Optimization of antisense drug design against conservative local motif in simulant secondary structures of *HER*-2 mRNA and QSAR analysis [1]

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ABSTRACT

AIM: To study the role of mRNA secondary structure stability in antisense drug design and obtain better antisense candidates against *neu*/*HER*-2/*erbB*-2 mRNA than previous report. **METHODS:** Program RNAstructure was utilized to simulate the secondary structures of *HER*-2 mRNA. Then 21 antisense phosphorothioate oligodeoxynucleotides (S-ODN) targeting different parts of secondary structural motif were designed. HA4 was set as positive control. Mean 50 % inhibitory effects (IC_{50}) of S-ODN on proliferations of SK-BR-3 breast cancer cells were evaluated. The expression of target mRNA was detected by RT-PCR. The multiple regression and quantitative structure-activity relationship (QSAR) analysis was preformed by SPSS software. **RESULTS:** One optimal and two suboptimal secondary structures of target mRNA were obtained. Nine out of 11 S-ODN against completely conservative local motif (LM) (conservative among all simulant secondary structures) got lower or similar IC_{50} values compared with HA4. On the other hand, 2 out of 3 S-ODN against relatively conservative LM (conservative between any two simulant secondary structures) got lower or similar IC_{50} values compared with HA4. Only 2 out of 5 S-ODN targeting variable LM (variable among different predicted secondary structures) had acceptable activities. Average IC_{50} of S-ODN against completely conservative LM was significantly lower than that of S-ODN against diverse LM. QSAR analysis suggested that stability, base number of bulge loops, and target free energies ΔG^{o}_{T} were statistically significant. In the multiple regression, R was 0.967, P=0.005. **CONCLUSION:** Antisense drug design against conservative LM was helpful for improving the positive rate. Several S-ODN candidates better than positive control were screened.

INTRODUCTION

The *HER-2*/*neu* gene, also called c-*erbB*-2, encodes a 185000-M_r glycoprotein with intrinsic tyrosine kinase activity[1]. Overexpression of the *HER*-2 oncogene, and its protein product, 185^{HER-2} (p185), is seen in approximately 30 % of human breast carcinoma[2]. Overexpression of *HER*-2 is a negative prognostic factor following tumor resection and may be associated with increased resistance to cancer chemotherapy[3]. Herceptin, one kind of humanized antibody to HER-2, has been shown to have an activity as a single anti-

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tumor agent in a phase II trial of heavily pretreated patients with advanced breast cancer, especially in those patients who expressed HER-2 at the highest levels [4]. This is the first time to set an oncogene product as a therapeutical target in the treatment of breast cancer. However, tumor cells that over-express p185 can shed soluble antigenic fragments from the cell membrane, which may interfere with monoprimarily cytostatic[5]. Several studies have demonstrated that antisense ODN targeting at mRNA HER-2 can specifically down-regulate *HER*-2 mRNA expression in breast cancer cells resulting in inhibitory effects on the proliferation of cancer cells[6]. Therefore, besides monoclonal antibodies, antisense ODN targeting HER-2 appears to be another potent tool in inhibiting HER-2 expression at mRNA level.

Our previous work suggested that computer aided mRNA secondary structure prediction is helpful for optimizing the antisense drug design [7]. However, the computer software for the mRNA secondary structure simulation often offered us quite a few predicted secondary structures with different structural free energies. In these structures, the optimal one was with the lowest free energy, others with higher free energies were suboptimal structures. Among the optimal and suboptimal structures, some local structural regions (we called them "local motif, LM" [8]) were completely invariable, or we can say they were conservative, and other LM were variable. So whether the conservative LM or the variable LM should be selected as an antisense target is still confused. In this study, we tried to elucidate the difference between the conservative and variable LM when they are acting as antisense targets. HA4, which was reported in previous literatures as the antisense S-ODN targeting *HER*-2 mRNA that have been concentrated plenty of investigations[6,9], was engaged as the positive control to provide reasonable comparison.

MATERIALS AND METHODS

Prediction of secondary structure of target mRNA and calculation of reaction free energy (ΔG^{o}_{R}) The computer program RNAstructure (version 3.25, 2001) was kindly permitted to get, update, and use by Prof Tunner DH (Department of Chemistry, University of Rochester, New York 14627) after our registration. The whole human *HER*-2 mRNA sequence (GenBank entry code: XM 049824) was obtained from GenBank. The primary sequence was input to the window of RNAstructure, and the parameters were set (maximum energy different was set at 10 %, maximum number of structures was 20), then RNAstructure window calculated the secondary structure of the mRNA based on the principle of minimizing free energy. Finally, the optimal and suboptimal secondary structures of the mRNA would be output. The methods of calculation of reaction free energy (ΔG^{o}_{R}) were based upon the methods previously described [7].

Design and synthesis of the antisense S-ODN On the basis of predicted structures of the mRNA, twenty-one 20-mer S-ODN were designed directly against the local secondary structural element bulge loops, internal loops, hairpins, and knots, which have lower free energies. The designed anitsense S-ODN were divided into three groups according to the target locations. The first group was against the completely conservative LM (complete common local structures of three simulant *HER*-2 mRNA secondary structures). Two other groups were against variable LM (complete different local structures of three simulant *HER*-2 mRNA secondary structures) and relatively conservative LM (common local structures of either two of three simulant *HER*-2 mRNA secondary structures) respectively (Tab 1). The anti-*HER*-2 S-ODN were named as HA, the number following HA denoted the initiation sites (5' to 3' on *HER*-2 mRNA) of target sequence of the S-ODN, and the number in blankets indicated the length of S-ODN (Tab 1). All of these antisense S-ODN were synthesized by SBS Genetech Co, Ltd (Beijing, China). The HA4 was set as positive control, and the random and scrambled sequences were used as negative controls.

Cell culture High *HER*-2 expressed SK-BR-3 breast cancer cell line was provided by the cell bank of Chinese Academy of Sciences. The cells were cultured in RPMI-1640 (Gibco, BRL) containing 10 % fetal calf serum (FCS, Hyclone) in 37 ºC, 5 % CO_2. The cells were routinely passaged when 85 % - 90 % of cells were confluent.

Treatment of cells in culture and IC_{50} evaluation About 30 thousands of SK-BR-3 cells for each well were seeded in 96-well plates (NUNC, Denmark) until 95 % were confluent. At this time, the S-ODN of required concentration were then transfected into cells by Lipofectamine™ 2000 (Invitrogen, Gibco, BRL) according to the instruction of the manufacturer directions. Six concentration series (13-1250 nmol/L) were set to evaluate the *in vitro* activities of every S-ODN and 3-4 duplicated wells per concentration were performed. The

positive control HA4 was set as working standard on every plate, and each S-ODN was tested for 3-5 times in order to get reliable results. After 24-48-h incubation at 37 ºC, 5 % CO_2, the culture medium containing S-ODN was poured and thiazolyl blue (MTT, SERVA) solution (0.5 g/L) was then added and incubated for another 4 h, then Me_2SO was used to dissolve the precipitation. The absorbance at 570 nm (reference wavelength was set at 450 nm) was determined by Wellscan MK-2 microplate reader (Labsystems, Finland). Then the concentration *vs* absorbance at 570 nm of each S-ODN were plotted, and 50 % inhibitory concentrations (IC_{50}) and slopes of S-ODN were calculated by the method of logit analysis using MicroCal Origin software. Mean IC_{50} value (mean±SD, n=3-5 experiments in duplicate) of S-ODN was used as the major criterion of S-ODN inhibitory potency.

Reverse transcription-polymerase chain reaction (RT-PCR) On the basis of the inhibitory effects of antisense S-ODN on SK-BR-3 proliferations, 200 nmol/L of HA824, HA974, HA418, HA193, HA901, and positive control HA4, random control scramble were set to observe their specific inhibitory effects on the expressions of *HER*-2 mRNA in SK-BR-3 breast cancer cells. The Trizol reagent was used to extract the total mRNA from SK-BR-3 cells transfected by different antisense S-ODN for about 9 h. Then the RT-PCR reaction was accomplished by a one-step RT-PCR with Platinum *Taq* kit (Invitrogen SuperScript™). Briefly, total RNA (3 μg), human-specific primers for *HER*-2 and glyceraldehydes-3-phosphate (G3PDH) were added into reverse transcription-polymerase chain reaction system (pre-degeneration for 1 cycle: 30 min at 55 ºC, 2 min at 94 ºC; PCR for 38 cycles: 15 s at 94 ºC, 30 s at 56 ºC, 1 min at 72 ºC, with 7-min extension). The *HER*-2 primers were: forward 5'- CAA TGG AGA CCC GCT GAA C -3' and reverse 5'- CAG TGC GCG TCA GGC TCT -3'; the G3PDH primers were: forward 5'- ACC ACA GTC CAT GCC ATC AC -3' and reverse 5'- TCC ACC ACC CTG TTG CTG TA -3'. The primers were synthesized by Augct Company (Beijing, China). The PCR products were subjected to electrophoresis in 1.5 % agarose gel (Gibco, BRL) containing ethidium bromide and photographed under ultraviolet light.

QSAR analysis by multiple regression and statistics The IC_{50} value (Tab 1, column 12) was set as dependent variable, and other factors (column 3-11) were set as independent variables, then multiple regression was executed by the backward elimination method (Criterion POUT=0.1000), the forward method or the remove method by SPSS (SPSS for windows, computer program, Version 10.0, SPSS Inc; 1999). Statistical inferring was obtained by *t* test, *F* test or tests related to the multiple regression performed by SPSS.

RESULTS

Secondary structures of *HER*-2 mRNA and calculation of relevant ΔG^{o}_{37} We eventually obtained one optimal simulant secondary structure (with the lowest free energy of -471.7 kcal/mol) and two suboptimal simulant secondary structures (with the free energies of -471.4 and -471.3 kcal/mol, respectively) of human *HER*-2 mRNA. Calculation of free energy related to S-ODN, target mRNA sequence, drug-target-formed duplex, and reaction were according to our previous description[7].

Inhibitory effect on proliferation of SK-BR-3 cells Nine out of 11 S-ODN against the completely conservative LM got lower or similar IC_{50} values compared with HA4 (Tab 1); to those S-ODN targeting relatively conservative LM, 2 out of 3 S-ODN got lower or similar IC_{50} values compared with HA 4 (Tab 1); anyway, to those S-ODN targeting variable LM, only 2 of 5 S-ODN got acceptable potencies (Tab 1). There was statistic difference between IC_{50} of antisense S-ODN targeting completely conservative LM and diverse LM (variable and relatively conservative LM) of three simulant secondary structures (Tab 2).

Reverse transcription-polymerase chain reaction The inhibitory effects of S-ODN on the expressions of *HER*-2 mRNA in SK-BR-3 cells were generally in accord with their IC_{50} results. HA824, HA418, and HA974 200 nmol/L significantly inhibited *HER*-2 mRNA expressions in SK-BR-3 cells, while random control scramble, HA193, HA901, and HA130, which has similar IC_{50} value with HA4 did not have significant effects on *HER*-2 mRNA expression (Fig 1).

QSAR analysis by multiple regression The methods were just as we previously described[7]. Variables of all S-ODN were analyzed. By the backward elimination method of multiple regression in SPSS software, the eliminated variable were ΔG^{o}_{D}, knots, internal loops, hairpin loops, ΔG^{o}_{S}, ΔG^{o}_{R}. The stability, base number of bulge loops, and target ΔG^{o}_{T} had statistically significant *t* values (Tab 3). While when we only analyzed variables of S-ODN targeting completely conservative LM of three simulant secondary structures,

Tab 1. Secondary structure, free energy, and inhibitory effect (proliferation of SK-BR-3 cells *in vitro*) of antisense S-OND 200 nmol/L targeting *HER*-2 mRNA. *n*=3-5 experiments in duplicate. Mean±SD. [b]*P*<0.05, [c]*P*<0.01, potency stronger *vs* HA4. [e]*P*<0.05, [f]P<0.01, potency weaker *vs* HA4. (Free energy parameters for RNA were in NaCl 1 mol/L, at 37 ºC)

Antisense S-OND	Structural characteristics of target sequences					Free energy ΔG^o (37 ºC, kcal·mol^{-1})				Inhibitory potency	
	Stability	Bulge loops	Internal loops	Hairpins	Knots	S-OND (ΔG^o_S)	Targets (ΔG^o_T)	Duplexes (ΔG^o_D)	Reaction (ΔG^o_R)	IC_{50} (nmol·L^{-1})	Inhibition (%)
Same structures of three *HER*-2 mRNA secondary structures											
HA 355 (20)	3	3	10+9+13	0	0	-0.7	5.4	-39.1	-43.8	65±43	84±8
HA 418 (20)	3	3	10+6	12	0	-5.2	-0.5	-46.8	-41.12	62±24	78±4
HA 824 (20)	3	3+5	18+6	6	0	-1.2	11.5	-37.4	-47.68	47±26[c]	68±18
HA 844 (20)	3	5	18+6	6	0	-2.8	11.1	-37.5	-45.79	74±44	80±8
HA 901 (20)	3	0	0	7	17	-1.8	6.3	-38.1	-42.6	518±93[f]	46±8
HA 910 (20)	3	0	0	0	17+16	0	4.7	-34	-38.7	356±220[e]	47±12
HA 974 (20)	3	3	11	6	17+16	0	1.7	-40.4	-42.1	69±34	84±9
HA 1125 (20)	3	0	0	5	21	-3.1	-2.2	-40	-34.7	153±55	61±7
HA 1223 (20)	3	6	13	7	12	-0.8	7.6	-39.4	-46.25	63±14	82±2
HA 1239 (20)	3	6	13	7	0	-3	4.2	-41.3	-42.54	64±23	75±9
HA 426 (20)	3	3	0	12	0	-2.9	1.2	-42.2	-40.53	63±21	72±11
Same structures of two of three *HER*-2 mRNA structures											
HA 1027 (20)	2	0	0	9	33	-1.7	8	-42.7	-49.01	>1000[f]	54±19
HA 1067 (20)	2	8	10	0	12	0	5	-40.6	-45.6	97±51	64±11
HA 260 (20)	2	0	0	7	27	-2.3	7.8	-38.5	-44.03	49±22[b]	80±9
Different structures of three *HER*-2 mRNA structures											
HA 41 (20)	1	0	0	18	0	-2.75	-2.8	-43.7	-38.15	>1000[f]	62±13
HA 151 (20)	1	0	8	0	27	-5.6	-13.7	-43.1	-23.81	295±266	53±5
HA 12 (20)	1	0	6+8+8	0	0	-2	-21.1	-42.3	-19.2	47±15[c]	89±2
HA 130 (20)	1	4	0	8	17	-1.8	7.7	-41.4	-47.3	69±24	74±4
HA 193 (20)	1	3	7+12	0	27	-0.28	-2.2	-41	-38.52	>1000[f]	40±13
Positive control											
HA 4 (20)	3	0	6+6	0	11	0	-9	-27.8	-18.8	97±27	79±7
Scramble control											
Scramble (20)	1	0	0	0	0	1.2	-	-	-	216±75[e]	55±6

Tab 2. Mean IC_{50} comparison between antisenses S-ODN target completely conservative and diverse LM of three simulant secondary structures of *HER*-2 mRNA. [b]*P*<0.05 *vs* completely conservative LM.

S-ODN target	S-ODN number	IC_{50}/ nmol·L^{-1}
Completely conservative LM	11	147±43
Diverse LM	8	466±165[b]

the results indicated that other variables such as internal loops, hairpin loops, S-ODN ΔG^o_S and reaction ΔG^o_R still had statistically significant *t* values (Tab 4).

DISCUSSION

In this study, S-ODN we designed were divided into three circumstances: target completely conservative LM, relatively conservative LM, and variable LM of three simulant *HER*-2 mRNA secondary structures. We believed that completely conservative LM were the most stable structures of the whole simulant mRNA secondary structures. S-ODN designed based on these structures might be more reliable. The results indicated that to those S-ODN targeting completely conservative LM, 8 out of 9 S-ODN with lower ΔG^o_R got lower or similar IC_{50} values, one of S-ODN with higher ΔG^o_R got relatively higher IC_{50} value, one of S-ODN with lower ΔG^o_R got relatively higher IC_{50} value as compared with HA4. To those S-ODN targeting relatively conserva-

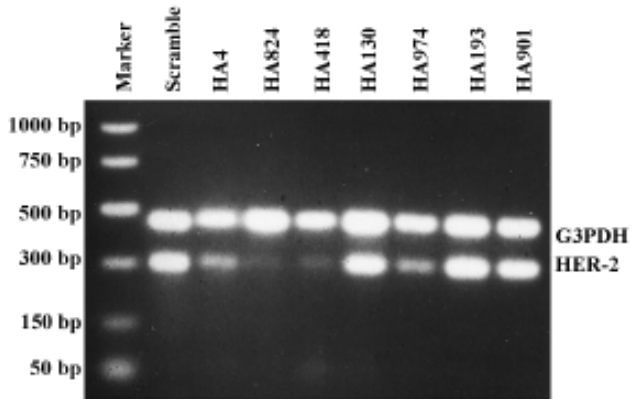

Fig 1. RT-PCR of G3PDH and *HER*-2 cDNA after treatment with random control scramble, positive control HA4, and antisense S-ODN HA824, HA418, HA130, HA974, HA193, HA910 (200 nmol/L for 9 h) demonstrates down-regulation of *HER*-2 mRNA expression in SK-BR-3 breast cancer cells by HA4, HA824, HA418, and HA974.

tive LM, 2 out of 3 S-ODN got lower or similar IC_{50} values compared with HA4. Anyway, to those S-ODN targeting variable LM, only 2 out of 5 S-ODN got similar or lower IC_{50} values compared with HA4 (Tab 1). The QSAR analysis for all S-ODN also suggested that the stability had statistically most significant *t* value, other variables are insignificant except base number of bulge loops and targets ΔG^{o}_{T}. Nevertheless, when we only analyzed variables of S-ODN targeting the completely conservative LM, we found that some other variables such as internal loops, hairpin loops, $\Delta G^{o}_{S,}$ and ΔG^{o}_{R} still had statistically significant *t* values. Because of inadequate cases made us hard to further analyze deviation between S-ODN targeting relatively conservative LM and variable LM, the standard deviations of mean IC_{50} values for most of S-ODN were a little higher and need to further accumulate the experimental data, the present results seem to indicate a tendency that is more conservative local structures with more number of internal loops, hairpin loops, and lower ΔG^{o}_{R} as S-ODN targets had more possibility to get the positive results.

Anyway, getting reliable and accurate secondary structure to find optimal hybridization sites of antisense is always the key issue for RNA secondary structure prediction[10]. Although the success rate for prediction of mRNA secondary structures is improving[11], existing approaches to compute RNA structures do not provide unambiguous predictions of optimal targets in RNA for antisense oligonucleotides. Chen *et al* have predicated the common secondary structures of RNAs by

Tab 3. Variables remained in the equation after the multiple regression with the backward elimination method by SPSS software (for all S-ODN). *n*=21 S-ODN. Mean±SD. Multiple *R*=0.741, *F*=6.882, *P*=0.003.

Variables	B	β	*t* values	Significance of *t*
(Constant)	1079±200		5.398	0.000
Stability	-289±79	-0.782	-3.630	0.002
Base number of bulge loops	-63±22	-0.528	-2.829	0.012
Targets ΔG^{o}_{T}	28±8	0.792	3.368	0.004

Tab 4. Variables remained in the equation after the multiple regression with the backward elimination method by SPSS software (for S-ODN only target completely conservative LM). *n*=11 S-ODN. Mean±SD. Multiple *R*=0.967, *F*=14.464, *P*=0.005.

Variables	B	β	*t* values	Significance of *t*
(Constant)	-1895±421		-4.500	0.006
Base number of bulge loops	-52±12	-0.924	-4.313	0.008
Base number of internal loops	-14±3	-1.090	-4.586	0.006
Base number of hairpin loops	-32±10	-0.802	-3.272	0.022
S-ODN ΔG^{o}_{S}	-61±22	-0.634	-2.753	0.040
Reaction ΔG^{o}_{R}	-58±11	-1.389	-5.334	0.003

genetic algorithm approach; anyway, they selected different RNA sequences[12]. We selected one 1316-bp length of *HER*-2 mRNA to find conservative local structures in three predicated optimal and suboptimal structures. These conservative local structures may be more stable and accessible to the real secondary structure.

We also found that one of S-ODN targeting completely conservative LM with lower ΔG^{o}_{R} was relatively ineffective in inhibiting the proliferation of SK-BR-3 cells. It is well known that many factors are involved in the interactions between antisense S-ODN and mRNA such as chemical stability, secondary structure of the oligonucleotide and the proximity of the binding site to a functional site on the RNA such as the CAP or translational start site[13]. In addition, the question of the role of structure in the binding of a particular oligonucleotide is far more unclear. Tertiary structures have also been shown to affect the affinity and rate of oligonucleotide hybridization[14]. So conservative LM, which might dedicate significant weight, are not only criterion for antisense design.

"Non-sequence specific antisense effect" for some of antisenses is generally one kind of side effect that has the activity of nonspecifically inhibiting the proliferation of some cell lines. To rule out such kind of effects, we detected the specific inhibitory effects of some S-ODN we designed on *HER*-2 mRNA expression. The results indicated that most of S-ODN which had lower or similar IC_{50} values compared with HA4 had the characteristic of specifically inhibiting the *HER*-2 mRNA expressions. Only one of antisense S-ODN, HA130, which had similar IC_{50} value with HA4, did not get similar result. It is necessary to note that this antisense S-ODN targets variable LM of three *HER*-2 mRNA simulant secondary structures. This suggested that antisense targeting variable LM might increase the possibility of non-sequence specific effect.

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REFERENCES

1 Schechter AL, Stern DF, Vaidyanathan L, Decker SJ, Drebin JA, Greene MI, *et al*. The neu oncogene: an erb-B-related gene encoding a 185000-M_t tumour antigen. Nature 1984; 312: 513-6.

2 Menard S, Tagliabue E, Campiglio M, Pupa SM. Role of *HER*2 gene overexpression in breast carcinoma. J Cell Physiol 2000; 182: 150-62.

3 Pegram MD, Finn RS, Arzoo K, Beryt M, Pietras RJ, Slamon DJ. The effect of *HER*-2/*neu* overexpression on chemotherapeutic drug sensitivity in human breast and ovarian cancer cells. Oncogene 1997; 15: 537-47.

4 Miles DW. Update on *HER*-2 as a target for cancer therapy: herceptin in the clinical setting. Breast Cancer Res 2001; 3: 380-4.

5 Brodowicz T, Wiltschke C, Budinsky AC, Krainer M, Steger GG, Zielinski CC. Soluble *HER*2/neu neutralizes biologic effects of anti-*HER*2/neu antibody on breast cancer cell *in vitro*. Int J Cancer 1997; 73: 875-9.

6 Roh H, Pippin JA, Green DW, Boswell CB, Hirose CT, Mokadam N, *et al*. *HER*2/*neu* antisense targeting of human breast carcinoma. Oncogene 2000; 19: 6138-43.

7 Song HF, Tang ZM, Yuan SJ, Zhu BZ. Application of secondary structure prediction in antisense drug design targeting protein kinase C-α mRNA and quantitative structure activity relationship analysis. Acta Pharmacol Sin 2000; 21: 80-6.

8 Song HF, Tang ZM. "Target secondary structural motif" in the action of antisense oligodeoxynucleotides. Acta Pharm Sin 2001; 36: 585-9.

9 Roh H, Hirose CB, Boswell CB, Pippin JA, Drebin JA. Synergistic anti-tumor effects of *HER*2/*neu* antisense oligodeoxynucleotides and conventional chemotherapeutic agents. Surgery 1999; 126: 413-21.

10 Smith L, Andersen KB, Hovgaard L, Jaroszewski JW. Rational selection of antisense oliogonucleotide sequences. Eur J Pharm Sci 2000; 11: 191-8.

11 Mathews DH, Sabina J, Zuker M, Turner DH. Expanded sequence dependence of thermodynamic parameters improves prediction of RNA secondary structure. J Mol Biol 1999; 288: 911-40.

12 Chen JH, Le SY, Maizel JV. Prediction of common secondary structures of RNAs: a genetic algorithm approach. Nucleic Acids Res 2000; 28: 991-9.

13 Baker BF, Lot SS, Condon TP, Cheng-Flournoy S, Lesnik EA, Sasmor HM, *et al*. 2'-*O*-(2-methoxy) ethyl-modified anti-intercellular adhesion molecule 1 (ICAM-1) oligonucleotides selectively increase the ICAM-1 mRNA level and inhibit formation of the ICAM-1 translation initiation complex in human umbilical vein endothelial cells. J Biol Chem 1997; 272: 11994-2000.

14 Paillart JC, Skripkin E, Ehresmann B, Ehresmann C, Marquet R. *In vitro* evidence for a long range pseudoknot in the 5'-untranslated and matrix coding regions of HIV-1 genomic RNA. J Biol Chem 2002; 277: 5995-6004.