Study towards the improvement of the enantioselective hydrolysis of Naproxen esters by sheep liver acetone powder

Héctor Luna*, Ana Pacheco, Aida Solís, Herminia I. Pérez, Norberto Manjarrez, Julia Cassani

Departamento de Sistemas Biológicos, Universidad Autónoma Metropolitana-Xochimilco, Calzada del Hueso 1100, Col. Villa Quietud, Delg. Coyoacan, México, D.F. CP 04960, MEXICO.

Liver acetone powders (LAPs) have been suggested as easy to handle and low cost crude esterase sources, which can be used to carry out estereoselective biotransformations involving carboxylic acids, esters and alcohols. (*S*)-Naproxen [(*S*)-2-(6-methoxy-2-naphtyl)-propionic acid] is 28-fold more active than the corresponding (*R*)-enantiomer as antiinflamatory agent, for this reason (*R,S*)-naproxen is a suitable commercial candidate as a model for the study of the application of crude sheep liver acetone powder (SLAP) for its resolution. After the evaluation of the effect of pH, reaction time, co-solvents, substrate/SLAP ratio and temperature we concluded that the best results (72% ee and 21% conversion) were obtained when the reaction was performed at room temperature for 48h at pH 7, with dioxane (15%), utilizing 1:1 substrate/SLAP ratio.

*Corresponding Author: Tel: +52 (55)5483-7255; Fax: +52 (55)5483-7237; E-mail: lchm1964@correo.xoc.uam.mx

Financial support: Consejo Nacional de Ciencia y Tecnología (CONACyT) Grant num. 37272-N

INTRODUCTION

Esterases and lipases are the most widely used enzyme biocatalysts in organic chemistry to conduct ester hydrolysis, esterification, transesterification, intraesterification and acyl transfer from esters to other nucleophiles such as amines, thiols and hydroperoxides [1]. The biological activity can differ for each enantiomer, and thus, enantioselective resolution using lipases has been extensively applied for the preparation of valuable chiral intermediates, in the synthesis of pharmaceuticals and agrochemicals [2-5]. Since commercially available enzymes are in general expensive, liver acetone powders (LAPs) have been suggested as easy to handle and low cost crude esterase sources to carry out biotransformations involving carboxylic acids, esters and alcohols [6-12]. The use of LAPs has been also extended to some industrial applications [13-17].

On the other hand, most government regulations ask for warranting elimination of the unwanted enantiomer from the pharmaceutical formulations, consequently there is an increasing need of efficient methods for the synthesis of the optically pure compounds [19-22]. As an example, (*S*)-Naproxen [(*S*)-2-(6-methoxy-2-naphtyl)-propionic acid] is 28-fold more active than the

corresponding (*R*)-enantiomer [18], for this reason (*R, S*)-naproxen is a suitable commercial candidate as a model of study for the application of crude hydrolytic enzymatic preparations for its resolution. Goswani reported the use of liver enzymes from diverse animals including rat, rabbit, sheep, seal, mouse, horse, goat, cat, chicken, cattle and dog, for the enantioselective hydrolysis of naproxen esters; being the dog acetone powder the one which gave the best enantioselectivity (74% enantiomeric excess(ee)) towards the *R*-isomer [23]. In this paper we describe studies of the reaction parameters for the resolution of naproxen esters (methyl, ethyl and butyl), under different reaction conditions, in order to determine the influence of factors such as co-solvent, pH, temperature and chain length of the alkyl group, on the percentage of conversion and enantioselectivity of hydrolysis using crude sheep liver acetone powders (SLAP) as biocatalyst.

MATERIALS AND METHODS

The liver was purchased from a local market. *n*-Butanol was analytical grade, methanol and hexane were HPLC grade. ^{1}H-NMR spectra were determined in $CDCl_3$ using a Varian Mercury VX 400 MHz spectrometer, with TMS as internal standard. Infrared spectroscopy was determined in a Paragon 1000 spectrometer (Perkin-Elmer) as KBr disk. The reactions were monitored by GC using an Agilent Chromatograph Mod. 6890 with an HP-5 column (Agilent Technologies) and a FID detector, injector at 250° temperature, Nitrogen was the carrier gas, at 1 mL/min. The reactions were monitored by HPLC using an Agilent 1100 Chromatograph, with a Chiracel-OD column (Chiral Technologies), with detection at 260 nm, with hexanes /isopropanol, 94:6 as eluent, at 25° C and a flow of 0.8 mL/min.

Sheep liver acetone powder (SLAP) preparation

In a blender vessel were added approximately 250 g of clean sheep liver and covered completely with acetone, the mixture was grounded at high speed. The brown mash was filtered and the residue was subjected to the same process twice more; the filtrate was discharged. The solid residue was left in the fume hood for the complete evaporation of the residual acetone, yielding a light brown fine powder, this crude material was kept in tightly close jars in the freezer (4° C) [24].

Ester preparation

Racemic naproxen (1 g, 4.67 mmol) was dissolved in methanol, ethanol or butanol (5 mL), concentrated sulfuric acid (0.3 mL) was slowly added under stirring [25]. The reaction mixture was refluxed for 5 h and then stirred at room temperature overnight. After that the reaction mixture was diluted with 20 mL of CH_2Cl_2 and washed with a saturated $NaHCO_3$ solution (10 mL), followed by brine (10 mL); the organic layer was dried with Na_2SO_4 and evaporated; the solid was recrystalized from hexane. In a similar manner the ethyl and butyl esters were prepared.

Methyl naproxenate: white powder; yield: 87%; mp: 64-65° C; IR(KBr) λ_{max}/cm^{-1}: 2976, 1738, 1605, 1201 cm^{-1}; ^{1}H-NMR ($CDCl_3$): δ/ppm 7.70 (d, *J*= 8.6 Hz, 2H), 7.65 (d, *J*= 2.0 Hz, 1H), 7.39 (dd, *J*= 2.0, 8.6 Hz, 1H), 7.14 (d, *J*= 2.8 Hz, 1H), 7.11 (dd, *J*= 2.8, 6.8 Hz, 1H), 3.90 (s, 3H), 3.87 (c, *J*= 7.6 Hz, 1H), 3.66 (s, 3H), 1.57 (d, *J*= 6.8 Hz, 3H); ^{13}C-NMR ($CDCl_3$): δ/ppm 174.7, 157.2, 135.3, 133.3, 128.9, 128.6, 126.8, 125.9, 125.6, 118.7, 105.3, 55.1, 51.9, 45.2, 18.5; Chiral HPLC:

R_t : 7.0 (*R*-) and 7.7 min (*S*-); GC: R_t 4.3 min (racemic).

Ethyl naproxenate: white powder; yield: 83%; mp: 66-68° C; IR ν 2977, 1727, 1604, 1180 cm^{-1}; ^{1}H-NMR ($CDCl_3$) : δ 7.69 (d, *J*= 8.4, 3H), 7.40 (dd, *J*= 2, 8.4 Hz, 1H), 7.14 (d, *J*= 2.4 Hz, 1H), 7.11 (m, 1H), 4.08 (m, 2H), 3.91 (s, 3H), 3.83 (c, *J*= 6.8 Hz, 1H), 1.56 (d, *J*= 6.8 Hz, 3H), 1.21 (t, *J*= 6.8 Hz, 2H); ^{13}C-NMR ($CDCl_3$): δ/ppm 174.7, 157.2, 135.3, 133.3, 128.9, 128.6, 126.8, 125.9, 125.6, 118.7, 105.3, 55.1, 51.9, 45.2, 18.5.

Butyl naproxenate: white powder; yield: 57%; mp: 50-52° C; IR ν 2954, 1726, 1606, 1193 cm^{-1}; ^{1}H-NMR ($CDCl_3$) : δ 7.69 (d, *J*= 8.4, 2H), 7.66 (d, *J*= 1.6 Hz, 1H), 7.40 (dd, *J*= 1.6, 8.4 Hz, 1H), 7.14 (d, *J*= 2.4 Hz, 1H), 7.11 (dd, *J*= 1.6, 5.6 Hz, 1H), 4.08 (m, 2H), 3.91 (s, 3H), 3.84 (c, *J*= 7.6 Hz, 1H), 1.57 (d, *J*= 7.6 Hz, 3H), 1.55 (m, 2H), 1.28 (m, 2H), 0.85 (t, *J*= 7.2 Hz, 3H); ^{13}C-NMR ($CDCl_3$): δ /ppm 174.3, 157.2, 135.5, 133.3, 128.9, 128.6, 126.8, 125.9, 125.6, 118.6, 105.2, 60.6, 55.1, 45.3, 18.6, 14.1; HPLC: R_t 6.0 (*R*-) and 6.5 min (*S*-); GC: R_t 8.3 min (racemic).

General procedure for the enzymatic hydrolysis

In a 30 mL vial, the racemic ester (100 mg) was dissolved in dioxane (1.5 mL), then a phosphate buffer solution (13.5 mL) was added under stirring, followed by the SLAP (100 mg). The reaction mixture was magnetically stirred at room temperature (~24° C) for 24h. The reaction mixture was filtered over celite, and washed three times with dichloromethane (5 mL). The phases were separated and the aqueous layer was extracted three more times with dichloromethane (5 mL each); the combined organic extracts were dried over sodium sulphate, filtered and evaporated. The crude product was analyzed by GC and HPLC, without any derivatization.

RESULTS AND DISCUSSION

Although a great variety of liver enzymatic preparations have been used as biocatalyst, for example those from pigeon, cat, dog, eel, horse, calf, guinea pig, mouse, goat, chicken, sheep, seal, rattlesnake, trout, turtle, lungfish, salmon, and lemon shark [6-17], there is not a generalization about the catalytic properties of any of these biocatalysts, in particular or even in groups species, to dictate some rules about the use of liver acetone powders (LAPs).

In a previous study the hydrolytic potential of LAPs from chicken, bovine, pig, rat, rabbit, cat, turkey and sheep over naproxen esters we demonstrated that, all these biocatalysts, except sheep liver acetone powder (SLAP) hydrolyzed enantioselectivelly the (*R*)-ester. The SLAP preferred the (*S*)-isomer with a 68% ee, as was observed by Goswami who reported only a 7% ee with a conversion of 18% [23]. Therefore in this study we decided to revise in more detail the influence of critical reaction parameters to carry out the naproxen esters hydrolysis (Figure 1), using the SLAP in order to control as well as perform the resolution of (*S*)-naproxen more efficiently. Most of the experiments were performed over the methyl ester, because it showed the best reactivity with the crude biocatalyst.

Effect of pH

Previously we have shown that the best pH for the reaction using SLAP as biocatalyst was in the range of 7-7.5 [26]. In the present study we re-examined the reaction in the same range of pH and also examined the reaction time necessary

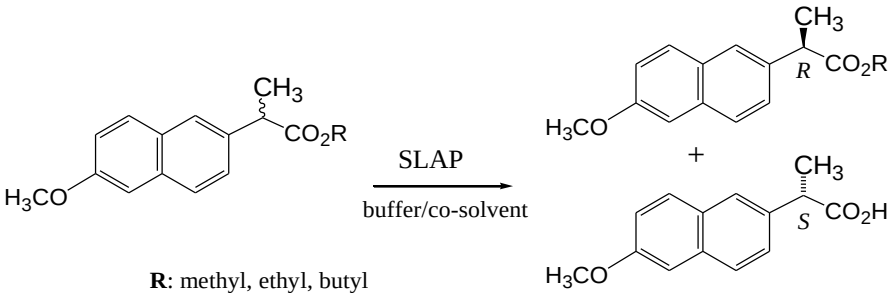

Figure 1. Enzymatic hydrolysis of naproxen esters catalyzed by SLAP, the upper hydrolysis product is the leftover from the racemic substrate and the lower compound is the real enantioselective hydrolysis product, which is the *S*-enantiomer.

to reach a reasonable reaction conversion. We observed that the best enantioselectivity took place at pH 7.0.The conversion was higher after 48h of reaction at room temperature than after 24h, although the enantioselectivity remained very similar as shown by the enantiomeric ratio (E) values (6.5 and 6.6 respectively); longer reaction times conduced to decrease of enantioselectivity. These experiments were done using a substrate/SLAP ratio of 1:1 (w/w) and 10% dioxane as co-solvent. For further experiments we decided to work at pH 7.0; in order to avoid any of the competitive chemically catalyzed reaction, which was observed during blank experiments carried out in absence of enzymatic preparation.

Effect of the co-solvent

Because methyl naproxenate was not soluble in water, it was necessary to add a co-solvent to make the reaction mixture homogeneous, because it is generally understood that the enzymatic reactions are faster in solution. It is also known that the presence of co-solvents exerts some influence on reactions catalyzed by pig liver esterase (PLE), in both conversion and enantioselectivity [27]. From previous work was evident that dioxane, acetonitrile or DMSO were acceptable co-solvents [26]; therefore we evaluate the effect of different proportions of those solvents, but just between 5-15%, because previously we found a decline of the reaction conversion at higher co-solvent concentrations.

Table 1 Effect of co-solvent in the hydrolysis of racemic methyl naproxenate.

Co solvent	Conc (%)[a]	%ee (% conv.)	E[b]
Dioxane	5	57 (39)	4
	10	63 (31)	5
	15	72 (21)	7
Acetonitrile	5	57 (45)	5
	10	57 (34)	5
	15	no rxn	nc[c]
DMSO	5	49 (59)	3
	10	50 (64)	4
	15	56 (74)	7

Reaction conditions: 24° C., sust./SLAP ratio 1:1, 48 h, magnetic stirring.

a: v/v

b: calculated by Selectivity program. Faber, K.; Höning, H.; Kleewin, A. <http://borgc185.kfunigraz.ac.at>.

c: nc= Not calculated due to no conversion.

From the results shown in Table 1 it is observed that acetonitrile exerted a strong effect on conversion; the reaction conversion dropped dramatically when the ratio was increased to 10% or 15%, indicating a great sensibility to this solvent. However, with dioxane the effect was

less significant, and with DMSO the increase of concentration conduced to slight increase in conversion.

Regarding the enantioselectivity, the best results were obtained using dioxane and DMSO at 15% and acetonitrile at 5%, according to the enantiomeric ratio (E) (Table 1). It is important to mention that blank reactions using these co-solvents, without biocatalyst, gave very low conversions and enantioselectivities, these facts strongly suggested that the enantioenrichment of the product mixture from the biocatalyzed reactions was indeed due to a biocatalytic process.

Effect of temperature

It is well known that a decrease of temperature usually produces lower reaction rate, with improved enantioselectivity. As expected, the evaluation at lower temperature (4-5° C) gave slightly better enantioselectivity (E=7), as shown in Table 2.

Table 2. Effect of temperature in the hydrolysis of racemic methyl naproxenate.

Reaction temp.	% ee (% conv.)	E
4-5° C	73 (15)	7
24° C	71 (13)	6
44° C	48 (1.3)	3

Reaction conditions: subst/SLAP ratio 1:1(w/w), pH 7.0, 15% dioxane, magnetic stirring.

When the reaction was done at higher temperature (44° C), the enantioselectivity was very low (E= 3), and it is possible that the low conversion (just 1.3%) could be due to inactivation of the biocatalyst and the enantioenrichment produced took place before the inactivation. It is interesting to note that at 0° C, the enantioselectivity was very high (E >200), but the conversion was less than 5%, after 73 h of reaction.

Effect of the substrate / SLAP ratio

In this study we used a crude biocatalyst where the concentration of the specific enzyme is unknown, so it was necessary to adjust the substrate / SLAP ratio (w/w) to improve conversion and enantioselectivity. From the Table 3 it is clear that an increase in the amount of biocatalyst conduced to an increase of the reaction rate, but it is remarkable that there is also a decrease in enanatioselectivity. At low temperature (4-5º C) with the substrate/ SLAP ratio of 1:4 the conversion was 38% with 76% ee.

Table 3. Effect of the substrate / SLAP ratio in the hydrolysis of racemic methyl naproxenate.

Subst./SLAP ratio (w/w)	1:0.25	1:0.5	1:1	1:2	1:4
% ee (% conv.)	traces	traces	72 (21)	52 (20)	68 (26)

Reaction conditions: pH 7.0, 24° C, 15% dioxane, magnetic stirring, 48 h.

Because the reaction with the ratios of 1:0.25 and 1:0.5 (substrate/SLAP) showed only traces of product (Table 3), we carried out an experiment for longer reaction time (167 h) and besides dioxane we also used acetonitrile (AcCN), which exhibited better ester dissolution. For the ratio 1:0.25, the conversions were 11 and 3% whereas 69 and >99% ee, for dioxane and AcCN respectively. For the ratio 1:0.5, the rate of the conversion were 20 and 10%, but the enantioselectivity changed to 67 and 72% ee, respectively for the same co-solvents.

Effect of the ester chain length

The effect of chain length of the alcoholic moiety was also considered, because it is well

known that esterases work better with short chain esters. So we tried the methyl, ethyl and *n*-butyl esters of racemic naproxen. As seen in Table 4, the best results in both, conversion and enantioselectivity, were for the methyl ester, under the reaction conditions used; in this occasion the reaction time was extended trying to improve the conversion. During our experiments about the co-solvent effect we noticed an increase of conversion when 15% DMSO was used (Table 1), so we decided to try this co-solvent in a reaction with the butyl ester to see if an increase in the rate of the reaction would occur at 5º C, and indeed the conversion after 144h of reaction was 30%, but the enantioselectivity did not improve (29% ee, and E~ 2); a very interesting fact of this reaction was that the hydrolysis took place over the "*R*" isomer, instead of the *S*-enantiomer.

Table 4. Effect of the ester chain length in the hydrolysis of racemic naprox esters.

Naproxen ester	% Conv.	% ee	E
Methyl	12	88	16
Ethyl	2	56	nc
n-Butyl	4	25	nc

Reaction condition: 15% dioxane, 1:1 sust./SLAP ratio, 4-5° C, 98 h. nc: not calculated due to low conversion

In previous work we reported a 4% conversion and 68% ee after the hydrolysis of methyl naproxenate using SLAP as biocatalyst under the following reaction conditions: pH 7.5, 10% dioxane, room temperature and 1:1 substrate/SLAP ratio for 24h. After the evaluation of the effect of pH, reaction time, co-solvents, ratio substrate/SLAP and temperature it was observed that better results, (21% conversion and 72% ee) were obtained when the reaction was performed at pH 7, 48h, dioxane (15%), 1:1 sustrate/SLAP ratio, and at room temperature (~24° C).

In conclusion we can say that the best naproxen ester for the resolution using SLAP was methyl, because ethyl and butyl gave low conversions. We also demonstrate that, after the proper tunning of reaction conditions, SLAP can have potential application for the preparation of chiral antiinflamatory agents, chemically related to naproxen.

ACKNOWLEDGEMENT

We thank the financial support of Consejo Nacional de Ciencia y Tecnología (CONACyT) Grant num. 37272-N. We also thank Dr. Ignacio Regla, UNAM; for the racemic Naproxen donation.

REFERENCE

[1] Poppe L, Novák L: From Selective Biocatalysis: Hydrolases , New York, VCH, 1992:67-155.

[2] Patel RN. 2004. Enzymatic Synthesis of Chiral Intermediates for Drug Development. Adv. Synth. Catal. 343(6-7):527.

[3] Ikunaka M. 2004.Biocatalysis from the perspective of an industrial practitioner: let a biocatalyst do a job that no chemocatalyst can. Cat. Today. 96(3):93.

[4] Higa HH, Manzi A, Varki A. 1989. O-acetylation and de-O-acetylation of sialic acids. Purification, characterization, and properties of a glycosylated rat liver esterase specific for 9-O-acetylated sialic acids J. Biol. Chem., 264:19435-19442.

[5] Dominguez de Maria P, Garcia Burgos CA, Bargeman G, van Gemert R. 2007. Pig liver esterase (PLE) as biocatalyst in organic synthesis: from nature to cloning and to practical applications Synthesis (10):1439-1452.

[6] Basavaiah D, Rama Krishna P, Bharathi TK. 1990. Convenient enantioselective hydrolysis of racemic *trans*-1-acetoxy-2-aryloxycyclohexanes by crude pig liver acetone powder (PLAP). Tetrahedron Lett. 31:4347-4348;

[7] Basavaiah D, Bhaskar Raju S. 1994. Bovine liver acetone powder (BLAP) catalyzed synthesis of chiral C-8 allyl alcohols: An application of substrate specificity approach. Tetrahedron 50(14):4137-4148.

[8] Basavaiah D, Dharma Rao P. 1994. Biocatalytic approach to optically active Baylis-Hillman reaction products. Synth. Comm., 24(7):925-929.

[9] Comini A, Forzato C, Nitti P, Pitacco G, Valentin E. 2004. Chemoenzymatic synthesis of enantioenriched 5-oxo-tetrahydro-3-furancarboxylic acid derivatives. Tetrahedron: Asymmetry, 15:617-625.

[10] Sánchez R, Luna H, Pérez HI, Manjarrez N, Solís A. 2001. Evaluation of animal liver acetone powders for the resolution of 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid., Tetrahedron: Asymmetry 12:1399-1401.

[11] Basavaiah D. 2001. Applications of liver acetone powders in enantioselective synthesis. Arkivoc. (8):70-82.

[12] Solís A, García S, Pérez HI, Manjarrez N, Luna H. 2008. Screening of liver acetone powders in the resolution of 1-phenylethanols and 1-phenylpropanols derivatives. Tetrahedron: Asymmetry. 19(7):549-553.

[13] Holton RA, Vu Ph, 2003. Enzymatic process for the resolution of enantiomeric mixtures of beta-lactams (FSU Research Foundation, Inc) US patent 6,548,293.

[14] Goswami A. 1994. Process for hydrolyzing stereoisomer esters of trans chrysanthemic acid using liver enzymes derived from horse, rabbit, pigeon or cat (Rhone-Poulenc Inc.) US patent 5,281,534.

[15] Liu KK. 2004. Enzymatic resolution of selective estrogen receptor modulators (Pfizer Inc.) US patent 6,828,134.

[16] Flavin MT, Xu Z-Q, Khilevich A, Rizzo JD, Chen W, Lin L, Kucherenko V, Sheinkman AK, Boulander WA. 2000. Method for the preparation of (+)-calanolide A and intermediates thereof (Sarawak MediChem Pharmaceuticals, Inc.) US patent 6,043,271.

[17] Raju MS, Huh N. 1994.Resolution of hydroxychroman-2-carboxylic acid esters by enantiomeric hydrolysis (Rhone-Poulenc Chimie) US patent 5,348,973.

[18] Roszkowski AP, Rooks WH II, Tomolins AJ, Miller LM. 1971. Antiinflamatory and analgetic propierties of d-2-(6'-methoxy-2'-naphtyl)-propionic acid (Naproxen) J. Pharmacol. Exp. Therp. 179(1):114-123.

[19] Sonawanae HR, Bellur NS, Ahuja JR, Kulkarni DG. 1992. Recent developments in the synthesis of optically active □-arylpropanoic acids: An important class of non-steroidal anti-inflammatory agents. Tetrahedron: Asymmetry 3(2) :163-192.

[20] Combes G, Coen E, Dehgani F, Foster N. 2005. Dense CO2 expanded methanol solvent system for synthesis of naproxen via enantioselective hydrogenation. J. Supercritical Fluids 36(2):127-136.

[21] Cretich M, Chiari M, Carrea G. 2001. Stereoselective synthesis of (S)-(+)-naproxen catalyzed by carboxyl esterase in a multicompartment electrolyzer. Biochem. & Biophys. Methods 48(3):247-256.

[22] Potluri SH, Ramulu AR, Pardhasaradhi V. Synthesis of new unsymmetrical optically active (S)-(+)-naproxen dendrimers. 2004. Tetrahedron 60(48):10915-10920.

[23] Goswani A. 1991. Stereospecific resolution by hydrolysis of esters of 2-arylpropionic acids by liver enzymes (Rhone-Poulenc, Inc.) US patent 5,175,100.

[24] Sánchez R, Luna H, Pérez HI, Manjarrez N, Solís A. 2001. Evaluation of animal liver acetone powders for the resolution of 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid. Tetrahedron: Asymmetry 12: 1399-1401.

[25] Koul S, Parshad R, Taneja SC, Qazi GN. 2003**.** Enzymetic resolution of Naproxen Tetrahedron: Asymmetry. 14(16**)**:2459-2465.

[26] Pacheco A, Luna H, Solís A, Pérez HI, Manjarrez N. 2006. Screening of liver acetone powders in the enantioselective hydrolysis of Naproxen esters. J. Mex. Chem. Soc. 50(3):137-142.

[27] Guanti G, Banfi L, Narisano E, Riva R, Thea S. 1986. Enzymes in asymmetric synthesis: Effect of reaction media on the PLE catalyzed hydrolysis of diesters. Tetrahedron Lett. 27(38): 4639-3642.