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Fast and easy in vitro screening assay for cholesterol biosynthesis inhibitors in the post-squalene pathway

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ABSTRACT

A whole-cell assay for screening cholesterol biosynthesis inhibitors in the post-squalene pathway has been developed. HL 60 cells were incubated for 24 h with test substances. The nonsaponifiable lipids were extracted by means of liquid–liquid extraction using *tert*-butylmethylether. The raw extracts were purified by dispersive solid phase extraction using a primary–secondary amine material (PSA) and dried using sodium sulphate. The sterols were derivatized using *N*-trimethylsilylimidazole. GLC/MS analysis was carried out in less than 12.5 min using fast GLC mode. The obtained sterol patterns indicated which enzyme had been inhibited. Specific sterol patterns which reflect the different enzyme inhibitions were obtained using established inhibitors of cholesterol biosynthesis like AY 9944, NB 598, clotrimazole, aminotriazole and DR 258, a Δ24-reductase inhibitor prepared in our working group. For characterizing IC_{50} values we used sodium 2-^{13}C-acetate and quantified the incorporation of it into cholesterol relative to control levels after the samples had been normalized to their protein content.

1. Introduction

Reduction of cholesterol levels is clearly associated with a decrease in mortality and morbidity in cardiovascular diseases [1]. The statins, inhibitors in the early stage of cholesterol biosynthesis, are generally used for the therapy of elevated cholesterol levels and are tolerated well [2]. Despite these facts, distal cholesterol biosynthesis also offers some interesting targets for cholesterol lowering therapy, for example, lanosterol synthase (OSC), for which a partial inhibition had been shown to have beneficial effects [3]. Not only does cholesterol biosynthesis play an important role in the development of cardiovascular diseases or the fluidity of biological membranes, but cholesterol also plays an important role in embryogenesis [4], which, for example, is expressed in the Smith–Lemli–Opitz syndrome, a severe developmental disorder which is caused by a deficiency of the enzyme 7-dehydrocholesterol reductase [5,6]. Usually inhibition of late cholesterol biosynthesis has been studied using scintillation counting with prior separation of the accumulating substances via TLC [7,8] or HPLC [9–11]. Stable isotope methods have also been used to study cholesterol biosynthesis (see below). The TLC methods have the disadvantage of limited resolution capability, whilst the HPLC methods afford very long run times of up to 45 min. Furthermore, both methods share the disadvantage that radioactive substances have to be used and that no structural information about the accumulating sterols can be collected. Harwood et al. [12] reported the identification and quantification of cholesterol precursors as their trimethylsilyl ethers after liquid–liquid extraction from plasma and liver samples using GLC/MS analysis. LC/MS could also be a suitable method, which would also allow the direct

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determination of cholesterol without derivatization [13], but LC/MS has the disadvantage of a lower resolution capability compared to GLC/MS. Furthermore, LC/MS mass spectra are sometimes very difficult to interpret and contain very less structural information. Moreover, LC/MS spectra cannot be compared to the most popular mass spectra libraries like NIST™, and up to date only very few LC/MS spectra libraries are available. Compared to LC/MS, GLC/MS analysis might have the disadvantage of sample breakdown, especially when labile substances are analyzed; this has to be claimed as an advantage of LC/MS analysis. But GLC/MS provides good separation of the accumulating sterols (Fig. 1) and information-rich mass spectra which are comparable to the standard mass spectra libraries. This gives the user the additional possibility to identify the unexpectedly accumulating sterols. Because of these facts, we choose GLC/MS for our whole-cell screening assay. To ensure short analysis times, fast GLC mode was employed, using a 0.15 mm i.d. column (15 m), which allows short run times of less than 12.5 min and shows good resolution capability [14]. For sample cleanup we selected dispersive solid-phase extraction using PSA and sodium sulphate. This procedure has recently been described by us for the extraction of different drugs from whole blood samples [15]. For the determination of IC_{50} values, we wanted to refuse radioactive labeled substances; hence we used stable isotope technology and quantified ^{13}C-acetate incorporation into the target molecule cholesterol. Stable isotope technology has previously been used to study cholesterol biosynthesis by means of isotopomer spectral analysis (ISA) [16]. The technique has already been used to study cholesterol biosynthesis in HepG2 cells [17,18] and human subjects [19,20]. Holleran et al. used ISA to determine the effects and the IC_{50} value of tamoxifen (antiestrogenic drug), a dual action inhibitor of $\Delta^{8/7}$-isomerase and Δ^{24}-reductase in distal cholesterol biosynthesis [18]. We then applied stable isotope technology to our screening assay to clearly separate the "newly" formed cholesterol from natu-

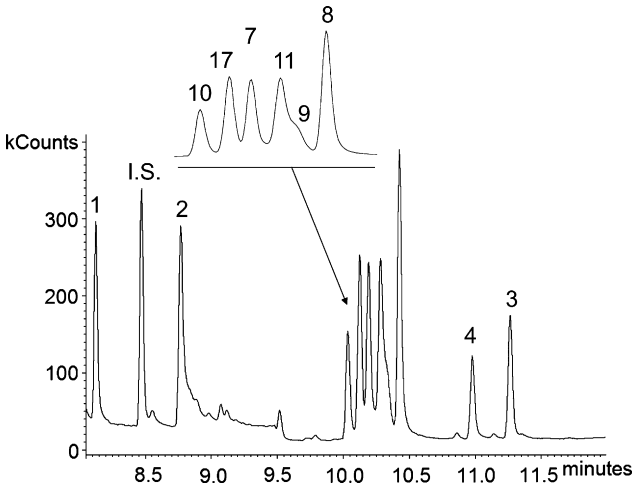

Fig. 1 – TIC chromatogram showing all available standard substances; substance concentration was 500 ng/ml, except 4, 3 ~250 ng/ml and 2 2 μg/ml; I.S. internal standard cholestane, 1 squalene, 2 monoepoxysqualene, 3 lanosterol, 4 dihydrolanosterol, 7 zymostenol, 8 lathosterol, 9 7-dehydrocholesterol, 10 cholesterol, 11 desmosterol, 17 cholesta-8,14-dien-3β-ol.

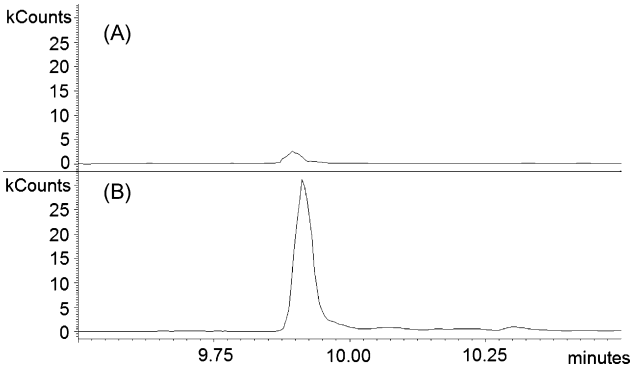

Fig. 2 – XIC (372–379 + 462–469 *m/z*); unlabeled control sample (A) and labeled control sample (B).

rally occurring matrix cholesterol, which was possible due to ^{13}C-acetate incorporation. The labeled cholesterol content of treated samples (enzyme inhibition) is then quantified relative to untreated control samples (Fig. 2). Regardless of the enzyme that is inhibited, an IC_{50} value referring to cholesterol can be determined and there is no need to quantify all cholesterol precursors, because the IC_{50} value refers to the overall inhibition of ^{13}C-acetate incorporation into cholesterol and describes a general inhibitory effect of cholesterol biosynthesis. This has the further advantage that IC_{50} values for substances inhibiting several enzymes of cholesterol biosynthesis can be determined in a single assay. To determine specific sterol patterns due to inhibition of single enzymes (Fig. 3), we incubated HL 60 cells for 24 h with different established inhibitors; this long incubation period was chosen to take regulatory effects as they have especially been described for lanosterol synthase (OSC) inhibitors into account [21].

The following inhibitors have been used to gather reference chromatograms for the different enzyme inhibitions.

AY 9944 has been proven to inhibit Δ^{14}-reductase and $\Delta^{8/7}$-isomerase at higher concentrations and 7-dehydrocholesterol reductase (7-DHCR) at lower concentrations [22,23]. NB 598 is a well-known inhibitor of squalene epoxidase [24,25]. For the inhibition of OSC, we used BIBX 79 [21]. Clotrimazole is known as an inhibitor of sterol C14-demethylase especially in fungi, but in human cells also clotrimazole has been shown to inhibit sterol C14-demethylase potently [8,26]. Aminotriazole has been shown to inhibit the C4-demethylase complex at high concentrations [27,28]. Ergosterol and other Δ^{22}-sterols were described as inhibitors of Δ^{24}-reductase [10], but we used DR 258 (Fig. 4), an ergosterol derivative prepared in our working group (unpublished data) which showed the same effects as described for ergosterol.

For the inhibition of lathosterol oxidase, no selective inhibitor is available up to now (Fig. 3).

2. Experimental

2.1. *Analysis and materials*

GLC/MS analysis was carried out on a Varian Saturn 2200 ion trap and a GC 3800 equipped with a CP 8400 autosampler and

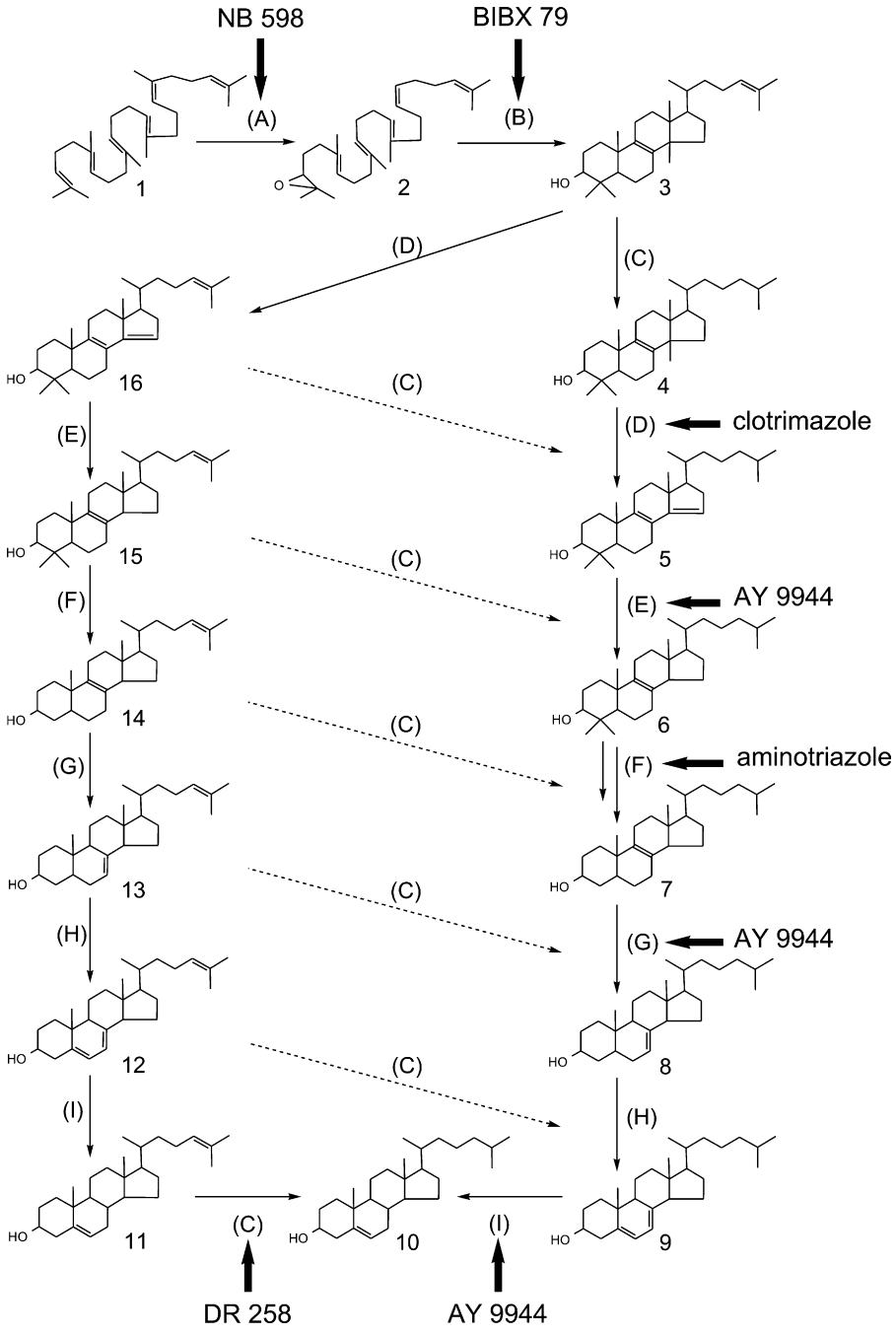

Fig. 3 – Cholesterol biosynthesis and target enzymes for different late cholesterol biosynthesis inhibitors [5]: (A) squalene epoxidase, (B) lanosterol synthase, (C) Δ^{24}-reductase, (D) sterol C14-demethylase, (E) Δ^{14}-reductase, (F) C4-demethylase, (G) $\Delta^{8/7}$-isomerase, (H) lathosterol oxidase, (I) 7-dehydrocholesterol reductase. 1 Squalene, 2 monoepoxysqualene, 3 lanosterol, 4 dihydrolanosterol, 5 4,4-dimethylcholesta-8,14-dien-3β-ol, 6 4,4-dimethylcholesta-8-en-3β-ol, 7 zymostenol, 8 lathosterol, 9 7-dehydrocholesterol, 10 cholesterol, 11 desmosterol, 12 cholesta-5-7-24-trien-3β-ol, 13 cholesta-7,24-dien-3β-ol, 14 zymosterol, 15 4,4-dimethylcholesta-8,24-dien-3β-ol, 16 4,4-dimethylcholesta-8,14,24-trien-3β-ol.

an 1177 split/splitless injector (Varian, Darmstadt, Germany). NMR spectra were recorded in $CDCl_3$ using the solvent peaks as standard on a JEOL GSX 400 or JNMR GX 500, and high resolution mass spectra were recorded on a GC Mate II (JEOL, Peabody, MA, USA). Melting points were determined on a Büchi B 540 apparatus (Büchi, Flavil, Switzerland). Desmosterol and cholestane were obtained from Steraloids Inc. (Birmingham, UK), *N*-trimethylsilylimidazole (TSIM) and autosampler vials were purchased from Macherey Nagel (Düren, Germany), clotrimazole was from Synopharm (Barsbüttel, Germany), Bradford color solution for protein determination was from Carl Roth GmbH + Co. KG (Karlsruhe, Germany), RPMI 1640 medium and fetal bovine serum (FBS) were from PAA Laboratories GmbH (Cölbe, Germany), Medium für HL 60 Zellen

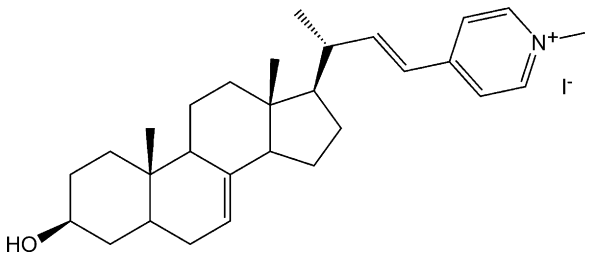

Fig. 4 – Structure of DR 258.

(lipid free medium) and lipoprotein deficient serum (LPDS) were purchased from PAN Biotech (Aidenbach, Germany), the HL 60 cell line was from DSMZ (Deutsche Sammlung für Mikroorganismen und Zellkulturen, Braunschweig, Germany). Culture flasks and 24-well plates were from Peske (Aindling-Arnhofen, Germany), PSA was purchased from Varian (Darmstadt, Germany), and BIBX 79 was a kind gift from Boehringer Ingelheim Pharma GmbH & Co. KG. All other chemicals were purchased from Sigma–Aldrich (Schnelldorf, Germany). *tert*-butylmethylether (TBME) was distilled before use.

2.2. *Cell culture*

HL 60 cells were maintained in RPMI 1640 medium containing 10% FBS without antibiotics at 37 °C in a humidified atmosphere containing 5% CO_2.

2.3. *Analysis of cholesterol biosynthesis inhibition and ^{13}C-acetate incorporation by GLC/MS*

HL 60 cells (1×10^6) were incubated in 24-well plates in the presence or absence of the different inhibitors in 1 ml of lipid-free medium containing 1% LPDS without antibiotics. The drugs were dissolved in absolute ethanol and added up to a final concentration of 1.0% ethanol. Aminotriazole was dissolved in incubation medium and subjected to sterile filtration and then added to the cells to reach the final test concentration. After a 24 h incubation period (conditions as stated under cell culture) the content of each well was transferred into a 2 ml plastic tube and the wells were washed with 750 μl of phosphate-buffered saline (PBS). The cells were centrifuged at $540 \times g$ for 5 min and washed once with 1 ml of PBS. One milliliter of 1 M NaOH was added to each tube and saponification was carried out for 60 min at 70 °C. Fifty microliters of internal standard solution (cholestane in TBME, 10 μg/ml) and 700 μl of TBME were added and the tubes were shaken vigorously for 1 min and centrifuged at $9200 \times g$ for 5 min. The extraction was repeated with another 750 μl of TBME in the same manner. The combined organic extracts were vigorously shaken for 30 s over 35 mg of dried sodium sulphate and 5 mg of PSA and centrifuged for 5 min at $9200 \times g$. One milliliter of the purified extract was transferred into an autosampler vial and evaporated to dryness under a mild stream of nitrogen. To each vial, 950 μl of TBME and 50 μl of TSIM were added. Silylation reaction was carried out for 1 h at room temperature. The trimethylsilyl ethers were analyzed on a Varian Factor Four 5-MS 15 m × 0.15 mm × 0.15 μm column. Temperature program started at 50 °C held for 1.5 min, then ramped to 260 °C with 55 °C/min, then ramped to 305 °C with 7 °C/min, and finally to 310 °C with 50 °C/min and held for 0.5 min. MS was operated in full scan mode 7–9.5 min 60–450 *m*/z and 9.5–12.0 min 100–550 *m*/z (EI, 70 eV). Injector temperature was maintained at 260 °C and 2 μl of the samples were injected splitless (splitless time 1.5 min). Helium of 99.999% purity was used as carrier gas at a constant flow rate of 0.7 ml/min. Transfer line temperature was 270 °C, ion trap temperature 200 °C. The accumulating sterols were identified by comparison with commercially available authentic substances or reference substances prepared by us (see below). In the cases where no reference substance was available, the sterols were identified on the basis of their mass spectral data by comparison with NIST™ 2005 database or literature [28–30]; this was only necessary for 4,4-dimethylcholesta-8-en-3β-ol (**6**), cholesta-5,7,24-trien-3β-ol (**12**) and 4-methylcholesta-7-en-3β-ol (**18**).

For the determination of labeled cholesterol, the protocol was altered in the following manner: To each incubation well, 10 μl of a sterile sodium 2-^{13}C-acetate solution (6.25 mg/ml) was added directly before substance addition, leading to a final ^{13}C-acetate concentration of 62.5 μg/ml. After saponification 3×25 μl aliquots were taken for protein determination following the method of Bradford [31], using bovine serum albumine as standard. Quantification of the labeled cholesterol was carried out by analyzing the ions 372–379 and 462–469 *m*/z. For cholestane (internal standard) 217 and 357 *m*/z were chosen as quantifier ions. The percentage inhibition (see Fig. 5 for the calculation formula) relative to untreated control samples (0% inhibition) was plotted against the logarithmic inhibitor concentration using Graph Pad Prism 4. A bottom level constant equal to 0 was set as constraint using a sigmoidal dose-response model with a variable slope. All samples were normalized to their protein content taking into account the number of cells. For each concentration the percentage inhibition was determined in triplicate.

2.4. *Validation*

The validation was carried out according to DIN 32645 [32]. Squalene (**1**) (non-sterol) and lathosterol (**8**) (sterol) were chosen as model substances. A bulk extract from 50×10^6 cells was prepared, and samples of 1 ml extract volume corresponding to 1 million cells were spiked with different amounts (six levels, 20–1000 ng) of **1** and **8**, the samples were worked up as described above. The result of an unspiked control sample was subtracted before calculations were done. For determining precision of ^{13}C-acetate incorporation into cholesterol, cells were incubated and worked up in the described quantitative manner at a concentration of 0.2 μM clotrimazole and without

$$\%\,inhibition = \left[1 - \left(\frac{A_S \times A_{I.S.C} \times PC_C}{A_C \times A_{I.S.S} \times PC_S}\right)\right] \times 100$$

Fig. 5 – Calculation formula for the percentage inhibition; A_S area sample; $A_{I.S.C.}$ area internal standard control; PC_c protein content control; A_c area control; $A_{I.S.S.}$ area internal standard sample; PC_S protein content sample.

addition of clotrimazole ($n = 6$). For determining the selectivity for the measurement of unlabeled versus labeled cholesterol, control samples corrected for their protein content were compared to each other in triplicate.

2.5. *Preparation of reference substances*

Reference substances which were not commercially available were prepared and characterized according to literature or as described below. Monoepoxysqualene (**2**) was prepared and characterized according to ref. [33], and cholesta-8,14-dien-3β-ol was prepared as described in ref. [34] and purified by recrystallization from methanol/dichloromethane and characterized according to ref. [35]. Zymostenol (**7**) was prepared from cholesta-8,14-dien-3β-ol (**17**) as described for 4,4-dimethylcholesta-8-en-3β-ol (**6**) in ref. [36] and characterized according to refs. [37,38]. Lathosterol (**8**) was prepared and characterized according to ref. [39]. **8** was recrystallized three times from methanol/dichloromethane before use. Dihydrolanosterol (**4**): 200 mg lanosterol from sheep wool was dissolved in 5 ml toluene/ethyl acetate (1:1) and hydrogenated using 40 mg palladium on charcoal (10%) for 17 h at room temperature. The crude product was filtered through celite, evaporated to dryness under vacuum and recrystallized from methanol/dichloromethane to give **4**. ^{1}H and ^{13}C NMR data matched those previously described for the substance [40]. HRMS: calculated 428.4008, found 428.4018; mp 147 °C.

3. Results

3.1. *Qualitative results*

The following substances accumulated upon treatment with standard inhibitors, or were used for the validation procedure (**8**). The substances were identified through comparison with standard substances or according to literature and NIST™ 2005 mass spectral database (Table 1).

For the qualitative identification of an enzyme inhibition, the accumulation of the described sterols was analyzed visually and no quantification of the accumulating sterols was carried out in the case of qualitative test procedure, because enzyme inhibition normally leads to a very significant change in the obtained sterol patterns compared to control (Fig. 6).

Under treatment with the inhibitor NB 598, accumulation of squalene (**1**) could be observed, as expected. In the case of BIBX 79, we could only detect the accumulation of monoepoxysqualene (**2**) at high inhibitor concentration (10 μM). Clotrimazole caused an accumulation of dihydrolanosterol (**4**) besides a weak accumulation of lanosterol (**3**). AY 9944 led to the accumulation of 7-dehydrocholesterol (**9**) at a concentration of 0.1 μM; at higher concentrations of 1 and 10 μM, zymostenol (**7**) and cholesta-8,14-dien-3β-ol (**17**) accumulated. Aminotriazole treatment resulted in the accumulation of 4-methylcholesta-7-en-3β-ol (**18**) and 4,4-dimethylcholesta-8-en-3β-ol (**6**), as expected [24]. Under treatment with DR 258, desmosterol (**11**) and cholesta-5,7,24-trien-3β-ol (**12**) accumulated, as described for ergosterol [10] (Fig. 6).

Table 1 – Relative retention times (RRT), qualifier ions and match factors

Substance	RRT	Characteristic ions *m/z* (%)	Match factor
(I.S.)	1.000	357 (62) 217 (100) 203 (25)	989
(1)	0.957	95 (31) 81 (81) 69 (100)	995
(2)	1.036	121 (39) 95 (37) 81 (100)	985
(10)	1.184	458 (52) 368 (100) 329 (79)	980
(17)	1.195	456 (38) 351 (100) 182 (41)	949
(7)	1.203	458 (100) 353 (48) 213 (49)	982
(11)	1.214	456 (27) 253 (100) 129 (79)	950
(9)	1.220	366 (34) 351 (100) 325 (60)	982
(8)	1.230	458 (100) 255 (55) 213 (45)	*n.d.*
(12)	1.249	364 (28) 349 (100) 323 (54)	[29]
(18)	1.269	472 (100) 382 (35) 227 (65)	[28]
(4)	1.296	395 (100)[a]	999
(6)	1.319	486 (43) 396 (81) 381 (100)	[28]
(3)	1.329	498 (13) 393 (100) 241 (15)	913

[a] All other ions showed intensities smaller than 10% of the base peak; **I.S.** internal standard cholestane, **1** squalene, **2** monoepoxysqualene, **3** lanosterol, **4** dihydrolanosterol, **6** 4,4-dimethylcholesta-8-en-3β-ol, **7** zymostenol, **8** lathosterol, **9** 7-dehydrocholesterol, **10** cholesterol, **11** desmosterol, **12** cholesta-5-7-24-trien-3β-ol, **17** cholesta-8,14-dien-3β-ol, **18** 4-methylcholesta-7-en-3β-ol; n.d. not determined, the match factor describes the conformance of the mass spectral data for the accumulated sterol under enzyme inhibition and the standard substance analyzed by us, in the case no match factor is given the substance was identified according to literature, **8** was used for the validation procedure.

3.2. *Quantitative results*

3.2.1. ^{13}C-Acetate incorporation

To demonstrate the effects of ^{13}C-acetate incorporation on mass spectral data, we compared the mass spectrum of labeled versus unlabeled cholesterol (**10**) (Fig. 7). Because of the high amounts of matrix cholesterol, the effect is mostly overlaid by unlabeled cholesterol, but the clear differentiation between both substances can be seen in the extracted ion chromatogram using the ion choice 372–379 + 462–469 *m*/z (Fig. 2). The first values of the ion choice 372 and 462 *m*/z were chosen because at least a difference of *m*/z = 4 between labeled and unlabeled cholesterol was necessary to gain high selectivity. The later values of *m*/z = 379 and 469 were chosen, because a maximum of 11 ^{13}C-acetate molecules can occur in one molecule of cholesterol. The selectivity for the deter-

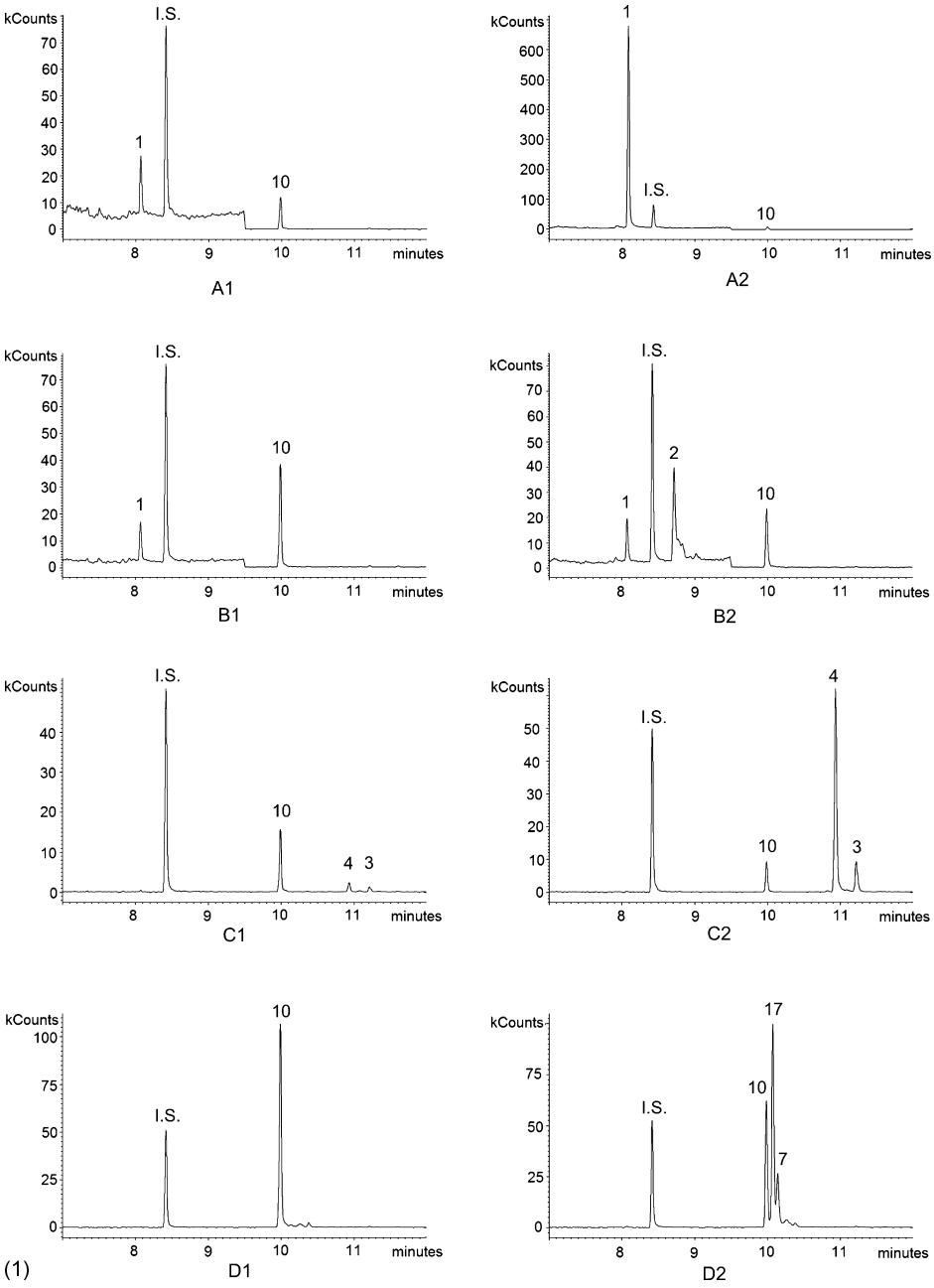

Fig. 6 – Extracted ion chromatograms of the accumulating sterols after treatment with inhibitors; A1 control; A2 NB 598, 40 nM (extracted ions *m*/z 217 + 357 + 69 + 81 + 95); B1 control; B2 BIBX 79, 10 μM (*m*/z 217 + 357 + 81 + 95 + 121); C1 control; C2 clotrimazole, 0.2 μM (*m*/z 217 + 357 + 498 + 393 + 241 + 395); D1 control; D2 AY 9944, 10 μM (*m*/z 217 + 357 + 456 + 351 + 182 + 458 + 353 + 213); E1 control; E2 aminotriazole, 25 mM (*m*/z 217 + 357 + 472 + 382 + 227 + 486 + 396 + 381); F1 control; F2 AY 9944, 0.1 μM (*m*/z 217 + 357 + 366 + 351 + 325); G1 control; G2 DR 258, 1 μM (*m*/z 217 + 357 + 456 + 253 + 129 + 364 + 349 + 323). I.S. Cholestane, 1 squalene, 2 monoepoxysqualene, 3 lanosterol 4 dihydrolanosterol, 6 4,4-dimethylcholesta-8-en-3β-ol, 7 zymostenol, 9 7-dehydrocholesterol, 10 cholesterol, 11 desmosterol, 12 cholesta-5,7,24-trien-3β-ol, 17 cholesta-8,14-dien-3β-ol, 18 4-methylcholesta-7-en-3β-ol.

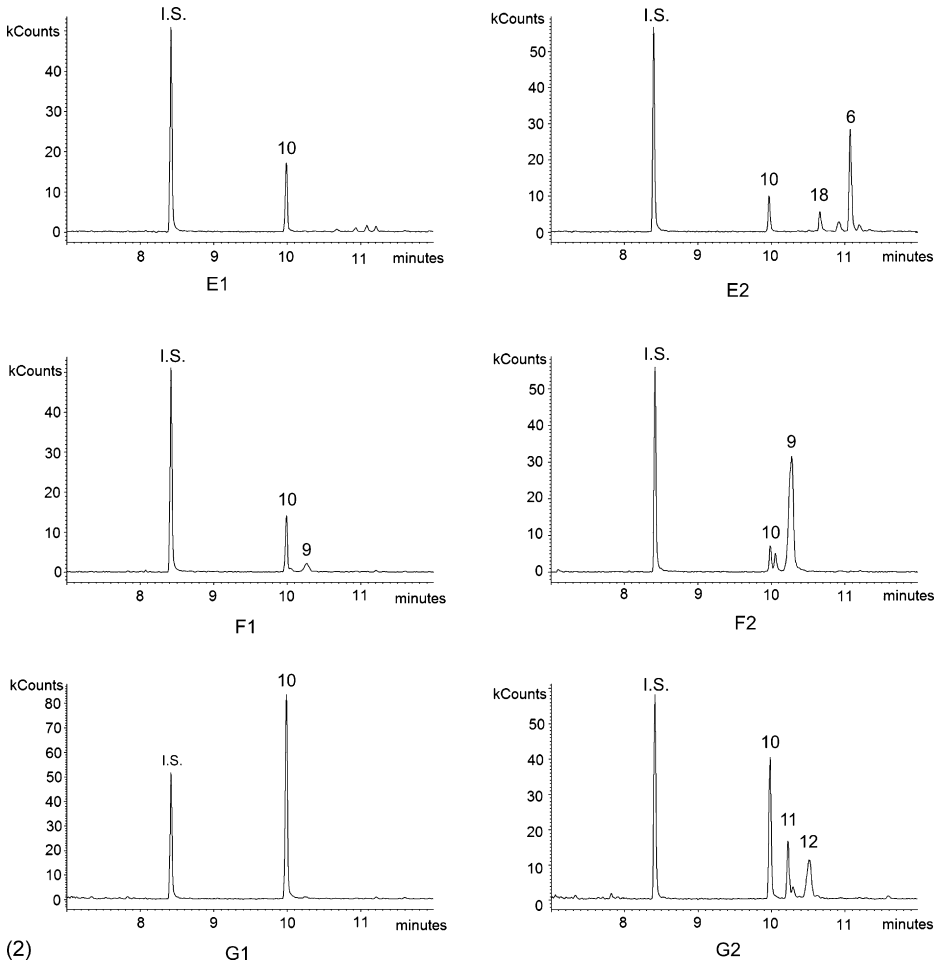

Fig. 6 – (*Continued*).

mination of labeled versus unlabeled cholesterol, respectively the ion choice, is given in the validation section. A clear imaging of the labeling effect can be shown for the accumulating precursors. In this case no matrix effects overlay the imaging of the labeling effect. This is shown for the main fragment of dihydrolanosterol which accumulated under treatment with

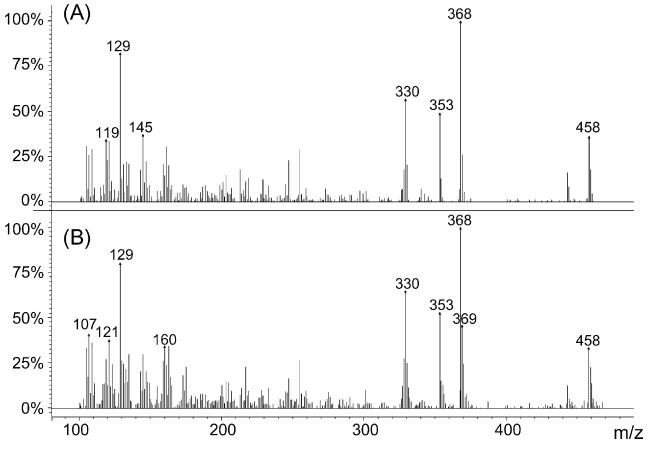

Fig. 7 – Mass spectra of unlabeled cholesterol (A) vs. ^{13}C labeled cholesterol (B).

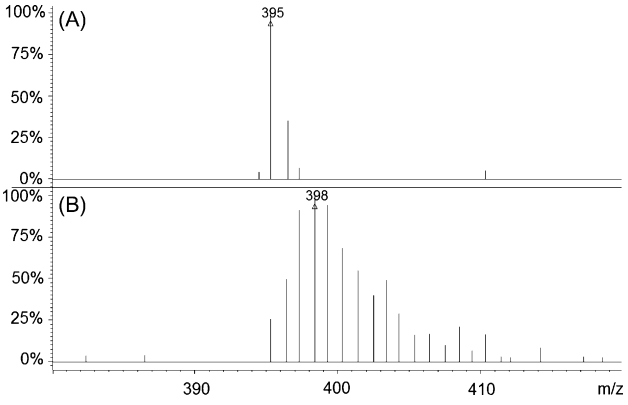

Fig. 8 – Mass spectra of the main fragment of unlabeled (A) vs. ^{13}C labeled (B) dihydrolanosterol.

0.2 μM clotrimazole (Fig. 8). Without the matrix effect it is obvious that approximately a gaussian curve is formed (Fig. 8).

3.2.2. *Determination of the IC_{50} values of NB 598 and clotrimazole*

The IC_{50} values (inhibitor concentration which results in a 50% inhibition of ^{13}C-acetate incorporation into cholesterol;

Table 2 – Goodness of fit for the determined dose response curves and IC_{50} values of NB 598 and clotrimazole

Substance	R^2	IC_{50} (nM)
NB 598	0.969	1.92
Clotrimazole	0.907	128

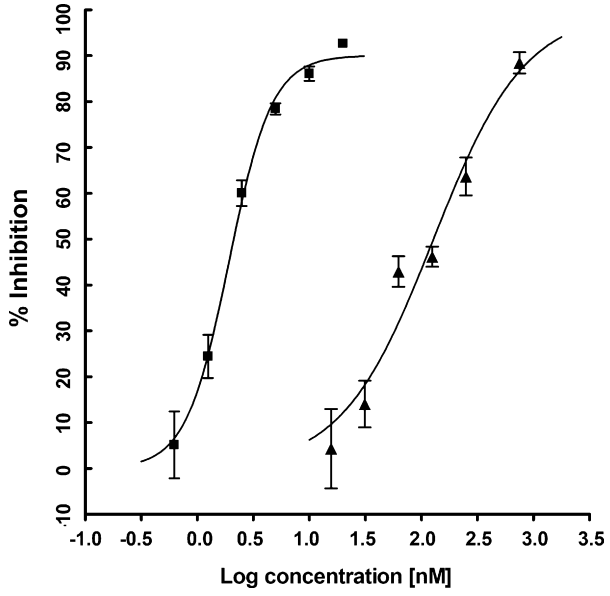

Fig. 9 – Dose response curves for NB 598 (■) and clotrimazole (▲); error bars show ± 1 standard error of the mean (S.E.M.).

Table 2) of clotrimazole and NB 598 were determined as described above in triplicate (Table 2, Fig. 9). The percentage inhibition was plotted against the logarithmic inhibitor concentration as described above. The goodness of fit for both curves is given in Table 2.

3.2.3. *Validation results*

Tables 3 and 4 show the validation results for squalene and lathosterol. The precision ($n = 6$) for the ^{13}C-acetate incorporation under control conditions and under inhibition with clotrimazole was determined as follows (Table 5).

The selectivity for the *m/z* choice 372–379 and 462–469 was determined for labeled versus unlabeled cholesterol and was greater than 92% (Fig. 2)

Table 3 – Linearity, recovery and precision for squalene and lathosterol

Substance	Linearity R^2 (20–1000 ng)	Recovery (%)	Method precision (%) ($n = 6$; 200 ng; C.V.)
Squalene	0.996	91	6.5
Lathosterol	0.996	105	7.6

C.V.: Coefficient of variation.

Table 4 – LOD, LOQ and slope for squalene and lathosterol

Substance	LOD (ng)	LOQ (ng)	Slope (u/ng)
Squalene	27	76	138
Lathosterol	47	127	191

LOD, limit of detection; LOQ, limit of quantitation; [u/ng], units per ng.

Table 5 – Precision of the ^{13}C-acetate incorporation into cholesterol

Conditions	Precision (%) ($n = 6$; C.V.)
Control conditions	23
0.2 μM clotrimazole	28

C.V.: Coefficient of variation.

4. Discussion

Enzyme inhibition with specific inhibitors led to the accumulation of different substances. The accumulating substances were identified and can serve as marker substances for the different enzyme inhibitions. With the presented sterol patterns of the previously described enzyme inhibitors, the identification of other substances acting as inhibitors in the post-squalene pathway of cholesterol biosynthesis is possible, indicating which enzyme has been inhibited. It has to be annotated that an accumulation of lathosterol should be representative for an inhibition of lathosterol oxidase [41]. Because no selective inhibitor for lathosterol oxidase was available, we could not investigate this thesis. The precursor accumulation was as follows.

On treatment with the squalene epoxidase inhibitor NB 598, an accumulation of the substrate squalene (**1**) was observed, as expected [24,25]. In the case of BIBX 79, we could clearly identify monoepoxysqualene (**2**) which accumulated at high inhibitor concentration (10 μM). In contrast, Mark et al. [21] reported on the accumulation of **2** besides diepoxysqualene and epoxycholesterol at concentrations from 0.01 to 1 μM. These differences can maybe traced back to the different cell lines used, but on the other hand it does not seem crucial for our screening assay to identify diepoxysqualene and epoxycholesterol because **2** and the gathered chromatogram can be used as reference for the inhibition of OSC. Under treatment with clotrimazole, accumulation of dihydrolanosterol (**4**) besides a weak accumulation of lanosterol (**3**) was obtained, as expected [8,26]. At a concentration of 10 μM AY 9944, zymostenol (**7**) and cholesta-8,14-dien-3β-ol (**17**) accumulated. These findings stand in contrast to the expected accumulation of 4,4-dimethylcholesta-8,14-dien-3β-ol (**5**), as described by Fernández et al [22]. To clearly verify the identity of **17**, we prepared the substance as described above and compared RRT and mass spectra of the accumulating sterol and the synthetic standard. Both RRT and mass spectra clearly matched which testifies that the accumulating sterol is **17** and not **5**. This result can be explained by the further metabolism of **5** through the C4-demethylase complex leading to **17**. Under aminotriazole treatment, 4,4-dimethylcholesta-8-en-3β-ol (**6**)

and 4-methylcholesta-7-en-3β-ol (**18**) accumulated, as previously described for in vivo experiments [28], both substances have been identified due to their mass spectral data, which was especially possible because of the previous description of their accumulation under enzyme inhibition with aminotriazole [28]. At a concentration of 0.1 μM AY 9944, the only accumulating sterol was 7-dehydrocholesterol (**9**), which is in accordance with the previously published results [22]. DR 258 (1 μM) showed an accumulation of desmosterol (**11**) besides cholesta-5,7,24-trien-3β-ol (**12**), which corresponds to the previously described results for ergosterol, having the same target enzyme [10]. **12** had been identified due to its mass spectral data according to ref. [29].

The IC_{50} values of clotrimazole and NB 598 could be determined without the need to use radioactive labeled substances through the mass spectrometric determination of labeled cholesterol. The labeling of the target molecule cholesterol was achieved by the addition of sodium 2-^{13}C-acetate. The obtained IC_{50} value for clotrimazole (1.28×10^{-7} M) fits quite well the previously described IC_{50} value of about 1.5×10^{-8} M [26], which was determined using human fibroblasts and ^{14}C-acetate. To show the comparability of our assay to assays using ^{14}C-acetate and HepG2 cells, we determined the IC_{50} value of NB 598 which was 1.92 nM (Table 2, Fig. 9). The IC_{50} values of NB 598 previously reported are 7.2 nM [24] and 0.75 nM [25]. The former value was determined using a HepG2 cell homogenate and [^{3}H]squalene, and the latter value was determined using HepG2 cells and ^{14}C-acetate. The value obtained with our assay fits quite well the previously determined values, suggesting that our presented assay can equally be used. In general, the IC_{50} values of NB 598 and clotrimazole previously described and determined with our assay fit quite well.

In conclusion, inhibition of all of the enzymes involved in late cholesterol biosynthesis except sterol-C5-desaturase (no selective inhibitor had been available) can be analyzed qualitatively and quantitatively in a single assay. With the assay described here, fast (more than 50 samples a day) identification of late cholesterol biosynthesis inhibitors is possible. The presented chromatograms can be used as qualitative standards for the different enzyme inhibitions. New substances showing the same sterol patterns as the described inhibitors can be stated as having the same target enzyme.

The quantitative test procedure allows determining an overall IC_{50} value for cholesterol biosynthesis, regardless of the type of enzyme(s) that was inhibited. Due to the use of ^{13}C-acetate, radioactive substances can be refused owing to safety and costs.

Taken together, we have worked out a fast and easy in vitro screening assay for cholesterol biosynthesis inhibitors in post-squalene pathway that should be useful for the first identification and characterization of new selective cholesterol biosynthesis inhibitors; nevertheless, single enzyme assays should always be used for further substance characterization.

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