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REVIEW ARTICLE

CANCER THERAPEUTICS: SMART AND SMARTER

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ABSTRACT

Conventional cancer therapeutics target rapidly proliferating cells by introducing widespread damage into DNA, but do so nonselectively, damaging normal as well as malignant cells. As a result, maximally tolerated doses are often not curative and the treatment regimens are often highly toxic. In recent years targeted "smart therapies" have been developed to provide selective tumor cell killing and reduced toxicity, with striking success in a small number of cases. This approach has been constrained, however, by the difficulty of identifying molecular targets unique to malignant cells, the small number of tumor types targeted by a given drug, and the tendency of malignant cells to escape through further changes in the targeted protein or the development of alternative signaling pathways in the tumor cells. These limitations, combined with the high cost of drug development, seem likely to reduce the eventual impact of such therapies, despite the excitement they have generated in the scientific and pharmaceutical communities. A conceptually more powerful approach would be to target an innate DNA damage response pathway, common to all cell types, activated by dysregulated cell growth to selectively kill malignant cells. An inducible DNA damage response first identified in bacteria and termed the SOS response appears to provide this opportunity. In mammalian cells, a telomere-based mechanism functions to protect normal cells against repeated exposures to DNA-damaging agents and to eliminate damaged cells, but is ineffective in malignant cells. Telomere homolog oligonucleotides, termed T-oligos, appear to mimic single-stranded telomeric DNA, interact at telomeres with the Werner protein and initiate signaling through the ATM and ATR kinases. The p53 tumor suppressor protein and many other DNA damage response proteins are then activated and/or upregulated, leading in normal cells to a variety of protective responses, but in malignant cells to apoptosis or senescence, depending on cell type. Surviving cells undergo differentiation, lose angiogenic capacity and increase their susceptibility to low-dose conventional chemotherapeutics. Overall, at least in mouse models, there is a broad and coordinated response that greatly reduces tumor burden without detectable adverse effects on normal cells and tissues. These preclinical observations suggest the possibility of a next generation of smarter therapies that rely on an SOS-like response, evolutionarily perfected to protect higher organisms from malignancy.

PAST AND PRESENT CANCER TREATMENT

Conventional cancer therapeutics target rapidly proliferating cells, usually by introducing damage into DNA (1-3). Such agents have dramatically increased survival for certain malignancies and remain the cornerstone of cancer therapy more than 50 years after their introduction to the clinic. Nevertheless, their very mechanism of action renders them highly toxic for proliferating normal cells and often results in life-threatening bone marrow suppression, as well as mucositis and hair loss. Nonproliferating tissues are also damaged and, depending on the specific agent(s) used, patients may experience disabling peripheral neuropathies, "chemo-brain", cardiomyopathy, hepatitis and numerous other organ-specific toxicities (1, 4). Perhaps most cruelly, patients who survive have a high risk of second, and even third and fourth, malignancies (5-7), the consequence of their treatment with DNA-targeting mutagenic oncological drugs.

Since the approval of imatinib mesilate (Gleevec®) for the treatment of chronic myeloid leukemia (CML) in 2001, targeted "smart therapies" have become the goal of many oncology drug development programs. By selectively inhibiting the constitutively active Abelson (ABL) cytoplasmic tyrosine kinase, created by the chromosome translocation 9;22 to form the signature Philadelphia chromosome, imatinib restores normal proliferation to the affected cells, returning peripheral white blood cell counts to normal and delaying (but ultimately not preventing) progression to lymphoblastic crisis and patient death (1, 8). The specificity of the drug for the ABL tyrosine kinase (and less so for two other tyrosine kinases, SCFR, or c-kit, and the platelet-derived growth factor receptor, or PDGF-R) and the

uniqueness of the mutated kinase for CML cells versus normal proliferating cells provide an excellent therapeutic index. Hence, imatinib is not only efficacious but, like other targeted therapies (9), produces far fewer adverse effects than conventional chemotherapeutics (1).

Because the molecular abnormalities underlying malignancy have been extensively elucidated in recent years, many potential therapeutic targets now exist. These include aberrant growth factor receptors, kinases in the intracellular signaling pathways involved in cell growth, and proteins that regulate programmed cell death or apoptosis, as well as proangiogenic factors (1, 10-13). Recently approved "smart therapies" and their targeted proteins are listed in Table I, and many others are currently in clinical trials (9-13). However, while they are clearly promising, early experience with this new class of oncology drugs has suggested that their utility may be limited by rapid tumor escape through further mutation of the targeted protein, rendering it insensitive to the inhibitor (14), and/or utilization of redundant new signaling pathways by the tumor cells, presumably through selection for rare cells harboring additional, untargeted mutations (11-13, 15, 16). In addition, these drugs are far from lacking in side effects (9). A final disadvantage of "smart therapies" is that they tend to target only one malignant cell type or, more often, only a subset of malignancies of that cell type that rely on the targeted protein for their growth advantage (11-13). Clinically identical malignancies with a different profile of mutated or dysregulated genes may be completely unaffected. High development costs and the anticipated limited market for each have led to extremely high pricing for at least the initial smart drugs and may ultimately limit the impact of these agents even beyond their intrinsic constraints.

In the U.S., at present, cancer is newly diagnosed in more than 1.4 million people and kills more than 565,000 annually, incidence and mortality rates that have not improved overall since the War on Cancer was declared in the 1960s. The shortcomings, conceptual and practical, of current cancer therapies are all too apparent and there is broad consensus that fundamentally new approaches are needed (17, 18).

NATURE'S APPROACH TO GENOME PROTECTION: THE SOS RESPONSE

The need for organisms to protect their genomic integrity has existed from the beginning of life on earth. Maintenance of at least most protein functions is required for the survival of each unicellular organism and the integrated, balanced function of millions of cells is required for the survival of higher organisms. Among the possible catastrophes in higher organisms is the dysregulated growth and aberrant function of a subset of cells, termed cancer.

In bacteria, nature provided not only a constitutive DNA repair capacity but also an inducible repair capacity, termed the SOS response (19, 20). When bacterial DNA is damaged, for example by ultraviolet (UV) irradiation, damaged DNA can act as a block to replication, generating long regions of single-stranded (ss) DNA at stalled replication forks. This ssDNA binds and activates the RecA protease that then cleaves LexA, a gene repressor protein (21). Loss of LexA shifts the dynamic equilibrium between promoter-bound and -unbound cleaved repressor, leading to a derepression of approximately 20 LexA-regulated genes, including DNA repair enzymes (22-24). As a result, soon after the DNA damage occurs and for several hours thereafter, if additional DNA damage is incurred, it is repaired more rapidly and a higher proportion of the bacteria survive. Of note, the induced repair enzymes include two, the DNA polymerases UmuC and UmuD, known to have low fidelity, and hence the SOS repair is more error-prone than constitutive repair (25, 26). This fact can be interpreted as advantageous or at least not disadvantageous to a single-celled organism with high reproductive capacity in a presumptively hostile (DNA-damaging) environment, facilitating mutations that might confer a selective advantage.

Table I. *Representative FDA-approved smart therapies, their molecular targets and clinical indications.*

Drug	Drug class	Molecular target(s)	Indication(s)
Bevacizumab (Avastin)	mAb	VEGF	Colorectal cancer
Sorafenib (Nexavar)	TKI	VEGF	Renal cell cancer
Sunitinib (Sutent)	TKI	VEGF	Renal cell cancer
Cetuximab (Erbitux)	mAb	EGFR	Colorectal cancer
Trastuzumab (Herceptin)	mAb	HER2	Breast cancer
Rituximab (Rituxan)	mAb	CD20	B-cell non-Hodgkin's lymphoma
Gemtuzumab ozogamicin	Immunotoxin	CD33	Acute myelogenous leukemia
Imatinib (Gleevec)	TKI	BCR/ABL, PDGF-R, c-kit	Chronic myeloid leukemia, Gastrointestinal stromal tumors
Erlotinib (Tarceva)	TKI	EGFR	Non-small cell lung cancer, Pancreatic cancer
Gefitinib (Iressa)	TKI	EGFR	Non-small cell lung cancer
Bortezomib	Proteosome inhibitor	Proteosome	Multiple myeloma

Adapted principally from Refs. 12, 13, 15. mAb, monoclonal antibody; TKI, tyrosine kinase inhibitor; VEGF, vascular endothelial growth factor; EGFR, epidermal growth factor receptor.

EVIDENCE FOR A TELOMERE-BASED MAMMALIAN SOS RESPONSE

For more than 20 years after the discovery of the SOS response, it was widely thought to be restricted to bacteria. However, multiple discrete observations beginning in the 1960s suggested that mammalian cells, after exposure to DNA damage of various kinds, subsequently manifested enhanced DNA repair capacity (27, 28). In 1997, on the basis of these apparently disparate observations and our own demonstration of inducible repair capacity in both keratinocytes and fibroblasts (29), we suggested and then demonstrated that mammals, including humans, also increased their DNA repair capacity in response to acute DNA damage (29, 30). This SOS-like response, mediated at least in part by the transcription factor and tumor suppressor protein p53 (29), is substantial in magnitude: the level of many DNA repair enzymes increases 2- to 3-fold within 24 h (30) and the repair rate for new DNA damage doubles during this time (29, 31). In contrast to the error-prone inducible DNA repair in bacteria, however, the mammalian SOS response is not associated with increased mutation frequency (32, 33). Furthermore, unlike the bacterial response, the mammalian SOS response is extremely complex, involving an interplay of numerous growth and differentiation pathways that together provide a remarkably coordinated anticancer defense. Many of these responses, or at least their endpoints of apoptosis and senescence, are lost in malignant cells but can be restored by provision of the natural initiating signal, as described below.

Work undertaken in our laboratory to understand tanning, the pathway leading from UV exposure to increased photoprotective epidermal melanin pigment several days later, identified thymidine dinucleotides (abbreviated pTT) as capable of increasing melanogenesis (pigment production) in the absence of UV exposure (34). Thymidine dinucleotides are the obligate substrate for thymine dimers, the UV photoproduct that accounts for up to 75-80% of UV-induced DNA damage (35). We observed that pTT, when provided to cultured pigment cells or topically applied to rodent skin, increased the level and activity of tyrosinase, the rate-limiting enzyme in melanogenesis, and led to increased epidermal melanin content with the formation of nuclear "caps", biochemically and histologically mimicking UV-induced tanning (34, 36). Tanning is now known to be mediated in large part by p53 (37-39), consistent with the long-presumed evolutionary role of tanning as a DNA-protective response in skin. Work with cultured pigment cells demonstrated that pTT also increases the binding of α-melanocyte-stimulating hormone to the melanocortin MC_1 receptor (40), another event expected to enhance melanogenesis (41-43). Moreover, in cultured keratinocytes pTT upregulates the superoxide dismutases (SOD) 1 and 2 (44), as also observed after UV irradiation (45), and increases cell survival in the face of oxidative stress, protective effects also found to be p53-regulated (44). Finally, pTT treatment of cultured human cells, human skin explants and intact rodent skin also leads to a marked upregulation and/or activation of numerous DNA repair enzymes and DNA damage response proteins, as well as to enhanced rate of repair for subsequently experienced DNA damage (30, 31, 36). Thus, mammalian cells and tissues are clearly capable of inducing a protective response after DNA damage that is functionally analogous to the bacterial SOS response, but more complex and multifaceted (46). Moreover, it can be induced in the absence of initial DNA damage (47-49) by providing cells with the presumptive damage signal, a DNA oligonucleotide.

pTT is not unique among oligonucleotides in its ability to upregulate and activate p53 and stimulate protective SOS-like responses. Many, but not all, oligonucleotides induce similar effects (50), and homology to the TTAGGG telomere repeat sequence is a critical feature (51) Complementary or unrelated sequences are completely ineffective, although like telomere homolog oligonucleotides (termed T-oligos) they rapidly concentrate in the nucleus of cultured cells (51, 52). Fully telomere homologous 11- to 20-base oligos demonstrate a far greater molar efficacy than pTT in stimulating SOS-like responses (53).

WHY BASE THE MAMMALIAN SOS RESPONSE IN THE TELOMERE?

Telomeres, tandem repeats of TTAGGG and its complement in all mammalian cells, cap the ends of chromosomes (54). In humans, telomeres comprise approximately 8,000-10,000 base pairs and have a single-stranded 3'-overhang of TTAGGG repeats estimated to be 100-400 bases in length (55, 56). Except at times of telomere replication, the telomere forms a large loop, held in place by insertion of the overhang into the proximal duplex DNA (57). Experimental disruption of the loop structure by sequestration of the telomere-binding protein TRF2 causes loss of the 3'-overhang and activation of the ATM kinase, which in turn activates p53 and leads to cellular senescence or apoptosis, depending on cell type (58). Experimental removal of the TTAGGG-binding protection of telomeres protein 1 (POT1) causes similar responses, but mediated through ATR rather than ATM (59, 60). We have suggested that exposure of TTAGGG repeats, followed by interaction of this sequence with a nuclear sensor protein, might be a mechanism by which critical telomere shortening induces senescence after multiple rounds of cell division; and that acute DNA damage might similarly expose TTAGGG repeats either by telomere loop disruption or, less dramatically, by separation of the strands in the duplex DNA by introduction of base damage or by influx of proteins to repair the damage (38, 49).

It has been observed that DNA-damaging agents, including but not restricted to UV irradiation, introduce damage throughout the genome, but proportionately more damage into telomeres (61). This is the logical consequence of the telomere sequence TTAGGG. Dithymidines (one-third of the repeat sequence) are the preferred substrate for UV-induced damage (35, 62, 63) and guanine (one-half of the repeat sequence) is the preferred substrate for oxidative damage, while damage due to chemical carcinogens targets either guanine or adjacent adenine–guanine bases (64-67). We therefore suggest that exposure to DNA-damaging agents would logically result in relatively low threshold signaling through such a telomere-based pathway, leading to proliferative senescence or apoptosis if extensive, or to SOS-like responses if more limited, all of which reduce the risk of cancer development and generally safeguard genomic integrity. The highly conserved TTAGGG repeat sequence, present in all mammalian species (68, 69), thus appears to be an inspired choice on nature's part for assuring prompt induction of protective responses at times of environmental challenge.

MOLECULAR MECHANISM OF TELOMERE SEQUENCE-INITIATED SIGNALING

Over the past eight years, the signaling pathways through which T-oligos induce SOS-like responses in mammalian cells have been substantially elucidated (Fig. 1). It is presumed that T-oligos mimic and exaggerate responses normally induced by exposure of endogenous guanine (G)-rich telomere sequences. These presumptive and far more subtle innate signaling events have yet to be experimentally demonstrated, but it is interesting to note that telomeres were recently demonstrated to elicit DNA damage responses at times of replication (70, 71), consistent with a role for single-stranded TTAGGG-specific DNA in these responses. The molecular cascade initiated by T-oligos in normal and malignant cells has come into focus gradually, with certain events still hypothetical, as indicated below. Nevertheless, strong parallels to the bacterial SOS response are apparent (Fig. 2). In the following section we will review preclinical data suggesting that this chain of events, presumptively initiated by acute DNA damage or critical telomere shortening at the time of replicative senescence, can be harnessed as an evolutionarily perfected anticancer mechanism for clinical application using T-oligos.

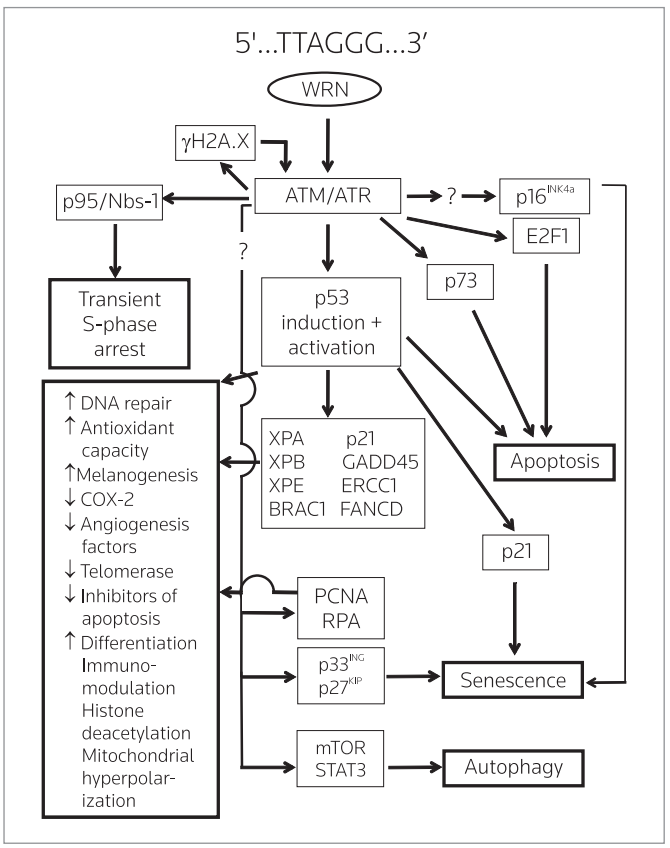

Figure 1. T-oligo mechanism of action. T-oligos rapidly concentrate in the nucleus and during S phase interact at telomeres with the Werner protein WRN (47). This leads to activation of the ATM (52, 130) and ATR (92) kinases (see Figs. 2 and 3 for a more detailed but not yet experimentally confirmed diagram of events). ATM then modifies the p95/Nbs-1 protein, causing an S-phase arrest (130). ATM also phosphorylates the histone variant protein H2A.X (131, 132), forming γH2A.X, which creates a scaffold for co-localizing multiple DNA damage response proteins that are then activated by ATM and/or ATR (133). Prominent among these is p53 (29, 30), which may then act at distant sites to transcriptionally regulate its many target proteins. p53 levels also increase (29, 30), presumably through stabilization of the protein. Additional proteins, not known to be p53-regulated, are also phosphorylated (activated) or upregulated either directly by ATM or ATR, or by an intermediate protein in the signaling cascade (30, 49, 51, 83). Simultaneous signaling through multiple overlapping pathways leads to apoptosis, senescence, autophagy or a variety of other cancer-preventive behaviors, with the dominant effects depending on cell type and the presence or absence of replicative stress.

T-oligos rapidly concentrate in the nucleus, as shown by FACS analysis and fluorescence microscopy using fluorescein-labeled oligos (47, 51, 52). Although the amount reaching the nucleus after supplementation of a culture with 20 μM of a 16-base T-oligo (a highly effective dose) is unknown, the following calculation suggests that it is likely very high in comparison to the amount of TTAGGG repeats present, for example, in the form of 100- to 400- (assumed average: 200) base 3′-overhangs on 46 chromosomes:

> 46 chromosomes/nucleus x two 3′-overhangs/chromosome x 200 bases/overhang = 18,400 bases/nucleus of telomeric TTAGGG repeats.
>
> 20 μM T-oligo = 20 μmol/L. Avagadro's number = 6.023×10^{23} molecules/mol. 20 μmol x 6.023×10^{23} molecules/mol ≈ 120×10^{17} molecules/L.
>
> Assume 16 bases/T-oligo: $16 \times 120 \times 10^{17}$ molecules/L = $19{,}200 \times 10^{16}$ bases/L of TTAGGG repeat sequences available for nuclear uptake.
>
> For 2 mL of medium (per 35 mm dish), 2 mL x $19{,}200 \times 10^{16}$ bases/L = $38{,}400 \times 10^{13}$ bases. Hence, the ratio of TTAGGG repeat sequences present in the medium as 16-mers to those in a cell's telomeric 3′-overhang DNA is [$38{,}400 \times 10^{13}$ bases] ÷ [18,400 bases/nucleus of telomeric TTAGGG repeats] ≈ 2×10^{13}.

Hence, to double the nuclear concentration of ss TTAGGG repeats, 1 in 2×10^{13} T-oligos present in the culture dish would need to reach the nucleus. To increase the concentration 1,000-fold, uptake of approximately 1 in 2×10^{10} T-oligo molecules would be required. The subsequent model therefore presumes a high concentration of T-oligos throughout the nucleus for a period of at least several hours and possibly for days, based on a calculated half-life of a 12-base T-oligo once it enters the nucleus (72), where it appears far more stable than in serum-containing medium (73).

As proliferating cells proceed through the S phase, telomeres, like the rest of the genome, must be replicated. This requires separation of the DNA strands that comprise the duplex chromosome, creating single-stranded TTAGGG repeats (Fig. 2). In vitro studies have shown that such G-rich DNA tends to form G-quadruplexes as a result of hydrostatic bonding among G-residues either within a single strand or between separate strands (Fig. 3) (74). These structures inhibit the replication of affected DNA and are believed to be resolved by the Werner protein (WRN) (75-77), mutated in the progeroid cancer-prone Werner syndrome (78). We propose that T-oligos (single-stranded oligonucleotides with TTAGGG or similar sequences) readily interact with the endogenous telomeric sequences to form G-quadruplexes (Fig. 3) that must then be resolved by the helicase and 3′→5′ exonuclease activities of WRN

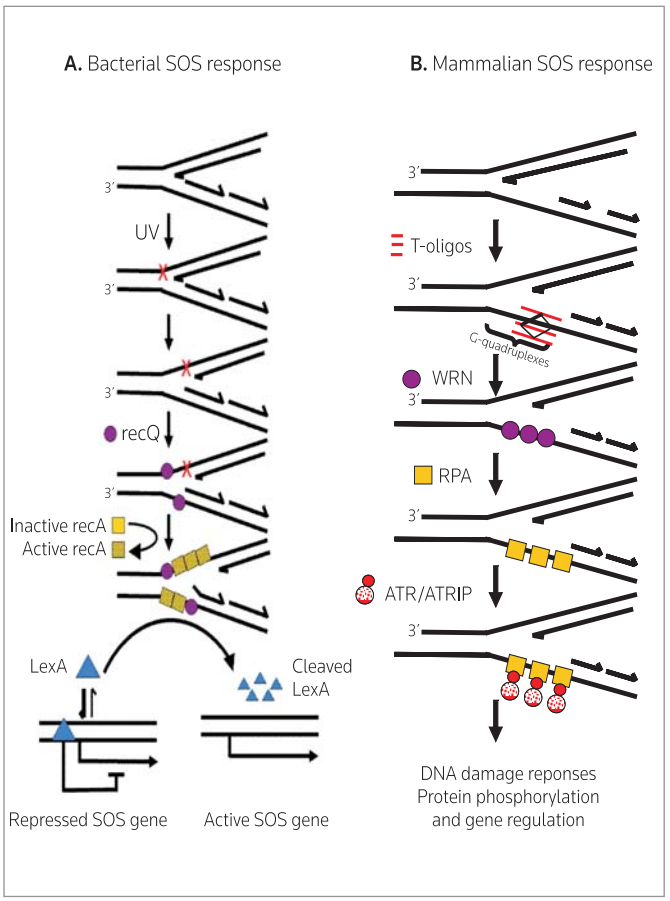

Figure 2. Comparison of the prokaryotic SOS response and the eukaryotic SOS-like response to replicative stress. (**A**) When prokaryotic cells undergoing DNA replication are damaged by genotoxic agents, DNA lesions (depicted as a red "X" in the template for leading strand synthesis) block the progression of the replicating polymerase and generate regions of single-stranded DNA between the lesion and the replication fork (134, 135). The RecQ helicase is then thought to bind to these regions, unwind the DNA helix in the 3′- to 5′-direction, and thus generate more single-stranded DNA (136). Additionally, it is thought that the RecQ helicase can also act on the lagging strand template and unwind Okazaki fragments, generating additional single-stranded DNA (136). The RecA protease domain is activated by binding to these regions of single-stranded DNA and active RecA then cleaves the LexA repressor (21), reducing its intranuclear concentration and hence shifting the equilibrium to increased expression of *LexA* target genes involved in the SOS response. (**B**) In mammalian cells, we hypothesize that as the replication fork moves into telomeric DNA even in the absence of local DNA damage, regions of single-stranded TTAGGG repeats form in the template for the lagging strand, before convergence and joining of the Okazaki fragments. This DNA would be likely to form G-quadruplex structures because of its high guanine content. If additional G-rich DNA, in the form of T-oligos, is present at this time, we propose that the formation of these structures would be exaggerated, forming extensive blocks to lagging strand DNA synthesis. Although these structures impede DNA polymerases and exclude the binding of single-stranded DNA-binding proteins such as RPA, they are a preferred substrate for the mammalian RecQ helicase WRN (75-77) and can be unwound and resolved by this helicase, allowing RPA to bind (87). RPA can then recruit the ATR/ATRIP complex, with subsequent activation of this kinase (88) and induction of DNA damage responses that lead to cell cycle arrest, chromatin modification and induction of genes involved in DNA repair (92-97).

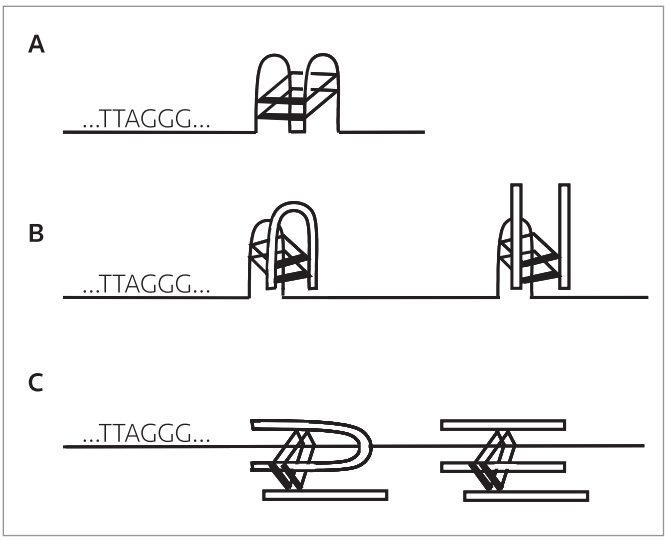

Figure 3. G-quadruplexes in telomeric DNA: spontaneous and T-oligo-mediated. (**A**) Single-stranded G-rich DNA, generated in the telomere at times of DNA replication or repair (see text) is believed to form G-quadruplexes spontaneously at sites where bends in the DNA strand place four guanines in proximity to each other, allowing hydrostatic bonds to form (74). These structures are understood to be resolved by WRN to allow DNA replication by appropriate polymerases (75-77). (**B**) When a high concentration of G-rich telomere homolog oligonucleotides (▭) are present in the vicinity of the telomere, we postulate that single bends in the telomere, stochastically far more probable than adjacent bends, may pair with one bent or two linear oligos to form G-quadruplexes that will also require resolution by WRN, thus exaggerating and/or prolonging WRN-initiated signaling (see Fig. 2). (**C**) Even more favorable stochastically, one S-shaped T-oligo (unlikely for oligos with 16 or fewer bases), one bent and one linear T-oligo, or three linear T-oligos may align themselves along a linear stretch of single-stranded telomeric DNA to form a G-quadruplex, with the same consequences as in (B). In each scenario, G-quadruplexes may "stack" if there are sufficient juxtaposed G residues on each strand, for example TTAGGG in the telomere sequence.

(79) before chromosome replication can resume and be completed (Fig. 2). This hypothesis is consistent with the observation that only 3′-hydrolyzable T-oligos initiate DNA damage-like signaling (47), despite the fact that T-oligos with non-WRN-degradable linkages also concentrate in the nucleus and can form G-quadruplexes (47, 80). More critically, we have shown that T-oligo signaling does not occur in cells containing mutated WRN or in otherwise responsive cells in which WRN is knocked down by small interfering RNA (siRNA) (47). Thus, the key events following T-oligo treatment are understood to be: 1) concentration of T-oligos in the nucleus; 2) formation of G-quadruplexes between transiently generated single-stranded G-rich telomeric DNA and nearby T-oligos during S phase; followed by 3) WRN-mediated G-quadruplex resolution, likely involving nucleolytic digestion of the T-oligos; and 4) generation of linear single-stranded telomeric DNA that can signal through ATM/ATR to induce DNA damage responses. This scenario is consistent with the recent report that DNA damage responses may occur following experimental co-localization of repair proteins at chromatin in the absence of actual DNA damage (81, 82). This postulated mechanism of action also provides a possible explanation for the striking selectivity of T-oligo-induced apoptosis, senescence or

autophagy for malignant versus normal proliferating cells (52, 83-85): malignant cells are known to have dysregulated DNA synthesis characterized by firing of origins of replication more than once per cell cycle (86), which is expected to greatly increase the replication stress generated by T-oligo treatment.

Research in numerous laboratories has delineated the molecular events that occur at sites of linear single-stranded DNA, and we hypothesize that these events occur at areas of just-resolved G-quadruplexes (Fig. 2). Replication protein A (RPA) rapidly attaches (87) and attracts the ATR kinase and ATR-interacting protein (ATRIP) (88). This complex in turn phosphorylates the histone variant protein H2A.X, forming γH2A.X (89), as well as multiple effector proteins, including ATM and p53 (90, 91). This creates an environment in which damage (if present) can be repaired and polymerases such as pol δ, present in T-oligo-induced foci (92) and known to cooperate with WRN, can bind the template and synthesize a complementary strand (93-95). Many of the activated proteins initially present within the γH2A.X foci may diffuse away, however, and function at distant sites (96). In the case of p53, for example, this may lead to transcriptional up- or downregulation of target genes or to proapoptotic signaling (97). Importantly, the large number of activated proteins leads to simultaneous signaling through multiple overlapping DNA damage response pathways. Because only a subset of these pathways is required to initiate apoptosis or other therapeutic effects, even malignant cells lacking expression of several major effector proteins may remain responsive to T-oligo treatment (52, 85).

Table II. *Malignant cell types demonstrated to be T-oligo-responsive.*

Cell type	Number of cell lines tested	Cellular response(s)*
Melanoma	10	A,S
Fibrosarcoma	1	S
Osteosarcoma	1	A,S
Breast carcinoma	8	A,S
Ovarian carcinoma	6	A
Squamous cell carcinoma (oral)	2	A
Cervical carcinoma	1	A
B-cell lymphoma	2	A
Leukemia	7	A
Prostate carcinoma	2	A
Lung carcinoma	8	A
Glioblastoma	6	Autophagy
Colon carcinoma	7	A

*A = apoptosis, S = senescence. Not all cell lines have been examined for both of these responses. However, all responses observed in one or more lines in the investigators' laboratory are listed as characterizing that cell type. Some cell lines show both responses, for example an 80% reduction in cell yields through apoptosis with senescence of the remaining viable cells. Autophagy was examined only in glioblastoma cells (83). For the 60 cell lines tested in the NCI-60 panel (84), only cell yields are reported, but many of the same lines were also tested in the investigators' laboratory. Data supporting the apoptotic or senescent response of the cell lines have been reported (51-53, 84, 85, 98), but most of the data on which the table entries are based are still unpublished. Note: All cell lines listed above are human. Several murine and canine cell lines have also been studied and are responsive, as anticipated from the conservation of the TTAGGG telomeric repeat sequence through all mammalian species, but these data are unpublished.

HARNESSING THE SOS RESPONSE: PRECLINICAL EXPERIENCE

Treatment of normal human cells and skin explants with T-oligos (a single dose added to culture medium at time 0) results in transient growth arrest, accompanied by enhanced DNA repair capacity and other protective responses (29-31, 36). Topical pTT treatment of mouse skin during a 6-7-month course of UV irradiation greatly reduces the development of squamous cell and basal cell carcinomas in genetically predisposed mice (32, 33). pTT also reduces the mutation frequency in a *lacZ* reporter transgene harvested from either cultured cells (48) or acutely or chronically irradiated skin (32) and reduces the number of tumor cell nests containing mutated p53 (33), a second indicator of decreased mutagenesis in the face of repeated DNA damage.

To determine the effect of T-oligos on already transformed cells, a large number of established malignant cell lines were treated with 11- to 16-base T-oligos (Table II). After a single supplementation at time 0, all cell lines showed growth arrest within 24 h and within 3-4 days underwent extensive apoptosis and/or senescence, depending on cell type (51-53, 84, 85, 98). Cells lacking functional p53, p16 or both pathways (52, 84, 85) frequently responded as well as those with less flagrant dysregulation and none of 14 types of malignant cells studied to date has proven refractory.

To examine the effect of T-oligos in vivo, mouse models of human melanoma (MM-AN) (85), breast carcinoma (MCF7, BT-20) (52) and glioblastoma (83) xenografts, as well as a syngeneic murine B-cell lymphoma model (84), were employed. In all instances T-oligos (11- or 16-base 100% telomere homologs) produce striking antitumor effects, leading to apoptosis, senescence and/or autophagy of the malignant cells (52, 83-85). These events occur selectively in the malignant cells both in vitro and in vivo, without detectable adverse effects in mice autopsied even after repeated systemic administration at doses sufficient to greatly reduce tumor burden (52, 85). In these models T-oligos are effective as monotherapy, but at one-tenth the dose also dramatically sensitize tumor cells to killing with comparably reduced doses of conventional chemotherapeutics (84). In the most extensively examined model, in which MM-AN human melanoma cells are injected into the flank or peritoneum of immunodeficient mice, tumor burden is reduced by approximately 90% in T-oligo-treated versus control mice and residual tumor nodules show ongoing apoptosis, as determined by TUNEL assay (85). Within 48-72 h T-oligo treatment also downregulates the inhibitor of apoptosis protein IAP/Livin to undetectable levels (85), deacetylates histone proteins (99), and reduces both mRNA and protein levels of multiple proangiogenic factors (100, 101). In addition, T-oligos increase the expression of multiple differentiation markers (e.g., tyrosinase, TRP-1, gp100 and MART-1 in the case of melanoma cells [85]), consistent with a more differentiated, less proliferative phenotype and increased vulnerability to immunotherapy or spontaneous host immune response to the tumor. In mouse skin and human skin explants, T-oligos also downregulate cyclooxygenase-2 (COX-2) (33, 102), a proinflammatory factor implicated in tumor promotion (103). The effect is observed within 24 h (102) and persists through 7 months of chronic UV irradiation in a mouse model of photocarcinogenesis (33).

POTENTIAL PROBLEMS WITH AN OLIGONUCLEOTIDE THERAPEUTIC

Despite the promise of various oligonucleotide-based therapies, to date few DNA or RNA therapeutics have entered clinical practice. In addition to the high cost of goods relative to conventional small molecules, delivery to target tissue(s), poor cellular uptake for double-stranded oligonucleotides, the requirement for sustained oligo levels and immune-mediated off-target effects have proven to be major obstacles (104, 105). It is anticipated that T-oligo therapy will share some but not all of these potential barriers to clinical utility.

RNA-interfering siRNAs (106, 107), small double-stranded RNA molecules that activate a sequence-specific ribonuclease or translational silencing mechanism (108), offer perhaps the closest parallel to T-oligos in that they rely on an innate protective cellular mechanism. However, unlike the situation with single-stranded DNA (109-112), cells have no uptake mechanism for double-stranded RNA, and siRNA therapeutics require a sophisticated carrier system to reach their intracellular targets (113). Moreover, they must be targeted to the cells of interest to avoid rapid clearance by the kidney and digestion by serum ribonucleases (113), as well as undesired effects on nonmalignant cells. In contrast, striking anticancer effects of T-oligos were observed in mouse models after i.v. or i.p. injection in saline alone, and marked reduction in tumor burden can be achieved without detectable toxicity in any organ system (52, 84, 85). Finally, siRNAs result in decreased levels of a single mRNA and protein, while T-oligos modify the levels and phosphorylation (activation) state of multiple gene products involved in growth and survival, increasing the probability that cancer cells will respond initially and throughout a sustained treatment course.

Oligonucleotides may also regulate gene expression by antisense and antigene mechanisms. Antisense DNAs interact with the targeted mRNA and prevent translation by one of several mechanisms. Specifically, the mRNA–DNA duplex can be a substrate for the endogenous enzyme RNase H that digests the RNA component of the duplex, leading to extensive mRNA degradation. Alternatively, binding of the antisense oligonucleotide to the complementary target mRNA can block sequences necessary for splicing or translation (104, 105). As antigene technology, the DNA oligonucleotide must enter the nucleus and form a triplex helix with the target gene to inhibit transcription (114). DNA oligonucleotides as antisense/antigene therapy present the same challenges of stability and targeting as siRNAs, but in addition, oligos containing the CpG motif can be immunostimulatory and can activate the NF-kappa-B pathway (115), leading to induction of proinflammatory cytokines (116). Although T-oligos have been tested to date primarily in immunocompromised SCID mice in which immune-mediated toxicities would likely not be observed, T-oligos do not contain the CpG sequences implicated in these antisense-associated toxicities.

Finally, DNA oligonucleotides that inhibit the enzyme telomerase have been extensively studied and are currently in human clinical trials for several malignancies (117-121). Telomerase is a ribonucleoprotein complex composed of two essential components, the RNA component hTERC and the catalytic component hTERT (122-124) that catalytically adds TTAGGG repeats to the 3′-end of telomeres and thus maintains telomere length (118-121). The majority of human cancers constitutively express high levels of telomerase (125), making it an attractive target for cancer therapy. However, germline and stem cells, as well as normal proliferating cells, also express telomerase, raising the possibility of serious mechanism-related adverse effects for this proposed cancer therapy. However, a modified DNA oligonucleotide that binds the active site of telomerase by base-pairing with the telomerase RNA has been shown to be effective in animal models of malignancy (117), and in cancer cells that rely on telomerase for telomere maintenance (80-90% of tested cell lines) this oligonucleotide leads to gradual telomere shortening and eventual cell death due to telomere dysfunction (126, 127). Telomerase inhibitors have also shown additive or supra-additive anticancer effects when used in combination with chemotherapeutic agents (126, 128). In contrast to T-oligos that must be 3′-hydrolyzable to exert anticancer effects (47) and do not act as telomerase inhibitors (38), these oligonucleotides are more effective when the DNA backbone is stabilized to increase serum half-life, a presumptive advantage for drug development. However, this relative advantage may be counterbalanced by the requirement that telomerase inhibition and hence oligonucleotide levels must be sustained for clinical efficacy (117). In contrast, T-oligos are active, at least in vitro in serum-containing medium, after a single supplementation and lead to progressive apoptosis or senescence of treated cancer cells over 3-5 days (51-53, 83-85, 98), a period at least 12- and perhaps 30-fold longer than the measured half-life of such oligos in serum-containing medium (73). These data are consistent with the understood mechanism of action of T-oligos (see Figs. 1-3) in which interaction of the oligos with WRN triggers a chain reaction involving sequential transcriptional upregulation and phosphorylation of effector proteins expected to self-perpetuate over several days. They further suggest that the major concern for oligonucleotide stability in the clinical setting may be less pertinent for T-oligos than for the other oligonucleotide-based therapies now in development.

THE FUTURE: A SMARTER THERAPY?

We have discovered that the Werner protein, through postulated resolution of G-quadruplexes that form in single-stranded G-rich telomeric DNA, initiates DNA damage-like signaling involving many, if not all, DNA damage response pathways, providing a new target for the development of cancer drugs. T-oligos, molecules that reinforce and exaggerate this innate signaling, represent the first drug candidate in this class and WRN-binding small molecules (129) represent a second. More important than the novel target, however, is that the WRN-initiated signaling appears to have evolved to enhance the survival of individual cells and the organism overall at times of environmental assault on genomic integrity, similar to the bacterial SOS response (46), nature's first attempt to strengthen genome-protective defenses at vulnerable times.

The mammalian responses consist of enhanced DNA repair capacity (both more rapid and as accurate repair, in contrast to the bacterial response); but also encompass increased melanogenesis and antioxidant capacity, reduction in the proinflammatory character of the tissue environment, and reduced angiogenic capacity of already transformed cells. The induced signaling broadly promotes differentiation, including downregulation of inappropriately expressed fetal proteins such as IAP/Livin that block apoptosis and upregulation of potential immunotherapy targets (85). It is tempting to speculate that this mammalian response represents the outcome of millions of

years of evolution directed at cancer prevention and elimination of transformed cells. Signaling through multiple redundant and overlapping pathways simultaneously minimizes the opportunity for selection of resistant cells and assures that virtually all malignant cells are responsive (Fig. 4). The highly selective killing of malignant cells, in contrast to equally proliferative normal cells, is an additional major advantage over conventional cancer therapies. Furthermore, the protective responses induced in normal cells should reduce the risk of secondary malignancies and other adverse effects if patients are treated additionally with conventional cancer therapy.

"Smart therapies" now under development result from the scientific community's hard-won identification of a new signaling molecule or a new abnormality in such a molecule in a specific cancer cell type, the targeting of which will impede the growth of the cancer. However, such approaches may also have severe limitations and unanticipated adverse effects. In contrast, signaling initiated through WRN/telomere interactions is the culmination of millions of nature's successful and unsuccessful experiments linking numerous genome-protective and anticancer responses in a coordinated manner to a single trigger event. These resulting characteristics of T-oligos and possible follow-on WRN-binding small molecules promise a next generation of smart**er** cancer therapies.

DISCLOSURE

The authors are inventors on patents that protect the use of T-oligos to prevent and treat cancer and are equity holders in SemaCo, a company formed to commercialize this intellectual property.

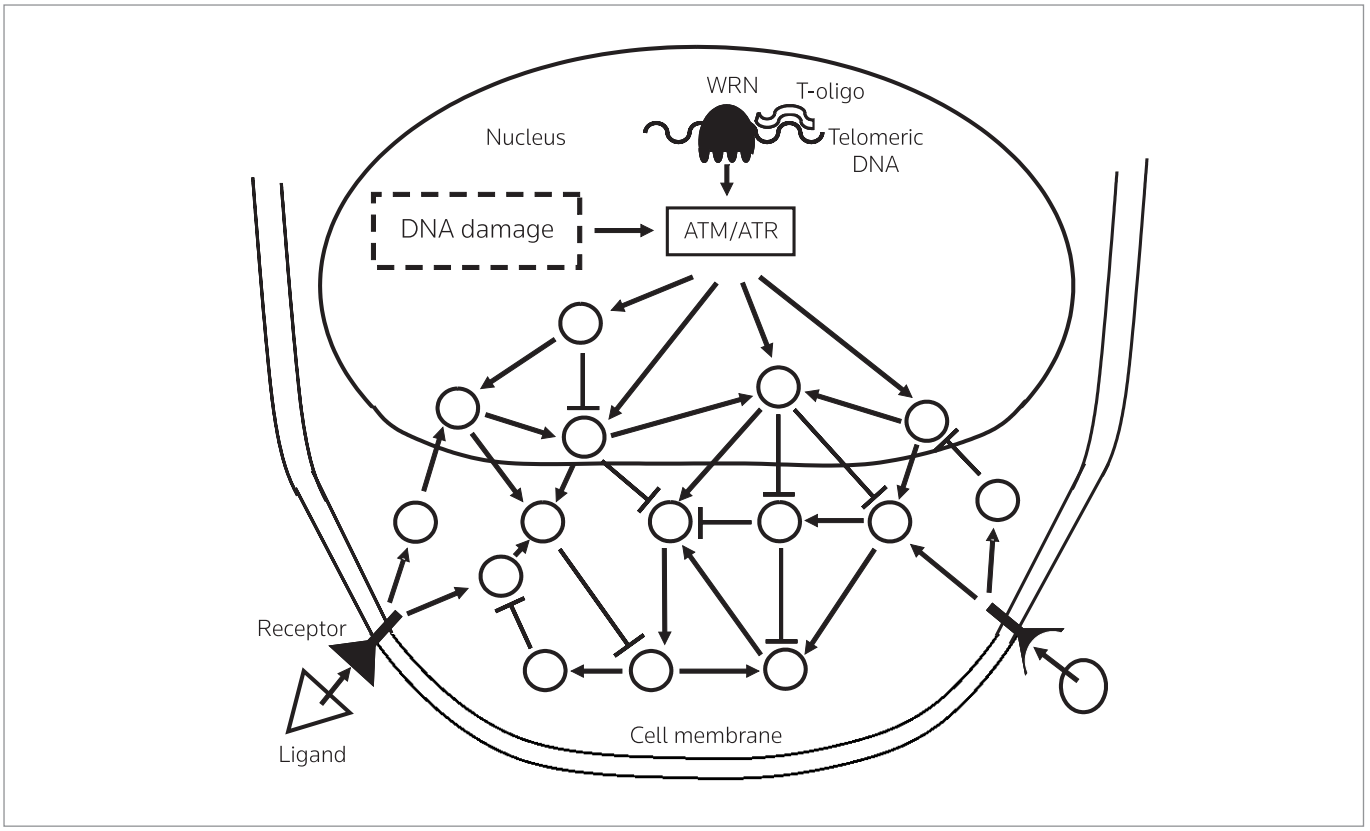

Figure 4. DNA damage signaling and growth-regulatory pathways in cancer cells: targets for intervention. Ionizing radiation and conventional chemotherapeutics target one or more steps in cell division, often by introducing damage into DNA to impede DNA replication and initiate DNA damage signaling, leading to apoptosis or senescence. Treatment is limited by acute toxicity to normal proliferative cells and by long-term DNA damage even to nondividing normal cells. Courses therefore must often stop short of curative doses, in which case the treatment may promote "escape" of the malignant cells through further mutations. Targeted smart therapies, in contrast, target usually one protein known to be central to growth and/or survival of a particular type of cancer cell and, preferably, unique to that malignant cell type (see Table I for examples). Such targets are represented diagrammatically by extracellular growth factor receptors (solid black) or as open circles representing tyrosine kinases, p53 or other tumor suppressor or proapoptotic molecules, or oncoproteins. Smart therapies are intended to prevent signaling (in most cases) through the targeted inappropriately active pathway, but not through often redundant pathways that circumvent the block and may become more active in compensation. The smart drug may also become ineffective by further mutation of the targeted protein, with selection of those malignant cells now displaying a growth or survival advantage. In contrast, interaction of WRN with T-oligo/telomeric DNA G-quadruplexes (see Figs. 1-3) initiates DNA damage signaling in the absence of actual DNA damage, simultaneously through both ATM and ATR to essentially all the cell's downstream effector pathways, leading selectively in dysregulated replication stressed (malignant) cells to apoptosis or senescence. Signaling occurs to a far lesser degree in normal cells and results instead in various DNA-protective responses. Loss of responsiveness in any one, or even many, of the signaling pathways does not reduce the therapeutic efficacy, and there is no malignant cell type restriction, as all cells harbor DNA damage response pathways.

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