Chapter 18

South African Herbal Extracts as Potential Chemopreventive Agents: Screening for Anticancer Splicing Activity

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Abstract

RT-PCR is an invaluable tool for the detection and characterization of mRNA. Cancer cell lines are treated with crude plant extracts and RNA is extracted and purified with DNase prior to RT-PCR. RT-PCR first-strand cDNA synthesis is done using random primers and can be refrigerated at 4 °C. PCR from the stored cDNA is performed using transcript-specific primers and electrophoresed on a molecular grade agarose gel to separate the splice variants.

Key words Anticancer splicing activity, Herbal plant extracts, RNA extraction, First-strand cDNA synthesis, DNase treatment, Agarose gel electrophoresis

1 Introduction

Traditional medicine use has a long history and is still the major source of medicine in developing countries. Approximately 70 % of the South African population consults traditional healers, perpetuating the need for scientific appraisal of traditional medicine as a means to establish its efficiency and safety [1]. Also, pharmacological and phytochemical insights into several plants have led to the discovery of novel chemicals and therefore novel drugs. The discovery of these drugs stresses the importance of using natural products and their derivatives to provide new target molecules for drug development.

Since the publication of the human genome, research has shown that the number of genes is considerably lower than that predicted from the known protein catalogue [2]. Posttranslational modification, such as splicing of immature messenger RNA (pre-mRNA), is fundamental for generating mature mRNAs ready to be translated into proteins. Additionally, through alternative splicing, a single gene is capable of generating multiple transcripts from a

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common mRNA precursor and this mechanism allows for proteome complexity. This event leads to the production of distinct protein isoforms, which might have diverse and even antagonistic functions. Several genome-wide analyses indicate that more than 50 % of human genes present alternative spliced forms, suggesting that this mechanism has a major role in the generation of protein diversity [8]. Alternative splicing is important in normal development as a means of creating protein catalogue or diversity in complex organisms [9, 10]. Alteration of the normal process in cancer cells results in the production of previously nonexisting mRNAs or in the modification of tissue-specific ratios between normal mRNA isoforms [2]. Defects in mRNA splicing are crucial in the development of diseases [4–6]. The most common forms of splicing defects are genomic splice site mutations in more than 12 different types of cancers [7]. To be a cause of cancer an abnormal alternatively spliced product must presumably be expressed at a significant level compared with properly spliced product [3]. An individual splice form seen exclusively in cancer but not in healthy cells could be a candidate for a diagnostic, prognostic, or predictive biomarker. An association between differential expression of splicing isoforms and tumour progression has been shown for several proteins, such as MDM2 and survivin [2]. Currently, the analysis of cancer-specific alternative splicing is a promising step forward in basic and translational molecular biology [2].

Thus we have screened medicinal plants for anticancer splicing activity using semiquantitative RT-PCR in cancer cell lines.

2 Materials

Prepare all solutions with ultrapure water. Autoclave all solutions for tissue culture.

2.1 Culture Medium

Prepare culture medium for appropriate cell lines by adding the appropriate medium, serum, and supplemental reagents required for growth according to the cell line manufacturer.

2.2 Stock Solution for SRPIN 340

SRPIN 340 (28 mM in 100 % DMSO-Kept in a –20 °C freezer).

1. Make 400 µl 10 %/DMSO (28 mM SPRPIN 340).
2. Dilute to 10 % DMSO with tissue culture media before use. (40 µl 100 % DMSO/2,8 mM SRPIN 340 + 360 µl medium-400 µl 10 % DMSO/2800 µM SRPIN 340).

2.3 Plant Extract Preparation

1. Prepare a stock solution of 100 mg/ml in 10 ml tubes.
2. Filter-sterilize and keep in small aliquots (100 µl Eppendorfs) at –20 °C until use.

2.4 PCR Primers

1. Experimental primers.

   Forward primer: Transcript specific.

   Reverse primer: Transcript specific.

   Forward primer GC content ±50 %.

   Reverse primer GC content ±50 %.

2. VEGFxxx/VEGFxxxb PCR control.

   Forward Exon 7b:

   5′GGC AGC TTG AGT TAA ACG AAC G-3′

   Oligo %GC = 50 %.

   Oligo is 25 nt bases long.

   Reverse Exon 8b:

   3′UTR BamHI + GG (5′-G/GATCC-3′):

   5′-CCA GGA AAG ACT Gat aca gaa cga -3′

   Oligo % GC = 48 %.

   Oligo is 25 nt base long.

   130 bp VEGFxxxb, 64 bp VEGFxxx

3. HnRNPA2 primers.

   HnRNPA2 ex1-forward primer: 5′-GCG GCA GTA GCA GCA GCG CC-3′

   HnRNPA2 ex3-reverse primer: 5′-CTT ACG GAA CTG TTC CTT TTC TC-3′

4. MKNK2 PCR (EXON 13a and 13b) primers.

   MKNK2 ex11-forward primer: 5′-CCA AGT CCT GCA GCA CCC CTG G-3′

   MKNK2 ex13a-reverse primer: 5′-CAT GGG AGG GTC AGG CGT GGT C-3′

   MKNK2 ex13b-reverse primer: 5′-GAG GAG GAA GTG ACT GTC CCA C-3′

3 Methods

3.1 Tissue Culture

1. Grow cells in T75 flasks in an appropriate medium until confluent.
2. Trypsinize cells as follows: add 3 ml trypsin plus 3× (9 ml) culture medium with serum in a T75 flask.
3. Keep cells at the CO_2 incubator at 37 °C shortly until cells have detached from the flask.
4. Spin the cells down for 3 min at 3000 rpm (700 × *g*) in a microfuge at 4 °C (*see* **Note 1**).

5. Take out the media by suction using a pipette. Add 5 ml tissue culture medium and resuspend cells. Disperse or separate clumps of cells by using a syringe and a needle.
6. Take 10 µl cells in 90 µl medium/PBS/H2O (10× dilute).
7. Count on a hemocytometer. Seed approximately 40,000–80,000 cells per well (24-well plate).
8. Incubate cells at 37 °C in a CO_2 incubator until 50 % confluent and then treat the cells with your plant extracts/compounds for 24 h.

3.2 SRPIN 340 Control Experiments

Do the experiment in triplicates in a 24-well plate.

1. Seed cells until they are 50 % confluent before treatment.

   Dilution: Provide 6 × 5 ml tubes (Table 1).
2. Transfer 3000 µl media into each tube. From tubes 2–6 discard 1, 5, 10, 54, and 107 µl respectively and then replace with 1, 5, 10, 54, and 107 µl 2800 µM SRPIN 340, respectively, to attain concentrations from tubes 1–6 of 0, 1, 5, 10, 50, and 100 µM, respectively.
3. Do the treatment in triplicates for 24 h and stop the reaction by washing with 1× PBS three times before RNA extraction.

3.3 Plant Extract Treatments

1. Dilution: Provide 5 × 5 ml tubes (Table 2).
2. Transfer 500 ml media into each tube. From tubes 2–5 discard 50, 100, 150, and 250 µl, respectively, and then replace with

Table 1
SRPIN 340 dose–response control experiments

Tubes (5 ml)	1	2	3	4	5	6
Final concentration of SRPIN 340 in µM	0	1	5	10	50	100
Medium in µl	3000	2999	2995	2990	2946	2893
10 % SRPIN 340/2800uMDMSO in µl	0	1	5	10	54	107

Table 2
Herbal extract dose–response experiments (*see* Note 9)

Tubes (5 ml)	1	2	3	4	5
Final concentration of herbal extract in mg/ml	0	1	2	3	5
Medium in µl	5000	4950	4900	4850	4750
100 mg/ml herbal extract stock solution in µl	0	50	100	150	250

50, 100, 150, and 250 μl of the 100 mg/ml stock solution of plant extract, respectively, to attain concentrations from tubes 1–5 of 0, 1, 2, 3, and 5 mg/ml, respectively.

3. Treat cells on 24-well plates for 24 h.
4. Wash the cells three times with 1 ml 1× PBS to stop the reaction.

   Control: We always have an extra 5 ml tube of 10 μM SRPIN 340 (15 μl in 4.5 ml medium) for control treatment.

3.4 RNA Extraction with Tri Reagent

1. Move cells from the Class 11 tissue culture laboratory incubator to a fume hood.
2. Remove the medium and wash cells ×2 with 1 ml per each well of 1× PBS. Add 125 μl per each well of TRI Reagent (Sigma T9424)(*see* **Note 2**).
3. Lyse the cells by mixing the solution up and down several times with a pipette (mix the first well and then move the lysate to the replica well of the same clone).
4. Collect the homogenous lysate in a fresh 1.5 ml Eppendorf tube and leave the samples in incubation for 5 min at room temperature to ensure the complete dissociation of nucleoprotein complexes.
5. Add 100 μl of Chloroform (Sigma C2432) per 500 μl of TRI Reagent used and close tightly.
6. Shake vigorously by hand for 15 s and leave in incubation for 15 min at room temperature.
7. Centrifuge at 12,000 rpm (12,000 × *g*) for 15 min at 4 °C.
8. Transfer the upper aqueous phase to a fresh tube (three phases: a pink organic, lower phase (protein), a white/grey interphase (DNA), a colorless upper aqueous phase (RNA)).
9. Add 500 μl of cold isopropanol (Fluka 59304) per ml of TRI Reagent used, close tightly, shake vigorously for 15 s, and leave in incubation for 10 min at room temperature.
10. Pellet the RNA by centrifugation at 12,000 rpm (12,000 × *g*) for 10 min at 4 °C. Discard the supernatant and wash the gel-like pellet by adding 1 ml of cold 75 % ethanol per ml of TRI Reagent used.
11. Gently wash the samples and centrifuge at 10,000 rpm (7500 × *g*) for 5 min at 4 °C.
12. Discard the supernatant and briefly dry the pellet for 5–10 min at room temperature (do not overdry).
13. Dissolve the RNA by adding 20–25 μl of DEPC H_2O and resuspend the pellet. Store the RNA at −20 °C.

3.5 DNase Treatment of RNA Samples Prior to RT-PCR

1. Quantify your RNA using the nanodrop or any other sensitive quantification tools.
2. Set up the DNAse digestion reaction as follows: RNA in water or TE buffer (use 5 μg RNA) in case you lose RNA during the procedure. RQ1 RNase-free DNase 10× reaction buffer 1 μl. RQ1 RNase-free DNase 1 μl (*see* **Note 3**).
3. Add nuclease-free water to a final volume of 10 μl. Incubate at 37 °C for 1 h. Add 1 μl of RQ1 DNase stop solution to terminate the reaction. Incubate at 65 °C for 10 min to inactivate the DNase. Add all of the treated RNA to the RT-PCR reaction.

3.6 RT-PCR First-Strand cDNA Synthesis

1. Use 1 μg (1000 ng) of total RNA in sterile RNase-free microcentrifuge. Anneal primers as recommended by the manufacturer (Table 3) (*see* **Note 4**).
2. Heat tube to 70 °C for 5 min, cool quickly on ice for 5 min.
3. Add components to annealed primer/template in the order shown in Table 4.
4. Fill up with Sigma water to 50 μl (50 μl PCR reaction volume).
5. Perform cDNA synthesis as shown in Table 5.

Table 3
Annealing of primers

Reagent	Final concentration	Volume per tube	Mix (x__)
Hexamer/random primer (500 μg/mL)	0.5 μg/ul	2.5 μl	__μl
RNAsin (40 U/μl)	40U/50 μl	1 μl	__μl
Total volume	_____	4 μl	__μl

Table 4
Reverse transcription components

Reagent	Final concentration	Volume per tube	Mix (x__)
M-MLV RT reaction buffer	5×	10 μl	__μl
dNTPs (10 mM)	0.5 mM	2.5 μl	__μl
M-MLV RT (h-) 200 U/μl	200 U	1 μl	__μl
Total volume	_____	_____	_____

Table 5
cDNA synthesis

PCR step	Temperature (°C)	Time	Cycle
Initial incubation	37	90 min	1
Inactivation	70	15 min	1
Refrigeration	4	Forever	–

Table 6
PCR reaction setup

Reagent	Final concentration	Volume per tube	Mix (x__)
PCR master mix Promega (2×)	1×	25 µl	__µl
Forward primer (20 µM)	1 µM	2.5 µl	__µl
Reverse primer (20 µM)	1 µM	2.5 µl	__µl
H_2O	_____	__µl	__µl
Total	–	__µl	__µl

3.7 PCR Reaction (Alternative Splicing)

1. Set up the PCR reactions as in Table 6 (*see* **Notes 5 and 6**).
2. Add the PCR Master Mix last and mix well before starting the PCR programs (Table 7). Transcript-specific primers may need optimization of PCR conditions.
3. Use 5–10 µl of cDNA as template.

3.7.1 Negative Controls

1. Add H_2O (Sigma) instead of cDNA.
2. Add H_2O (Sigma) instead of reverse and forward primers.

3.7.2 VEGF Controls

Known DNA samples and the experimental cDNA (Table 8) were used.

3.7.3 HnRNPA2 PCR (EXON 2) Control Experiments

The experimental cDNA (Table 8) was used.

3.7.4 MKNK2 PCR (EXON 13a and 13b) Control Experiments

The experimental cDNA (Table 8) was used.

3.8 Agarose Gel Electrophoresis

Weigh out 2 g of agarose gel (*see* **Note 7**). Dissolve in 100 ml 1× TBE by heating on low to medium in a microwave (2 % agarose gel for ease of separation of splice variants). Cool it down to about

Table 7
PCR amplification for VEGF controls

PCR step	Temperature (°C)	Time	Cycle
Initial denaturation	95	2 min	1
Denaturation	95	1 min	30
Annealing	55	1 min	
Extension	72	1 min	
Final extension	72	5 min	1
Refrigeration	4	Hold	–

Table 7b
PCR amplification for MKNK2 controls

PCR step	Temperature (°C)	Time	Cycle
Initial denaturation	95	2 min	1
Denaturation	95	1 min	30
Annealing	60	1 min	
Extension	72	1 min	
Final extension	72	5 min	1
Refrigeration	4	Hold	–

Table 7c
PCR amplification for HNRNPA2/B1 controls (*see* Note 8)

PCR step	Temperature (°C)	Time	Cycle
Initial denaturation	95	2 min	1
Denaturation	95	1 min	30
Annealing	55	1 min	
Extension	72	1 min	
Final extension	72	5 min	1
Refrigeration	4	Hold	–

Table 8
DNA templates for experiments and controls

Reagent	Final concentration	Volume per tube
Template tubes after RT-PCR first-strand synthesis	–	10 µl
VEGF165 in pcDNA3 (200 ng/µl)	200 ng	1 + 9 µl Sigma H_2O (10 µl)
VEGF165b in pcDNA3 (200 ng/µl)	200 ng	1 + 9 µl Sigma H_2O (10 µl)

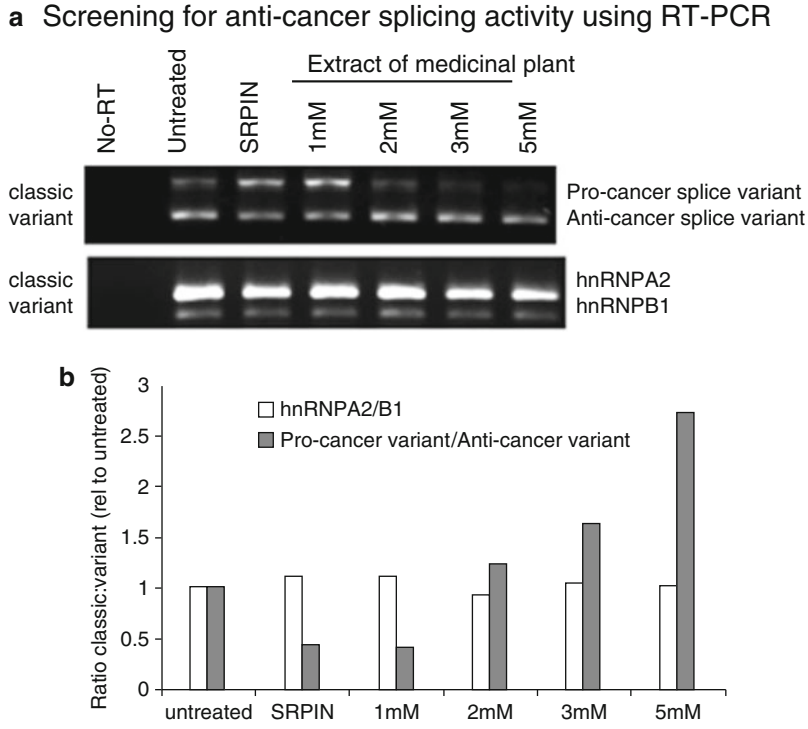

Fig. 1 There is a dose–response on gene splice variants. Cells were treated with a medicinal plant extract for 24 h, followed by mRNA extraction and RT-PCR. The dose–response is clearly visible after treatment with the plant extract when the anticancer splice variant becomes clearly evident. There is no dose–response on HnRNPA2 splice variants—no effect of the herbal extract to the HnRNPA2 splicing factor (**a**). (**b**) clearly shows the increase in ratio of the classic: variant in reference to untreated cells

50 °C and add 5 µl of 100 mg/ml ethidium bromide and pour the gel into a tray. When the gel has set, immerse the gel to a running buffer (1× TBE). Load samples in a loading dye including a lane of an appropriate molecular weight marker. Run at a voltage of 100 V for 10 min and then at 70 V until the dye front is ¾ way down the gel. Stop the gel and view it using the Gel Doc Image analyzer and capture the image for analysis (Fig. 1).

4 Notes

1. Tissue culture: Make sure that when you seed cells for treatment with herbal extract, the concentration of the cells in the wells are equivalent. Keep an autoclaved stock solution of 20× PBS to dilute it to 1× whenever needed.
2. RNA extraction: You can use the Tri Reagent (Sigma T9424) or the Trizol Reagent (Invitrogen 15596-018) for RNA extraction using exactly the same RNA extraction protocol.
3. DNase treatment: RQ1 RNase-free DNase 10× reaction buffer, RQ1 RNase-free DNase, and RQ1 DNase stop solution are all obtainable from Promega.
4. RT-reaction/first-strand cDNA synthesis: Random/Hexamer/OligodT: use the Promega C1181-20 μg. RNAsin, M-MLV Reaction buffer, and M-MLV RT (H-) are obtainable from Promega. dNTPs (10 mM) are from Fermentas R0191.
5. PCR reaction: PCR Master Mix: you can use the Promega or the Roche FastStart Universal SYBR Green Master Mix.
6. Oligos: Use Invitrogen Primers if possible. Tm for both primers should be as close as possible, i.e., 50 %.
7. Agarose Gel electrophoresis: Use the Bioline Molecular grade agarose powder.
8. hnRNPA2 RT-PCR control: This should be done for all treatments with herbal extracts.
9. Always include 10 μM SRPIN 340 treatment when doing herbal extracts treatment as a control experiment.

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References

1. Puckee T, Mkhize M, Zama M et al (2002) African traditional healers: what health care professionals need to know. Int J Rehabil Res 25:247–251
2. Pajares MJ (2007) Alternative splicing: an emerging topic in molecular and clinical oncology. Lancet Oncol 8:349–357
3. Venables JP (2004) Aberrant and alternative splicing in cancer. Cancer Res 64:7647–7654
4. Faustino NA, Cooper TA (2003) Pre-mRNA splicing and human disease. Genes Dev 17: 419–437
5. Garcia-Blanco MA, Baranik AP, Lasda EL (2004) Alternative splicing in disease and therapy. Nat Biotechnol 22:535–546
6. Pagani F, Baralle FE (2004) Genomic variants in exons and introns: identifying the splicing spoilers. Nat Rev Genet 5:389–396

7. Hoimila R, Fouquet C, Cadranet J et al (2003) Splice mutations on the p53 gene case report and review of the literature. Hum Mutat 21: 100–102
8. Hu CK, Madore SJ, Moldover B et al (2001) Predicying splice variant from DNA chip expression data. Genome Res 11:1237–1245
9. Black DL (2003) Mechanisms of alternative pre-messenger RNA splicing. Annu Rev Biochem 72:291–336
10. Colapietro P, Gervasini C, Natassi F et al (2003) Exon 7 skipping and sequence alterations in exonic splice enhancers (ESEs) in a neurofibromatosis patient. Hum Genet 113:551–554