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Research paper

The effect of formulation and concentration of cholesteryl butyrate solid lipid nanospheres (SLN) on NIH-H460 cell proliferation

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Abstract

Experimental factorial design was used to evaluate the influence of two factors involved in producing cholesteryl butyrate (chol-but) solid lipid nanospheres (SLN), microemulsion formulation and microemulsion/water ratio, on the effect of the SLN on the proliferation of NIH-H460, a non-small-cell lung carcinoma; six experimental settings were tested. The cells were treated with scalar concentrations of cholesteryl butyrate (from 0.008 to 1.000 mM) for each experimental condition; NIH-H460 cell growth was inhibited in all cases. The best experimental setting provided complete inhibition at 0.125 mM chol-but, while at the same concentration sodium butyrate provided only 38% inhibition.

Keywords: Solid lipid nanospheres; Cholesteryl butyrate; NIH-H460 cell proliferation; Factorial design; Colloidal systems

1. Introduction

Butyric acid, a short-chain fatty acid naturally present in the human colon, is a compound that regulates cell proliferation and induces differentiation [1]. Its salts, especially the sodium salt, exert an antiproliferative activity on a wide range of neoplastic cells [2,3]. In particular, sodium butyrate has been shown to modulate the expression of some carcinogenesis-related oncogenes, such as c-myc, c-fos and H-ras [4,5], and of some genes controlling the apoptotic program, such as p53 and bcl-2 [6,7]. However, its rapid metabolism and excretion (half-life about 5 min) limit its clinical potential [8,9].

Non-small-cell lung cancer, which accounts for 75% of all lung cancers and is characterised by poor overall prognosis, remains difficult to treat, mainly due to its pathobiologic features (high aggressiveness, poor differentiation, rapid cell growth). A more effective therapeutic approach aimed at inhibiting the proliferative and metastatic potential of residual tumour cells following surgery or other treatment is necessary, and compounds able to regulate cell proliferation have been proposed as alternative or additional therapeutic measures. There is thus much interest in identifying innovative treatments able to inhibit the proliferative and metastatic potential of residual tumour cells. In this perspective, cholesteryl butyrate (chol-but) solid lipid nanospheres could be an alternative approach for butyric acid delivery: in a study [10] on the non-small-cell lung carcinoma cell line NIH-H460 we demonstrated that chol-but in solid lipid SLN was a potentially effective approach for delivering butyric acid to tumour cells, and that it could prolong cell exposure to the drug. SLN can be obtained by dispersing warm oil-in-water (O/W) microemulsion in a cold aqueous medium [11]; the resulting dispersion can be sterilised by autoclaving [12]. The lipid matrix of SLN was chol-but, used as prodrug to deliver butyric acid. The main components of the microemulsion were chol-but, Epikuron 200®, taurocholate and water. Chol-but inhibits 90% of cell growth (IC_{90}) at 0.19 mM, a concentration six times lower (1.2 mM) than that necessary to obtain a similar effect with free sodium butyrate. The chol-but/Epikuron 200® ratio was varied, obtaining different inhibition curves.

Due to the different inhibition that was achieved by changing only the chol-but/Epikuron 200® ratio, we performed some preliminary tests to examine the influence of this ratio and of dilution. By modifying both parameters, samples

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with various chol-but concentrations and with different antiproliferative activities were obtained.

The main aim of the present study was to investigate the possibility of obtaining a lower inhibitory concentration in the cell cultures. On the basis of preliminary experiments, we used experimental design to evaluate the influence of the two main factors involved in SLN preparation, the microemulsion formulation (chol-but percentage) and the warm microemulsion/water ratio, and to identify the best conditions in which to prepare SLN.

2. Materials and methods

2.1. Materials

Epikuron 200® (containing about 95% of soy phosphatidylcholine) and taurocholate were kind gifts from Lucas Meyer (Hamburg, Germany) and P.C.A. (Basaluzzo, Italy), respectively. Chol-but and butanol were purchased from Fluka (Buchs, Switzerland).

2.2. Preparation of SLN dispersions at different percentages of chol-but

Epikuron 200® and chol-but were melted at 85°C and a warm aqueous solution of biliar salt and butanol added to obtain a clear system. For the experimental design three different microemulsions were prepared; they contained the same components but different percentages of chol-but. The warm microemulsions were dispersed in cold water, producing SLN; different ratios of microemulsion/water were studied. The six experimental conditions considered are listed in Table 1. All dispersions of SLN were washed twice with water by the ultrafiltration system (TCF 2A-Amicon Grace, Beverly, MA, USA; membrane Amicon Diaflo YM 100) and then sterilised by autoclaving (15 min at 121°C, 1 atm).

2.3. Characterisation of SLN

After sterilisation, the mean diameter and polydispersity index (P.I.) of SLN were measured by photon correlation spectroscopy using a N4MD instrument (Coulter, Hialeah, FL, USA) at a fixed angle of 90°. The wavelength of the laser light (He/Ne) was 632.8 nm and measurements were recorded at 25°C. Size was also measured before autoclaving, obtaining similar results (data not shown).

2.4. Determination of chol-but concentration of the dispersions

The chol-but concentration of the six dispersions prepared was determined using high pressure liquid chromatography ultraviolet equipment, as described by Duncan [13]. A portion of sterilised dispersion (50–100 μl) was dissolved in the mobile phase and analysed.

2.5. Cell line and culture conditions

NIH-H460 cells [14] were grown as a monolayer in RPMI 1640 (Bio Whittaker, Verviers, Belgium) supplemented with 10% fetal calf serum (v/v), cultured in T-75 cm^2 plastic flasks (Corning Industries, Corning, NY, USA), maintained at 37°C in 5% CO_2 humidified atmosphere, and passaged weekly. At the beginning of the experiments, cells in the exponential growth phase were removed from the flasks with a 0.05% trypsin-0.02% EDTA solution.

2.6. Cell proliferation experiments

Cells were seeded in a 24 well/plate (10 000 cells/well) in RPMI 1640 medium with 10% fetal calf serum. They were allowed to attach for 24 h, after which seeding medium was removed and replaced by experimental medium. Cells were maintained for 6 days in medium supplemented with increasing concentrations of SLN (final chol-but range, from 0.008 to 1.000 mM) or sodium butyrate (range from 0.016 to 1.000 mM) (ICN Biomedicals, South Chillicothe, OH, USA). Each experiment was carried out at least twice, and each sample was run in quadruplicate using a 4×4 factorial design. At the end of the experiment the antiproliferative effect was evaluated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) [15] and the absorbance was measured at 550 nm using an ELx800 photometer (Bio-Tek instruments Inc., Wiooski, VT, USA). Wells containing all admixtures except cells were used as blanks. The results were expressed as percentages of inhibition versus control (i.e., cell growth in the absence of SLN or sodium butyrate).

Table 1
Experimental conditions and chemical-physical characterisation of chol-but SLN dispersions

Exp. Setting	% chol-but in microemulsion	microemulsion/ dispersion water ratio	mean diameter (nm)	P.I.[a]	chol-but (mM)
#1	6	1:10	190	0.37	12.5
#2	12	1:10	130	0.44	23.4
#3	6	1:80	200	0.52	1.8
#4	12	1:80	138	0.46	3.5
#5	9	1:18	148	0.54	10.5
#6	9	1:45	158	0.40	4.7

[a] Polydispersity index.

2.7. Factorial design

The effect of the chol-but percentage in the microemulsion (CB%) and that of the warm microemulsion/water ratio (Ratio) were investigated by a 2-level full factorial design (2^2) [16,17], which allowed us to evaluate the effect and interaction of two experimental factors. The two different chol-but percentages (6 and 12%) were classified as −1 and +1, respectively, and the two different warm microemulsion/water ratios (1:10 and 1:80) as −1 and +1. To better discriminate the effect of CB% and Ratio by final chol-but concentration, cells were treated with serial SLN dilutions corresponding to different scalar concentrations of chol-but (0.008, 0.016, 0.032, 0.064, 0.125, 0.250, 0.500, 1.000 mM). The four experimental conditions included in the factorial design were repeated for each chol-but concentration. The main effect of CB% and Ratio and their interaction were calculated by the Yates algorithm [17]. The effect of CB% and Ratio and their interaction, i.e. the average effect exerted by these variables on the experimental response, passing from their low value (coded as −1) to their high value (coded as +1), was calculated [16].

To evaluate the linearity of the relation between CB% and Ratio we investigated the effect of a further experimental condition, in which CB% and Ratio assumed the central value of the experimental interval [18]. CB% thus assumed the value of 9%, whereas since we did not know 'a priori' whether Ratio should be considered in terms of water fraction or SLN fraction, we adopted two alternative values as central experimental condition: 1:45 (45 being the central value between 10 and 80) and 1:18 (1:18 = 0.056 being the central value between 1:10 and 1:80 = 0.0125), considering Ratio in terms of the former and of the latter, respectively.

3. Results and discussion

SLN have been studied by various researchers [19,20]. Müller [21–23] has proposed two different preparation methods: the hot and the cold dispersion techniques, and has also studied the enzymatic degradation of SLN [24].

In the present study, solid lipid nanospheres with different compositions were prepared in order to achieve the best inhibitory effect on NIH-H460 cell proliferation.

Table 1 reports the experimental conditions and the results of chemical-physical characterisation of chol-but SLN dispersions; the mean diameters and polydispersity indices determined after sterilisation are given together with the millimolar chol-but concentrations of the six dispersions (experimental conditions #1–6) used to prepare the eight final dilutions tested on cells.

The effect exerted by all experimental conditions (the complete factorial design plus the two central conditions) on NIH-H460 cell growth in comparison with that exerted by sodium butyrate alone is given in Fig. 1 and the corresponding absorbance variations in treated versus untreated sample (control) in Table 2. The chol-but concentration-dependent inhibitory effects were determined for all experimental conditions except the lowest (0.008 mM) and highest (1.000 mM) concentrations, where none of the tested conditions produced any difference in inhibitory effect. When the experimental conditions were compared in terms of IC_{50} (the concentration inhibiting cell growth by 50%), experimental condition #3, i.e. 6% chol-but, microemulsion/water ratio 1:80, was the most effective, with an IC_{50} value (0.075 mM) about seven times lower than sodium butyrate alone (0.539 mM). When dose-response curves were analysed as a function of chol-but percentage (6% or 12%) the microemulsion/water ratio significantly contributed to the final inhibitory effect. Experimental conditions #1 and #2 (microemulsion/water ratio 1:10) showed IC_{50} values (0.244 and 0.645 mM, respectively) above those (0.075 and 0.103 mM, respectively) of corresponding experimental conditions #3 and #4 (microemulsion/water ratio 1:80), in which the SLN were dispersed in more water. When we analysed the effect of chol-but percentage was isolated from the microemulsion/water ratio, the highest effect was found to be with the lowest percentage. Experimental conditions #1 and #3 (6% chol-but) seemed to produce greater inhibition than corresponding experimental conditions #2 and #4, with 12% chol-but. For central experimental condition #5 (9% chol-but, microemulsion/water ratio 1:18) an IC_{50} value (0.157 mM) was observed, which was intermediate between experimental conditions #1 (0.244 mM) and #4 (0.103 mM). On the contrary, central experimental condition #6, with 9% chol-but and microemulsion/water ratio 1:45, showed an IC_{50} value (0.083 mM) almost identical to that of experimental condition #3 (0.075 mM).

Table 3 reports the main effects of the experimental factors and their interaction; values underlined are statistically significant; the results should be interpreted taking those in Table 2 into consideration.

For the lowest and highest chol-but concentrations (0.008 and 1.000 mM) CB% and Ratio had no apparent effect. The reason for this is different for the two concentrations: (1) at 0.008 mM there was almost no inhibition, so no effect of the experimental factors was observed; (2) at 1.000 mM, inhibition was complete, so again no effect of the experimental factors was observed.

At the 0.016 and 0.032 mM concentrations there was some CB% × Ratio interaction; it was of positive sign, which means that the effect is synergistic: the strongest inhibition is obtained for discordant settings of the two factors, i.e. when CB% was high and Ratio low or CB% low and Ratio high.

The significant effect pattern changed at the 0.064 mM concentration: Ratio became relevant, although not as relevant as the interaction. This effect is mainly due to experimental condition #3 of the factorial design, which represents the best choice for Ratio and CB% at the concentration of 0.064 mM.

At the 0.125 and 0.250 mM concentrations the effect of

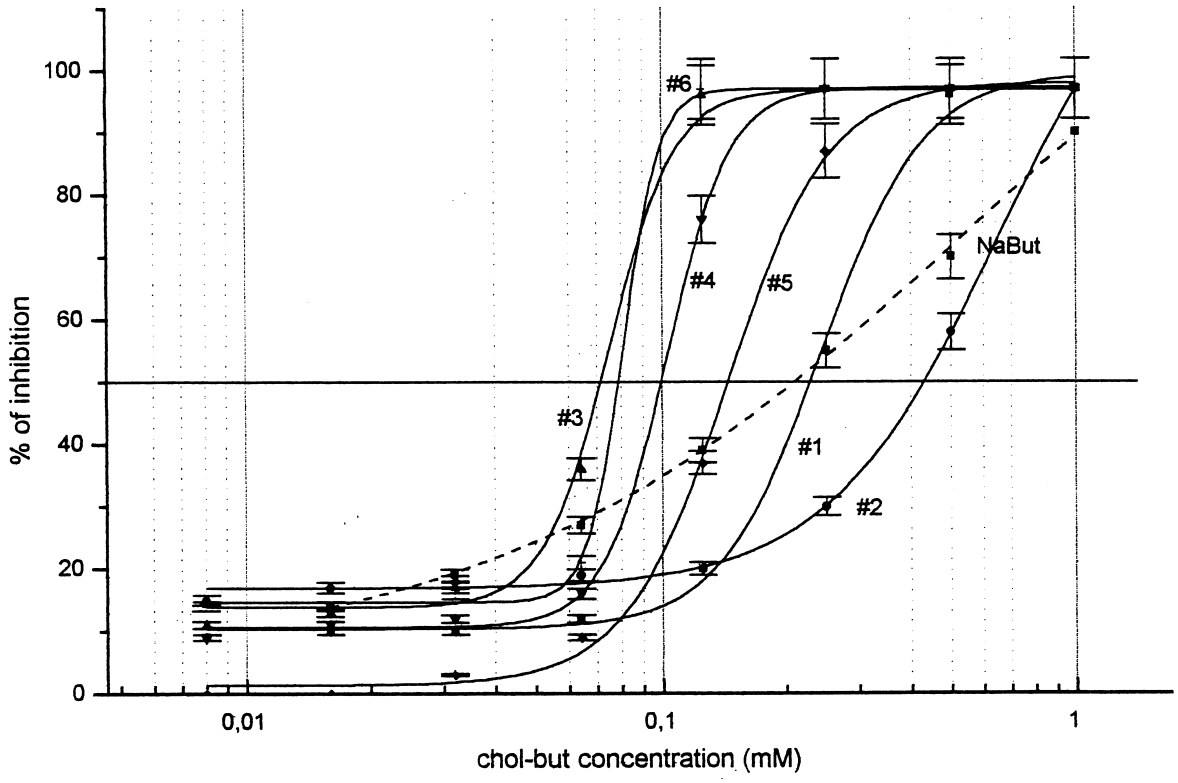

Fig. 1. The effect of all experimental conditions (#1–6) (the complete factorial design plus the two central conditions) on NIH-H460 cell growth in comparison with the effect exerted by sodium butyrate alone. Experimental conditions #1 and #2 had a microemulsion/water ratio of 1:10 and 6 or 12% of chol-but, respectively. Experimental conditions #3 and #4 had a microemulsion/water ratio of 1:80 and 6 or 12% of chol-but, respectively. Experimental conditions #5 and #6 had 9% of chol-but and a microemulsion/water ratio of 1:18 or 1:45, respectively. Results are expressed as percentage inhibition versus control. Experimental points are the means of four replicates. For details see Section 2.

the interaction was no longer statistically significant, while the effect of Ratio became the most important; the pattern of experimental results (Table 2) changed completely. Experimental condition #3 continued to perform better than the others: inhibition was already complete at 0.125 mM. The effect of Ratio is negative (as for 0.064 mM) which means that the best inhibition is obtained when Ratio is high (1:80). From the point of view of application to clinical trials, 0.125 mM is the lowest concentration worth investigating. At the 0.250 mM concentration either set of experimental conditions at high Ratio completely inhibited cell growth.

At the 0.500 mM concentration the pattern was rather similar to that at 0.250 mM. The difference was determined by a change in experimental condition #1 where it produced complete inhibition. The order of inhibition was the same as for 0.250 mM but conditions #1, #3 and #4 level out at complete inhibition. From the point of view of the experimental factor effect, such a pattern in the experimental results is highly confusing: the two factors and their interaction all appear to be significant. Experimental condition #1 is discriminated in the opposite direction to experimental condition #3 at the 0.064 mM chol-but concentration: experimental condition #1 is the worst experimental setting for the SLN dispersion.

Table 2
Absorbance variation in treated versus untreated sample (control) at final chol-but concentration for each of the six sets of experimental conditions

Exp.	CB%[a]	Ratio[b]	0.008 mM	0.016 mM	0.032 mM	0.064 mM	0.125 mM	0.250 mM	0.500 mM	1.000 mM
#1	−1	−1	0.901	0.912	0.901	0.884	0.802	0.451	0.044	0.027
#2	+1	−1	0.846	0.832	0.818	0.802	0.807	0.696	0.420	0.034
#3	−1	+1	0.887	0.871	0.828	0.642	0.036	0.031	0.031	0.031
#4	+1	+1	0.885	0.908	0.879	0.839	0.239	0.029	0.029	0.032
#5	0	0*	1.017	0.972	0.997	0.914	0.726	0.132	0.029	0.026
#6	0	0**	0.864	0.861	0.836	0.794	0.032	0.032	0.029	0.032

[a] CB% (% chol-but in microemulsion): -1, 6%; +1, 12%; 0, 9%.
[b] Ratio (microemulsion/dispersion water ratio): -1, 1:10; +1, 1:80; 0*, 1:18; 0**, 1:45.

Table 3
Effects of the factors CB% and Ratio and the interaction between the two factors[a]

	0.008 mM	0.016 mM	0.032 mM	0.064 mM	0.125 mM	0.250 mM	0.500 mM	1.000 mM
Offset	0.880	0.881	0.857	0.792	0.471	0.302	0.131	0.031
CB%	−0.028	−0.021	−0.015	0.057	0.104	0.122	0.188	0.004
Ratio	0.013	0.018	−0.006	−0.102	−0.667	−0.543	−0.202	0.001
CB% × Ratio	0.026	0.058	0.067	0.139	0.099	−0.123	−0.189	−0.002

[a] For explanation see text. Underlined values are statistically significant.

At the highest concentration (1.000 mM) the levelling effect also affects experimental condition #2: no effect of the experimental factors can be calculated since all dispersions completely inhibit cell growth.

In two-level factorial design, the central experimental setting is usually applied to identify non-linear behaviour in the system investigated. This is achieved by comparing the experimental and estimated results in the centre of the experimental domain. If the difference between these values is large it means that the system does not behave linearly. In this study we did not know whether Ratio should be considered in terms of the water fraction, in which case the centre would be 1:45, or the SLN fraction, with the centre at 1:18.

The estimated response in the centre of the domain is offset in Table 3; when this value is compared with the experimental values the experimental result closest to the calculated result probably indicates how Ratio should actually be considered. In our case none of the alternative central experiment settings showed response values similar to the estimate throughout the chol-but concentration range. The exception is at the low chol-but concentrations: from 0.008 to 0.064 mM, where the result achieved at 1:45 is not very different from the estimated value. At 0.125 and 0.250 mM the data probably undergo a plateau effect, so that at the centre inhibition is greater than predicted by the factorial model. At higher concentrations, levelling of inhibition makes it difficult to discriminate between estimated and experimental results.

Fig. 1 shows the results of the six experimental conditions performed to achieve optimisation, together with the results for sodium butyrate. As can be observed, experimental condition #3, which was the best experimental setting, achieved full inhibition at 0.125 mM, at which concentration sodium butyrate only produced 38% inhibition.

4. Conclusions

This research provided the following results: (1) inhibition of NIH-H460 cell growth was observed with all cholesteryl butyrate-SLN formulations; (2) the factorial design showed that antiproliferative effects can be related to the two experimental factors investigated, i.e. chol-but percentage and warm microemulsion/water ratio; (3) at low chol-but concentrations (0.016 and 0.032 mM) there was a synergistic effect of the two factors (interaction) which disappeared at higher concentrations; (4) at higher concentrations (0.125 and 0.250 mM) the Ratio effect became predominant; (5) at the highest concentrations (0.500 and 1.000 mM) the effect levelled out at complete cell growth inhibition; (6) there was a plateau effect in the central region; (7) the best experimental setting was experimental condition #3 (CB% = 6%, Ratio = 1 : 80) which gave complete inhibition at 0.125 mM, while at the same concentration sodium butyrate provided only 38% inhibition.

However, it must kept in mind that despite cell culture being a suitable experimental model to investigate the effect of a drug on cell growth, morphology and gene expression, it is an artificial system, in which the active principle is directly in contact with the cells. Since the effect observed in such a system would presumably be higher than that observable in vivo, further in vivo studies are necessary to verify the ability of SLN to release butyric acid over a prolonged period of time.

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