

Tissue-specific triple ribozyme vectors for prostate cancer gene therapy

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Summary

Ribozymes are naturally occurring catalytic RNAs which can be targeted to bind and cleave other RNA molecules with high specificity. Ribozyme-mediated inhibition of gene expression at the message level represents a powerful therapeutic tool. Implement of ribozymes in gene therapy has focused mainly on HIV and cancer. Use in HIV protocols has been widespread due to the wealth of targets offered by a well-characterized RNA retrovirus. Ribozyme utility in cancer has centered on oncogene inhibition in tumors with well defined genetics. As molecular mechanisms of prostate cancer are poorly understood, ribozyme targets must be universal with either delivery or expression firmly restricted to the area of interest. Overall, continued research on ribozyme design, delivery vehicles, tissue specificity, and the genetic makeup of disease will allow ribozymes to fulfill their considerable potential as therapeutic agents.

I. Introduction

The regulation of gene expression through inhibition at the protein, message, or DNA level offers not only insight into biologic function but also a powerful treatment modality in disease. Antisense oligonucleotides have previously been demonstrated to have utility at this level of regulation (Reviewed in Helene and Toulne, 1990). More recently, research in ribozyme design and implement in therapy has expanded exponentially. Ribozymes are RNA enzymes capable of specifically binding to and cleaving complementary RNA molecules allowing for inhibition of gene expression at the message level.

A. Ribozyme background

The discovery that certain naturally occurring RNAs had catalytic self-cleaving activity (Cech and Bass, 1986; Altman, 1987; Forster and Symons, 1989) changed our conception about the origin of life (Joyce, 1989). RNA catalysis proved that enzymatic activity was not restricted to proteins. The original ribozyme was a self-splicing intervening sequence in the pre-rRNA of *Tetrahymena thermophila* whose only requirement for activity was the presence of a divalent metal cation (Cech and Bass, 1986).

However, ribozymes originating from the plant viroids satellite RNA of tobacco ringspot virus (sTobRV) (Buzayan et al., 1986) and lucerne transient streak virus (Forster and Symons, 1987) are better suited for manipulation of cleavage activity and specificity. The catalytic domains were termed "hammerhead" or "hairpin" owing to their secondary structure. Uhlenbeck (1987) showed that the hammerhead ribozyme could cleave *in trans*. Haseloff and Gerlach (1988) defined the consensus sequences of the hammerhead ribozyme required for site-specific cleavage of target RNA through *in vitro* mutagenesis studies of the plus strand of sTobRV. These early studies paved the way for research on the design of hammerhead ribozymes against any gene containing a cleavage target.

B. Hammerhead ribozyme design

The basic hammerhead ribozyme (see **Figure 1**) is composed of a catalytic core of 24 conserved bases and three self associating helices. Helix I and III serve as substrate recognition and binding sites by acting in antisense to the target sequence. The site of cleavage for the ribozyme was initially shown to require a NUX sequence where N is any nucleotide and X is A, C, or U

(Ruffner et al., 1990) with GUC predominantly favored as the cleavage site in naturally occurring self-cleavage

(Haseloff and Gerlach, 1988). The ribozyme cleaves 3' of this ribonucleotide triplet with the presence of divalent

Figure 1. Hammerhead ribozyme. The basic design is composed of three hybridizing helices and a conserved catalytic core. Helices I and III recognize and bind the target RNA sequence by antisense base pairing. Cleavage occurs 3' of the NUX sequence.

cations fulfilled by Mg⁺² or Mn⁺² as the only requirement (Pyle, 1993).

The overall cleavage reaction consists of three phases. (1) The ribozyme flanking (recognition) sequences bind to the RNA substrate by Watson-Crick base pairing to properly align the catalytic core. (2) Phosphodiester cleavage results in the formation of a 2',3' cyclic phosphate on the 5' fragment and a free 5'-hydroxyl on the 3' fragment. (3) Substrate dissociation and product release from the flanking sequences allow for multiple turnover of the ribozyme (Reviewed in Scanlon et al., 1995).

Identification of variables affecting these steps to improve the efficiency of the ribozyme is an active area of research. Ribozymes differing in number and sequence of RNA in their hybridizing helices were shown to vary greatly in cleavage kinetics (Fedor and Uhlenbeck, 1990). The optimal length of ribozyme flanking sequences is still controversial. The goal is minimized mismatch pairing with maximized product dissociation (the cleavage step is unaffected by length of recognition sequence). Increasing the number of paired bases resulted in stronger binding and slow product release which limited turnover (Herschlag and Cech, 1990). Strong binding also reduced specificity by slowing the dissociation of mismatched RNA substrates compromising ribozyme discrimination (Herschlag, 1991). Although a target sequence length of 15 bases may be considered unique in the human genome, multiple studies have demonstrated the optimal length to be 12-14 bases (Bertrand et al., 1994; Goodchild and Kohli, 1991). In addition, the RNA binding protein hnRNP1 and the nucleocapsid protein of HIV-1 enhanced the rate of binding and product release if the length of the flanking sequences was not more than 14

bases (Bertrand and Rossi, 1994). However, some findings support longer flanking sequences for activity *in vivo* (Crisell et al., 1993).

Another variable affecting hybridization is substrate structure (Scarabino and Tocchini-Valentini, 1996). Hammerhead ribozymes have demonstrated reduced efficiency in cleaving long RNA transcripts (presumably due to secondary/tertiary structure considerations) (Bertrand et al., 1994). But, enhanced cleavage of longer RNA transcripts was achieved using oligonucleotide facilitators which interact with the substrate at the termini of the ribozyme (Jankowsky and Schwenzler, 1996). Other substrate concerns can be addressed by accurate predictions of target secondary structure to identify susceptible NUX-containing cleavage sites for ribozyme design.

The next stage in improved ribozyme efficiency (without taking into account the rate-limiting step of delivery) is increasing intracellular activity of the ribozyme. There are two general methods for introducing ribozymes into the cell: (1) incorporation of DNA that codes for the ribozyme into viral or non-viral vectors, or (2) delivery by lipid or other means of chemically synthesized ribozymes. Under the latter technique, the ribozymes are very susceptible to ribonuclease attack once inside the cell. A number of structural modifications can improve stability. Substitution of deoxynucleotides in helices I and III increased stability and catalytic activity (Taylor et al., 1992). The introduction of phosphorothioate linkages in stems I, II, and III further protected from degradation (Shimayama et al., 1993).

With the first method, a gene encoding the ribozyme is cloned into a vector to take advantage of the cellular

transcriptional machinery for ribozyme production following delivery. Cloning along with erratic initiation or termination of transcription introduces anomalous flanking sequences both upstream and downstream of the ribozyme recognition sequences capable of interfering with catalytic activity by non-specific hybridization or

increased secondary structure (Fedor and Uhlenbeck, 1990). Several strategies utilizing *cis*-acting ribozymes have been proposed to generate a consistent RNA transcript with defined 5' and 3' ends (Ruiz et al., 1997; Ventura et al., 1993; Chowrira et al., 1994; Taira et al., 1991). In a

Figure 2. Autocatalytic double ribozyme vector.

The 5' and 3' *cis*-acting ribozymes were designed to bind and undergo self-cleavage at GUC sequences by homology of 13 bases thereby releasing the internal *trans*-acting ribozyme with defined 5' and 3' ends. The DNA template for the internal ribozyme can be inserted into the BglII site.

prominent design, the 5' and 3' *cis*-acting ribozymes undergo autocatalytic self-cleavage thereby releasing the internal *trans*-acting ribozyme as a short uniform RNA (see **Figure 2**) (Clawson et al., 1997). Under this approach, *trans*-acting ribozyme sequences targeting either single or multiple cleavage sites can be connected in tandem with flanking *cis*-acting ribozymes to counteract two inherent difficulties. (1) A large excess of ribozyme over substrate is necessary for a significant reduction in target RNA (Cotten and Biernstiel, 1989; Cameron and Jennings, 1989). (2) It may be beneficial to target more than one site of a given substrate due to the presence of genetic variability in target sites and the inaccessibility of certain sites (Reviewed in Sarver et al., 1990; Rossi et al., 1990). These multimeric ribozymes are liberated as individual units in a cell by autocatalysis to either increase ribozyme concentration or target multiple sites (Ohkawa et al., 1993).

II. Ribozymes in gene therapy

Research in ribozyme technology coupled with that in delivery vehicles implicates ribozymes as potential therapeutic agents. Ribozyme-mediated inhibition of gene expression at the message level allows the mRNA of nearly any gene to be targeted for destruction. Possible applications are only limited by molecular knowledge of

the disease. The majority of ribozymes implemented for therapy thus far have focused on HIV and cancer.

A. HIV-1

HIV-1 is a retrovirus with an RNA genome and, consequently, has numerous potential ribozyme cleavage sites. Ribozyme activity against HIV-1 infection would theoretically be effective at two stages in the life cycle of the virus: (1) directly following infection when the viral genome is still in RNA form, and (2) following integration when viral transcript production begins (Reviewed in Akhtar and Rossi, 1996; Yu et al., 1994). As an added benefit, ribozymes are designed to cleave viral RNA leaving cellular transcripts unaffected.

In early studies, a ribozyme directed against gag and expressed in HeLa CD4+ cells challenged with HIV-1 reduced gag transcript and p24 levels (Sarver et al., 1990). Other studies have shown ribozyme-mediated protection from HIV-1 infection by targeting the 5' leader sequence (regulatory region conserved in most viral strains) (Weerisinghe et al., 1991; Dropulic, 1992). A hairpin ribozyme also targeted to this region under the control of a strong promoter decreased p24 levels and drastically reduced proviral DNA formation (Yu et al., 1993; Yamada et al., 1994).

Because a base change in the recognition or cleavage sequence of the target could abrogate ribozyme effect, the high mutation rate of HIV-1 resulting from the error-prone activity of its reverse transcriptase (Roberts et al., 1988) poses a problem for ribozyme therapy. However, the use of multimeric ribozymes to simultaneously target different sites can offset this potential concern. In this regard, multiple hammerhead ribozymes with different flanking sequences targeted against the HIV-1 genome increased cleavage in proportion to the number of connector units (Ohkawa et al., 1993). In conclusion, ribozymes exhibit significant promise in gene therapy of AIDS; but, as with all gene therapy, improvements in a number of aspects such as delivery are required for successful clinical translation.

B. Cancer

Cancer is a multi-step process involving sequential genetic activations and inactivations of certain key elements involved in cell proliferation, differentiation, and apoptosis. The altered genes fall into one of two groups: oncogenes or tumor suppressor genes. Oncogenes are often overexpressed or mutated in the signal transduction pathway leading to uncontrolled cell growth. Because the process works through an RNA intermediate, targets are available for ribozyme activity. The majority of ribozyme gene therapy of cancer has focused on inhibition of specific oncogene expression in tumors with a relatively defined genetic basis.

The ras family of G proteins plays a vital role in the signal transduction pathway which converts extracellular signals into an intracellular response mediated by nuclear transcription factors. The ras gene is mutated in approximately 90% of pancreatic adenocarcinomas (Almoguera et al., 1988), approximately 50% of adenocarcinomas of the colon (Bos et al., 1987), and approximately 45% of melanomas (Ball et al., 1994). Because of this high prevalence, the ras oncogene is a popular target for knockout in gene therapy. A hammerhead ribozyme targeted to a predominant mutation in the 12th codon of H-ras (GGC--GUC) can discriminate the mutated from the normal gene and exert profound anti-neoplastic effects (Kashani-Sabet et al., 1992). EJ bladder carcinoma cells transfected with the ras ribozyme under transcriptional control of the human β -actin promoter showed a decreased level of H-ras gene expression, reduced cell growth, and diminished tumorigenicity in nude mice (Kashani-Sabet et al., 1992; Tone et al., 1993). In human melanoma cells, transfection of the ras ribozyme resulted in decreased H-ras gene expression, reduced cell proliferation, and also exhibited a more differentiated phenotype (Ohta et al., 1994/1996). Transfection into NIH3T3 cells transformed by the mutant H-ras gene demonstrated decreased focus formation and protection from future transformation (while not affecting normal 3T3 growth) (Koizumi et al., 1992; Funato et al., 1994).

The ras ribozyme displays great therapeutic potential due to its prevalence in a number of tumors and the ability to target a tumor-specific mutation while leaving normal cells unaffected.

The c-fos protein complexes with c-jun to form the AP-1 transcription factor at the distal end of many signal transduction pathways and regulates a number of genes involved in proliferation, apoptosis, and drug resistance. The c-fos oncogene plays a leading role in the development of resistance to chemotherapeutic drugs which currently represents a serious limitation in the treatment of a number of cancers. Resistance to cisplatin (one of the most widely used anti-neoplastic agents) appears to be caused by overexpression of the c-fos oncogene (Scanlon et al., 1989/1990; Kashani-Sabet et al., 1990a/1990b). A ribozyme designed to cleave c-fos was transfected into an ovarian carcinoma cell line resistant to cisplatin resulting in regained sensitivity to cisplatin treatment due to down-regulation of c-fos as well as c-fos regulated genes which direct DNA synthesis and repair (DNA polymerase β , topoisomerase I, and metallothionein IIB) (Scanlon et al., 1991/1994; Funato et al., 1992).

The MDR drug resistant phenotype is caused by overexpression or amplification of the *mdr-1* gene which also has an AP-1 site signifying regulation by c-fos (Teeter et al., 1991; Gottesman and Pasten, 1993). *mdr-1* encodes a membrane p-glycoprotein involved in drug efflux. The same drug resistant cell line found to overexpress c-fos also overexpresses *mdr-1*; and transfection of the c-fos ribozyme decreased *mdr-1* levels along with levels of c-fos which restored sensitivity to MDR agents (Scanlon et al., 1994). Multiple groups have also produced *mdr-1* ribozymes and showed their efficacy in restoring drug sensitivity in a number of cell lines (Kobayashi et al., 1994; Holm et al., 1994; Scanlon et al., 1994; Kientopf et al., 1994).

Another prime ribozyme target is the *bcr-abl* gene present in more than 95% of chronic myelogenous leukemias (CML) (Kurzrock et al., 1988). The Philadelphia chromosome results from the reciprocal translocation between chromosomes 9 and 22 fusing the *bcr* and *abl* genes to form an augmented tyrosine kinase with transforming ability. Several groups targeted the breakpoint of the fusion gene for ribozyme cleavage resulting in varying levels of reduced cell growth in CML cell lines transfected with the ribozyme (Snyder et al., 1993; Shore et al., 1993; Lange et al., 1994). However, ribozyme-mediated cleavage of *bcr-abl* may not be entirely specific to the fusion gene (Wright et al., 1993). Several groups have overcome wild type mRNA cleavage and therefore regained tumor-specificity by altering the recognition sequences of the ribozyme (Kearney et al., 1995; James et al., 1996).

The studies reviewed here of oncogene inhibition by ribozymes clearly demonstrate their future role as anti-neoplastic agents in gene therapy protocols. They have proven their superiority to antisense approaches (currently used in several clinical trials) due to multiple catalytic turnover (Reviewed in Kijima et al, 1995). And potential targets in cancer therapeutics are limited only by our current understanding of the disease.

a. Prostate Cancer

Prostate cancer is the most frequently diagnosed cancer in U.S. males and the second leading cause of cancer death (Parker et al., 1997). Although already a major health problem with predicted dramatic increases in incidence and mortality over the next 15 years, the molecular mechanisms behind the disease's initiation and progression remain largely a mystery. Therapeutic modalities thus far are limited and largely ineffective. Current treatment of prostate cancer includes watchful waiting, radical prostatectomy, radiation therapy, hormonal therapy, and limited chemotherapy. Recurrence rates and mortality, especially with non-localized late stage disease are very high (Reviewed in Cersosimo and Carr, 1996). Further, approximately one-third of patients are in advanced stages of the disease at the time of diagnosis (Reviewed in Konety and Getzenberg, 1997). Additional therapeutic options are clearly necessary. Though a number of genes such as Rb and p53 have been found mutated in late stage disease (Kubota et al., 1995;

Dahiya et al., 1996), a consensus sequential series of activations and inactivations of oncogenes and tumor suppressor genes has not been elucidated in prostate cancer as in other cancers such as in the colon (Cho and Vogelstein, 1992). Because specific genetic alterations are relatively unknown, target selection for ribozyme gene therapy must move beyond inhibition of oncogene expression. The target must be more universal to affect all prostate cancers and not just subsets harboring a specific oncogene or tumor suppressor gene dysfunction.

In this regard, the transcription of ribosomal DNA is completely dependent on activated RNA polymerase I (RNA pol1) and its associated factors. Ribosomal genes comprise only 1% of the genome but account for 40% of total cellular transcription (Reviewed in Moss and Stefanowsky, 1995). This immense undertaking is a prerequisite for growth and development (Reviewed in Moss and Stefanowsky, 1995). Indeed, the tumor suppressor gene Rb may repress proliferation by disrupting RNA pol1-mediated transcription (Cavanaugh et al., 1995). Similarly, a ribozyme targeted to bind and cleave the message of RNA pol1 would compromise cellular viability by preventing proper formation of the protein synthesis machinery through a shutdown or reduction in ribosomal RNA production (see **Figure 3**). Increased effect should be observed in neoplastic cells due to their augmented growth rate.

Figure 3. RNA polymerase I targeted ribozyme. A ribozyme targeted to bind and cleave the message of RNA polymerase I leads to a shutdown or significant reduction in ribosomal RNA transcription. The disruption in the protein synthesis machinery results in diminished cellular growth and compromised viability.

Figure 4. RNA polymerase I secondary structure prediction. To identify potential cleavage sites for the *trans*-acting ribozyme, the sequence of the RNA pol1 transcript was searched for the presence of the nucleotide triplet GUC. Secondary RNA structure was then predicted using the program MFOLD and susceptible regions of the transcript sequence containing GUC were identified. The region at nucleotide 457-459 indicated by the arrow was chosen as the target cleavage site.

A ribozyme was targeted to subunit AC40 of RNA pol1 (see **Figure 4**) and cloned into the BglIII site between the 5' and 3' autocatalytic ribozymes to form a RNA pol1 targeted triple ribozyme (TR) (Clawson et al., 1997). *In vitro*, the construct displayed predicted autocatalytic cleavage in *cis* to release the internal ribozyme targeted to cleave RNA pol1 in *trans* (Clawson et al., 1997). Transfection of TR into mouse fibroblast cells resulted in a 50% decrease in RNA pol1 message levels (50% transfection efficiency) (Clawson et al., 1997). Further, the diminution of RNA pol1 mRNA caused by the activity of the triple ribozyme was sufficient to markedly reduce cell population (below that of a mutant non-catalytic version of the ribozyme acting in antisense fashion) thus implicating ribosomal transcription as an important mediator of cell proliferation and viability (Clawson et al., 1997). Thus, ribozyme-mediated knockout of this housekeeping gene has cell killing ability. However, application of the RNA pol1 triple ribozyme to cancer gene therapy, in general, and prostate cancer gene therapy, in particular, requires highly restricted targeting of the construct to the tissue of interest to prevent collateral damage in other tissues.

b. Tissue Specificity

Targeting can occur at two levels: (1) targeted delivery by restricted entry of DNA into cells, or (2) targeted expression by restricted transcription of the introduced DNA. Through implementation of these strategies both a local and systemic response to primary and distant tumors can be achieved. In the former approach, research is focused on engineering surface components of viral and non-viral vectors to recognize specific target cell types or tissues (Reviewed in Miller and Vile, 1995; Harris and Lemoine, 1996). The latter approach of directed expression of the therapeutic gene to specific cell types can be accomplished by coupling to a tumor- or tissue-specific promoter.

Tumors often sustain the ability to produce proteins specific for the tissue from which the neoplasm arose (Hart, 1996). To exploit this transcriptional specificity, essential promoter regions are defined and used to restrict expression of the exogenous gene of interest. Although transcriptional targeting is considered to be tissue-specific, a number of promoters can also be thought of as tumor-specific if the normal tissues are not essential for viability or accessible to the introduced DNA (Deonarain et al., 1995). Promoters of potential use for gene therapy of cancer include α -fetoprotein for liver tumors and c-erbB2 for breast or pancreatic tumors (Deonarain et al., 1995), tyrosinase or tyrosinase related protein for melanoma and polymorphic epithelial mucin for ovarian carcinoma (Hart, 1996), and surfactant protein promoter for lung cancer (Smith et al., 1994). The tyrosinase promoter was used to express a ribozyme targeted to the ras mutation in human

melanoma cells and found to be superior in suppressing the melanoma phenotypes over a conventional promoter (Ohta et al., 1996).

The expression of certain proteins is strongly restricted to prostate tissue. Prostate-specific antigen (PSA) is a serine protease with homology to kallikreins (Watt et al., 1986). It is considered the most sensitive marker available for diagnosis and management of prostate cancer (Cersosimo and Carr, 1996). The biologic role of PSA is to lyse seminal fluid, and it was recently found to cleave the insulin-like growth factor binding protein-3 which then releases insulin-like growth factor serving as a mitogen (Cohen et al., 1994). Of therapeutic interest is the nearly exclusive production of PSA by both normal and, in most cases, neoplastic luminal prostatic epithelial cells (Reviewed in Peehl, 1995).

The ability of the androgen regulated promoter region of the PSA gene to target heterologous gene expression to prostate tissue is of critical import. Transgenic mice bearing a 6-kb PSA promoter region driving expression of a reporter construct demonstrated prostate-specific expression mirroring the expression patterns seen in humans (Cleutjens et al., 1997). A shorter segment of the PSA promoter was able to drive expression of a reporter in LNCaP cells without activity in lines of non-prostatic origin (Lee et al., 1996; Pang et al., 1995). Overall, the PSA promoter has significant potential in prostate cancer gene therapy; and research continues in the identification of promoter regulatory elements necessary for tissue specificity and maximum expression (Zhang et al., 1997; Pang et al., 1997).

Similarly, rat probasin is a hormonally regulated protein predominantly found in the dorsolateral region of the prostate (Spence et al., 1989). Transcriptional activity of probasin (mediated by androgens) begins in the prostate between 2 and 7 weeks of age and increases with sexual maturity (Matusik et al., 1986). Transgenic studies with a minimal rat probasin promoter region driving a reporter construct revealed highly specific expression restricted to the lateral, dorsal, and ventral lobes of the prostate with very limited expression also observed in the anterior prostate and seminal vesicles but nowhere else in the body (Greenberg et al., 1994). Maximal expression occurred in the mouse dorsolateral prostate, significant due to the region's correlation to the peripheral zone of the human prostate where the majority and most invasive forms of human cancer arise (Reviewed in Price, 1963). Subsequent research demonstrated that a larger fragment of the probasin promoter containing androgen and zinc regulatory regions was able to direct higher levels of expression specifically to the prostate in transgenic mice (Yan et al., 1997). Probasin promoter expression patterns in human tissue have yet to be resolved; but, at minimum, the promoter sequence has utility in the transcriptional

targeting of prostate cancer gene therapy constructs for evaluation of effect in animal model systems.

Under the aforementioned classification system for transcriptional targeting, prostate tissue is considered non-essential; thereby defining prostate-specific promoters as tissue-specific and tumor-specific (Deonarain et al., 1995). The coupling of cytotoxic genes to prostate-specific promoters is a feasible approach to prostate cancer therapy. The cell killing effects of the previously designed RNA

Figure 5. Seap assay--probasin promoter. TRAMP cells were transfected with PBSeap or Seap Basic (control) in triplicate. Samples of culture media were taken daily and the seap assay performed. RLU/sec values of the PBSeap and Seap Basic transfected cells were averaged and control transfected cell values subtracted. Initiation of maximal expression was observed on day two post-transfection.

Figure 6. Transfection assay--probasin-RNA pol1 triple ribozyme (turnover rate). TRAMP cells were plated at 8×10^5 for either mock transfection with liposome only (Lipo Cntrl), transfection with the probasin-driven triple ribozyme vector (PBTR), or transfection with the identical construct without the internal RNA pol1 ribozyme (PBDR). The population and turnover rate of the PBTR transfected cells were lower at all time points. (Note: transient transfection with efficiency of 25-30%)

pol1 targeted triple ribozyme can be restricted to prostatic epithelium and neoplastic derivatives if placed under transcriptional control of the minimal probasin promoter. A recently developed mouse prostate tumor cell line (TRAMP) provides an opportunity to evaluate the probasin-driven triple ribozyme construct (PBTR). The probasin promoter was able to drive expression of heterologous genes in this cell line as judged by transient transfection of a probasin-driven Seap reporter construct. Initiation of maximal expression occurred between day one and two with slightly increasing intensity thereafter (see **Figure 5**) (Voeks et al., 1997). Transfection of the prostate tumor cells with the PBTR construct reduced cellular proliferation beginning between day one and two as compared to control transfected cells (see **Figure 6**) (Voeks et al., 1997). Because transfection was transient and at low efficiency, profound deviation in cell number only occurred for a brief time before returning to normalcy (presumably when the transduced cells were removed from the population).

To evaluate the therapeutic potential of PBTR on tumor tissue *in vivo* (TRAMP xenografts), the PBTR construct was administered by direct intra-tumoral injection using a cationic liposomal carrier as vehicle. Both single administration and repeated administration on three consecutive days resulted in a consistent reduction of tumor growth between day two and day six post-administration followed by a resumption of normal tumor growth (Voeks et al., 1997). Delivery of reporter constructs under the same conditions indicated that gene

transfer by liposomal vehicle was low (unpublished). However, the reduced tumor burden following injection of the probasin-driven triple ribozyme offers the promise of an enhanced, longer-lasting effect by employing a more efficient delivery vehicle.

Prostate epithelial cell-specific knockout of a key gene in cellular growth and viability has the capability of treating cancer confined to the prostatic capsule and, more importantly, the potential to track metastasized tumor cells without realizing collateral damage in other tissues. The initial reduction in tumor burden by liposomal delivery of the probasin-driven triple ribozyme indicates the possibility for translation of prostate-specific RNA pol1 ribozyme therapy to human cancer if increased levels of cell killing can be attained with improved delivery. Also, additional promoters can be utilized should PB not display similar properties in human cancer cells or lack the ability to drive expression in advanced stages of the disease.

III. Conclusions

The rapid emergence of ribozyme technology for the specific control of gene expression harbors great potential in gene therapy. Antisense approaches have been widely utilized in therapeutic research and a number of clinical trials initiated. Ribozymes hold clear advantages over antisense methodology evidenced by their increasing application in the treatment of disease, most notably HIV and cancer. At present, there are two ongoing HIV ribozyme clinical trials, and widespread ribozyme entry into a broad range of clinical trials should soon occur. However, additional research is necessary to successfully advance ribozyme therapy to the clinical setting. For instance, extensive *in vivo* animal-based evaluation is required. Ribozyme activity and specificity need to be further optimized. Even with stronger research emphasis, delivery remains the major rate-limiting step in gene therapy protocols. Also, improved molecular knowledge of malignancies will identify additional ribozyme targets. And advances in targeting through vector design coupled with elucidation of key transcriptional elements will greatly improve ribozyme therapy. The future of ribozymes in the growing field of gene therapy appears promising.

References

- Akhtar, S., and Rossi, J.J. (1996). Anti-HIV therapy with antisense oligonucleotides and ribozymes: Realistic approaches or expensive myths? **J. Antimicrob. Chemother.** 38, 159-165.
- Almoguera, C., Shibata, D., Forrester, K., Martin, J., Arnheim, N., and Peurcho, M. (1988). Most human carcinomas of the exocrine pancreas contain mutant c-K-ras genes. **Cell** 53, 549-554.
- Altman, S. (1989). Ribonuclease P: an enzyme with a catalytic RNA subunit. **Adv. Enzymol. Relat. Areas Mol. Biol.** 62, 1-36.
- Ball, N.J., Yohn, J.J., Morelli, J.G., Norris, D.S., Golitz L.E., and Hoefler J.P. (1994). RAS mutations in human melanoma: a marker of malignant progression. **J. Invest. Dermatol.** 102, 285-290.
- Bertrand, E., Pictet, R., and Grange, T. (1994). Can hammerhead ribozymes be efficient tools to inactivate gene function? **Nucleic Acids Res.** 22, 293-300.
- Bertrand, E.L., and Rossi, J.J. (1994). Facilitation of hammerhead ribozyme catalysis by the nucleocapsid protein of HIV-1 and the heterogeneous nuclear ribonucleoprotein A1. **EMBO J.** 13, 2904-2912.
- Bos, J.L., Fearon, E.R., Hamilton, S.R., Verlaan-de Vries, M., Van Boom, J.H., van der Eb, A.J., and Vogelstein, B. (1987). Prevalence of ras gene mutations in human colorectal cancers. **Nature** 327, 293-297.
- Buzayan, J.M., Gerlach, W.L., and Bruening, G. (1986). Non-enzymatic cleavage and ligation of RNAs complementary to a plant virus satellite RNA. **Nature** 323, 349-352.
- Cameron, F.H., and Jennigs, P.A. (1989). Specific gene suppression by engineered ribozymes in monkey cells. **Proc. Natl. Acad. Sci. USA** 86, 9139-9143.
- Cavanaugh, A.H., Hempel, W.M., Taylor, L.J., Rogalsky, V., Todorov, G., and Rothblum, L.I. (1995). Activity of RNA polymerase I transcription factor UBF blocked by Rb gene product. **Nature** 374, 177-180.
- Cech T.R., and Bass B.L. (1986). Biological catalysis by RNA. **Annu. Rev. Biochem.** 55, 599-629.
- Cersosimo, R.J., and Carr, D. (1996). Prostate cancer: current and evolving strategies. **Am. J. Health Syst. Pharm.** 53, 381-396.
- Cho, K.R., and Vogelstein, B. (1992). Genetic alterations in the adenoma-carcinoma sequence. **Cancer Suppl.** 70, 1727-1731.
- Chowrira, B.M., Pavco, P.A., and McSwiggen, J.A. (1994). In vitro and in vivo comparison of hammerhead, hairpin, and hepatitis delta virus self-processing ribozyme cassettes. **J. Biol. Chem.** 269, 25856-25864.
- Clawson, G.A., Schalles, S.L., Ren, L., and Norris, J.S. (1997). Construction and deployment of targeted triple ribozyme constructs. **Biochemistry**, submitted.
- Cleutjens K.B., van der Korput, H.A., van Eekelen, C.C., Sikes, R.A., Fasciana, C., Chung, L.W., and Trapman, J. (1997). A 6-kb promoter fragment mimics in transgenic mice the prostate-specific and androgen-regulated expression of the endogenous prostate-specific antigen gene in humans. **Mol. Endocrinol.** 11, 1256-1264.
- Cohen, P., Peehl, D.M., Graves, H.C., and Rosenfeld, R.G. (1994). Biological effects of prostate specific antigen as an IGF binding protein-3 protease. **J. Endocrinol.** 142, 407-415.
- Cotten, M., and Birnstiel, M.L. (1989). Ribozyme mediated destruction of RNA in vivo. **EMBO J.** 8, 3861-3866.

- Crisell, P., Thompson, S., and James, W. (1993). Inhibition of HIV-1 replication by ribozymes that show poor activity in vitro. **Nucleic Acids Res.** 21, 5251-5255.
- Dahiya, R., Deng, G., Chen, K.M., Chui, R.M., Haughney, P.C., and Narayan, P. (1996). p53 tumour-suppressor gene mutations are mainly localized on exon 7 in human primary and metastatic prostate cancer. **Br. J. Cancer** 74, 264-268.
- Deonarain, M.P., Spooner, R.A., and Epenetos, A.A. (1995). Genetic delivery of enzymes for cancer therapy. **Gene Ther.** 2, 235-244.
- Dropulic, B., Lin, N.H., Martin, M.A., and Jeang, K.T. (1992). Functional characterization of a U5 ribozyme: intracellular suppression of human immunodeficiency virus type 1 expression. **J. Virol.** 66, 1432-1441.
- Fedor M.J., and Uhlenbeck O.C. (1990). Substrate sequence effects on "hammerhead" RNA catalytic efficiency. **Proc. Natl. Acad. Sci. USA** 87, 1668-1672.
- Forster A.C., and Symons R.H. (1987). Self-cleavage of plus and minus RNAs of a virusoid and a structural model for the active sites. **Cell** 49, 211-220.
- Funato, T., Shitara, T., Tone, T., Jiao, L., Kashani-Sabet, M., and Scanlon, K.J. (1994). Suppression of H-ras-mediated transformation in NIH3T3 cells by a ras ribozyme. **Biochem. Pharmacol.** 48, 1471-1475.
- Funato, T., Yoshida, E., Jiao, L., Tone, T., Kashani-Sabet, M., and Scanlon, K.J. (1992). The utility of an anti-fos ribozyme in reversing cisplatin resistance in human carcinomas. **Adv. Enzyme Regul.** 32, 195-209.
- Goodchild, J., and Kohli, V. (1991). Ribozymes that cleave an RNA sequence from human immunodeficiency virus: the effect of flanking sequence on rate. **Arch. Biochem. Biophys.** 284, 386-391.
- Gottesman, M.M., and Pastan, I. (1993). Biochemistry of multidrug resistance mediated by the multidrug transporter. **Annu. Rev. Biochem.** 62, 385-427.
- Greenberg N.M., DeMayo, F.J., Sheppard, P.C., Barrios, R., Lebovitz, R., Finegold, M., Angelopoulou, R., Dodd, J.G., Duckworth, M.L., Rosen, J.M., and Matusik, R.J. (1994). The rat probasin gene promoter directs hormonally and developmentally regulated expression of a heterologous gene specifically to the prostate in transgenic mice. **Mol. Endocrinol.** 8, 230-239.
- Harris, J.D., and Lemoine, N.R. (1996). Strategies for targeted gene therapy. **Trends Genet.** 12, 400-405.
- Hart, I.R. (1996). Tissue specific promoters in targeting systemically delivered gene therapy. **Semin. Oncol.** 23, 154-158.
- Haseloff, J., and Gerlach, W.L. (1988). Simple RNA enzymes with new and highly specific endoribonuclease activities. **Nature** 334, 585-591.
- Helene, C., and Toulme, J.J. (1990). Specific regulation of gene expression by antisense, sense and antigene nucleic acids. **Biochim. Biophys. Acta** 1049, 99-125.
- Herschlag, D. (1991). Implications of ribozyme kinetics for targeting the cleavage of specific RNA molecules in vivo: more isn't always better. **Proc. Natl. Acad. Sci. USA** 88, 6921-6925.
- Herschlag, D., and Cech, T.R. (1990). DNA cleavage catalysed by the ribozyme from tetrahymena. **Nature** 344, 405-409.
- Holm, P.S., Scanlon, K.J., and Dietel, M. (1994). Reversion of multidrug resistance in the P-glycoprotein-positive human pancreatic cell line (EPP85-181RDB) by introduction of a hammerhead ribozyme. **Br. J. Cancer** 70, 239-243.
- James, H., Mills, K., and Gibson, I. (1996). Investigating and improving the specificity of ribozymes directed against the bcr-abl translocation. **Leukemia** 10, 1676.
- Jankowsky, E., and Schwenzer, B. (1996). Efficient improvement of hammerhead ribozyme mediated cleavage of long substrates by oligonucleotide facilitators. **Biochemistry** 35, 15313-15321.
- Joyce, G.F. (1989). RNA evolution and the origins of life. **Nature** 338, 217-224.
- Kashani-Sabet, M., Funato, T., Tone, T., Jiao, L., Wang, W., Yoshida, E., Kashfian, B.J., Shitara, T., Wu, A.M., and Moreno, J.G., et al. (1992). Reversal of the malignant phenotype by an anti-ras ribozyme. **Antisense Res. Dev.** 2, 3-15.
- Kashani-Sabet, M., Lu, Y., Leong, L., Haedicke, K., and Scanlon, K.J. (1990a). Differential oncogene amplification in tumor cells from a patient treated with cisplatin and 5-fluorouracil. **Eur. J. Cancer** 26, 383-390.
- Kashani-Sabet, M., Wang, W., and Scanlon, K.J. (1990b). Cyclosporin A suppresses cisplatin-induced c-fos gene expression in ovarian carcinoma cells. **J. Biol. Chem.** 265, 11285-11288.
- Kearney P., Wright, L.A., Milliken S., and Biggs J.C. (1995). Improved specificity of ribozyme-mediated cleavage of bcr-abl mRNA. **Exp. Hematol.** 23, 986-989.
- Kiehintopf, M., Brach, M.A., Licht, T., Petschauer, S., Karawajew, L., Kirschning, C., and Herrmann, F. (1994). Ribozyme-mediated cleavage of the MDR-1 transcript restores chemosensitivity in previously resistant cancer cells. **EMBO J.** 13, 4645-4652.
- Kijima, H., Ishida, H., Ohkawa, T., Kashani-Sabet, M., and Scanlon, K.J. (1995). Therapeutic applications of ribozymes. **Pharmacol. Ther.** 68, 247-267.
- Kobayashi, H., Dorai, T., Holland, J.F., and Ohnuma, T. (1994). Reversal of drug sensitivity in multidrug resistant tumor cells by an MDR1 (PGY1) ribozyme. **Cancer Res.** 54, 1271-1275.
- Koizumi, M., Kamiya, H., and Ohtsuka, E. (1992). Ribozymes designed to inhibit transformation of NIH3T3 cells by the activated c-Ha-ras gene. **Gene** 117, 179-184.
- Konety, B.R., and Getzenberg, R.H. (1997). Novel therapies for advanced prostate cancer. **Semin. Urol. Oncol.** 15, 33-42.
- Kubota, Y., Fujinami, K., Uemura, H., Dobashi, Y., Miyamoto, H., Iwasaki, Y., Kitamura, H., and Shuin, T. (1995). Retinoblastoma gene mutations in primary human prostate cancer. **Prostate** 27, 314-320.
- Kurzrock, R., Gutterman, J.U., and Talpaz, M. (1988). The molecular genetics of Philadelphia chromosome-positive leukemias. **N. Engl. J. Med.** 319, 990-998.
- Lange, W., Daskalakis, M., Finke, J., and Dolken, G. (1994). Comparison of different ribozymes for efficient and specific

- cleavage of BCR/ABL related mRNAs. **FEBS Lett.** 338, 175-178.
- Lee, C.H., Liu, M., Sie, K.L., and Lee, M.S. (1996). Prostate-specific antigen promoter driven gene therapy targeting DNA polymerase- α and topoisomerase II α in prostate cancer. **Anticancer Res.** 16, 1805-1812.
- Matusik, R.J., Kreis, C., McNichol, P., Sweetland, R., Mullin, C., Fleming, W.H., and Dodd, J.G. (1986). Regulation of prostatic genes: roles of androgens and zinc in expression. **J. Biochem. Cell Biol.** 64, 601-607.
- Miller, N., and Vile, R. (1995). Targeted vectors for gene therapy. **FASEB J.** 9, 190-199.
- Moss, T., and Stefanovsky, V.Y. (1995). Promotion and regulation of ribosomal transcription in eukaryotes by RNA polymerase I. **Prog. Nucleic Acids Res. Mol. Biol.** 50, 25-66.
- Ohkawa, J., Yuyama, N., Takebe, Y., Nishikawa, S., and Taira, K. (1993). Importance of independence in ribozyme reactions: kinetic behavior of trimmed and of simply connected multiple ribozymes with potential activity against human immunodeficiency virus. **Proc. Natl. Acad. Sci. USA** 90, 11302-11306.
- Ohta, Y., Kijima, H., Ohkawa, T., Kashani-Sabet, M., and Scanlon, K.J. (1996). Tissue-specific expression of an anti-ras ribozyme inhibits proliferation of human malignant melanoma cells. **Nucleic Acids Res.** 24, 938-942.
- Ohta, Y., Tone, T., Shitara, T., Funato, T., Jiao, L., Kashfian, B.I., Yoshida, F., Horng, M., Tsai, P., and Lauterbach, K., et al. (1994). H-ras ribozyme-mediated alteration of the human melanoma phenotype. **Ann. N.Y. Acad. Sci. USA** 716, 242-253.
- Pang, S., Dannull, J., Kaboo, R., Xie, Y., Tso, C.L., Michel, K., de Kernion, J.B., and Belldgrun, A.S. (1997). Identification of a positive regulatory element responsible for tissue-specific expression of prostate-specific antigen. **Cancer Res.** 57, 495-499.
- Pang, S., Taneja, S., Dardashti, K., Cohan, P., Kaboo, R., Sokoloff, M., Tso, C.L., Dekernion, J.B., and Belldgrun, A.S. (1995). Prostate tissue specificity of the prostate-specific antigen promoter isolated from a patient with prostate cancer. **Hum. Gene Ther.** 6, 1417-1426.
- Parker, S.L., Tong, T., Bolden, S., and Wingo, P.A. (1997). Cancer statistics. **CA Cancer J. Clin.** 47, 5-27.
- Peehl, D.M. (1995). Prostate specific antigen role and function. **Cancer Suppl.** 75, 2021-2026.
- Price, D. (1963). Comparative aspects of development and structure in the prostate. In **Biology of the Prostate and Related Tissues**, E.P. Vollmer and G. Kauffman, eds. (Washington D.C., US Government Printing Office), 1-28.
- Pyle, A.M. (1993). Ribozymes: a distinct class of metalloenzymes. **Science** 261, 709-714.
- Roberts, J.D., Bebenek, K., and Kunkel, T.A. (1988). The accuracy of reverse transcriptase from HIV-1. **Science** 242, 1171-1173.
- Rossi, J.J., Cantin, E.M., Zaia, J.A., Ladne, P.A., Chen, J., Stephens, D.A., Sarver, N., and Chang, P.S. (1990). Ribozymes as therapies for AIDS. **Ann. N.Y. Acad. Sci.** 616, 184-200.
- Ruffner D.E., Stormo G.D., and Uhlenbeck O.C. (1990). Sequence requirements of the hammerhead RNA self-cleavage reaction. **Biochemistry** 29, 10695-10702.
- Ruiz, J., Wu, C.H., Ito, A.Y., and Wu, G.Y. (1997). Design and preparation of a multimeric self-cleaving hammerhead ribozyme. **Biotechniques** 22, 338-345.
- Sarver, N., Cantin, E.M., Change, P.S., Zaia, J.A., Ladne, P.A., Stephens, D.A., and Rossi, J.J. (1990). Ribozymes as potential anti-Hiv-1 therapeutic agents. **Science** 247, 1222-1225.
- Scanlon, K.J., Ishida, H., and Kashani-Sabet, M. (1994). Ribozyme-mediated reversal of the multidrug-resistant phenotype. **Proc. Natl. Acad. Sci. USA** 91, 11123-11127.
- Scanlon, K.J., Jiao, L., Funato, T., Wang, W., Tone, T., Rossi, J.J., and Kashani-Sabet, M. (1991). Ribozyme-mediated cleavage of c-fos mRNA reduces gene expression of DNA synthesis enzymes and metallothionein. **Proc. Natl. Acad. Sci. USA** 88, 10591-10595.
- Scanlon, K.J., Kashani-Sabet, M., Miyachi, H., Sowers, L.C., and Rossi, J.J. (1989). Molecular basis of cisplatin resistance in human carcinomas: model systems and patients. **Anticancer Res.** 9, 1301-1312.
- Scanlon, K.J., Ohta, Y., Ishida, H., Kijima, H., Ohkawa, T., Kaminski, A., Tsai, J., Horng, G., and Kashani-Sabet, M. (1995). Oligonucleotide-mediated modulation of mammalian gene expression. **FASEB J.** 9, 1288-1296.
- Scanlon, K.J., Wang, W.Z., and Han, H. (1990). Cyclosporin A suppresses cisplatin-induced oncogene expression in human cancer cells. **Cancer Treat. Rev. Suppl.** 17, 27-35.
- Scarabino, D., and Tocchini-Valentini, G.P. (1996). Influence of substrate structure on cleavage by hammerhead ribozyme. **FEBS Lett.** 383, 185-190.
- Shimayama, T., Nishikawa, F., Nishikawa, S., and Taira, K. (1993). Nuclease-resistant chimeric ribozymes containing deoxyribonucleotides and phosphorothioate linkages. **Nucleic Acids Res.** 21, 2605-2611.
- Shore, S.K., Nabissa, P.M., and Reddy, E.P. (1993). Ribozyme-mediated cleavage of the BCRABL oncogene transcript: in vitro cleavage of RNA and in vivo loss of P210 protein-kinase activity. **Oncogene** 8, 3183-3188.
- Smith, M.J., Rousculp, M.D., Goldsmith, K.T., Curiel, D.T., and Garver, R.I. Jr. (1994). Surfactant protein A-directed toxin gene kills lung cancer cells in vitro. **Hum. Gene Ther.** 5, 29-35.
- Snyder, D.S., Wu, Y., Wang, J.L., Rossi, J.J., Swiderski, P., Kaplan, B.F., and Forman, S.J. (1993). Ribozyme-mediated inhibition of bcr-abl gene expression in a Philadelphia chromosome-positive cell line. **Blood** 82, 600-605.
- Spence, A.M., Sheppard, P.C., Davie, J.R., Matuo, Y., Nishi, N., McKeehan, W.L., Dodd, J.G., and Matusik, R.J. (1989). Regulation of a bifunctional mRNA results in synthesis of secreted and nuclear probasin. **Proc. Natl. Acad. Sci. USA** 86, 7843-7847.
- Taira, K., Nakagawa, K., Nishikawa, S., and Furukawa K. (1991). Construction of a novel RNA-transcript-trimming

- plasmid which can be used both in vitro in place of run-off and (G)-free transcriptions and in vivo as multi-sequences transcription vectors. **Nucleic Acids Res.** 19, 5125-5130.
- Talyor, N.R., Kaplan, B.E., Swiderski, P., Li, H., and Rossi, J.J. (1992). Chimeric DNA-RNA hammerhead ribozymes have enhanced in vitro catalytic efficiency and increased stability in vivo. **Nucleic Acids Res.** 20, 4559-4565.
- Teeter, L.D., Eckersberg, T., Tsai, Y., and Kuo, M.T. (1991). Analysis of the Chinese hamster P-glycoprotein/multidrug resistance gene pgpl reveals that the AP-1 site is essential for full promoter activity. **Cell Growth Differ.** 2, 429-437.
- Tone, T., Kashani-Sabet, M., Funato, T., Shitara, T., Yoshida, E., Kashfian, B.I., Horng, M., Fodstad, O., and Scanlon, K.J. (1993). Suppression of EJ cells tumorigenicity. **In Vivo** 7, 471-476.
- Uhlenbeck, O.C. (1987). A small catalytic oligoribonucleotide. **Nature** 328, 596-600.
- Ventura, M., Wang, P., Ragot, T., Perricaudet, M., and Saragosti, S. (1993). Activation of HIV-specific ribozyme activity by self-cleavage. **Nucleic Acids Res.** 21, 3249-3255.
- Voeks, D.J., Staley, M.C., Greenberg, N.M., Matusik, R.J., Clawson, G.A., and Norris, J.S. (1997). Prostate cancer gene therapy using targeted expression of ribozymes against the AC40 subunit of RNA pol1. In **Cancer Gene Therapy**, L. Savage, ed. (Southborough, MA, IBC), in press.
- Watt, K.W., Lee, P.J., M'Timkulu, T., Chan, W.P. and Loor, R. (1986). Human prostate-specific antigen: structural and functional similarity with serine proteases. **Proc. Natl. Acad. Sci. USA** 83, 3166-3170.
- Weerasinghe, M., Liem, S.E., Asad, S., Read, S.E., and Joshi, S. (1991). Resistance to human immunodeficiency virus type 1 (HIV-1) infection in human CD4+ lymphocyte-derived cell lines conferred by using retroviral vectors expressing an HIV-1 RNA-specific ribozyme. **J. Virol.** 65, 5531-5534.
- Wright, L., Wilson, S.B., Milliken, S., Biggs, J., and Kearney, P. (1993). Ribozyme-mediated cleavage of the bcr/abl transcript expressed in chronic myeloid leukemia. **Exp. Hematol.** 21, 1714-1719.
- Yamada, O., Yu, M., Yee, J.K., Kraus, G., Looney, D., and Wong-Staal, F. (1994). Intracellular immunization of human T cells with a hairpin ribozyme against human immunodeficiency virus type 1. **Gene Ther.** 1, 38-45.
- Yan, Y., Sheppard, P.C., Kasper, S., Lin, L., Hoare, S., Kapoor, A., Dodd, J.G., Duckworth, M.L., and Matusik, R.J. (1997). A large fragment of the probasin promoter targets high levels of transgene expression to the prostate of transgenic mice. **Prostate**, in press.
- Yu, M., Ojwang, J., Yamada, O., Hampel, A., Rappaport, J., Looney, D., and Wong-Staal, F. (1993). A hairpin ribozyme inhibits expression of diverse strains of human immunodeficiency virus type 1. **Proc. Natl. Acad. Sci. USA** 90, 6340-6344.
- Yu, M., Poeschla, E., and Wong-Staal, F. (1994). Progress towards gene therapy for HIV-1 infection. **Gene Ther.** 1, 13-26.
- Zhang, S., Murtha, P.E., and Young, C.Y. (1997). Defining a functional androgen responsive element in the 5' far upstream flanking region of the prostate-specific antigen gene. **Biochem. Biophys. Res. Commun.** 231, 784-788.