Targeting Stat3 in cancer therapy

Naijie Jing[a] and David J. Tweardy[a]

Stat3 is constitutively activated in many human cancers where it functions as a critical mediator of oncogenic signaling through transcriptional activation of genes encoding apoptosis inhibitors (e.g. Bcl-x_L, Mcl-1 and survivin), cell-cycle regulators (e.g. cyclin D1 and c-Myc) and inducers of angiogenesis (e.g. vascular endothelial growth factor). This article reviews several approaches that have been pursued for targeting Stat3 in cancer therapy including antisense strategies, tyrosine kinase inhibition, decoy phosphopeptides, decoy duplex oligonucleotides and G-quartet oligodeoxynucleotides (GQ-ODN). The GQ-ODN strategy is reviewed in somewhat greater detail than the others because it includes a novel system that effectively delivers drug into cells and tissues, addresses successfully the issue of specificity of targeting Stat3 versus Stat1, and has demonstrated efficacy *in vivo*. *Anti-Cancer Drugs* 16:601–607 © 2005 Lippincott Williams & Wilkins.

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[a]Department of Medicine and Cancer Center, Baylor College of Medicine, Houston, TX, USA.

Correspondence to N. Jing, Department of Medicine and Cancer Center, Baylor College of Medicine, One Baylor Plaza N520, Houston, TX 77030, USA.
Tel: +1 713 798-3685; fax: +1 713 798-8948;
e-mail: njing@bcm.tmc.edu

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Introduction

Signal transducer and activator of transcription (STAT) proteins were originally discovered as latent cytoplasmic transcription factors a decade ago [1]. There are seven known mammalian STAT proteins, Stat1, 2, 3, 4, 5a, 5b and 6, which are involved in cell proliferation, differentiation and apoptosis [2–13]. STAT proteins contain several domains: a tetramerization domain, a coil-coil domain, a DNA-binding domain, a linker domain, a Src homology (SH)2 domain, a critical tyrosine reside near the C-terminal end (position 705 in Stat3) and a C-terminal transactivation domain [14]. STAT proteins are activated in response to binding of a number of ligands to their cognate cell surface receptors, especially cytokines [e.g. interleukin (IL)-6] and growth factors (e.g. epidermal growth factor; Fig. 1). STAT proteins exist as monomers or N-terminal head-to-head dimers in the cytoplasm of non-stimulated cells [15]. Binding of cytokine or growth factor to its surface receptor results in receptor dimerization and transphosphorylation by receptor-intrinsic or receptor-associated tyrosine kinases. STAT proteins are recruited to specific phosphotyrosine residues within receptor complexes through their SH2 domain, become phosphorylated on the tyrosine residue within their C-terminus, and dimerize through reciprocal interactions between the SH2 domain of one monomer and the phosphorylated tyrosine of the other. The activated dimers translocate to the nucleus, where they bind to DNA-response elements in the promoters of target genes and activate specific gene expression programs [16–18]. The X-ray crystal structures of Stat1 and Stat3 homodimers bound to DNA have been reported, revealing many, but not all, details regarding dimerization and DNA binding [19,20].

Stat3 was originally termed acute-phase response factor (APRF) because it was first identified as a DNA-binding activity within IL-6-stimulated hepatocytes that was capable of selectively interacting with an enhancer element in the promoter of the acute-phase response genes [21–24]. The protein was first purified to homogeneity, and its cDNA isolated and sequenced from the livers of IL-6-treated mice [25]. Receptors linked to Stat3 activation include receptors for granulocyte colony-stimulating factor (G-CSF) and for the IL-6 cytokine family members, as well as other type I and type II cytokine receptors, receptor tyrosine kinases, and G-protein-coupled receptors [26].

Stat3 activation downstream of these receptors has been demonstrated in various *in vitro* systems to influence multiple cell fate decisions, including proliferation [27], differentiation [28–30] and apoptosis [6,31]. Targeted disruption of the mouse Stat3 gene leads to embryonic lethality at 6.5–7.5 days [10], indicating that Stat3 is essential for early embryonic development, possibly involved in gastrulation or visceral endoderm function [13]. Tissue-specific deletion of Stat3 has confirmed earlier *in vitro* results and demonstrated that Stat3 plays a crucial role in a variety of biological functions, including cell growth, suppression and induction of apoptosis, and cell motility [32].

Rational of targeting Stat3 for cancer therapy

Stat3 as a novel molecular target for cancer drug discovery has been well described previously [16–18]. Consideration of Stat3 as a target for anti-cancer drug design is based upon the findings that Stat3 is a critical mediator of oncogenic signaling. Stat3 was also shown to be

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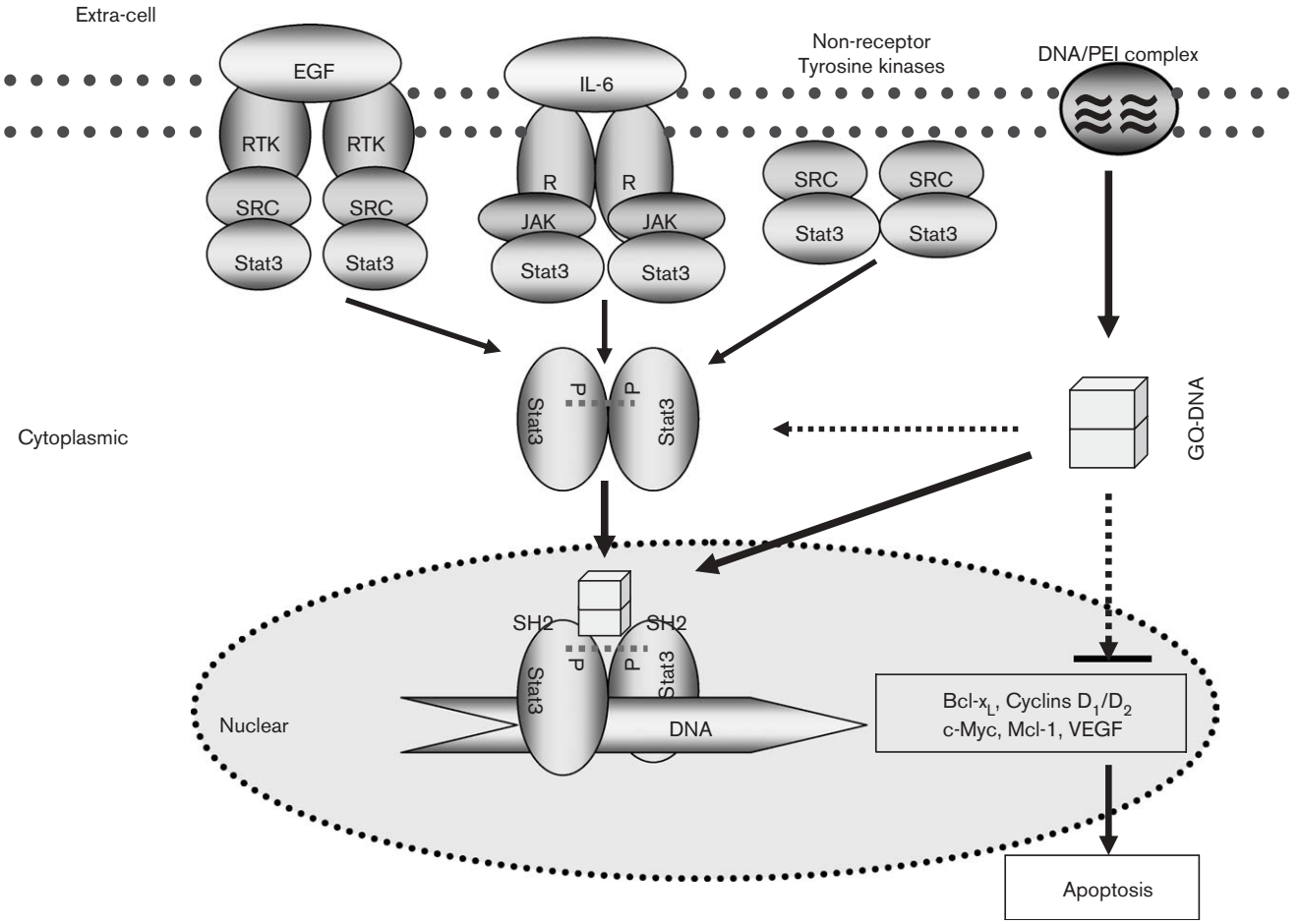
Fig. 1



Stat3 signaling pathway and its inhibition by GQ-ODN. Stat3 in the cytoplasm of unstimulated cells becomes activated by recruitment to phosphotyrosine motifs within complexes of growth factor receptors (e.g. EGFR), cytokine receptors (e.g. IL-6R) or cytoplasmic tyrosine kinases (e.g. Src) through their SH2 domain. Recruitment of Stat3 to these complexes juxtaposes them to activated tyrosine kinases, resulting in their phosphorylation on Y705. Tyrosine phosphorylation of Stat3 induces formation of tail-to-tail Stat3 dimers, which translocate to the nucleus, where they bind to DNA response elements within the promoters of target genes and activate transcription. Stat3 activation can be blocked at several stems in this pathway (see text for details). Shown here is one strategy—GQ-ODN. Using ODN/PEI complexes, GQ-ODN are delivered into the cytoplasm where the high K^+ concentration (140 mM) induces them to form G-quartets. G-quartets diffuse into the nucleus and interact with Stat3 SH2 domains. This interaction destabilizes Stat3 homodimers, interferes with expression of Stat3-regulated genes, such as Bcl-x_L and Mcl-1, and triggers apoptosis.

constitutively activated in fibroblasts transformed by oncoproteins such as v-Src [33,34] and to be essential for v-Src-mediated transformation [35,36]. Overexpression of a constitutively active form of Stat3 in immortalized rat or mouse fibroblasts induced their transformation and conferred the ability to form tumors in nude mice [37]. Stat3 is activated in many human cancers, including 82% of prostate cancers [38], 70% of breast cancers [39], more than 82% of squamous cell carcinoma of the head and neck [40], and 71% of nasopharyngeal carcinoma [41]. Stat3 participates in oncogenesis through up-regulation of genes encoding apoptosis inhibitors (Bcl-x_L, Mcl-1 and survivin), cell-cycle regulators (cyclin D_1 and c-Myc) and inducers of angiogenesis [vascular endothelial growth factor (VEGF)] [18]. Bcl-x_L is an anti-apoptotic protein within the Bcl-2 family that inhibits apoptosis by binding pro-apoptotic proteins and preventing cytochrome *c* release [42–44]. High levels of Bcl-x_L expression were associated with advanced disease and poor prognosis in several tumor systems [45]. Mcl-1 also represents a survival factor for human cancer cells [46,47]. Suppression of the expression of Bcl-x_L and Mcl-1 proved to be useful in cancer therapy by inducing cell apoptosis. Survivin is a protein that regulates both cell cycle and apoptosis, and has been show to be overexpressed in numerous human cancers [48]. Inhibition of Stat3 activity reduced the expression of survivin in primary effusion lymphoma-induced cell apoptosis [49]. Stat3 makes an essential contribution to the regulation of cyclin D_1 and c-Myc in v-Src transformation [50].

Overexpression of cyclin D_1 serves to drive oncogenesis through its ability to regulate cell-cycle progression [51]. The c-Myc protooncogene is overexpressed in Burkett's lymphoma, and in carcinomas such as breast and colon where it contributes to increased cellular proliferation and inhibition of differentiation [52–54]. Stat3 also induces VEGF expression, resulting in an increase in tumor angiogenesis [55]. VEGF has been suggested to play a crucial role in invasion and metastasis of human cancers such as ovarian carcinoma [56]. Thus, interrupting constitutive Stat3 signaling in tumor cells would be expected to downregulate expression of several important classes of oncogenic proteins.

Approaches for targeting Stat3 for cancer therapy

As outlined below, Stat3 can be targeted at one or more steps. Strategies to target Stat3 include decreasing Stat3 levels, reducing tyrosine phosphorylation of Stat3 and receptor complexes, reducing Stat3 recruitment to receptor complexes and its dimerization, and reducing Stat3 binding to promoters. While progress has been made pursuing each strategy, no anti-tumor drug whose main mode of action is targeting of Stat3 has yet to reach the clinic.

Targeting Stat3 to reduce protein levels within cells

Antisense oligodeoxynucleotide (ODN) and plasmids

Introduction of Stat3 antisense ODN into cells to cause the selective degradation of Stat3 mRNA has been shown to inhibit tumor growth in cells from prostate carcinoma [38] and LGL leukemia [57]. In squamous cell carcinoma of the head and neck, injection of a Stat3 antisense plasmid (complexed with liposomes) into tumors in a mouse xenograft model resulted in decreased constitutive Stat3 activity, decreased Bcl-x_L levels and increased apoptosis, compared to tumors treated with liposomes alone [58].

Targeting phosphorylation of the receptor complex and Stat3: tyrosine kinase inhibition

AG-490

AG-490 is a tyrosine kinase inhibitor that exhibits specifically for JAK2. Systemic administration of AG-490 in SCID mice demonstrated that AG-490 selectively blocked leukemic cell growth *in vitro* and *in vivo* by inducing apoptosis [59]. AG-490 also reduced Stat3 DNA-binding activity, resulting in inhibition of U266 myeloma cell and mycosis fungoides tumor cell growth [60]. Administration of AG-490 reduced levels of activated Stat3, causes transient regression of murine myeloma/plasmacytoma tumors and induced apoptosis of tumor cells *in vivo*. Also, combination therapy with AG-490 and IL-12 induced greater anti-tumor effects than either agent alone [61].

JSI-124 (Cucurbitacin I)

JSI-124 is a JAK/Stat3 inhibitor identified within the NCI Diversity Set [62]. It is a natural product isolated from plants within the Cucurbitaceae and Cruciferae families, and has been used as a folk remedy in China and India for centuries. JSI-124 is a selective inhibitor of members of the JAK family. JSI-124 was reported to reduce the levels of constitutively activated Stat3 in many cancer cells, and to reduce Stat3 DNA-binding activity and Stat3-mediated gene transcription. JSI-124 administration inhibited growth of nude mice tumor xenografts including lung adenocarcinoma (A549) and breast cancer (MDA-MB-468) in which Stat3 is constitutively active, and significantly increased the survival duration of these mice [62].

Targeting Stat3 SH2 interactions to inhibit Stat3 recruitment to activated receptor complexes and Stat3 dimerization: phosphopeptide ligand-based approaches

Strategies to target Stat3 by identifying phosphopeptide inhibitors of Stat3 SH2 binding to phosphotyrosine ligands have been pursued by several groups including our own [63–66]. We demonstrated that a phosphododecapeptide (PDP) based on the sequence surrounding Y1068 within the EGFR was able to directly bind non-phosphorylated Stat3, and to inhibit DNA binding of phosphorylated and activated Stat3 *in vitro* [65]. In addition, we demonstrated that this PDP inhibited ligand-stimulated Stat3 activation and transforming growth factor (TGF)-α/EGFR-mediated autocrine growth when delivered into cancer cells [65]. More recently, we investigated the structural basis for the unique specificity of Stat3 SH2 for YXXQ-containing phosphopeptides [68]. We tested three models of this interaction based on computational analysis of available structures and sequence alignments—two of which assumed an extended peptide configuration and one in which the peptide had a β turn. Using peptide immunoblot affinity assays and mirror resonance affinity analysis, we demonstrated that only phosphotyrosine (pY) peptides containing +3 Q (not L, M. E or R) bound to wild-type Stat3. Examination of a series of wild-type and mutant Stat3 proteins demonstrated loss of binding to pYXXQ-containing peptides only in Stat3 mutated at K589 or R607, whose side-chains interact with the pY residue, and Stat3 mutated at E638, whose amide hydrogen bonds with oxygen within the +3 Q side-chain when the peptide ligand assumes a β turn. These findings support a model for Stat3 SH2 interactions that could form the basis for anti-cancer drugs that specifically target Stat3.

Turkson *et al.* [63] demonstrated that pY peptides based on the sequence PYLKTK surrounding Y705 within Stat3 inhibited Stat3 DNA binding and pulled down Stat3 from lysates of unstimulated cells. Furthermore, addition of a membrane translocation sequence to the C-terminus of PYLKTK revealed the ability of this peptide to inhibit Stat3 activation within cells and suppress v-Src-mediated transformation. Alanine-scanning mutagenesis of the peptide mapped the essential sequence for inhibition of

Stat3 DNA binding to the tripeptide sequence PpYL; the tripeptide demonstrated potency in inhibiting Stat3 DNA binding equivalent to full-length peptide.

Ren *et al.* [64] examined a series of pY-containing peptides shown to bind Stat3 for the ability to inhibit DNA binding of recombinant Stat3. The peptide sequences were located within Stat3 itself or within receptors shown to activate Stat3 (IL-6R, LIFR, EGFR, mIL-10R and G-CSFR). These studies revealed a 1000-fold range of activity and that pY peptide based on Gp130 Y904 (pYLPQTV) demonstrated the greatest activity ($IC_{50} = 0.15\,\mu M$). The primary mode of inhibition of Stat3 DNA binding by pYLPQTV is likely destabilization of homodimers through competition with the Y705 region for binding to Stat3 SH2. Wiederkerhr-Adams *et al.* performed phosphopeptide library screening with recombinant Stat3 followed by confirmation of selected peptides using an indirect surface plasmon resonance inhibition assay [66]. These studies revealed that Stat3 binds peptides with the core motif pY-basic/hydrophobic-P/basic-Q. Neither the Ren *et al.* nor the Wiederkerhr-Adams *et al.* studies have been extended to demonstrate efficacy of the phosphopeptide ligands identified against tumors *in vitro* or *in vivo.*

Targeting Stat3 SH2 interactions to inhibit Stat3 recruitment to activated receptor complexes and dimerization: G-quartet oligonucleotides (GQ-ODN)

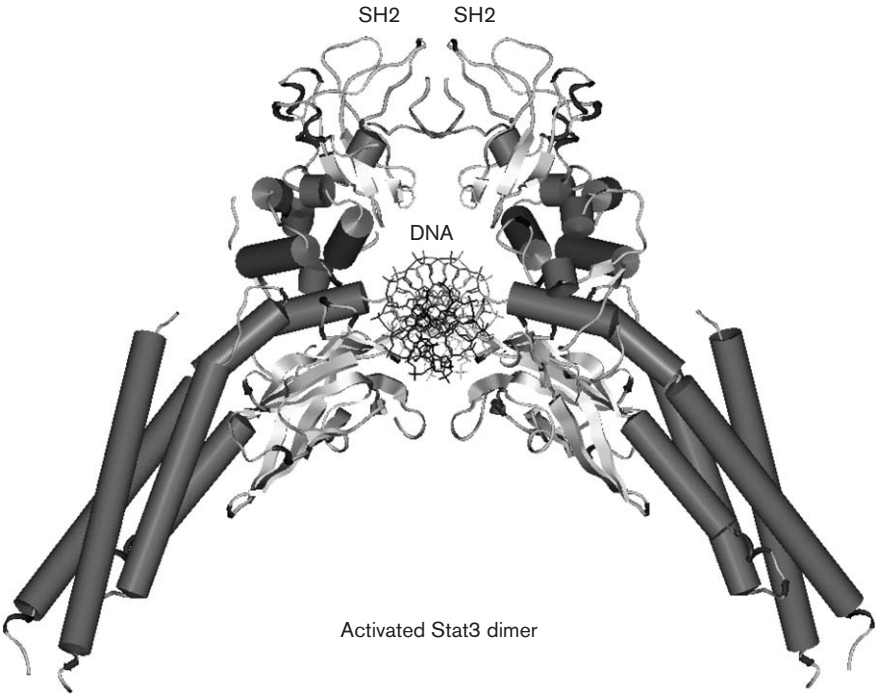
Recently, we developed GQ-ODN to be potent inhibitors of Stat3 activity that result in the decreased expression of Stat3-regulated genes, *bcl-x* and Mcl-1, in cancer cells [68]. GQ-ODN can directly interact with their target molecule, functioning in a manner similar to small molecule inhibitors. The size of the GQ-ODN (15 Å length and width) makes it possible for them to occupy sites within the SH2 domains of Stat3 homodimers. Docking computations resulted in a model in which GQ-ODN insert between the two groups of N643–Q648 amino acids of the Stat3 homodimer resulting in dimer destabilization. When administered intravenously using a novel drug-delivery system, the GQ-ODN T40214 and T40231 dramatically inhibited the growth of prostate, breast and other tumor xenografts in nude mice. Biochemical examination of tumors from mice treated with GQ-ODN demonstrated a significant decrease in expression of anti-apoptotic proteins, Bcl-2 and Bcl-x_L, and a markedly increase in apoptosis of tumor cells [69]. The G-quartet structure prevents single-strand endonucleases from accessing their cleavage sites, leading to a long oligonucleotide half-life *in vivo*.

Review of the structure of Stat3β dimer bound to duplex DNA (Fig. 2) [19] reveals several sites as possible targets for drug design, including the DNA-binding site (discussed below), the site of binding of the phosphotyrosine residue to the SH2 domain, and the region between N643 and Q648 that interacts within the Stat3 dimers. The approach we employed to target the N643–Q648 interaction site included few critical steps. The first step was to establish that this region was the primary binding site for GQ-ODN. For this purpose, GRAMM docking was employed. The structure of the GQ-ODN lead compound, T40214, was randomly docked 1000 times onto the structure of the Stat3 dimer without setting any constraints. A histogram of hydrogen bonds formed between T40214 and Stat3 was plotted as a function of each amino acid residue within Stat3. The results showed that 35% of total hydrogen bonds were distributed within the residues from 638 to 650; this region was considered to be the primary binding site for GQ-ODN. A series of modified GQ-ODN was designed and synthesized. Their binding to this site was determined by GRAMM docking analysis; their IC_{50} for inhibition of Stat3 activation was determined after intracellular delivery using electrophoretic mobility shift assay (EMSA). Combining the docking data with IC_{50}s, a linear correlation was observed between the percentages of GQ-ODN binding within the primary binding site of Stat3 and the IC_{50} of inhibition of Stat3 activation within cells. Based upon this correlation, a structure–activity relationship (SAR) between GQ-ODN and inhibition of Stat3 activity was established. The SAR indicates that the higher the percentage of GQ-ODN interacting within the binding site of Stat3, the greater its ability to inhibit Stat3 activity. Therefore, the percentage of hydrogen bond distribution within the primary binding site of Stat3 dimer provides critical information to evaluate if the designed inhibitor should undergo testing. Moreover, several specific SARs were determined by this analysis, including (i) the loop structure of GQ-ODN is a key factor that can be modified to improve drug activity, (ii) an increase in the length of the G-quartet stem causes a decrease in drug activity and (iii) substitution of residue T with C in the top-loop domain enhances drug activity.

Specifically targeting Stat3 among other STAT protein members is highly desirable since other STAT proteins, notably Stat1, which is activated along with Stat3 in many tumor systems, have potent pro-apoptotic function [70,71]. EMSA data demonstrated clearly that GQ-ODN T40214 strongly inhibited the activation of Stat3, but not Stat1 [70]. The mechanism of selectively targeting Stat3 was predicted based on the results of randomly docking GQ-ODN T40214 onto the crystal structures of both Stat3β and Stat1 homodimer 2000 times without setting any binding restrictions [68]. The results showed that the interactions between GQ-ODN and Stat3 were concentrated within the site of amino acids 638–650 of Stat3. In contrast, the interactions between GQ-ODN and Stat1 were spread out over the whole Stat1 structure.

Developing an effective delivery system is a critical step in all strategies designed to target Stat3. GQ-ODN with a large molecular size cannot directly penetrate through cell membranes. The principal difficulty of delivering

Fig. 2



Molecular structure of the Stat3β dimer binding with DNA duplex (modified from Becker *et al.* [19]). The dark cylinders and light sheets represent α-helixes and β-sheets, respectively. The DNA duplex is located near the center of the Stat3 dimer.

GQ-ODN into cells arises from the physical and structure properties of GQ-ODN [72]. G-quartet structure has a specific affinity for monovalent cations and the formation of the G-quartet structure strongly depends on the presence of potassium. Based upon the mechanism of potassium ions inducing the formation of G-quartet structure, a novel delivery system for GQ-ODN has been developed to increase the drug activity in cells and *in vivo* [72]. Polyethylenimine (PEI) is used as a vehicle to deliver GQ-ODN into cells. GQ-ODN are heat-denatured and cooled to room temperature at low K^+ concentration to maintain their random coil structure and increase their incorporation with vehicles. Once delivered into cells where the K^+ concentration is high, they form G-quartet structures and then G-quartets penetrate into the nucleus through nuclear pores [69]. This delivery system greatly increases the drug activity of GQ-ODN within cells. Intravenous administration of a single dose of fluorescein-labeled GQ-ODN T40214 plus PEI as vehicle to nude mice with human tumor xenografts resulted in uptake of GQ-ODN by tumors and normal tissues that persisted for 72 h.

Targeting Stat3 binding to promoters: decoy duplex oligonucleotides

Treatment of head and neck cancer cells *in vitro* with the Stat3 decoy inhibited proliferation and Stat3-mediated gene expression, but did not decrease the proliferation of normal oral keratinocytes [73]. The Stat3 decoy was composed of a 15mer double-stranded oligonucleotide based upon the Stat3 response element within the c-fos promoter. The Stat3 decoy bound specifically to activated Stat3 and blocked binding of Stat3 to a radiolabeled Stat3 binding element. While successful use of this agent has not been reported in nude mouse tumor xenografts, application of Stat3 decoy oligonucleotides to the skin inhibited the onset and reversed established psoriatic lesions in the K5. Stat3C mouse model of psoriasis recently developed that linked Stat3 activation within activated keratinocytes and immonocytes to development of this disease [74].

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