行政院國家科學委員會專題研究計畫成果報告

計畫名稱：建立脊髓性肌肉萎縮症之動物模式及治療方法之發展

計畫編號：NSC89-2320-B039-002

NSC89-2320-B-039-051

執行期限：87 **年** 8 **月** 1 **日至** 89 **年** 7 **月** 31 **日**

主持人：張建國 醫師

執行機構：中國醫藥學院附設醫院

聯絡方式：張建國

中國醫藥學院附設醫院

檢驗醫學部

台中市北區 404 **育德路** 2 **號**

Tel: 04-22052121 ext. 1565 Fax: 04-22033295 E-mail: d6781@www.cmch.org.tw

註：本計畫成果已發表於國際期刊：

Nature Genetics 24: 66-70, 2000.
PNAS 98(17): 9808-9813, 2001.

一. 中文摘要

脊髓性肌肉萎縮症（SMA**）是一種體染色體隱性遺傳病，它是因脊髓前角的運動元細胞退化導致肌肉麻痺及肌肉萎縮。目前尚未有治療的方法。過去的研究顯示此病的嚴重度與** SMN **蛋白質的量成反比。我們的研究發現** Sodium butyrate **可以增加** SMA **病人細胞株的** SMN **蛋白的量，其機轉是改變** SMN2 **基因的表現，我們利用它來治療** SMA **老鼠，結果顯示它可增加** SMN **蛋白質的量，而且改善** SMA **老鼠的症狀。我們給會產生** SMA **老鼠的懷孕老鼠服用，結果產生嚴重型** SMA **老鼠的量下降，而生下的** SMA **老鼠症狀也較輕，由以上的結果看來，**sodium butyrate **可能是一種治療人類脊髓性肌肉萎縮症有效的藥。**

關鍵詞：脊髓性肌肉萎縮症，治療，SMA **老鼠**

Abstract

Spinal muscular atrophy (SMA) is an autosomal recessive disease characterized by degeneration of the anterior horn cells of the spinal cord leading to muscular paralysis with muscular atrophy. No effective treatment of this disorder is presently available. Studies of the correlation between disease severity and the amount of SMN protein have shown an inverse relationship. We report that sodium butyrate effectively increases the amount of exon 7-containing SMN protein in SMA lymphoid cell lines by changing the alternative splicing pattern of exon 7 in the *SMN*2 gene. *In vivo*, sodium butyrate treatment of SMA-like mice resulted in increased expression of SMN protein in motor neurons of the spinal cord, and resulted in significant improvement of SMA clinical symptoms. Oral administration of sodium butyrate to intercrosses of heterozygous pregnant knockout-transgenic SMA-like mice decreased the birth rate of severe types of SMA-like mice, and SMA symptoms were ameliorated for all three types of SMA-like mice. These results suggest that sodium butyrate may be an effective drug for the treatment of human SMA patients.

Keywords: spinal muscular atrophy, treatment, SMA mouse

二. 緣由與目的

Proximal spinal muscular atrophy (SMA) is an autosomal recessive disease characterized by degeneration of anterior horn cells of the spinal cord leading to muscular paralysis with muscular atrophy. Clinical diagnosis of SMA is based on findings of progressive symmetric weakness and atrophy of the proximal muscles. Affected individuals are usually classified into three groups according to the age of onset and progression of the disease. Children with type I SMA are most severely affected and usually have SMA symptoms before the age of 6 months and rarely live beyond 2 years. Type II and type III SMA are milder forms and the age of onset of symptoms varies between 6 months and 17 years. SMA is one of the most common fatal autosomal recessive diseases in children with a carrier rate of 1-2% in the general population and an incidence of 1 in 10,000 newborns (1). No specific treatment is currently available for SMA patients.

Two survival motor neuron genes (*SMN*) are typically present on 5q13: *SMN1* (also known as SMN^T, *SMNtel*) and *SMN2* (also known as SMN^C, *SMNcen*). Loss of function mutations of both copies of the telomeric gene, *SMN1,* is correlated with the development of SMA (2-5). The nearly identical centromeric gene, *SMN2* appears to modify disease severity in a dose-dependent manner, as SMN protein levels from this gene are correlated with disease severity (6,7). However, the expressed amount of intact SMN protein from *SMN2* does not provide adequate protection from SMA (8).

Although *SMN1* and *SMN2* encode identical proteins, all three forms of proximal SMA are due to mutation in the *SMN1* gene, but not in the *SMN2* gene (2-5). The differences between these highly homologous genes are in their RNA expression patterns (9-12). Most *SMN2* transcripts lack exons 3, 5 or most frequently, exon 7, with only a small amount of full-length mRNA generated. On the other hand, the *SMN1* gene expresses mostly a full-length mRNA, and only a small fraction of its transcripts are spliced to remove exon 3, 5 or 7 (11,12). Recent studies have also shown that an AG-rich exonic splice enhancer (ESE) in the center of *SMN* exon 7 is required for constitutive inclusion of exon 7 (13). These findings also imply that the low levels of full-length SMN protein produced by *SMN2* are insufficient to protect against disease development (6,7). Clearly, the total amount of full-length oligomerization-competent SMN protein is a critical SMA determinant, and the amount of SMN protein correlates with the severity of pathologies (14). In addition, there is a strong correlation between the *SMN2* copy number and phenotype in human SMA and SMA-like mice (5,6,7,15,16).

We recently developed a SMA mouse model which genotypically and pheno-typically mimics human SMA (15). The severity of pathology in the knockout-transgenic mice is correlated with the amount of intact SMN protein. The difference between *SMN1* and *SMN2* gene expression is the number of full-length transcripts and the amount of SMN protein, and all 5q-linked SMA patients have at least a single intact copy of *SMN2*. Drugs that modify the pattern of *SMN2* transcript in SMA patients in order to increase full-length *SMN* mRNA expression and the amount of SMN protein may have a therapeutic effect on SMA patients. As a first step towards designing a therapeutic protocol for SMA patients, we used EBV transformed lymphoid cell lines from SMA patients to screen a series of drugs for their possible effect on the expression of the *SMN2* gene. One drug which was found to be effective was then used to treat our SMA-like mice to determine its potential for the treatment of human SMA.

SR proteins (Ser-Arg proteins) constitute a family of pre-mRNA splicing factors that are highly conserved throughout the metazoa (17,18). These proteins have multiple functions in splicing. Biochemical experiments have provided strong evidence that SR proteins play essential roles in general, or constitutive, splicing. They seem to be equally important in splicing

regulation, through their ability to modulate selection of alternative splicing sites in a concentration-dependent manner which contributes to activation (and repression) of splicing through interaction with elements in the pre-mRNA known as splicing enhancers (or silencers) (27-29). Recently, Lorson and Androphy demonstrated that an AG-rich exonic splice enhancer in the center of *SMN* exon 7 is required for inclusion of exon 7 (13), and Hofmann et al further demonstrated that Htra2-β1, an SR-like splicing factor, promoted the inclusion of *SMN* exon 7, stimulating full-length *SMN2* expression. Htra2-β1 specifically functioned through and bound to an AG-rich exonic splicing enhancer in *SMN* exon 7 (30). In the present study, we have explored the relationship between drug's effect and SR protein.

三. 結果與討論

Sodium butyrate changes the processing of *SMN2* gene transcripts

EBV transformed lymphoid cell lines from all three types of SMA patients were established and used for drug screening. Several drugs were tested to investigate their potential effect on the expression of the *SMN2* gene using RT-PCR. Among them, sodium butyrate was able to change the expression pattern of the *SMN2* gene. The amount of exon 7-containing *SMN* mRNAs increased in lymphoid cells cultured with 5 ng/ml to 500 μg/ml of sodium butyrate (Fig. 1*a*). The maximal effect was found after 4 hours of stimulation (Fig. 1*b*). Sodium butyrate-treated lymphoid cells from different types of SMA patients showed an increased number of full-length *SMN* transcripts (Fig. 1*c*). To better understand the mechanism involved in this change in full-length *SMN* transcript levels, separate RT-PCRs were used to examine the patterns of alternative splicing in exons 3, 5 and 7. We found that the alternative splicing pattern of exons 3 and 5 was unchanged after sodium butyrate stimulation (Fig. 1*d* and *e*), but that the alternative splicing pattern of exon 7 of the *SMN2* gene changed to the *SMN1* pattern (Fig. 1*f*). Therefore, addition of sodium butyrate in the culture resulted in an increased number of full-length *SMN* mRNA transcripts.

Sodium butyrate increases exon 7-containing SMN protein in SMA lymphoid cells

To determine whether sodium butyrate-induced expression pattern changes of *SMN2* resulted in an increased amount of exon 7-containing SMN protein, we used different concentrations of sodium butyrate to treat the lymphoid cell lines (3 cases each for type I, II, and III respectively) established from different types of SMA patients. In both cytosolic and nuclear fractions, Western blot analysis indicated that sodium butyrate also increased the intact SMN protein after 4 hours stimulation with 0.5 ng/ml to 500 μg/ml of sodium butyrate (Fig. 2). However, a decreasing effect was found in the cytosolic fraction when more than 5 μg/ml sodium butyrate was used.

Sodium butyrate increases specific SR proteins in SMA lymphoid cell lines

SR proteins are known to play an important role in the processes of alternative splicing of genes (17,18), and previous

studies have identified a splicing enhancer element in exon 7 of the *SMN* gene (10,13). To investigate sodium butyrate-induced expression pattern changes of *SMN2* involving the SR protein, we used different antibodies for SR proteins to detect SR protein expression patterns after sodium butyrate treatment. The results showed that two SR proteins of about 27 kDa were induced after treatment, which were detected using mouse anti-SR protein 16H3 antibody. However, no difference was found using the mouse anti-SRp20 antibody (Fig. 3*a*). These induced SR protein reactions were blocked by either a specific mitogen-activated protein kinase (MEK) inhibitor (PD98059) or an inhibitor of protein phosphatases (okadaic acid) (Fig. 3*b*). All (3 cases each for type I, II, and III) lymphoid cell lines, which were established from different types of SMA patients showed similar results.

Treatment of type 2 and 3 SMA-like mice with sodium butyrate

To investigate whether the *in vitro* effects of sodium butyrate on lymphoid cell lines also occur in SMA-like mice *in vivo*, we used sodium butyrate to treat type 2 and 3 SMA-like mice (15 mice each). Sodium butyrate was administered to SMA-like mice via a 0.8 mg/ml or 8 mg/ml solution available *ad libitum* in their drinking water for 1-12 weeks. The amount of sodium butyrate consumed by SMA-like mice was estimated to be approximately 4-80 mg/day. The sodium butyrate treated type 2 SMA-like mice survived 4-5 days longer than the untreated ones (Fig. 4). Most of the treated type 2 mice ultimately died from infection due to traumatic injury of the paralytic hindlimbs.

Our previous study showed that tails of untreated type 2 and 3 SMA-like mice had decreased diameters of muscle fibers, atrophy of muscle bundles, group atrophy and subcutaneous edema (15). In the present study, after sodium butyrate treatment, the tails of type 2 and 3 SMA-like mice showed nearly normal muscle patterns. Grossly, the tails of treated mice were slightly shorter than normal, and treated mice rarely developed chronic necrosis from the tip of the tail toward the root (2% for the treated group *vs.* 50% for the untreated group). Histopathologically, the tails of treated mice had few atrophied muscle bundles, and group atrophy and subcutaneous edema were rarely present (Fig. 5). Western blot analysis showed that the exon 7-containing SMN protein level was elevated in different tissues, including motor neurons of the spinal cord (Fig. 6*a* and *b*). Immunohistochemical studies showed that both exon 2 (Fig. 6*c* and *d*) and exon 7 (Fig. 6*e* and *f*) containing proteins were increased, which may have resulted from an increase in the total amount of intact SMN protein. Since the severity of the pathological changes in SMA patients and SMA-like mice is correlated with the amount of intact SMN protein present in the spinal cord (6,7,15,16), the effect of oral sodium butyrate, particularly in the spinal cord, may be of therapeutic value for SMA patients.

We also used 16 mg/day and 40 mg/day sodium butyrate solution for the treatment, and found no definite toxicity at 16mg/day sodium butyrate treatment, while

mice that received 40 mg/day sodium butyrate treatment died due to dehydration.

Treatment of intercross heterozygous knockout-transgenic mice after pregnancy

Since the survival time of type 1 and some type 2 SMA mice is short, evaluation of the therapeutic effect of sodium butyrate is difficult. To overcome these problems, sodium butyrate (4-80 mg/day) was administered *ad libitum* in drinking water to pregnant *Smn*+/-*SMN2* intercrossed mice, which had previously produced offspring of different types of SMA progeny, especially the severe form (15). Sodium butyrate treatment began on the 15th day postcoitum in order to avoid a possible teratogenic effect. A total of 21 pups with type 1, 22 with type 2, and 48 with type 3 were born from the treated group; and 35 pups with type 1, 17 with type 2, and 38 with type 3 were born from the untreated group (Table 1*a*). These results show that treatment with sodium butyrate from day 15 of pregnancy significantly ameliorated the clinical symptoms of the severe SMA phenotype, leading to milder types of SMA in offspring (Table 1*b*). In addition, fewer SMA-like mice were born in the untreated group, which may have been due to some severe type mice being aborted in the fetal stage or eaten by their mother after birth, and thus remaining uncounted.

The amount of exon 7-containing SMN protein has been shown to be an inverse indicator of disease severity in SMA patients and mice (6,7,15,16). Therefore, increasing the expression of intact SMN protein may have clinically therapeutic effects on SMA patients. In this study, we found that sodium butyrate treatment of human SMA lymphoid cell lines increased the expression of exon 7-containing SMN protein from the *SMN2* gene. The mechanism by which sodium butyrate affects SMN protein expression of the *SMN2* gene involves a change in its RNA splicing pattern of the gene. After sodium butyrate stimulation *in vitro* and *in vivo*, the transcription pattern of *SMN2* changed to an *SMN1*-like transcription pattern, which was nearly identical to the *SMN* pattern in healthy individuals. These findings may have important implications regarding the treatment of SMA patients.

Sodium butyrate has been shown to induce differentiation and apoptosis (19,20). There is evidence that sodium butyrate may act at the transcription level by increasing the acetylation of histones, thereby releasing constraints upon the DNA template and reactivating a number of genes (21,22). Sodium butyrate also increases the expression of fetal-globin genes in adult baboons, humans and other animals (23-25). *In utero* infusions of butyrate delay the developmental switch from γ- to β- globin gene expression in sheep fetuses (25). These effects of butyrate may occur through the inhibition of histone deacetylase (21,22,25,26). In the case of *SMN*, sodium butyrate may acetylate nucleosomal DNA and release other factors that control alternating splicing of exon 7 of the *SMN2* gene.

We demonstrated that sodium butyrate induced two specific SR proteins involved in inclusion of exon 7 for

full-length *SMN* expression of the *SMN2* gene. These reactions were blocked by either the MEK inhibitor or a phosphatase inhibitor. Our results strongly support that SR proteins are involved in *SMN2*exon 7 inclusion after sodium butyrate treatment.

Approximately 15% of all mutations that cause genetic diseases result from the defective splicing of pre-mRNA (31). A number of these mutations do not alter consensus splice sites or generate missense or nonsense mutations, yet do affect splice site selection (32,33). These mutations may cause skipping of exon(s) by disrupting the splicing enhancer(s). Our findings suggest that an approach similar to that used in our study may be effective in treating these kinds of genetic diseases as well.

Most of SMA patients gradually develop clinical symptoms after birth. We previously demonstrated that the *SMN2* in SMA-like mice expressed only a decreased or nearly normal amount of intact SMN protein in most tissues, except in motor neurons (15). This is why SMA is a disease which directly affects only the motor neurons. The motor neuron-specific splicing factors regulating the inclusion/exclusion of exon 7 in the fetal stage, which are shut-down in the spinal cord after birth, may account for the specific defect present in SMA. These factors may also play an important role in genotypic and phenotypic discrepancies. Sodium butyrate inhibits the deacetylation of these phenotype-related genes, modifying the clinical symptoms and signs of SMA in a fashion similar to the mechanism involved in fetal hemoglobin gene expression (21,22,26). However, there is a major difference between modification of the γ-globin gene and the *SMN2* gene after butyrate reaction. The transcription of *SMN2* gene is modified through the alternative splicing of exon 7 rather than directly through the inhibition of histone deacetylation. Gene modification after sodium butyrate treatment not only increased the transcription of *SMN2*, but also changed the splicing pattern of exon 7 of *SMN2* while the splicing pattern of exons 3 or 5 remained unchanged. This may be due to the influence on exon 7 inclusion of specific SR proteins that are induced by sodium butyrate treatment.

Sodium butyrate and related compounds have been used clinically to treat patients with sickle cell anemia and thalassemia for several years (34,35). The pharmacokinetics and toxicities of sodium butyrate are well documented; its toxicity is low and was well-tolerated in both human and animal studies (34-37). Our findings suggest that sodium butyrate is an excellent candidate for the treatment of human SMA. In the present study, although sodium butyrate had a therapeutic effect on SMA symptoms, a number of severe types of SMA mice were born to sodium butyrate treated pregnant mice and a few type 2 mice showed poor response after sodium butyrate treatment. This may have been due to incomplete treatment, or be because the increase in the amount of intact SMN protein after treatment was unable to sufficiently compensate, to provide the minimal requirement for motor neuron survival. It is also possible that the timing of treatment was too late after day 15 of

pregnancy.

In summary, SMA lymphoid cell lines and SMA-like mice were used to explore possible medication for the treatment of human SMA in this study. We found that sodium butyrate can effectively treat SMA-like mice by changing the expression pattern of *SMN2* and increasing the amount of full-length mRNA of *SMN2* both *in vitro* and *in vivo*. This is the first study to demonstrate a possible treatment for human SMA. The methods developed in this study may be useful in screening other candidate drugs for SMA treatment. We also demonstrated that the mechanism of action of sodium butyrate involves a modification of the splicing of exon 7 of the *SMN2* gene under the regulation of SR proteins. This study is the first to show that a deacetylase inhibitor can specifically modulate a disease-related defect gene to change its expression pattern, resulting in amelioration of the related symptoms. Our methods may also provide a useful approach for the treatment of other splicing-defect-related diseases (31,38).

四. 參考文獻

1. Talbot, K. (1999) *J. Inherit. Metab. Dis.* **22**, 545-554.
2. Lefebvre, S., Bürglen, L., Reboullet, S., Clermont, O., Burlet, P., Viollet, L., Benichou, B., Cruaud, C., Millasseau, P., Zeviani, M., *et al.* (1995) *Cell* **80**, 155-165.
3. Rodrigues, N. R., Owen, N., Talbot, K., Ignatius, J., Dubowitz, V. & Davies, K. E. (1995) *Hum. Mol. Genet.* **4**, 631-634.
4. Chang, J. G., Jong, Y. J., Huang, J. M., Wang, W. S., Yang, T. Y., Chang, C. P., Chen, Y. J. & Lin S. P. (1995) *Am. J. Hum. Genet.* **57**, 1503-1505.
5. Parsons, D. W., McAndrew, P. E., Iannaccone, S. T., Mendell, J. R., Burghes, A. H. M. & Prior, T. W. (1998) *Am. J. Hum. Genet.* **63**,1712-1723.
6. Coovert, D. D., Le, T. T., McAndrew, P. E., Strasswimmer, J., Crawford, T. O., Mendell, J. R., Coulson, S. E., Androphy, E. J., Prior, T. W. & Burghes, A. H. M. (1997) *Hum. Mol. Genet.* **6**, 1205-1214.
7. Lefebvre, S., Burlet, P., Liu, Q., Bertrandy, S., Clermont, O., Munnich, A., Dreyfuss, G. & Melki, J. (1997) *Nature Genet.* **16**, 265-269.
8. McAndrew, P. E., Parsons, D. W., Simard, L. R., Rochette, C., Ray, P. N., Mendell, J. R., Prior, T. W. & Burghes, A. H. M. (1997) *Am. J. Hum. Genet.* **60**, 1411-1422.
9. Bürglen, L., Lefebvre, S., Clermont, O., Burlet, P., Viollet, L., Cruaud, C., Munnich, A. & Melki, J. (1996) *Genomics* **32**, 479-482.
10. Monani, U. R., Lorson, C. L., Parsons, D. W., Prior, T. W., Androphy, E. J., Burghes, A. H. M. & McPherson, J. D. (1999) *Hum. Mol. Genet.* **8**, 1177-1183.
11. Gennarelli, M., Lucarelli, M., Capon, F., Pizzuti, A., Merlini, L., Angelini, C., Novelli, G. & Dallapiccola, B. (1995) *Biochem. Biophys. Res. Commun.* **213**, 342-348.
12. Jong, Y. J., Chang, J. G., Lin, S. P., Yang, T. Y., Wang, J. C., Chang, C. P., Lee, C. C., Li, H., Hsieh-Li, H. M. & Tsai, C. H. (2000) *J. Neurol. Sci.* **173**, 147-153.
13. Lorson, C. L. & Androphy, E. J. (2000)

Hum. Mol. Genet. **9**, 259-265.
14. Lorson, C. L., Strasswimmer, J., Yao, J. M., Baleja, J. D., Hahnen, E., Wirth, B., Le, T., Burghes, A. H. M. & Androphy, E. J. (1998) *Nature Genet.* **19**, 63-66.
15. Hsieh-Li, H. M., Chang, J. G., Jong, Y. J., Wu, M. H., Wang, N. M., Tsai, C. H., Li, H. (2000) *Nature Genet.* **24**, 66-70.
16. Monani, U. R., Sendtner, M., Coovert, D. D., Parsons, D. W., Andreassi, C., Le, T. T., Jablonka, S., Schrank, B., Rossol, W., Prior, T. W., *et al.* (2000) *Hum. Mol. Genet.* **9**, 333-339.
17. Fu, X. D. (1995) *RNA* **1**, 663-680.
18. Manley, J. L. & Tacke, R. (1996) *Genes Dev.* **10,** 1569-1579.
19. Cáceres, J. F., Stamm, S., Helfman, D. M. & Krainer, A. R. (1994) *Science* **265**, 1706-1709.
20. Wang, J. & Manley, J.L. (1995) *RNA* **1,** 335-346.
21. Tacke, R. & Manley, J.L. (1999) *Curr. Opin. Cell Biol.* **11,** 358-362.
22. Hofmann, Y., Lorson, C.L., Stamm, S., Androphy, E.J. & Wirth, B. (2000) *Proc. Natl. Acad. Sci. USA* **97**, 9618-9623.
23. Byrd, J. C. & Alho, H. (1987) *Brain Res.* **428**, 151-155.
24. Leder, A. & Leder, P. (1975) *Cell* **5**, 319-322.
25. Sealy, L. & Chalkley, R. (1978) *Cell* **14**, 115-121.
26. Candido, E. P. M., Reeves, R. & Davie, J. R. (1978) *Cell* **14**, 105-113.
27. Ginder, G.D., Whitters, M.J. & Pohlman, J.K. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 3954-3958.
28. Perrine, S. P., Swerdlow, P., Faller, D. V., Qin, G., Rudolph, A. M., Reczek, J. & Kan, Y. (1989) *Adv. Exp. Med. Biol.* **271**, 177-183.
29. Perrine, S. P., Rudolph, A., Faller, D. V., Roman, C., Cohen, R. A., Chen, S. J. & Kan, Y. W. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 8540-8542.
30. McCaffrey, P.G., Newsome, D.A., Fibach, E., Yoshida, M. & Su, M.S.-S. (1997) *Blood* **90**, 2075-2083.
31. Krawczak, M., Reiss, J. & Cooper, D.N. (1992) *Hum. Genet.* **90**, 41-54.
32. Santisteban, I., Arredondo-Vega, F. X., Kelly, S., Loubser, M., Meydan, N., Roifman, C., Howell, P. L., Bowen, T., Weinberg, K. I., Schroeder, M. L., *et al.* (1995) *Hum. Mol. Genet.* **4**, 2081-2087.
33. Jin, Y., Dietz, H. C., Montgomery, R. A., Bell, W. R., McIntosh, I., Coller, B. & Bray, P. F. (1996) *J. Clin. Invest.* **98**, 1745-1754.
34. Collins, A. F., Pearson, H. A., Giardina, P., McDonagh, K. T., Brusilow, S. W. & Dover, G. J. (1995) *Blood* **85**, 43-49.
35. Sher, G. D., Ginder, G. D., Little, J., Yang, S., Dover, G. J. & Olivieri, N. F. (1995) *N. Engl. J. Med.* **332**, 1606-1610.
36. Daniel, P., Brazier, M., Cerutti, I., Pieri, F., Tardivel, I., Desmet, G., Baillet, J. & Chany, C. (1989) *Clin. Chim. Acta.* **181**, 255-263.
37. Egorin, M. J., Yuan, Z. M., Sentz, D. L., Plaisance, K. & Eiseman, J. L. (1999) *Cancer Chemother. Pharmacol.* **43**, 445-453.
38. Philips, A. V. & Cooper, T. .A. (2000) *Cell Mol. Life Sci.* **57**, 235-249.