hôpital Avicenne, Service d’hématologie biologique, Bobigny 93009, France. 5Moscow State University, Moscow, Russia. 6CNRS UMR 7244, CSPBAT, Bobigny 93017, France. 7Université Paris 6, CNRS FRE 3207 ANBiop, Paris 75015, France.

Authors’ contributions
IS, PL, VM, SLC, JAHC and RF made substantial contributions to conception, design, and data acquisition, analysis and interpretation; NVB and FBM were involved in revising the manuscript for important intellectual content; and IS, PL, VM, JAHC, SLC, NVB, FBM and RF gave final approval of the version to be published. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

Received: 23 December 2011 Accepted: 16 March 2012
Published: 16 March 2012
47. Rouyez MC, Lestingi M, Charon M, Fichelson S, Buzyn A, Dusanter-Fourt I: IFN regulatory factor-2 cooperates with STAT1 to regulate transporter associated with antigen processing-1 promoter activity. J Immunol 2005, 174(7):3948-3958.

48. Chin YE, Kitagawa M, Kuida K, Flavell RA, Fu XY: Activation of the STAT signaling pathway can cause expression of caspase 1 and apoptosis. Mol Cell Biol 1997, 17(9):5328-5337.

49. Epling-Burnette PK, Zhong B, Bai F, Jiang K, Bailey RD, Garcia R, Jove R, Djeu JY, Loughran TP Jr, Wei S: Cooperative regulation of Mcl-1 by Janus kinase/stat and phosphatidylinositol 3-kinase contribute to granulocyte-macrophage colony-stimulating factor-delayed apoptosis in human neutrophils. J Immunol 2001, 166(12):7486-7495.

50. Geller J, Petak I, Szucs KS, Nagy K, Tillman DM, Houghton JA: Interferon-gamma-induced sensitization of colon carcinomas to ZD9331 targets caspases, downstream of Fas, independent of mitochondrial signaling and the inhibitor of apoptosis survivin. Clin Cancer Res 2003, 9(17):6504-6515.

51. Geller JI, Szekely-Szucs K, Petak I, Doyle B, Houghton JA: P21Cip1 is a critical mediator of the cytotoxic action of thymidylate synthase inhibitors in colorectal carcinoma cells. Cancer Res 2004, 64(17):6306-6313.

52. Hizver J, Rozenberg H, Frolow F, Rabinovich D, Shakked Z: DNA bending by an adenine-thymine tract and its role in gene regulation. Proc Natl Acad Sci USA 2001, 98(15):8490-8495.

53. Costa-Pereira AP, Tininini S, Strobl B, Alonzi T, Schlaak JF, Is 'harc H, Gesualdo I, Newman SJ, Kerr IM, Poli V: Mutational switch of an IL-6 response to an interferon-gamma-like response. Proc Natl Acad Sci USA 2002, 99(12):8043-8047.

54. Tanabe Y, Nishibori T, Su L, Arduini RM, Baker DP, David M: Cutting edge: role of STAT1, STAT3, and STAT5 in IFN-alpha beta responses in T lymphocytes. J Immunol 2005, 166(12):7486-7495.

55. Regis G, Pensa S, Boselli D, Novelli F, Poli V: Ups and downs: The STAT1:STAT3 seesaw of Interferon and gp130 receptor signalling. Semin Cell Dev Biol 2008, 19(4):351-359.

56. Santini GP, Cognet JA, Xu D, Singarapu KK, Herve du Penhoat C: Nucleic acid folding determined by mesoscale modeling and NMR spectroscopy: solution structure of d(GCGAAAGC). J Phys Chem B 2009, 113(19):6881-6893.

Cite this article as: Souissi et al: A STAT3-inhibitory hairpin decoy oligodeoxynucleotide discriminates between STAT1 and STAT3 and induces death in a human colon carcinoma cell line. Molecular Cancer 2012 11:12.