Urol Res (2002) 30: 137–147
DOI 10.1007/s00240-002-0248-3

INVITED EDITORIAL

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The role of antisense oligonucleotides in the treatment of bladder cancer

Received: 5 November 2001 / Accepted: 20 February 2002 / Published online: 23 May 2002

Abstract Both intravesical and systemic chemotherapy are limited in their efficacy in the treatment of bladder cancer patients. These limitations are centred around an inability to induce apoptosis in bladder tumour cells. This resistance to apoptosis induction is commonly associated with the overexpression of antiapoptotic proteins such as Bcl-2. Strategies to decrease the cellular expression of such proteins would enhance chemotherapy effectiveness. One such strategy is to use antisense oligonucleotides which are short sequence specific single stranded DNA or RNA molecules designed to bind to the RNA of the target protein. By binding to the target RNA, protein production is interrupted and target protein levels decease. When used to target antiapoptotic proteins, antisense oligonucleotides can therefore be used as a pre-treatment before chemotherapy to help chemosensitise the tumour cell. This review outlines the rationale for this strategy and the work done to date with antisense oligonucleotides in bladder cancer.

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The limitations of current modalities for the treatment of bladder cancer

Current treatment modalities for superficial bladder cancer

Treatment methods for Ta, T1 and *cis* bladder cancer include transurethral resection (TUR) with or without intravesical chemotherapy or immunotherapy. Intravesical therapy is most commonly used as prophylaxis to prevent recurrence of tumour following complete removal of all macroscopic disease by TUR. An additional goal of chemotherapy, which would be desirable but not at present achieved, is to reduce the incidence of disease progression and improve survival [57]. It is the decision of each urologist to balance the benefit of reduced recurrence against the risk of side effects of intravesical chemotherapy.

Intravesical therapy should be tailored to the patient and is most effective when tumour burden is minimized by TUR of papillary disease or fulguration of visible areas of *cis*. The suspected biologic behaviour of the patient's tumour remains an important determinant factor in the decision of intravesical therapy. Consequently, a G3 tumour at high risk for recurrence and progression constitutes an accepted indication for intravesical therapy. In the absence of other risk factors for progression, intravesical therapy is not required for GI/Ta lesions which have a progression rate of 4% [27]. However, multifocal Ta disease with or without *cis* is a relative indication for intravesical therapy [1].

The European Organisation for the Research and Treatment of Cancer (EORTC) reported that a single intravesical instillation of epirubicin within 6 h of surgical removal of a solitary Ta or T1 tumour significantly reduced recurrence rates from 41% to 29% [55]. It is plausible that this single instillation, if performed immediately after transurethral resection, may destroy viable floating tumour cells which otherwise may lead to implantation. The EORTC data was supported by the

results of an MRC trial showing that a single intravesical instillation of mitomycin-C reduced recurrences by up to 40% for a follow-up period of 5 years [73].

The Fifth International Consensus Conference on Bladder Cancer recommended that low risk tumours receive a single intravesical chemotherapy dose after surgery, intermediate risk tumours should receive intravesical therapy based on the prognostic factors of the tumour and that high risk patients receive intravesical BCG with maintenance or intravesical chemotherapy in selected patients [41]. These recommendations are similar to the Bladder Cancer Panel summary report on the management of non-muscle invasive bladder cancer (stages Ta, T1 and TIS) of the American Urological Association [66].

Intravesical immunotherapy, namely BCG is more effective than chemotherapy in the treatment of *cis*. With over 1,000 patients from several series, the average complete response rate of *cis* to BCG is in excess of 70% [35]. There is strong evidence in support of BCG for the primary treatment of *cis*. Lamm reviewed 34 series involving 1,354 patients with *cis* treated with BCG and reported complete responses in 70% of patients [35]. Although BCG may not prolong overall survival for *cis* disease, it appears to afford complete response rates of about 70%, thereby decreasing the need for salvage cystectomy [11]. *Cis* patients treated with BCG have increased complete response rates from 73% to 87% at 6 months when three additional instillations are given at 6 monthly intervals. Such maintenance BCG regimens increase long-term disease-free status from the expected 65% to 83% [30]. Direct randomized comparisons of BCG immunotherapy with intravesical chemotherapy have demonstrated a statistically significant decrease in tumour recurrence rate with BCG compared to chemotherapeutic agents [12, 36, 43, 45, 56, 80].

Current treatment modalities for invasive bladder cancer

Invasive tumours that are confined to the bladder muscle on pathologic staging after radical cystectomy are associated with approximately a 75% 5-year progression-free survival rate. Patients with more deeply invasive tumours experience 5-year survival rates of 20%–40% following radical cystectomy. When the patient presents with locally extensive tumour that invades pelvic viscera or with metastases to lymph nodes or distant sites, 5-year survival is uncommon, but considerable symptomatic palliation can still be achieved [72]. Among patients with invasive bladder cancer, treatment must be individualized accounting for general medical condition, extent of cancer, and the wishes of the patient.

Radical cystectomy and radical radiotherapy are the main treatment options. In T2 disease, a prospective, randomized trial did not show any survival advantage for preoperative radiotherapy and radical cystectomy compared with radical cystectomy alone [65]. Prospective randomized comparison of radiotherapy and chemoradiotherapy reported an improved rate of local control when cisplatin was given in conjunction with radiotherapy [8].

In T3–4a disease the outcome of either radical cystectomy and external-beam irradiation when used alone are poor. Combined preoperative irradiation followed by radical cystectomy modality treatment appears to reduce the rate of local recurrence and is associated with especially good results in patients whose resected bladders contain no pathologic evidence of cancer. However, similar results achieved with radical cystectomy alone in some series have brought this issue under scrutiny. The only prospective, randomized trial reported to date did not show any survival advantage for preoperative radiotherapy and radical cystectomy compared with radical cystectomy alone [65]. In the United States, external-beam irradiation has been generally reserved for patients who are poor medical candidates for radical cystectomy. Because the frequency of distant metastases is becoming apparent with improved local control of advanced bladder cancer, systemic preoperative or postoperative adjuvant chemotherapy is now under evaluation in clinical trials. Treatment with concurrent chemotherapy and radiotherapy has been associated with improved rates of local control compared with radiotherapy alone. A prospective, randomized trial showed an improved rate of local control when cisplatin was given in conjunction with radiotherapy [79].

Results from two clinical studies suggest that a combined modality treatment with neoadjuvant methotrexate, cisplatin, and vinblastine (MCV) followed by radiotherapy and concurrent cisplatin can result in high rates of tumour clearance and can allow bladder preservation in some patients [31, 71]. Currently, only a small fraction of patients with T4b or patients with nodal or metastatic bladder carcinoma can be cured. The prognosis of patients with T4 tumours is generally poor with entry into a clinical trial appropriate. Combination chemotherapy regimens that include cisplatin, methotrexate, and vinblastine, with or without doxorubicin (CMV or M-VAC) are encouraging and have induced some pathological complete responses [26, 69]. Recent data from a large randomised controlled trial of gemcitabine and cisplatin versus methotrexate, vinblastine, doxorubicin and cisplatin concluded that both regimens had similar efficacy but that the gemcitabine regimen was much less toxic [75]. Although response rates of up to 50% are reported with these neoadjuvant chemotherapy regimens [74], there has been no demonstrable increase in overall survival [4]. The joint EORTC and MRC trial failed to demonstrate any difference in overall survival between patients treated with neoadjuvant cisplantin, methotrexate and vinblastine compared with patients just treated with surgery or radiotherapy alone [4]. The only slight advantage for the chemotherapy patients was an 8% difference in metastasis free survival at median follow-up of 4 years but this is countered by a chemotherapy mortality of 1% [4]. This

data would suggest that invasive bladder cancer is at best only moderately chemosensitive and begs the question why does chemotherapy fail?

Why do the current treatment modalities fail?

Significant numbers of patients do not benefit from either intravesical chemotherapy for superficial disease or systemic chemotherapy for invasive disease. Inability to clear the bladder within 1 year is associated with a poor outcome [21]. Patients who fail to respond to chemotherapy or radiotherapy may fall into several categories depending on the pattern of the disease. Patients who fail intravesical treatment may persist or recur as continued superficial disease (stage Tis, Ta or T1 with or without progression) or as invasive disease. Tumour may also recur in the ureters or renal pelvis although this is rare. Patients who fail systemic chemo- or radiotherapy generally have progressive metastatic disease. The clinician is left with few curative options in such a situation. Alternative forms of therapy can be considered depending on the stage and grade of the tumour, but therapy is gradually aimed at palliation as opposed to curative treatment. Failure of therapy in these patients provides an insight into the molecular makeup of tumour cells. Because the failure of a course of therapy indicates that the tumour cells have the ability to resist apoptosis induced by the agent used. This means that the tumour cells possess proteins and/or pathways that have the ability to resist either drug target interaction and/or apoptosis induction. The aim of this article is to explain why the apoptotic pathway is central to understanding cancer cells and how antisense oligonucleotides can alter treatment responses by manipulating anti-apoptotic proteins.

Apoptosis

It is important to understand that anticancer agents are only effective at tumour cell killing when they induce apoptosis and that DNA damage alone does not always result in cell death.

It is the activation of genes involved in the apoptotic process which ensures cell death [13, 20]. Soon after apoptosis was described, Searle examined the effect of a number of chemotherapeutic agents including MMC, actinomycin, and cycloheximide and demonstrated that these agents caused apoptosis in normal and transformed cells [62]. It is now accepted that virtually all anticancer agents induce apoptosis [14, 15, 32, 46]. Induction appears to be independent of the initial target of these agents. Therefore, despite their diverse mode of action, agents that damage DNA directly, target microtubules, disrupt nucleotide biosynthesis or which convert topoisomerases into DNA damaging agents all ultimately are effective by inducing apoptosis [46]. The survival of DNA damaged cells is one of the key features in carcinogenesis and this principle ties in with cellular resistance to chemotherapy [28].

The apoptotic pathway is a multistep complex pathway that leads to programmed cell death. Initiation can be from a variety of stimuli ranging from drug induced DNA damage to receptor activated signals. These initial signals are then processed by a range of second messenger proteins such as p53 in the case of DNA damage or TRADD in the case of TNF receptor activated death signal. The key step in the irreversible triggering of apoptosis is activation of the caspases, a family of cysteine proteases that cause cell breakdown.

For effective intervention in the apoptotic process there must be successful modulation of the final execution pathways [16]. Several proteins are known to suppress the processing of the apoptotic signal to the caspases, these include Bcl-2, Bcl-x_L and the IAP (inhibitors of apoptosis) family. Bcl-2 prevents the release of mitochondrial Apaf-1 and cytochrome c and thus inhibits the assembly of the apoptosome, the multimeric complex that triggers cleavage and activation of caspase 9 (Fig. 1).

Apoptosis promoting therapies, such as Bcl-2 inhibition, could be useful as single agents or could amplify the effects of chemotherapeutic drugs [16].

This area stands at something of a crossroads: a significant amount of information has been generated on the molecular events involved in executing apoptosis but much remains unknown, especially in relation to its control. While respecting these current limitations manipulation of the Bcl-2 protein in bladder tumour cells has merit because several studies have shown that it is associated with increased recurrence and progression of disease as well as chemoresistance.

It would seem logical that reversing Bcl-2 expression would translate into enhanced treatment response. Successful downregulation of Bcl-2 would result in apoptotic signals being processed in a way that is favourable for the apoptosis inducing therapy, e.g. blockade of antiapoptotic proteins before chemotherapy to achieve a greater cell kill. Such molecular therapies or gene therapeutic approaches, however, are not without problems.

To achieve this goal, the gene therapy will need to be taken up by all tumour cells, be selectively activated in the tumour, selectively kill tumour cells with no bystander effects, have no adverse effect when expressed in normal cells, and be deactivated once the therapeutic goal is achieved. For selective targeting in bladder cancer gene therapy vectors to EGFR and 6beta4 integrin receptors have been shown to allow selective gene accumulation in bladder cancer cells [63]. An alternative is the recently cloned uroplakin gene, which codes for a transitional cell epithelium membrane protein, and has been reported to be selectively expressed in bladder epithelium [40]. The use of a uroplakin promoter in gene therapy constructs may allow selective manipulation of genes in TCCB.

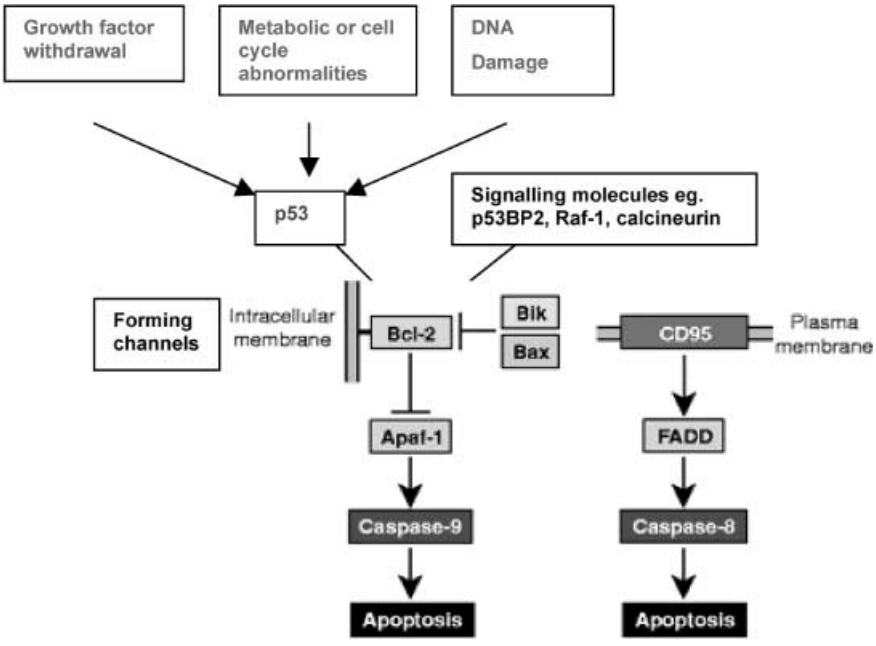

Fig. 1. Bcl-2 governs a crucial checkpoint in the apoptotic pathway bridging factors that have the ability to induce apoptosis such as growth factor withdrawal and DNA damage with the effectors of the process, the caspases. Overexpression of the Bcl-2 protein blocks the activation of caspases. Bcl-2 protein can function in three main ways, (1) binding to other proteins (2) dimerisation with family members and (3) pore formation. Bcl-2 can bind to other proteins such as caspase activator, Apaf-1, and also cellular signalling molecules such as Raf-1 and p53 binding protein. Bcl-2 dimerises with members of its own family (Bax and Bik) and can dock in the phospholipid membrane of intracellular organelles such as mitochondria to form channels. Here it controls release of caspase activators such as Apaf-1 and cytochrome c. Adapted from Adams and Cory [2]

Many things about gene therapy still need to be understood, such as transgene and vector expression, persistence of the new gene, toxicity and immunity [53]. Current efforts are aimed at designing a more sophisticated delivery systems to replace current regimens. The recent death of a patient after adenoviral vectoring of a congenitally absent liver enzyme has raised awareness of the potential problems of such gene therapy and consequently raised awareness of new methods such as antisense oligonucleotides [53].

Antisense oligonucleotides, structure and function

Traditional drugs generally, are designed to interact with protein molecules throughout the body that support or cause diseases. Antisense oligonucleotides function at the genetic level to interrupt the process by which proteins, and in the case of this work, anti-apoptotic proteins, are produced. The idea of using these short synthetic single stranded pieces of DNA to inhibit gene expression was first attempted in 1978 when the Rous sarcoma virus was down regulated by a 13 base oligonucleotide complementary to the 3′-reiterated terminal sequences in a chick embryo fibroblast [81]. Antisense oligonucleotides act in a highly selective manner, blocking the protein at its translation from messenger RNA (mRNA). Their goal is to alter gene expression resulting from the binding of the antisense oligonucleotide molecule to its unique gene sequence.

The information necessary to produce proteins in cells is contained in genes. Specific genes contain the DNA information to produce specific proteins. The DNA molecule is a ''double helix'' – a duplex of entwined strands. In each duplex, the bases or nucleotides namely adenine (A), thymidine (T), guanine (G), cytosine (C) are weakly bound or ''paired'' by hydrogen bonds to complementary nucleotides on the other strand (A to T, G to C). Such highly specific complementary base pairing is the essence of information transfer from DNA to its intermediary, messenger RNA (mRNA).

During transcription of information from DNA into mRNA, the two complementary strands of the DNA partly uncoil. The ''sense'' strand separates from the ''antisense'' strand. The ''antisense'' strand of DNA is used as a template for transcribing enzymes to assemble mRNA – a process called ''transcription.'' mRNA is single-stranded and its sequence of nucleotides is called ''sense'' because they result in a gene product. Normally, the unpaired mRNA nucleotides are ''read'' by transfer RNA anticodons as the ribosome proceeds to translate the message. However, RNA can form duplexes just as DNA does and this is the basis of the mechanism of action of antisense oligonucleotides.

Antisense oligonucleotides are composed of deoxynucleotides like those in DNA and there are usually only 15–20 of them, hence ''oligo'' and their sequence is 3′→5′ i.e. antisense; complementary to the sense sequence of a molecule of its target mRNA; e.g. 5′ C A U G 3′ mRNA 3′ G U A C 5′ antisense RNA.

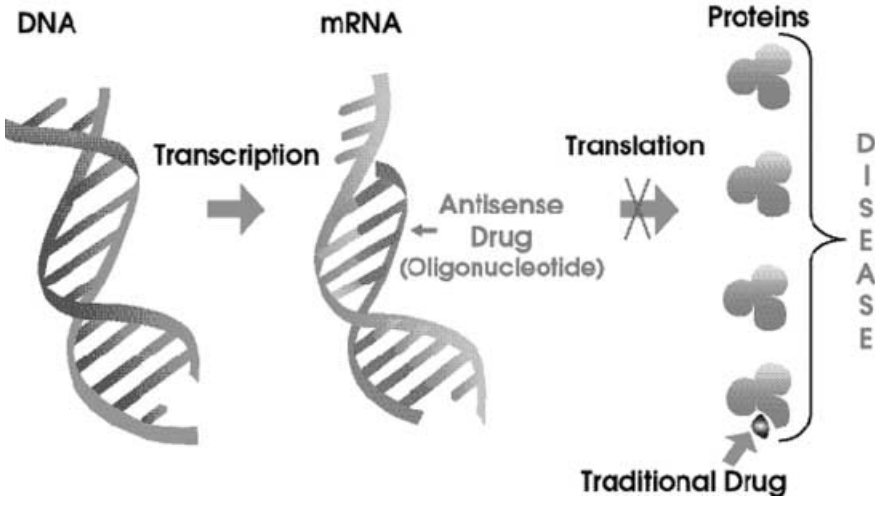

Fig. 2. Antisense oligonucleotides (*red*) bind specifically to their target site on mRNA (*green*) and as such inhibit translation in protein in comparison to traditional drugs which purely target proteins

For such Watson–Crick base pairing to occur, nucleic acid drugs must be complementary to the exposed regions in their target RNAs and must co-localize with them (Fig. 2).

When these requirements are met, true antisense effects are enhanced, and unwanted effects are minimized. When mRNA forms a duplex with a complementary antisense RNA sequence, translation is blocked. This may occur because the ribosome cannot gain access to the nucleotides on the mRNA or alternatively duplex RNA may be quickly degraded by ribonucleases in the cell. Other proposed mechanisms include triplex formation, blocking RNA splicing, preventing transport of the mRNA antisense complex into the cytoplasm, increasing RNA degradation, or blocking the initiation of translation. Approaches for achieving these types of responses would typically rely on a non-RNA cleaving mechanism of action (i.e. 'occupancy only'). There is strong evidence however that in several systems, including *Xenopus* oocytes and permeabilized cells, the target RNA is destroyed by the action of RNase H [24, 64]. RNase H activities cleave the RNA component of DNA-RNA hybrids. Despite the wide range of potential mechanisms for modulating RNA function with antisense, the vast majority of reports documenting true antisense effects in cells utilize RNase H mechanisms as the primary terminating event. In fact, all of the antisense-based drugs that have been investigated in the clinic to date are designed to function through an RNase H-terminating mechanism. Two primary reasons exist to explain this. First, RNase H is a very efficient mechanism for inducing antisense-mediated cleavage of RNA. RNase H enzymes are present in most, if not all, mammalian cells, subcellularly localized primarily in the nucleus, and RNaseH activity does not appear to be a rate-limiting reaction in cells. Antisense-mediated reduction of target RNA levels by RNase H is commonly on the order of 85–95% of control levels. The second reason why RNase H is such a commonly used terminating mechanism for antisense is because phosphorothioate oligodeoxynucleotides serve as efficient substrates when duplexed with RNA for RNase H enzymes.

Originally it was thought that hybridization of an antisense reagent to any portion of the target mRNA would suffice to block translation, but this reasoning was incorrect. Hybridization of an oligonucleotide to some portions of the mRNA sequence has no effect, while other regions provide insufficient inhibition of translation for the desired therapeutic response. In practice, it is necessary to explore many different regions of the target mRNA before a hybridization site is found that allows the antisense oligonucleotides to shut down translation. The internal structures of target RNAs and their associations with cellular proteins create physical barriers, which render most potential binding sites inaccessible to antisense molecules [61]. Theoretically, the minimum number of bases required for an antisense oligonucleotide to bind to a unique DNA sequences is 17 bases on average and is 13 bases to bind to a unique RNA sequence. To knock out a single gene, an intervention would have to distinguish a 17-base perfect match from one with a single-base mismatch [7].

Considerable controversy has arisen over the mechanism of action of antisense oligonucleotides. Most of the oligonucleotides also bind non-specifically to other molecules in the cell and appropriate control experiments were not always performed, particularly in the early days of antisense research. Even when appropriate control experiments are conducted, determining the specific mechanism of action can still be a challenge.

Because it is very difficult to predict what portions of an RNA molecule will be accessible in vivo, effective antisense molecules must be found empirically by screening a large number of candidates for their ability to act inside cells [61]. Sequences targeted to the RNA loop structures show logarithmically greater hybridisation than those in the areas flanking the loop [39].

All this highlights the need for detailed analysis of target selection for the antisense drug.

Antisense drug design

Cellular nucleases reduce the effectiveness of antisense oligonucleotides by degrading these molecules after administration but this can be overcome by altering the nature of the phosphodiester backbone, replacing an oxygen with sulphur to form phosphorothioates [19, 68]. This greatly increases the half-life of the antisense oligonucleotides especially in plasma. Unfortunately, phosphorothioate antisense oligonucleotides bind avidly to many proteins, forming complexes with dissociation constants one to three orders of magnitude lower than those of phosphodiesters. Increased arget-binding affinity of phosphothioate antisense oligonucleotides can be achieved by introducing 2′-O-methoxy-ethyl modifications to selected deoxyriboses [23]. Another aim of the modification of antisense oligonucleotides is to increase the lipophilicity of the molecule or the attachment of carrier groups in order to obtain increased uptake into the target cells. Several backbone modifications include benzylphosphonates, methylphosphonates, phenylphosphonates, and phosphorofluoridates [9].

The effectiveness of antisense oligonucleotides as therapeutic agents depends on their pharmacokinetics, tissue disposition, stability, elimination and safety profile [61, 67]. Pharmacokinetic data allow one to determine the frequency of administration and any potential toxicity associated with chronic administration. Phosphorothioate antisense oligonucleotides degrade from the 3′ end, the 5′ end, and both the 3′ and 5′ ends in a time- and tissue-dependent manner. After intravenous administration in mice, rats and monkeys, phosphorothioate antisense oligonucleotides are detected in plasma; they distribute rapidly and are retained in the majority of tissues [10].

The phosphorothioate (P = S) modification increased resistance to both exo- and endo-nucleases, but short tissue half-lives of ~4 h dictates the need for continuous intravenous infusions [44, 76]. In addition, as highly charged molecules, phosphorothioate antisense oligonucleotides are associated with non-specific activities, including immune stimulation and interaction with other proteins. As a class, phosphorothioate antisense oligonucleotides are well tolerated, and for the most part, toxicity has been non-sequence specific and attributable to the phosphorothioate backbone. General toxicities have included hypotension, tachycardia, hemodynamic changes at high doses, thrombocytopenia and leukopenia, effects on coagulation, activated partial thromboplastin time (APTT), reduction in serum complement activity, increases in transaminases, splenomegaly, renal dysfunction, and cytokine release syndromes including fever of unknown origin. In clinical trials, continuous or frequent intravenous infusions are required to administer conventional phosphorothioate antisense oligonucleotides because of their short tissue lives, which remains a major technical limitation. Therefore, over the last 10 years considerable effort has been made by numerous groups to improve the stability and efficacy of antisense oligonucleotides by modifications of the phophodiester-linkage, the heterocycle or the sugar. To overcome these drawbacks, ongoing research has focused on backbone modifications which provide a more attractive pharmacological profile than phosphorothioate antisense oligonucleotides. The incorporation of 2′-O-(2-methoxy)ethyl (2′-MOE) modifications into 21-mer phosphorothioate antisense oligonucleotides at the 5′ and 3′ ends of the backbone (MOE gapmer) increases hybridization affinity and resistance toward nuclease digestion, without losing RNAse H activation, and results in 20-fold higher activity [5, 44,52]. A 2′-MOE clusterin antisense oligonucleotide had >5 times longer tissue half-lives than phosphorothioate antisense oligonucleotides in human prostate PC3 tumours, and once weekly administration of MOE gapmers was equivalent to daily phosphothiaote oligonucleotides [82] (Fig 3). This preclinical data permits more convenient dosing regimens in clinical trials.

Antisense uptake

For antisense oligonucleotides to become effective therapeutic agents, they need to reach their intracellular target site. As outlined already, the accessibility of the Bcl-2 mRNA target site has been a major problem in antisense therapy and this has been confounded by difficulties in cellular uptake [61]. A recognised limiting factor in their efficacy is intracellular incorporation as they are negatively charged and diffusion across the lipid bilayer is theoretically unlikely. Cellular uptake of antisense oligonucleotides is known to be both time and temperature dependent while cell type, media and culture conditions are all known to effect uptake levels [42]. Phosphodiester and phosphorothioate antisense oligonucleotides are polyanions and as such they cannot passively diffuse across cell membranes. Phosphorothioate antisense oligonucleotides are taken up and broadly distributed in a wide variety of cell types in vitro, although the exact mechanism of uptake is unknown. An 80 kDa plasma membrane protein has been identified that binds AO [42] and as such uptake is felt to occur by either receptor mediated endocytosis or pinocytosis. Subsequent to internalization, antisense oligonucleotides reside in subcellular vesicular structures, i.e. endosomes and lysosomes. There are multiple strategies employed to enhance uptake of antisense molecules into cells. Alteration of the oligonucleotide backbone, addition of a cholesterol moiety and microinjection have all proved successful to some degree [9]. Other new strategies include attaching oligonucleotides to DNA-protein complexes or receptor ligands or cell-specific antibodies to incorporate them into particular cells. Liposomes resemble cell membranes in their structure and have been used to enhance uptake in vitro with varying degrees of success [70].

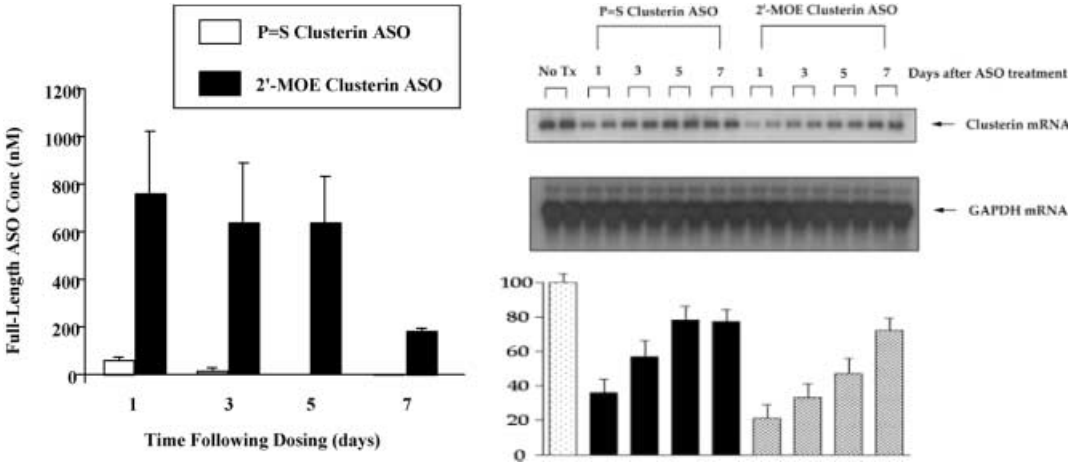

Fig. 3. Tissue half-life of ASO is prolonged by 2′-MOE modification. Capillary gel electrophoresis (CGE) was used to analyse time-dependent ASO metabolism in human prostate PC-3 tumours. *Left*, In vivo tissue half-life of ASO was increased >fivefold by the 2′-MOE modification, compared to conventional P=S ASO (>5 days versus <1 day). 90% of 2′-MOE modified ASO was detectable as full length material 1 week following dosing, whereas only 10% of P=S ASO was found as full-length sequence 1 day following dosing (data not shown) [79]. *Right*, Northern blotting illustrating changes in clusterin mRNA levels in PC-3 tumours after treatment of mice with either type of clusterin ASO for 5 days. Over a period of 7 days after the last ASO treatment, in vivo clusterin mRNA expression was more efficiently inhibited using 2′-MOE modified compared to conventional P=S Clusterin ASO. Clusterin mRNA-levels were quantified (after normalization to GAPDH mRNA levels) by laser densitometry. Each column represents the mean value of triplicate analysis with standard deviation. Numbers on the Y-axis represent arbitrary densitometric units

Problems with antisense therapy

As well as problems with uptake, various side effects have caused much scepticism following initial enthusiasm for antisense therapy. Inadequate intracellular compartmentalization, often fragmentary knowledge of intracellular behaviour and mechanism of action, and lack of specificity remain major challenges. The main drawbacks include avid protein binding of antisense, G quartet effects, CpG motif immune stimulation. The CpG motif effects include the stimulation of B-cell proliferation and the inhibition of viral entry into cells [67]. Some animals treated with antisense oligonucleotides have had significant side effects, some of which have been lethal. Primate studies using high doses of other phosphorothioate compounds not directed at Bcl-2 demonstrated hypotension and deaths if the drugs were given as a rapid intravenous bolus but not when given as an infusion [22].

Antisense directed at Bcl-2

The aim for antisense researchers is to show that an AO targeted against a gene downregulates that gene in a sequence specific manner with no other effects. It follows that all antisense experiments must be interpreted with adequate reference to control parameters. Whatever the mechanism, the important consideration in assessing antisense effects is to establish sequence specificity of the AO against control oligomers, and to demonstrate a decrease in the amount of protein produced by the gene targeted. This has been demonstrated for antisense Bcl-2 sequence G3139 using both sense and nonsense controls [10].

Pharmacokinetic data of phosphorothioate antisense Bcl-2 with intravenous or intraperitoneal infusions has established that plasma clearance by both routes is biphasic with an initial half-life of 15–25 min and a second half-life, related in part to protein binding, of 20–40 h [3, 29]. A similar finding with subcutaneous delivery of Bcl-2 antisense (G3139) was found in mice [58]. G3139 has a high volume distribution due to high protein binding, particularly to albumin. Organ distribution varies with good uptake into bone marrow, liver, spleen, kidney and lymphatics and minimal uptake into the central nervous system [58]. Subcutaneous antisense oligonucleotide infusions prolong the bioavailability of the molecule and increase steady state levels. Minimal toxicities, at a dose up to 10 mg/kg/day for 2 weeks, have been administered as an intravenous continuous infusion, avoiding the potential i.v. bolus toxicities and possibly providing greater bioavailability [58].

Initial attempts to manipulate the *bcl-2* gene with antisense consisted of transfecting a Bcl-2 antisense sequence in a plasmid into a human T-cell lymphoma cell line [59]. Downregulating Bcl-2 in a cell that is heavily dependent on Bcl-2 expression for its survival advantage, appears to commit the cell to an apoptotic death. Similar effects are seen with Bcl-2 antisense oligonucleotide on a human leukaemia cell line with high Bcl-2 expression [59, 60].

Patients with relapsing lymphoma and high Bcl-2 expression were enrolled for a phase I study to examine the toxicities of such antisense oligonucleotide treatment [78]. The G3139 phosphorothioate oligonucleotide was administered for 2 weeks subcutaneously to the abdomen as a continuous infusion using a portable syringe

driver [78]. Twenty-one patients have been entered into the trial over a 3 year period [78] and it has now been completed [77]. The toxicities seen have essentially been minimal with thrombocytopenia the only factor preventing dose increases. The other significant toxicity related to antisense therapy was a local skin reaction surrounding the infusion site that simply required resiting the line site every 3–4 days. Unlike chemotherapy responses, the effect of Bcl-2 antisense appears to be slower and more prolonged in duration, with continued reduction in lymphoma bulk in excess of 6 weeks from the end of the infusion [78]. The most impressive response was seen in one patient who had no lymph node masses larger than 1 cm by 6 weeks and had achieved a response in all lymph node sites. No other patients achieved a complete response; however, two have had partial responses and a further eight have had a durable stabilisation of the disease and at least two of this latter group have shown symptomatic improvement.

Antisense Bcl-2 in bladder cancer

We examined the uptake and effect of antisense Bcl-2 phosphothioate oligonucleotides in vitro in bladder tumour cells and in an ex vivo bladder tumour model [17, 18].

One of our most important findings while assessing AO effects was that when we demonstrated down-regulation of the Bcl-2 protein in the cell lines this did not always translate into enhanced chemosensitivity. In terms of response to pretreatment with antisense Bcl-2, the T24/83 was the most responsive cell line [17]. In T24/83 Bcl-2 protein expression levels were reduced to near zero, possibly to below a threshold level and mitomycin C induced apoptosis increased. This result concurs with a similar study from Japan in which T24 cells had increased chemosensitivity to adriamycin after pretreatment with antisense Bcl-2 [6]. This suggests the apoptotic pathway in T24 and T24/83 cells is Bcl-2 dependent. We found no synergistic effect in three other bladder cancer cell lines. This raises the possibility that alternative pathways which resist or bypass the apoptosis initiation exist in these cell lines. Alternatively, chemoresistance could be the result of other anti-apoptotic proteins substituting for Bcl-2 after its down-regulation. In this case, prime substitutes would include Bcl-xL or members of the Inhibitors of Apoptosis protein family.

We have also compared uptake in RT4 bladder cancer cells, normal pig urothelium and fresh human tumours in an ex vivo model. Using FITC-conjugated antisense oligonucleotide sequences, flow cytometric quantitation of antisense oligonucleotide uptake in various cell populations or subpopulations within tumours was possible. Uptake was predominantly nuclear but this was gradually traffficked into cytoplasm (Fig 4).

Uptake in normal pig urothelium was an important finding because the diffuse urothelial cancer, carcinoma in situ, could only be targeted by intravesical antisense therapy if antisense oligonucleotides permeated normal urothelium.

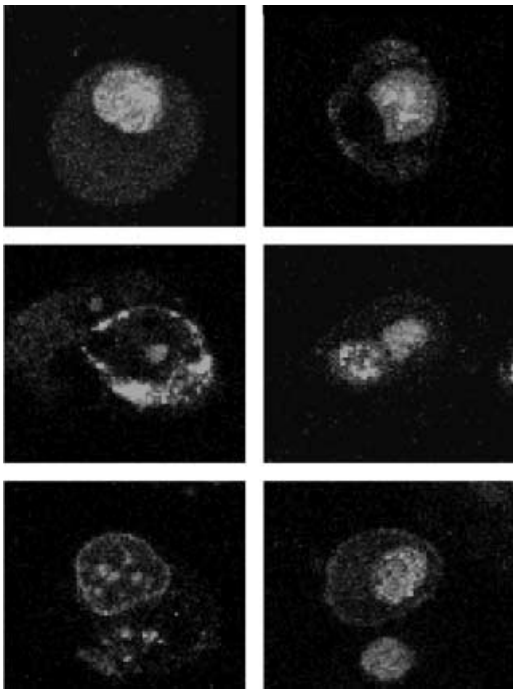

Fig. 4. Confocal microscopy of unfixed RT4 cells incubated with FITC-AO for 1 h and then harvested at 4 h intervals after incubation. Nuclear accumulation was best seen at 4 h while intensity of fluorescence was greatest at 12 h and then diminished over the remaining 12 h. Increased cytoplasmic staining was seen in the later time points

Alternative molecular targets in bladder cancer

Alternative antisense targets both inside and outside the Bcl-2 family are currently being evaluated by other groups. Inside the Bcl-2 family, the most obvious is Bcl-x_L [23]. A recent paper reported downregulation and increased chemosensitivity in T24 cells that were induced to stably overexpress Bcl- x_L [37].

Outside the Bcl-2 family, alternative targets include telomerase which functions to elongate telomeric DNA at the end of chromosomes. Antisense oligonucleotides against the RNA component of human telomerase has been shown to increase caspase based apoptosis in seven bladder cancer cell lines [34]. Antisense oligonucleotides to members of the Inhibitors of Apoptosis proteins including survivin have also been developed and these have a potential application in bladder cancer [54].

Bladder cancer research groups lag behind the level of research of antisense oligonucleotides in prostate cancer. Miyake et al. have successfully enhanced chemosensitivity using antisense Bcl-2 in the Shionogi and LNCaP tumour models [38, 50].

Antisense Bcl-2 oligonucleotides have shown minimal toxicity when administered as an IV infusion as part of a

Phase 1 study by the Vancouver Group in patients with hormone resistant prostate cancer. The patients were treated with a combination of antisense and mitoxantrone which conferred additive benefit [50]. Furthermore the Vancouver Group have moved onto antisense Bcl-2 with Taxotere in a Phase 2 trial also in hormone resistant prostate cancer. They are also assessing new targets such as insulin like growth factor binding protein 5 (IGFBP-5) and clusterin [50]. Clusterin functions like small heat shock proteins (Hsp) to chaperone and stabilize protein conformations at times of cell stress. Indeed, clusterin is substantially more potent than other Hsp's at inhibiting stress-induced protein precipitation [33]. Clusterin levels are regulated by HSP-1 and increase in prostate, bladder, renal, breast, ovary, and other tumours after various cell death triggers, including hormone, chemo-, and radiotherapy [25]. Forced overexpression of clusterin in human prostate LNCaP cells confers a hormone and chemoresistant phenotype [48, 49]. The upregulation of clusterin in human prostate cancer tissues after castration and the accumulating findings implicating clusterin in protection of apoptosis suggests that targeting clusterin upregulation precipitated by androgen ablation may enhance castration-induced apoptosis. Indeed, treatment of mice bearing Shionogi or PC3 tumours with clusterin ASOs decreased clusterin mRNA levels by 70% and enhanced apoptotic tumour regression after chemotherapy and hormone therapy [47, 48].

The ability of clusterin antisense oligonucleotides to chemosensitize bladder cancer was recently reported [51]. Clusterin mRNA was increased in a dose-dependent manner in human bladder cancer KoTCC-1 cells by cisplatin treatment. Although clusterin antisense oligonucleotides had no significant effect on growth of KoTCC-1 cells, they significantly enhanced cisplatin chemosensitivity of KoTCC-1 cells in a dose-dependent manner, reducing the IC_{50} by more than 50%. Similarly, in vivo systemic administration of clusterin antisense oligonucleotides and cisplatin significantly decreased the tumour volume compared with mismatch control oligos plus cisplatin. Furthermore, after the orthotopic implantation of KoTCC-1 cells, combined treatment with antisense clusterin and cisplatin significantly inhibited the growth of primary KoTCC-1 tumours as well as the incidence of lymph node metastasis.

Collectively these findings illustrate that clusterin upregulation after hormone or chemotherapy confers resistance to androgen ablation and chemotherapy in several tumour systems. A Phase I/II clinical trial to determine the serum and tissue pharmacokinetics and biologic activity (i.e. the ability of clusterin antisense oligonucleotides to inhibit the upregulation of clusterin after androgen ablation) of a 2nd generation clusterin antisense when combined with neoadjuvant hormone therapy prior to radical prostatectomy will begin in mid-2002.

Conclusions

We believe that antisense oligonucleotides are a feasible strategy which could be employed to modify apoptosis in bladder tumour cells. This, however, will only translate into a relevant clinical response in a selected number of patients. The antisense approach to down-regulating individual proteins within the apoptotic pathway seems a logical way to gain information about and solve the many conundrums of the pathway. However, it would seem that for true clinical benefit, manipulation of single anti-apoptotic proteins is insufficient and multimolecular manipulation of apoptosis may be a more favourable approach to increase chemosensitivity in a greater proportion of patients. Cell survival signals such as local growth factors and cell adhesion mediated signals often collaborate with Bcl-2 family members and other antiapoptotic proteins in protecting cells from apoptosis. Successful manipulation of apoptosis may need both extra and intracellular factors to achieve its goal. The most exciting application of antisense oligonucleotides will happen in the next few years as important molecular targets are identified from microarray analysis.

We would confidently predict that the treatments outlined at the start of this paper will in the future be significantly altered as molecular therapies become more sophisticated. Predicting the exact role of antisense oligonucleotides in the future treatment of bladder cancer is more difficult.

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