Arch. Pharm. Res. Vol.19, No.2, pp. 116-121, 1996

Percutaneous Absorption of Antisense Phosphorothioate Oligonucleotide *in vitro*

Young Mi Lee, Kyung Song, Sung Hee Lee, Geon Il Ko, Jae Baek Kim and Dong Hwan Sohn

College of Pharmacy, and Medicinal Resource Research Center, Wonkwang University, Iksan city, Cheon-Buk 570-749, Korea

(Recived December 16, 1995)

Antisense oligonucleotides seem to provide a promising new tool for the therapy. Choi *et al.* (1995) reported antisense phosphorothioate oligonucleotides (PS-ODN, 25 mer) complementary to TGF-β mRNA designed for scar formation inhibitor to eliminate scars, which was caused by undesired collagen deposition due to overexpression of TGF-β, in wounded skin. PS-ODN were evaluated *in vitro* for skin penetration using normal and tape-stripped damaged rat skin. The *in vitro* skin transports were carried out with partially modified PS-ODN (6S) and fully modified PS-ODN (25S). The cumulative amount of PS-ODN (6S) penetrated through normal rat skin was 0.234 ± 0.041 μg/cm^2 and that of tape-stripped damaged rat skin was 1.077 ± 0.301 μg/cm^2 over 8 hrs. PS-ODN (25S) can not be found in receptor medium through normal skin due to high molecular weight (Mol.Wt.=8,000) and polyanionic charge. However, the cumulative amount of PS-ODN (25S) penetrated across damaged rat skin in PBS was 0.340 ± 0.296 μg/cm^2 over 8 hrs. The absense of dermis raised the cumulative amount of PS-ODN (6S) penetrated through rat skin. And the fluxes of PS-ODN (6S) and PS-ODN (25S) at 8hrs across damaged rat skin were 134.63 ± 37.67 ng/cm^2 h, and 42.50 ± 36.95 ng/cm^2 h, respectively. While PS-ODN (25S) was stable in 10% heat inactivated fetal bovine serum (FBS) during 24 hrs, PS-ODN (6S) was less stable than PS-ODN (25S), but was markedly stable than unmodified phosphodiester. It is suggested that the cumulative amount of PS-ODN (6S) penetrated through damaged rat skin is larger than that of PS-ODN (25S) since the former is easier to degrade by nuclease than the latter and then is apt to penetrate into skin. Thus, PS-ODN represents a logical candidate for further evaluation due to the potential for delivery into the wounded skin.

Key words : Antisense oligonucleotide, Phosphorothioate, Transforming growth factor-β, Percutaneous absorption

INTRODUCTION

Antisense oligonucleotide technology (Uhlman *et al.*, 1990) presents an exciting new avenue for therapy of many significant diseases, including viral infections and cancer. However, before this can successfully accomplished, a number of potential barriers to antisense therapeutics must be overcome. Firstly, oligonucleotide should be adequately stabilized against nucleases in biological fluids such as blood, serum or plasma and in different cell compartments. Second set of problems is linked to the poor penetration of polyanionic charged oligonucleotide into cells. Most antisense compounds are relatively large molecules(ranging from about 500 to over 10,000 Mol. Wt.) and are relatively polar. A third series of problems arises from the difficulty of defining the most adequate target nucleic acid sequence. These molecules would need to remain stable and not degrade within the extracellular environment for a desired time, long enough to allow sufficient amount of the antisense oligonucleotide to enter cells and exert the desired biological activity (Shoji *et al.*, 1991). A number of publications report that the biological stability can be improved by modifying the oligonucleotides (Agrawal *et al.*, 1991; Hoke *et al.*, 1991; Akhtar *et al.*, 1991) and, despite their polarity and large molecular weights, these molecules are taken up by cells (Shoji *et al.*, 1991; Jaroszewski *et al.*, 1991) of the wound by various cell types. Among the cytokine, TGF-β and PDGF are highly fibrogenic cytokine (Whitby *et al.*, 1991). Multiple events involving TGF-β take place in tissue repair after injury (Cromack *et al.*, 1987; Sporn *et al.*,

Correspondence to: Dong Hwan Sohn, Ph. D., College of Pharmacy, and Medicinal Resource Research Center, Wonkwang University, Iksan, Cheon-Buk 570-749, Korea

1989). Recent data suggested that level of the TGF-β gene was significantly decreased (Choi *et al.*, 1995) after application of antisense oligonucleotide complementary to TGF-β in adult wound.

The local administration of PS-ODN (Sharples *et al.*, 1987; Derynck *et al.*, 1985) complementary to TGF-β mRNA has been elaborate to circumvent the complexity of targeted delivery (Saffran *et al.*, 1986), reduce the huge costs of therapy, and prompt to facilitate practical applications. These PS-ODN would need to remain stable and not degrade within the extracellular environment for a desired time, long enough to allow sufficient amount of the PS-ODN to enter cells and exert the desired biological activity. Nolen III *et al.* (1994) reported the percutaneous penetration of methyl phosphonate antisense oligonucleotides. Although methylphosphonate have better permeability than phosphorothioate, sequence specificity will be reduced by changing of physical properties. The ultimate purpose of the present research is to find out optimum formulations of PS-ODN available for the wounded skin administration. Preferentially, *in vitro* skin permeation across skin of PS-ODN (25 mer) was investigated.

MATERIALS AND METHODS

Materials

Antisense agent® and NAP-10 were purchased from Pharmacia LKB Biotechnology. ^{35}S-ATPγS and T4 polynucleotide kinase were purchased from Amersham International plc. Microcon-3 (Mol. wt. 3, 000 cut off) was purchased from Amicon, Inc. Other reagents were all of special reagent grade.

Synthesis of PS-ODN

PS-ODN (6S) were synthesized on an automated DNA synthesizer (Plodel, Gene assembler special, Pharmacia LKB Biotechnology, Uppsala, Sweden) at the Korea Biotechnology. The sequence of PS-ODN (6S) is as follows; (a) 5'-C(*)AG CCC(*) GGA GG(*)G CGG C(*)AT GGG(*) GGA(*) G-3', (b) 5'-XC(*)AG CCC(*) GGA GG(*)G CGG C(*)AT GGG(*) GGA(*) G-3'. Asterisk and X represent position of sulfur and fluorescence, respectively. All PS-ODN were lyophilized and purified by NAP-10 column (Pharmacia LKB Biotechnology). The PS-ODN (25S) was synthesized at ABI company, USA and purified using perfusion chromatography at PerSeptive Biosystems, USA.

^{35}S-labeling of PS-ODN

PS-ODN was 5'-end labeled with ^{35}S-γ ATP and T4 polynucleotide kinase (Amersham International plc.) in reaction mixture (Shaw *et al.*, 1991). A reaction mixture set up as follows; 2 μl oligonucleotide (20 pmoles), 4 μl 5×T4 polynucleotide kinase buffer containing 300 mM Tris Cl (pH 7.6), 50 mM $MgCl_2$, 1.65 μM ATP, 75 mM 2-mercaptoethanol, 2 μl ^{35}S-γ ATP (specific activity 1,000 Ci/mmole; 10 mCi/ml in aqueous solution) (20 pmoles), 10 μl ddH_2O, 2 μl T4 polynucleotide kinase (10 units/μl). It was mixed well and incubated for 45 minutes at 37°C. The remainder of the reaction was heated for 10 minutes at 68°C to inactivate the T4 polynucleotide kinase. Labeled PS-ODN was purified by Microcon-3 (Mol. wt. 3,000 cut off, Amicon, Inc.).

Skin preparation

On the day before the experiment, the hair of the dorsal area of rats was removed with an electric clipper and depilatory. On the next day, pieces of full thickness dorsal skin were excised from the rat. The rats were purchased from Korea Research Institute of Chemical Technology. The adherent fat and other debris were removed from the under surface. Damaged skin was prepared by removing the stratum corneum by repeated application of cellophane tape (about 15 times) until the skin glistened. Removal of dermis was accomplished by immersion at 60°C water for 1 minute. The dermal side of the skin was soaked in phosphate buffered saline (PBS, pH 7.4) for 4 hrs at 37°C.

In vitro skin transport studies

In vitro skin transport study was investigated according to the method of Nolen III *et al.* (1994). The receptor compartment of each franz diffusion cell (Crown Glass Co. USA) was filled with 10 ml of PBS (pH 7.4) containing 0.05% sodium azide. The jacked receptor compartments were kept at 37±0.1°C sufficient to maintain the temperature of the receptor just below the skin at 32±0.5°C. And the available surface area for penetration was 1.77 cm^2. Receptor compartment was mixed through out the experiment with teflon magnetic stirring bar driven by a constant speed (300 rpm) motor. ^{35}S-labeled PS-ODN (10 μg/100 μl) was uniformly applied to the donor side and occluded with a sheet of aluminum foil and parafilm. 100 μl samples were withdrawn periodically and replaced with an equal volume of fresh normal medium maintained at 37°C. The drug permeation was monitored for 24 hrs. The concentrations of fluorescence-labeled PS-ODN sample were determined by spectrofluorimetry (Ex. 460 nm, Em. 517 nm). The sample of ^{35}S-labeled PS-ODN (100 μl) was mixed with 6 ml of scintillation cocktail (Amersham International plc.) and the concentrations were det-

ermined using a liquid scintillation system (Beckman LS 7800).

Stability study of PS-ODN (6S) and PS-ODN (25S) in 10% fetal bovine serum (FBS)

Degradation study of PS-ODN was examined according to the methods of Akhtar *et al.* (1991) 5'-end [^{35}S] labeled PS-ODN (20 μg, specific activity 2×10^6 cpm) was incubated with individual 10% heat inactivated FBS at 37°C for 48 hrs in a reaction mixture containing 4 mM HEPES buffer (pH 7.9), 250 mM KCl, 0.5 mM EDTA, 0.1 mM DTT, 0.6 mM $MgCl_2$, 4% glycerol and 2%v/v poly vinyl alcohol. At timed intervals, aliquots (10 μl) of the reaction mixture were removed. Sample was loaded onto gel of 20% polyacrylamide/7M urea. Radioactive bands were visualized by autoradiography after a 3 days exposure at room temperature.

Analysis of data

The *in vitro* percutaneous permeation parameters were calculated from the penetration data by using the following equations (Chow *et al.*, 1984),

$$D = l^2/6T$$
$$Js = DKm\ Cs/l$$
$$Kp = D\ Km/l$$

where Js is the penetration rate, D denotes the diffusion constant within skin, Km is the skin/vehicle partition coefficient of PS-ODN, T represents the lag time calculated from the intercept of the cumulative amount of PS-ODN penetrated across skin with the time axis. Kp denotes the permeability coefficient through the stratum corneum and Cs is the PS-ODN concentration.

RESULTS AND DISCUSSION

Permeation of ^{35}S-labeled PS-ODN(6S) using rat skin

Table I shows the cumulative amount of ^{35}S-labeled PS-ODN (6S) penetrated through rat skin with the stratum corneum intact and with the stratum corneum removed by tape stripping. The cumulative amount of PS-ODN(6S) penetrated during 24 hrs were 0.498±0.044 mg/cm^2 in intact skin and 1.446±0.224 mg/cm^2 in damaged skin. The ratio of the cumulative amount of PS-ODN (6S) penetrated to initial loading dose during 24 hrs were 8.8% in normal skin and 25.6% in damaged skin. It was increased about 3 fold by removal of stratum corneum. This finding shows that the stratum corneum is largely responsible for controlling the permeation rate of PS-ODN (6S). Surprisingly, despite their polarity and large molecular weights, these molecules were penetrated through normal rat skin. It might be due to degradation of PS-ODN (6S) by nuclease within skin.

Table I. The cumulative amount (mg/cm^2) of PS-ODN (6S) penetrated across rat skin

Time (hr)	Normal skin	Damaged skin
1	0.011±0.002	0.096±0.115
4	0.089±0.078	0.657±0.226
8	0.234±0.041	1.077±0.301
12	0.466±0.058	1.185±0.215
24	0.498±0.044	1.446±0.224

Each value represent the mean±S.E.(n=3).

Permeation of ^{35}S-labeled PS-ODN (6S) in the presence of dermis

The effect of the dermis on the permeation of a PS-ODN (6S) through rat skin was also examined. Fig. 1 shows the cumulative amount of PS-ODN (6S) penetrated through rat skin with or without dermis in PBS at 8 hrs. A comparison of the cumulative amount of PS-ODN (6S) penetrated shows that skin without dermis was only slightly more permeable than skin with dermis. For example, the cumulative amount of PS-ODN (6S) penetrated across normal skin with dermis was 0.159±0.02 μg/cm^2 compared with 0.234±0.041 μg/cm^2 of skin without dermis. Therefore, the cumulative amount penetrated across the normal rat skin was decreased 1.47 fold by the presence of dermis. And in the case of damaged rat skin, the cumulative amount of PS-ODN (6S) penetrated across damaged skin were decreased 1.7 fold from 1.077±0.301 μg/cm^2 to 0.633±0.30 μg/cm^2 by the presence of dermis. These results suggest that PS-ODN (6S) may

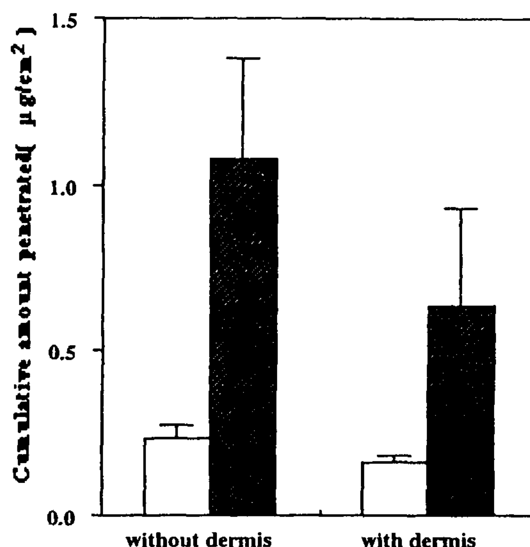

Fig. 1. The effect of rat skin with or without dermis on skin the cumulative amount of PS-ODN(6S) at 8hrs. Each value represent the mean±S.E (n=3). Key: □; Normal skin, ▨; Damaged skin

be remained considerably in dermal layer of skin.

Permeation of ^{35}S-labeled PS-ODN (25S) using rat skin

The penetration ability of PS-ODN (25S) was examined to describe the fact that PS-ODN (6S) penetrates through normal rat skin. The results are shown in Fig. 2. The ratio of the cumulative amount of PS-ODN (6S) penetrated to initial loading dose during 24 hrs were 8.8±0.74% in normal skin and 25.6±5.33% in damaged skin. However, PS-ODN (25S) was not detected in the receptor medium at 24 hrs through normal rat skin due to high molecular weight (Mol. Wt.=8,000) and polyanionic charge. Although there was a lag time of about 2.10±0.30 hr through tape-stripped damaged rat skin, PS-ODN (25S) was permeated 14.17% of loading dose over 24 hrs. The cumulative amount of PS-ODN (6S) penetrated through rat skin were considerably higher than those of PS-ODN (25S). As described previously, the removal of the stratum corneum generally led to higher permeation rate of PS-ODN.

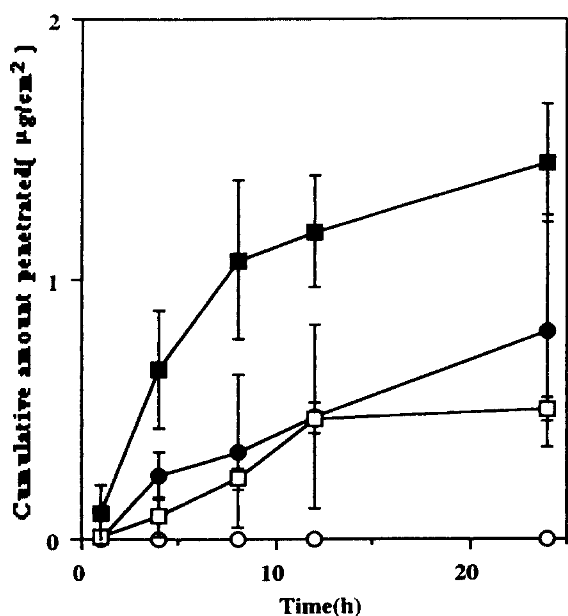

Fig. 2. *In vitro* permeation profiles of PS-ODN(6S) and PS-ODN(25S) across rat skin. Each point represents the mean± S.E. (n=3). Key: PS-ODN(25S)- ○; Normal skin, ●; Damaged skin, PS-ODN(6S)- □; Normal skin, ■; Damaged skin

Penetration parameters of PS-ODN (6S) and PS-ODN (25S) through damaged rat skin at 8hrs after permeation study was listed in Table II. In this paper, we focused on damaged skin because PS-ODN was developed as scar removing agent. The flux of PS-ODN (6S) through damaged rat skin is considerably higher than that of PS-ODN (25S). The flux of PS-ODN (25S) was about 3.17 times less than that of PS-ODN(6S). And lag time was increased from 0.70±0.02 hr to 2.10±0.30 hr. The lag time was estimated by extrapolation of the cumulative amount (mg/cm^2) penetrated across skin versus time plot at steady state to zero. Permeability coefficient (Kp) was decreased 3.17 times from 1.346±0.377×10^{-3} cm/h to 0.425±0.370×10^{-3} cm/h. Compared with the penetration data gathered with the PS-ODN (6S), the PS-ODN (25S) substantially reduced the amount permeated through rat skin. However, very little of the PS-ODN (25S) was found to permeate through the skin, suggesting that topical administration of the PS-ODN (25S) may lead to lower systemic delivery as compared with the PS-ODN (6S). It suggests that, despite their polarity and large molecular weights, both molecules can be penetrated through damaged rat skin. However, while PS-ODN (25S) was undetectable in the receptor medium at 24 hrs through normal rat skin, PS-ODN (6S) was penetrated across normal rat skin. It seemed that PS-ODN (6S) is less stable than PS-ODN (25S) in skin during skin transport.

Stability study of PS-ODN (6S) and PS-ODN (25S) in 10% FBS

Stability study of PS-ODN (6S) and PS-ODN (25S) were carried out in 10% heat inactivated fetal bovine serum (FBS), 37°C for 48 hrs to explain the increased the cumulative amount of PS-ODN (6S) penetrated across normal rat skin. Fig. 3 shows the comparative stability of PS-ODN (6S) and PS-ODN (25S) in 10% FBS. While the PS-ODN (25S) was stable during 24 hrs in 10% FBS, PS-ODN (6S) was less stable. However, PS-ODN (6S) was more stable than unmodified

Table II. Penetration parameters of PS-ODN (6S) and PS-ODN (25S) across damaged rat skin at 8hrs after permeation study

	Flux (ng/cm^2·h)	Lag time (h)	Permeability coefficient (Kp^a,cm/h × 10^{-3})	Diffusion constant (CD^b,cm^2/h × 10^{-6})	Partition coefficient (Km)^c
PS-ODN (6S)	134.63±37.67	0.70±0.02	1.346±0.377	2.438±0.070	1.776±0.46
PS-ODN(25S)	42.50±36.95	2.10±0.30	0.425±0.370	0.818±0.199	1.668±1.44

Each value represent the mean±S.E. (n=3).
Thickness of rat skin obtained using microscope is 32 μm.
[a]Kp denotes the permeability coefficient through the stratum corneum.
[b]D denotes the diffusion constant within skin.
[c]Km is the skin/vehicle partition coefficient of PS-ODN.

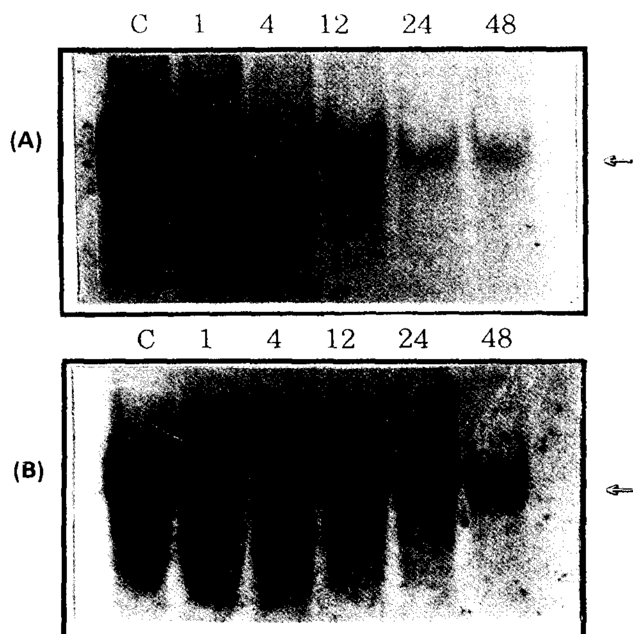

Fig. 3. Comparative stability of PS-ODN (6S) and PS-ODN (25S) in 10% FBS. ^{35}S-labeled PS-ODN (specific activity 2× 10^6 cpm) were incubated with 10% FBS at 37°C during 48 hrs. 10 μl of sample was load onto gel of 20% polyacrylamide/7M urea. Radioactive bands were visualized by autoradiography after a 3 days exposure at room temperature. (A) PS-ODN (6S), (B) PS-ODN (25S). Arrow denotes position of full length 25 mer PS-ODN. Numbers at top of gel represent time in hours.

phosphodiester. This can be also proved by the result that while PS-ODN (25S) across normal skin was not detected in receptor medium, PS-ODN (6S) was measurable during the transport. This results suggest that PS-ODN (6S) is easier to degrade by nuclease within skin than PS-ODN (25S) during *in vitro* skin transport study and then degraded PS-ODN is apt to penetrate into skin. However, since the nuclease within skin is more abundant than in serum, PS-ODN (6S) during skin transport will be more degraded than that presumed by this stability result. Thus, the increased the cumulative amount of PS-ODN (6S) penetrated across normal rat skin may be explained by these results. Additional experiments should be studied if some of the PS-ODN exist in epidermis and dermis. Moreover, stability test should be examined whether PS-ODN remained in skin and receptor medium after skin transport is intact.

In conclusion, these results indicate that development of topical dosage forms using more stable PS-ODN (25S) than PS-ODN (6S) for scar removing would be possible with minimal systemic exposure with the PS-ODN (25S).

ACKNOWLEDGEMENTS

This study was partially supported by grants from Il Yang Pharm.Co. and Ministry of Health and Welfare, Korea.

REFERENCES CITED

Agrawal, S., Temsamani, J. and Tang, J. Y., Pharmacokinetics, biodistribution, and stability of oligonucleotide phosphorothioates in mice. *Proc. Natl. Acad. Sci., USA*, 88, 7595-7599 (1991).

Akhtar, S., Kole, R. and Juliano, R.L., Stability of antisense DNA oligodeoxynucleotide analogs in cellular extracts and sera. *Life Sci.*, 49, 1793-1801 (1991).

Choi, B. M., Kim, S. U., Lee, S. Y. and Chung, H. T., Control of Scarring in adult wounds using antisense transforming growth factor β1. *Antisense Res. Dev.*, 5, 96 (1995).

Chow, D. S. L., Kaka, I. and Wang, T. I., Concentration-dependent enhancement of 1-dodecylazacycloheptan-2-one on the percutaneous penetration kinetics of triamcinolone acetonide. *J. Pharm. Sci.*, 73, 1794-1799 (1984).

Cromack, D. T., Sporn, M. B., Roberts, A. B., Merino, M. J., Dart, L. L. and Norton, J. A., Transforming growth factor β levels in rat wound chambers. *J. Sur. Res.*, 42, 622-628 (1987).

Derynck, R., Jarrett, J. A., Chen, E. Y., Eaton, D. H., Bell, J. R., Assoian, R. K., Roberts, A. B., Sporn, M. B. and Goeddel, D. V., Human transforming growth factor-β complementary DNA sequence and expression in normal and transformed cells. *Nature*, 316, 701-705 (1985).

Hoke, G. D., Draper, K., Freier, S. M., Gonzalez, C., Driver, V. B., Zounes, M. C. and Ecker, D., Effect of phosphorothioate capping on antisense oligonucleotides stability, hybridization and antiviral efficacy versus herpes simplex virus infection. *Nucleic Acids Res.*, 19, 5743-5748 (1991).

Jaroszewski, J.W. and Cohen, J. S., Cellular uptake of antisense oligodeoxynucleotides. *Adv. Drug Delivery. Rev.*, 6, 235-250 (1991).

Nolen III, H. W., Catz, P., and Friend, D. R., Percutaneous penetration of methyl phosphonate antisense oligonucleotides. *Int. J. Pharm.*, 107, 169-177 (1994).

Saffran, M., Kumar, G. S., Savariar, C., Burnham, J. C., Williams, F. and Neckers, D. C., A new approach to the oral administration of insulin and other peptide drugs. *Reports*, 233, 1081-1084 (1986).

Sharples, K., Plowman, G. D., Rose, T. M., Twardzik, D. R. and Purchio, A. F., Cloning and sequence analysis of simian transforming growth factor β cDNA. *DNA*, 6, 239-244 (1987).

Shaw, J. P., Kent, K., Bird, J. and Froehler, B., Modified deoxyoligonucleotides stable to exonuclease degradation in serum. *Nucleic Acid Res.*, 19, 747-

750 (1991).

Shoji, Y., Akhtar, S., Periasamy, A., Herman, B. and Juliano, R. L., Mechanism of cellular uptake of modified oligodeoxynucleotides containing methylphosphonate linkages. *Nucleic Acids Res.*, 19, 5543-5550 (1991).

Sporn, M. B. and Roberts, A. B., Transforming Growth Factor β; Multiple actions and potential clinical applications. *JAMA*, 262, 938-941 (1989).

Uhlman, E. and Peyman, A., Antisense oligonucleotides : A new therapeutic principle. *Chem. Rev.*, 90, 544-584 (1990).

Whitby, D. J. and Ferguson, M. W. J., Immunohistochemical localization of growth factors in fetal wound healing. *Dev. Biol.*, 147, 207-215 (1991).