Cholesterol level determines viability and mitogenicity, but it does not affect sodium butyrate-dependent sensitization of Colo 205 cells to TNF-α-induced apoptosis

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Abstract. Transient treatment of human adenocarcinoma COLO 205 cells with lipit raft (LR) modulators (MßCD, NY, IMP) was followed by the challenge with metabolic inhibitors and selected anti-cancer drugs. To overturn cholesterol chelation, the MßCD, NY treatment was followed by cholesterol conjugates (CHOL-MßCD or CHOL-PEG). The TNF-α- and P(Ser473)-PKB/Akt1/2-mediated effects initiated at LR were evaluated with regard to cell viability and mitogenicity. Cholesterol chelators reversibly reduced cell survival, whereas some of the tested compounds had weak effects (CIS, CLA), stimulated (EGCG) or reduced (NaB) cell survival. Cellular localizations of LR-associated molecules (ceramides, $G_{\alpha i\text{-}2}$ heterotrimeric protein, and TNF-R1) in different cellular compartments including the plasma membrane were observed in the respective photographs from TEM and SEM. Evidence from SEM also showed that TNF-R1 is clustered on the surface of COLO 205 cells without presence of cognate ligand but clustering is promoted by TNF-α, while it vanished after IMP treatment. COLO 205 cells remained immune to TNF-α-induced apoptosis unless NaB was added, in which case NaB-induced cell death was further potentiated by TNF-α. Combined NaB and TNF-α treatment was associated with marked changes in the expression of pro- and antiapoptotic proteins. In this study, we demonstrated that initial excess of prosurvival signals could be diminished by cholesterol chelators, whereas LR-independent cell survival could be targeted by NaB. Apparently, lipid rafts do not participate in NaB-dependent cell death.

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Introduction

The idea of lipid rafts (LR) over the years remained elusive and tricky to observe. Recent progress in experimental technologies such as fluorescence resonance energy transfer (FRET), fluorescence recovery after bleaching (FRAP), and finally fluorescence correlation spectroscopy (1) provided evidence that rafts are extremely small (5-20 nm in diameter), highly dynamic, and involve interactions of both proteins and lipids (2). They are thought to be plasma membrane lateral assemblies enriched in cholesterol and sphingomyelin (SPHING) which associate together, away from the more abundant unsaturated phospholipids (3). Some proteins prefer LR (glycosylphosphatidylinositol, GPI-anchored) while others (EGF receptors, EGF-R) do not (4). This led to the hypothesis that LR are basic platforms to organize signaling proteins. Actually, among others ß-secretase, flotillin or $G_{\alpha i\text{-}2}$ are constitutively expressed in raft nanodomains being routine markers of LR, while other proteins, i.e. TNF-R1 or PKB/Akt1/2 interact temporarily with LR (1). LR facilitate recruitment and activation of PKB/Akt1/2, upon phosphatidylinositol-3-kinase (PI3-K)-dependent phosphatidylinositol-3,4,5-triphosphate modification. Similarly, TNF-R1 receptor may oligomerize in response to ceramides freed from sphingomyelin by sphingomyelinases (SMA-ses) (5).

In the extensive search for the platform which segregates pro- and antiapoptotic signals, lipid rafts seem to be the most likely site for consideration. First, when the LR became the focus of attention (4,6) it became clear that both death and survival signals elicited by respective cytokines have to originate from these small entities (7). Of course, other than LR portion of plasma membrane also contributes to cell fate. In any case, LR are the only known nanodomains where the transmembrane proteins may interact through the raft clustering (8,9). In the initiation of signaling cascades it is essential for the receptors to assemble into polymers (dimers, trimers). For survival, the most prominent cascade is PI3-K/Akt1/2/GSK-3ß while cell death could be regularly evoked by TNF-α/TNF-R1/TRADD and other death ligands (FasL, TRAIL). Either system is frequently disturbed in tumor cells,

where the first is often up- and the latter is down-regulated (gain and/or loss of function). This is the case for COLO 205 cells, the cell line particularly hard for deletion and therefore suitable model for this study. Upon alteration of raft composition we examined two critically important signaling pathways, namely PI3-K/Akt1/2/GSK-3ß and TNF-α/ TNF-R1/TRADD in COLO 205 cells. Several factors (metabolic inhibitors, anti-cancer drugs) have been tested in order to find out whether they act through LR and how they affect the above-mentioned signaling cascades. To provide evidence at ultrastructural level, scanning (SEM) and transmission (TEM) electron microscopy were used.

Materials and methods

All reagents were cell culture tested, of high purity and unless otherwise stated they were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Plastics were from Corning-Costar Inc. (Cambridge, MA, USA), while sera (heat-inactivated), media, PBS and antibiotics were from Gibco Life Technologies (Grand Island, NY, USA). The cholesterol-methyl-ß-cyclodextrin (CHOL-MßCD) inclusion complex was prepared as described by Klein *et al* (10) and Gimple *et al* (11). Briefly, 300 mg of cholesterol was dissolved in 2-propanol and was added to a stirred solution of methyl-ß-cyclodextrin (MßCD, 5% w/v) on a water bath (80˚C). The mixture was stirred at 80˚C until the initially precipitating steroid was completely dissolved. The solution was freeze-dried and stored at room temperature for use at concentration of 100 μl/15 ml of medium. Also ready to use polyethylenglycol conjugated to cholesterol (CHOL-PEG, 100 μg/ml) was applied to run LR up with cholesterol.

Human colon adenocarcinoma cell line COLO 205 was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were maintained in exponential phase of growth in growth medium [GM, 100 ml/l fetal bovine serum (FBS)/Dulbecco's modified Eagle medium (DMEM) with Glutamax and antibiotic-antimycotic mixture]. The cells were grown at 37˚C, in a controlled, humidified 50 ml/l CO_2 atmosphere, on a multiwell (96) or tissue culture Petri dishes (100-mm diameter, BD Biosciences, Franklin Lakes, NJ, USA). During propagation, medium was changed every other day until cultures reached 80% confluence. One day (24 h) prior to the experiment, confluent cells were withdrawn from cell cycle by replacing GM with 20 g/l BSA/DMEM designated as control medium (CTRL). During the study freshly prepared media with or without experimental factors were used, whereas vehicle (0.1% DMSO) was added to the control system. Exactly 30 min prior to the application of water-soluble reagents, DMSO-dissolved reagents (cycloheximide, CHX; actinomycin D,- AD; PD098059 or LY294002) were applied.

Cell viability/proliferation was based on the ability of cells grown on multiplate wells to convert soluble MTT (3-(4,5-dimethylthiazol-2-yl)-2-5-diphenyltetrazolium bromide) into an insoluble purple formazan reaction product with minor modifications to protocol described by Jacobson *et al* (12).

Cell mitogenicity was determined by crystal violet (CV) assay on 96-multiwell plates. Upon completing the experiment cells were washed with PBS and fixed with two-step bath in ice-cold methanol (70% followed by 100%, v/v, 20 min, 4˚C). Next, cells were immersed in 0.2%, w/v crystal violet solution in dd.H_2O with ethanol 2%, v/v for 10 min. Subsequently, they were gently washed with dd.H_2O, air dried and incubated with SDS solution (1%, w/v in dd.H_2O). The absorbances for MTT and CV were measured at 570 nm with ELISA reader type Infinite 200 (Tecan, Austria). Percentage of viable or proliferating cells was measured by MTT conversion into purple formazan and quantity of CV bound to cellular DNA, respectively.

Rafts were isolated by extraction with Brij 98 at 37˚C, as previously reported by Drevot *et al* (13) with modification of Remacle-Bonnet *et al* (14). In brief, cells were harvested with a rubber policeman and gently sonicated (five 5-sec bursts, 5 W) in 1 ml of ice-cold buffer A (25 mmol/l HEPES, 150 mmol/l NaCl, 1 mmol/l EGTA, 10 mmol/l NaF, 5 mmol/l Na_3VO_4, 10 mmol/l NaP-P, and a mixture of protease inhibitors). The postnuclear supernatant was recovered after centrifugation at 800 x g at 4˚C for 10 min, and then spun at 100,000 x g in a SW41 rotor (Beckman Instruments) for 60 min at 4˚C and the supernatant was referred as the soluble fraction (S) containing solubilized membrane and cytosolic fractions. The membrane raft fraction in the pellet (R) was resuspended in buffer A containing 1% Brij 98, 0.3% deoxycholic acid, and 60 mmol/l *n*-octyl-ß-D-glucopyranoside (ODG), and nonsoluble material was removed by an additional centrifugation. Collected (S) and (R) fraction from an identical number of cells were analyzed by WB. Finally, for protein quantification, blots were scanned and analyzed by spot densitometry, and results were expressed as average value of pixels enclosed (AVG), calculated as the sum of all the pixel values after background correction divided by area.

In turn, for whole-cell lysates, the cells were cultured with or without experimental factors indicated in figure legends, harvested, washed, and lysed with RIPA lysis buffer (1X PBS, 10 ml/l Igepal CA-630, 5 g/l sodium deoxycholate, 1 g/l SDS) supplemented with 0.4 mM PMSF, 10 μg/ml of aprotinin and 10 μg/ml of sodium orthovanadate was added. To lyse the cell pellet, cells were broken up by repetitive triturating with the syringe with attached needle (21G, 0.8-mm diameter). Cell suspension was then left on ice (4˚C) for 30 min, and centrifuged for another 5 min (4˚C, 10,000 x g). Next, viscous solution was divided into smaller volumes and transferred to fresh Eppendorf tubes and stored at -80˚C until used. For protein quantification in the whole-cell lysates the protein-dye-binding method by Bradford (15) with commercial reagent was used (Bio-Rad Laboratories, Hercules, CA, USA).

Cell lysates (equal protein loads) were subjected to SDS-PAGE, transferred to polyvinylidene difluoride membrane, and immunoblotted with antibodies against phosphorylated and/or non-phosphorylated forms of proteins: actin, PKB/Akt-1/2 and P(Ser473)-PKB/Akt1/2, Bax, Bcl-2, Bid, caspase-3/procaspase-3, cIAP-1, ERK1/2 and P(Y204)-ERK1/2, GSK-3ß and P(Ser9)-GSK-3ß, $G_{\alpha i-2}$, survivin, TRADD (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Secondary polyclonal antibodies (Santa Cruz Biotechnology) raised against respective species and conjugated to horseradish peroxidase were used for detection, followed by enhanced chemiluminescence assay (Amersham International,

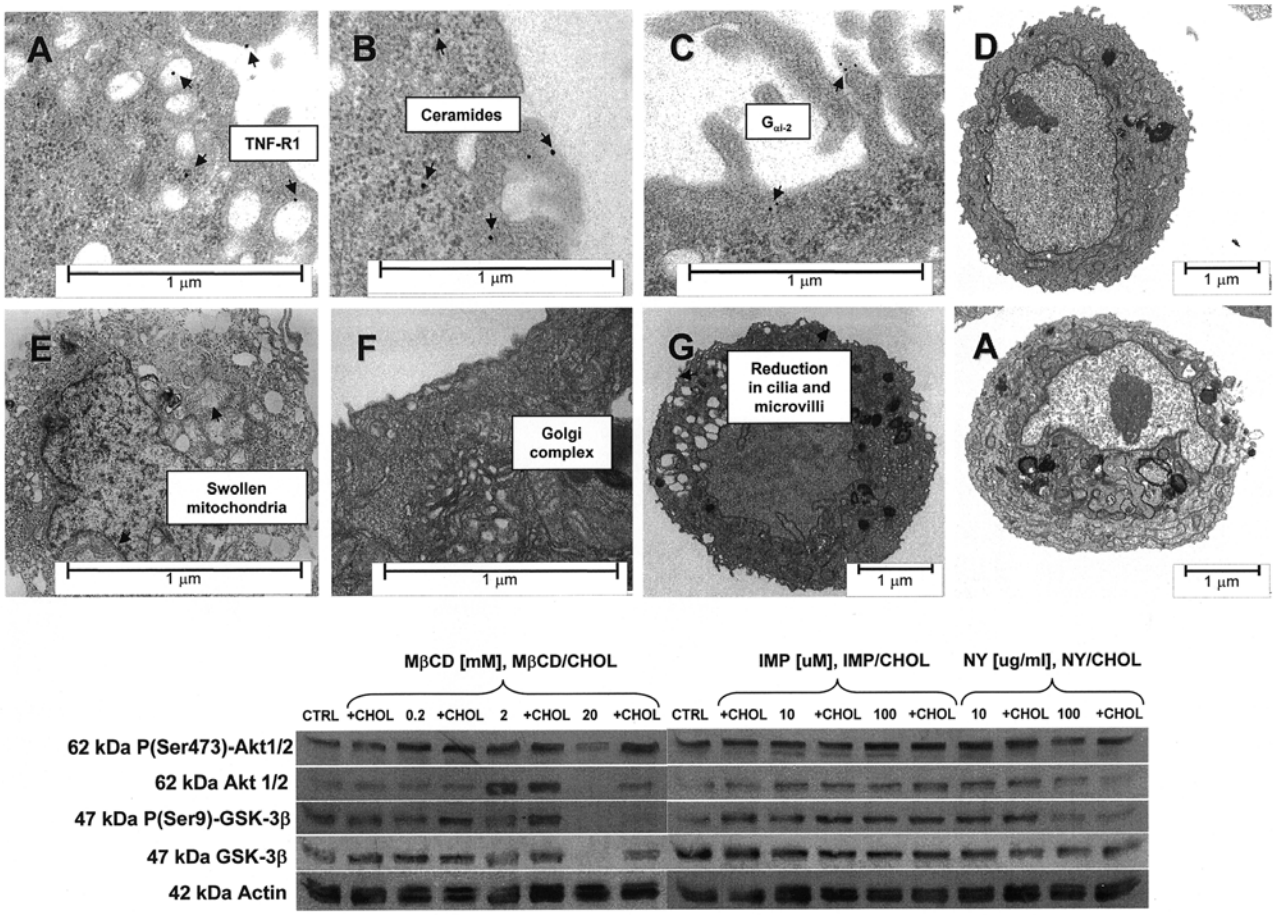

Figure 1. Top: Series of ultrastructural views of control (upper panel) and treated (lower panel) COLO 205 cells. Upper panel: immunocytochemical detection of TNF-R1, ceramides, and $G_{\alpha i\text{-}2}$ protein (TNF-R1 18 nm, cermide 18 nm, $G_{\alpha i\text{-}2}$ 12 nm diameter, respectively). Lower panel: morphological appearances of COLO 205 cells treated for 1 h with 2 mM of MßCD followed by replenishment of cholesterol with 1 h CHOL-MßCD (100 μl/ 15 ml, see Materials and methods). Bottom: Immunoblots from whole-cell lysates obtained from COLO 205 cells treated for 1 h with increasing concentrations of MßCD, 0.2, 2, 20 mM; IMP, 10, 100 μM; or NY, 10, 100 μg/ml. Afterwards, cells were recovered for 1 h by the addition of CHOL-PEG (100 μg/ml, see Material and methods). To demonstrate activity of survival system (PI3-K/Akt1/2) the expression levels of PKB/Akt1/2, P(Ser473)-PKB/ Akt1/2, GSK-3ß, P(Ser9)-GSK-3ß are shown.

Aylesbury, UK). After exposure, and processing the film was scanned and analyzed using Kodak EDAS 290/Kodak 1D 3.5 system.

Post-embedment immunostaining was performed on 4% paraformaldehyde:0.1% glutaraldehyde fixed cells with antibodies raised against $G_{\alpha i\text{-}2}$ or/and TNF-R1 or/and ceramide (Santa Cruz Biotechnology). Ultrathin sections were mounted on the formvar-coated nickel grids, air-dried and stained for 10 min with 4.7% uranyl acetate and for 2 min with lead citrate. Secondary antibodies conjugated with colloidal gold particles (Jackson Immunoresearch, West Grove, PA, USA) were used to demonstrate immunoreactivity of investigated proteins (TNF-R1 18 nm, cermide 18 nm, $G_{\alpha i\text{-}2}$ 12 nm diameter, respectively). The sections were examined and photographed with JEOL 1011XE electron microscope (Jeol, Tokyo, Japan).

The QuantomiX QX-102 system (QuantomiX Ltd. Rehovot, Israel) was applied to show the presence and distribution of TNF-α receptors (TNF-R1) on plasma membranes of wet COLO 205 cells after fixation (2% paraformaldehyde and 0.1% glutaraldehyde solution in PBS) and two-step immunodetection with colloidal gold particles (Jackson Immunoresearch). Gold particles (18 nm in diameter) were demonstrated and photographed with JEOL JSM3690LV scanning electron microscope (Jeol).

Statistical evaluation. Each experiment was repeated at least twice with identical results. The data are expressed as the means ± SE. Statistical significance was determined using one-way ANOVA for multiple comparisons followed by the Tukey multiple range test. $P<0.05$ was con-sidered significant. In the figures statistical differences from control values are indicated by asterisks ($^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$), whereas statistical differences between the means are ticked with different lower case letters. These analyses were performed using GraphPad Prism™ version 4.03 software (GraphPad Software Inc., San Diego, CA, USA).

Results

Short-term deprivation of plasma membrane cholesterol impairs cell survival, although it is not lethal to COLO 205 cells if followed by cholesterol replenishment. To evaluate the input of LR to the effects of examined substances, first we tested how the LR modulators impinge on the level phosphorylated (active) form of PKB/Akt1/2 known to be specific indicator of intact LR (1) and viable cells. Methyl-ß-cyclo-dextrin (MßCD) and nystatin (NY) were tested at different concentrations (0.2, 2, 20 mM) for MßCD and (10, 100 μg/ml) for NY. Additionally, imipramine (IMP) was used

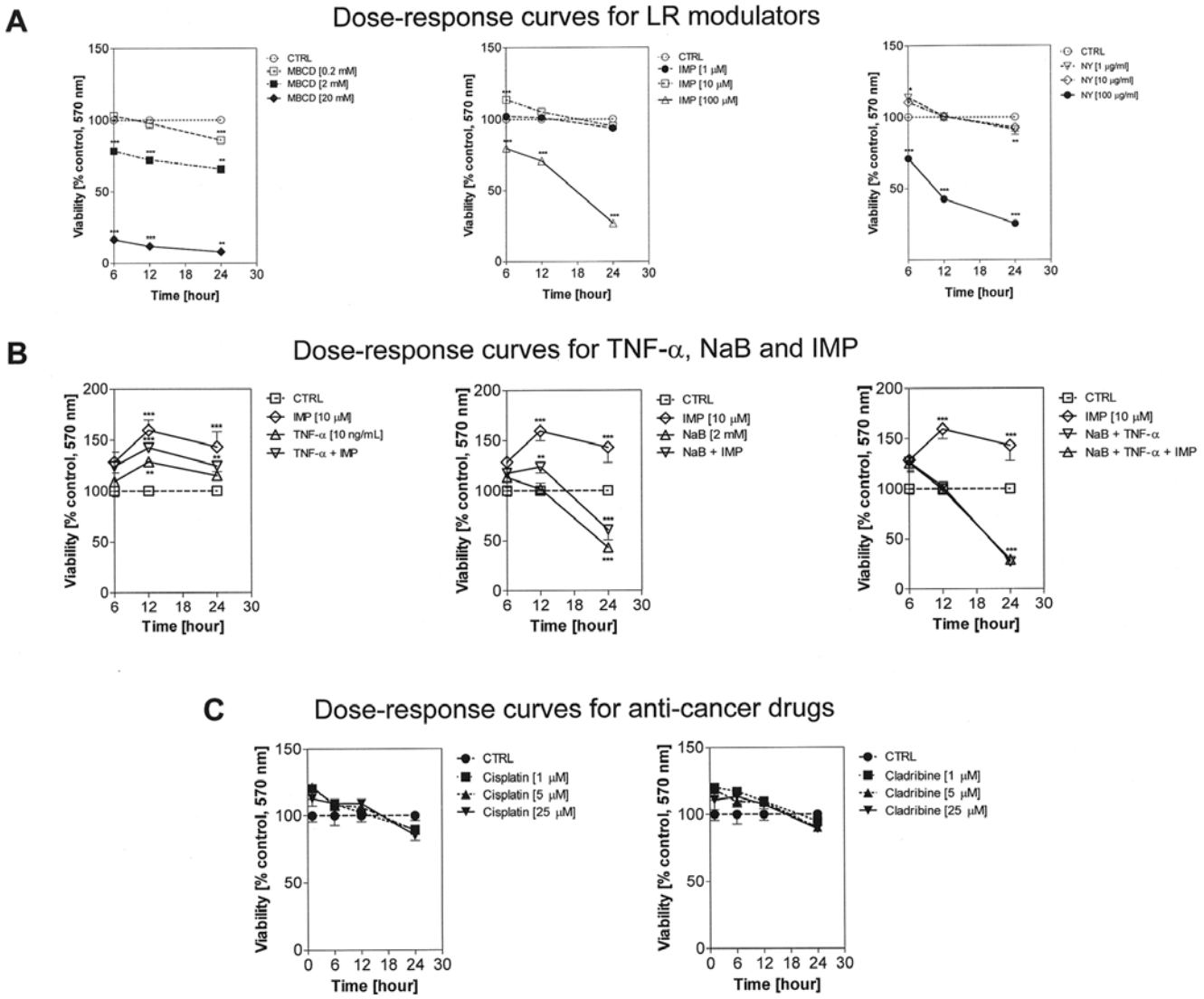

Figure 2. (A) The dose-response effect of each LR modulator on cell viability is shown. Highest concentrations used (20 mM of MßCD, 100 μg/ml of NY and 100 μM of IMP) were apparently cytotoxic. Moderate concentrations were chosen for further experiments. (B) The dose response-curves representing cell viability upon treatment with TNF-α and/or NaB and/or not IMP. IMP stimulated cell viability, also it reduced the cytotoxicity of NaB and elevated response to TNF-α. No effect of IMP was observed when both TNF-α and NaB were used together. (C) Anti-cancer drugs (cisplatin, cladribine) were used at increasing concentrations (1, 5, 25 μM) in order to determine if they reduce the metabolic activity of COLO 205 cells. It is clear from the presented curves, that none impaired the metabolic activity of COLO 205 cells. Asterisks indicate values statistically different from the control group (P<0.05).

(10, 100 μM) in order to inhibit acidic sphingomyelinase (aSMAse) (16). According to Remacle-Bonnet *et al* (14) cells might substantially be out of cholesterol (CHOL) upon 1 h administration with MßCD, but they do not die if they are subsequently washed and/or additionally re-loaded with CHOL. Cells might become briefly detached from substratum (2 mM MßCD, 1 h), though subsequent recovery make them functional at a molecular and ultrastructural level (Fig. 1). Similar effect was demonstrated for NY (100 μg/ml, data not shown). The 2 mM concentration of MßCD was selected for the subsequent study.

Cell viability is altered by LR modulators and to some extent by metabolic inhibitors. To determine cytotoxic effect of either factor they were initially tested in a dose- and time-dependent manner (0, 1, 6, 12, 24 h). In this study, each LR modulator was apparently cytotoxic after 6 h at concentrations of 2 and 20 mM, 100 μg/ml and 100 μM, for MßCD, NY and IMP, respectively (Fig. 2A, P<0.05). After 24 h of incubation the viability was reduced by 92.30±0.18% for 20 mM of MßCD, by 74.75±1.09% for 100 μg/ml of NY, and by 73.05±1.31% for 100 μM of IMP (Fig. 2A, P<0.001). The high concentrations of MßCD, NY and IMP were selected solely for acute experiments, whereas medium or low range concentrations were helpful to examine transient or chronic effects of LR modulators. Other metabolic inhibitors were tested accordingly. IMP (10 μM) stimulated cell survival, and it enhanced viability at the presence of TNF-α or NaB (Fig. 2B, P<0.001). IMP did not reduce the effect of both factors used concomitantly. Anti-cancer drugs (CIS and CLA) did not influence significantly viability of COLO 205 cells when added in therapeutic concentrations (1, 5 and 25 μM, Fig. 2C, P>0.05), however they moderately decreased cell survival in higher (toxic) concentrations (50, 100, 200 μM), particularly after 24 h of treatment (data not shown). On the contrary, EGCG did stimulate cell viability (Fig. 3A, P<0.001 for

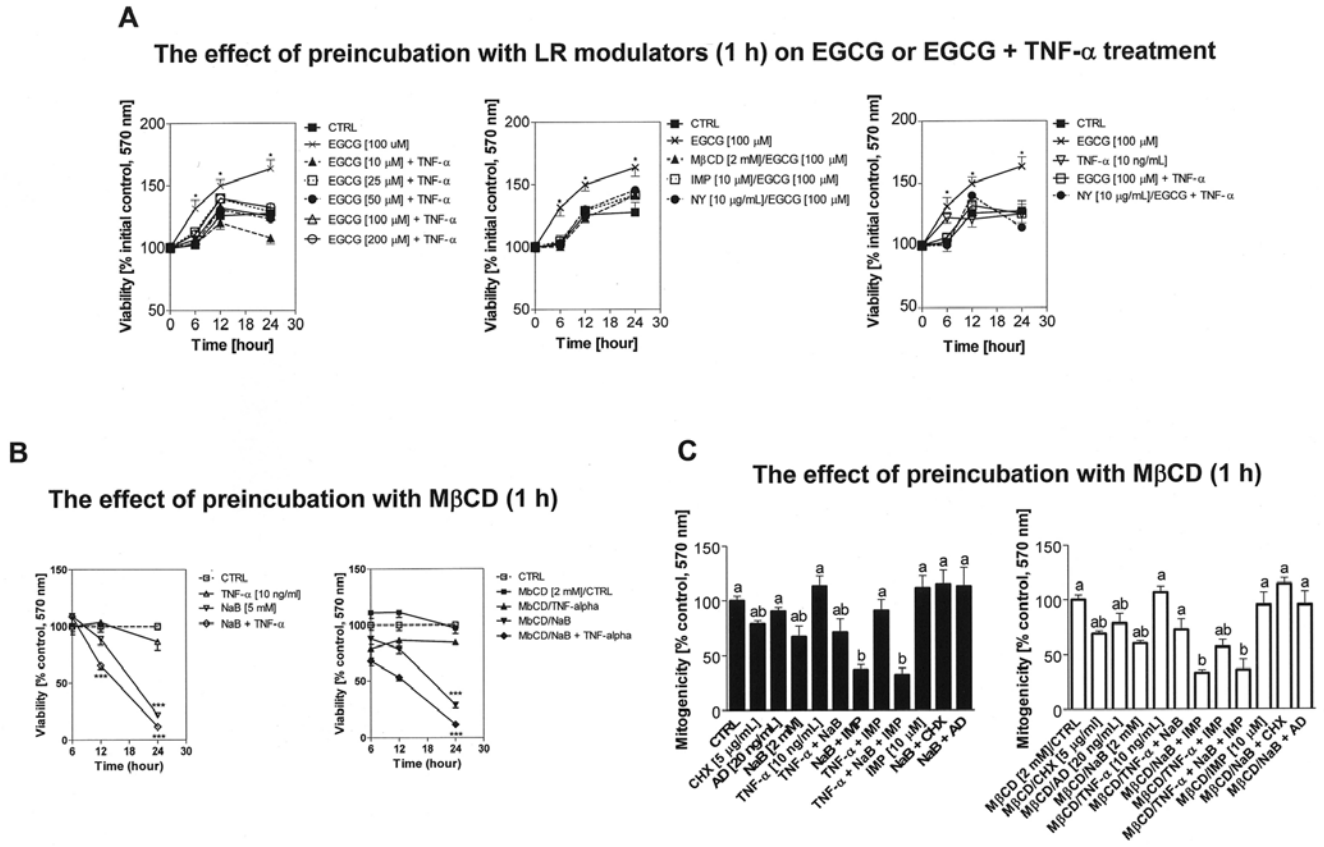

Figure 3. (A) Preincubation (1 h) with MßCD or NY or co-treatment with TNF-α both obstruct the effect of EGCG on viability/proliferation of COLO 205 cells. (B) Preincubation (1 h) with MßCD reduces the effect of TNF-α but does not affect the cytotoxicity of NaB and TNF-α. (C) Mitogenicity of COLO 205 cells is significantly retarded by NaB, regardless of IMP. Preincubation (1 h) with MßCD does not markedly affect the mitogenic responses to tested substances. Asterisks indicate values statistically different from the control group (P<0.05).

100 μM). Neither of the tested metabolic inhibitors (PD098059 at 50 μM, LY294002 at 50 μM, and AD at 20 ng/ml) affected cell survival by 24 h of experimental period as previously shown by Pajak *et al* (17). In contrast, profound and time-dependent cytotoxic effect was observed after NaB administration at this same time (Fig. 3C, P<0.001).

LR modulators sensitize/desensitize cellular responses to certain metabolic inhibitors. Previously, we reported that sodium butyrate (NaB) administered at physiological concentrations (2-5 mM) provoked apoptosis in COLO 205 cells (17). Additionally, when TNF-α was combined with NaB the latter facilitated TNF-α-mediated extrinsic apoptosis. In this study, we verified the involvement of LR in either effect, as well as other factors used by us. Close view did not show any effect of transient CHOL deprivation with MßCD (2 mM) on the NaB-dependent cell death and mitogenic response (Fig. 3B and C, P>0.05). In contrast, when NaB was added together with TNF-α, they both acted jointly to delete cells (Figs. 2B, 3B and 3C, P<0.001). Cytotoxic effect of NaB could be impeded by co-treatment with CHX and to some extent with AD, known inhibitors of translation and transcription, respectively (Fig. 4A and B, P<0.001). These observations prompted us to examine the expression levels of pro- and antiapoptotic proteins (see below). On the contrary, MßCD (2 mM) and NY (100 μg/ml), the IMP at 10 μM was shown to stimulate viability of COLO 205 cells. IMP affected TNF-α- but not NaB-dependent effect on DNA synthesis (Figs. 2B and 3C, P<0.001). Cholesterol deprivation with MßCD did not affect the cytotoxic outcome of common treatment with TNF-α and NaB (Fig. 3B, P>0.05) although it markedly reduced the effect of IMP on TNF-α-dependent mitogenicity (Fig. 3C, P<0.05).

Immunoreactivity of PKB/Akt1/2 and its substrate GSK-3β are affected by LR modulators. To set it off, the LR modulators were used in non-toxic concentrations. None of them (MßCD at 0.2 mM, NY at 10 μg/ml or IMP at 10 μM) affected the levels of certain immunoreactive proteins determined in whole-cell lysates after the short-term (1 h) treatment (Fig. 5A, P>0.05). Correspondingly, neither EGCG (100 μM), nor TNF-α (10 ng/ml), nor PD098059 (50 μM), nor LR modulators in low concentrations (0.2 mM, 10 ng/ml or 10 μM for MßCD, NY and IMP, respectively) brought any effect on Akt1/2, P(Ser473)-Akt1/2 and its substrate P(Ser9)-GSK-3ß (data not shown). On the contrary, when MßCD or NY were used in the concentrations of 2 mM and 100 μg/ml, respectively, they reduced the levels of P(Ser473)-PKB/Akt1/2 and P(Ser9)-GSK-3ß (Fig. 1B, P<0.05). These effects were fully reversible after cholesterol refill (Figs. 1B and 5B). Finally, when the lipid membrane fractions (non-raft, detergent soluble - S, and lipid raft, detergent resistant - R) were isolated, separated and proteins were immunoblotted, the levels of PKB/Akt1/2 phosphorylated at Ser473 were markedly

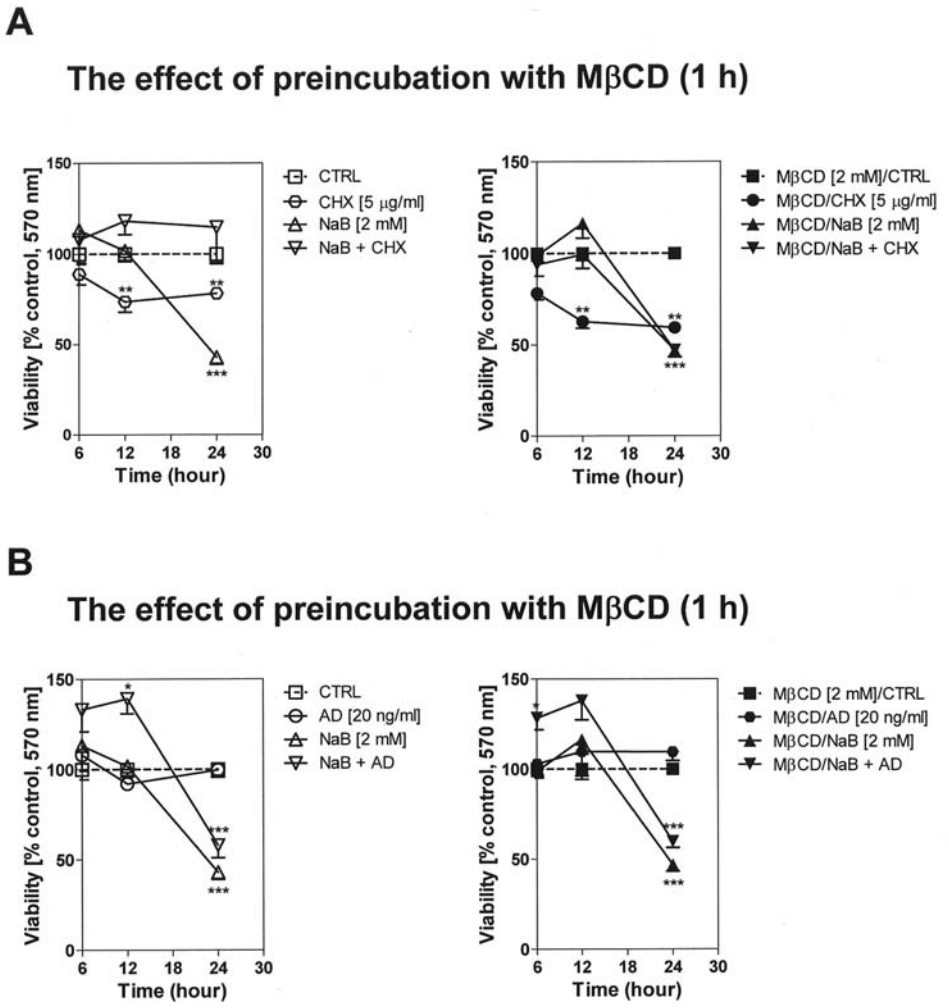

Figure 4. Protein synthesis inhibitor CHX (5 μg/ml) and transcriptional inhibitor AD (20 ng/ml) prevent cell death promoting activity of NaB. Preincubation (1 h) with MßCD does not affect the CHX- and AD-induced effects. $^{**}P<0.01$; $^{**}P<0.001$ values are statistically different from the control group ($P<0.05$).

diminished in both S and R fractions after 1 h of treatment with 2 mM MßCD (Fig. 5B). Stock up of the plasma membrane with either of the cholesterol conjugate CHOL-PEG at concentration of 100 μg/ml (or CHOL-MßCD, not shown) restored P(Ser473)-PKB/Akt1/2 to control level (Fig. 1B). This experiment clearly revealed how certain membrane fractions (non-raft vs. raft) could be transformed by the selected LR modulators in order to control the expression levels of particular LR anchored signaling proteins.

The expression levels of pro- and anti-apoptotic proteins are altered by TNF-α, NaB and imipramine. To find out how the translation inhibitor CHX protected COLO 205 cells against the NaB-induced cell death we determined the expression levels of regulatory proteins involved in apoptosis. We observed marked changes in the expression of anti-apoptotic (Bcl-2, cIAP-1, survivin, XIAP) vs. pro-apoptotic proteins (Bax, Bid) by 24 h of treatment with NaB and TNF-α or NaB alone (Fig. 5C). AD (20 ng/ml) prevented viability of COLO 205 cells merely up to 12 h, while non-specific translation inhibitor CHX protected cells from NaB toxicity during 24 h of treatment. At this time we found enhanced cytotoxicity exerted by TNF-α and NaB was accompanied by elevated levels of TRADD adaptor protein and were paralleled by marked decline of XIAP and Bcl-2 proteins. IMP given individually stimulated XIAP and Bax expression, even though the latter protein expression increased considerably after TNF-α and NaB administration (Fig. 5C). On the contrary, the expression of anti-apoptotic proteins Bcl-2, survivin and XIAP declined each time NaB was added. In the latter case IMP slightly prevented the expression of survivin (Fig. 5C). Importantly, marked reduction in Bid expression was observed after combined treatment of NaB with TNF-α giving a clue for understanding how they could act synergistically in apoptosis induction.

Post-embedding immunocytochemistry, scanning and transmission electron microscopy. This approach was used to demonstrate, that LR modulator-dependent ultrastructural modifications could be observed at subcellular level and that they are in causal relationship with the cell injury. The cells treated for 1 h with MßCD or NY (2 mM, or 100 μg/ml, respectively) showed loss of intracellular integrity (organelle swelling), reduced number of cilia and microvilli. After subsequent washing or cholesterol repletion the cells returned to normal appearance (Fig. 1A). Immunoreactivity of survival proteins points to the essential role of LR since they disappeared upon treatment with LR modulators, and

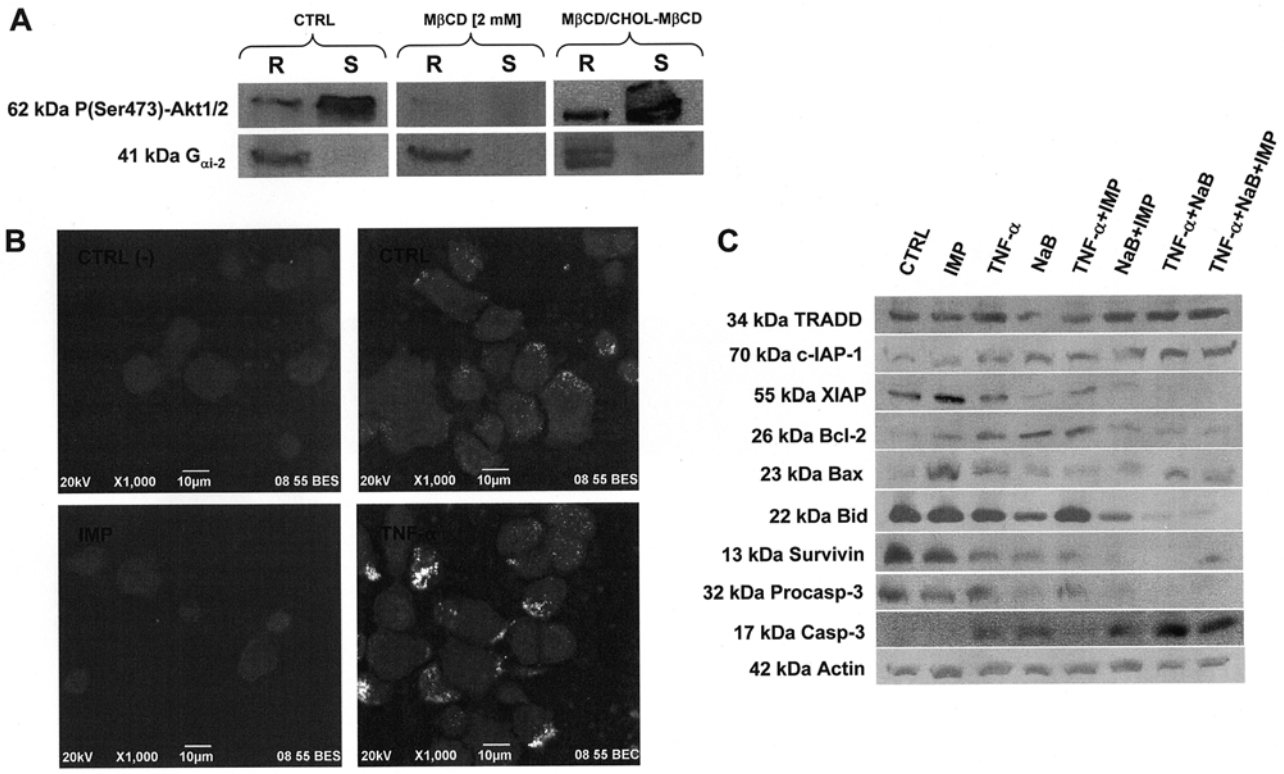

Figure 5. (A) Proteins in detergent soluble (S) and detergent resistant (R) membrane fractions were separated by SDS-PAGE and immunblotted with anti-$G_{\alpha i-2}$ and anti-P(Ser473)-PKB/Akt1/2 antiserum (see Material and methods). Pretreatment with MßCD (1 h) caused absolute loss of P(Ser473)-PKB/Akt1/2 expression levels in both fractions. Subsequent stock up of cholesterol (1 h with CHOL-MßCD, 100 μl/15 ml) led to restitution of P(Ser473)-PKB/Akt1/2 expression in both S and R membrane fractions. (B) Scanning electron microscopy of COLO 205 cells cultured in Quantomix QX-102 capsules. After the experiment, cells were fixed as described by the manufacturer. TNF-R1 receptors were detected with monoclonal Ab and visualized with gold nanoparticles (18-nm diameter) conjugated to second donkey anti-mouse Ab. Clusters of TNF-R1 are clearly visible. Short-term (5 min) of TNF-α treatment led to noticeable increase in the size and intensity of spots representing colloidal gold particles. IMP efficiently blocks the surface expression of TNF-R1. CTRL (-) is a negative control where solely second Ab were used. (C) Immunoblots from whole-cell lysates showing the expression levels of TRADD, cIAP-1, XIAP, Bcl-2, Bax, Bid, procaspase-3 and caspase-3. Equal protein loading was confirmed by verification of actin level.

reappeared after subsequent treatment with cholesterol conjugates. Localization of selected lipid raft proteins and constituents was demonstrated with immunogold particles conjugated to secondary IgG raised against species specific primary antibody. A two-step procedure was used to show the presence of ceramides, $G_{\alpha i-2}$ heterotrimeric protein, and TNF-R1 in different cellular compartments including plasma membrane (Fig. 1A). To show occurrence of TNF-R1 on the surface of COLO 205 cells they were also cultured in QuantomiX QX-102 capsules. Appropriate experiments were conducted, and cells were processed according to the manufacturer's manual and visualized. The photographs revealed uneven and patchy distribution of spots representing TNF-R1 (Fig. 5B). The spots were of several magnitudes larger than individual gold particles conjugated with secondary antibodies (18 nm).

Discussion

Neoplastic diseases are often caused by the proteins aberrantly expressed at plasma membrane (reviewed in ref. 18). Human colon adenocarcinoma COLO 205 cells are typical for malignancies which are incurable with current therapeutic approaches due to a high anti-cancer drug resistance, even though these cells are Pgp-negative (19). Thus, plasma membrane proteins involved in the anomalous cell viability and mitogenicity should be suitable goals in targeted therapy (20,21). In this study, we showed that approach, other than the typical anti-cancer drug treatment, is very promising.

Temporary removal of cholesterol from the plasma membrane (lipid rafts) inhibited activation of PKB/Akt1/2 (Ser473 phosphorylation) and was further substantiated by the lack of Ser9 phosphorylation of GSK-3ß (Fig. 5A). The latter kinase is a cognate substrate of PKB/Akt1/2, that upon PKB/Akt1/2-mediated Ser9 phosphorylation is unable to suppress cell survival (22). Inactivation of PKB/Akt1/2 (Fig. 1B) evoked reduction in cell viability (Fig. 2A, $P<0.001$), but this effect was reversed through the revitalization of cells with cholesterol replenishment as shown by ultrastructural examination (Fig. 1A). Neoplastic cells are principally affected by modulation of LR (23,24) since it has been known for a century that they hold elevated levels of cholesterol (25). The analysis showed reversible fluctuations of PKB/Akt1/2 Ser473 phosphorylation which were brought about in concert with chelation and subsequent repletion of plasma membrane cholesterol (Fig. 1B). CHOL-rich lipid rafts are essential for PKB/Akt1/2 kinase activation (26) and tumor cells are particularly sensitive to apoptosis upon CHOL depletion (24). Our observations are consistent with studies showing that LR are essential for maintaining

the activity of PI3-K/PKB/Akt1/2 signaling pathway (1,26) but they also indicate the importance of survival systems other than the LR-dependent one. Cho *et al* (27) showed in elegant study that when mice were given simvastatin, an inhibitor of HMG-CoA reductase (step-limiting enzyme in cholesterol synthesis), the decline in viability was followed by necrosis of xenografted COLO 205 cells.

COLO 205 cells are immune to extrinsic apoptosis induced by death ligands, although they express surface death receptors (TNF-R1, TNF-R2, TRAIL-R1, TRAIL-R2, Fas) as demonstrated by FACS (28) or by immunoprecipitation (29). The immune escape of COLO 205 cells from TNF-α-mediated apoptosis is in part the outcome of extensive shedding of TNF-R1 and TNF-R2 (30) and/or by cFLIP protein inhibitory effect on caspase-8 activation (31). The latter case was confirmed by sensitization of COLO 205 cells upon treatment with CHX, a non-specific protein synthesis inhibitor. Similarly, unresponsiveness of COLO 205 cells to TNF-α has been overcome by bisindolylmaleimide IX (32) or by NaB (17). NaB is of special interest, since this is a natural product of fiber fermentation in the gut, and it is a powerful apoptosis inducing agent (33). As we previously reported, NaB-mediated effect facilitated the TNF-α activity, although temporary CHOL depletion with MßCD did not limit this reaction (Fig. 3B, P>0.05). The decline in Bid expression by 24 h of NaB and TNF-α combined treatment suggests, that death promoting signal from TNF-R1 was amplified by NaB through the activation of Bid (tBid), and that 1 h MßCD pretreatment was unable to impede NaB-aided signal transduction from TNF-R1.

Growing body of evidence suggest that cholesterol plays a dual role in cell signaling. This sterol compound of LR is important both for survival and death signals (34,35). Depending on time and concentration the depletion of CHOL with MßCD or NY led to drop in metabolic activity of COLO 205 cells and finally to cell deletion (Fig. 2A, P<0.001), or occurrence of morphological changes (loss of microvilli, swollen mitochondria, degranulation of RER) which were reversed when CHOL was refilled (Fig. 1A). Interestingly, modifications of raft composition might change the upshot originated in LR. Both SPHING and CHOL seem to be indispensable for the maintenance of LR functionality and cell viability (1). Moreover, LR embedded in receptosomes and endosomes allow continuous intracellular signaling unless the signalosomes remain intact (36). Small diameter of LR (5-20 nm) make these entities unlikely to contain enough receptor proteins to elicit any signal.

It is generally believed that LR might cluster into larger platforms (37). We provide evidence (Fig. 5B) that TNF-R1 surface receptors form clusters if one admits that spots observed from TNF-R1 immunocytochemical study are several hundred nanometers in diameter. They were localized to the surface of leading edge in COLO 205 cells (Fig. 5B) suggesting that this is a chemoreceptive zone. If LR form a link between extrinsic and intrinsic apoptotic pathway, then it explains how TNF-α and NaB can act in accordance. There is room for sphingomyelinases, which by release of ceramides permit the formation of gangliosides (glycosphingolipids) known to activate intrinsic apoptosis (5). When IMP was added, cell viability increased (Fig. 2B, P<0.001), and NaB cytotoxicity was reduced (Fig. 2B). Noteworthy, there was no effect of IMP on NaB-dependent potentiation of TNF-α cell death promoting activity (Fig. 2B, P>0.05). The latter finding might be explained by the ceramide-independent apoptotic pathway triggered by combined treatment of TNF-α and NaB. This was not checked by, but some authors suggest the prominent role of neutral sphingomyelinase (nSMAse) in cell death evoked in tumor cells (38). nSMAse is not targeted by IMP but is activated by PUFA, and PUFA are known to make LR poorer in CHOL and SPHING but richer in phosphatidylcholine (39). Thus, PUFA and NaB may act differently (40) pointing to less clear outcome of IMP use. This was more evident in the measurement of mitogenicity, where DNA synthesis was lesser in IMP treatment in common with NaB and TNF-α (Fig. 3C). Perhaps the number of surface receptors is an important causal factor, since in the IMP treated COLO 205 cells no TNF-R1 receptors were detected (Fig. 5B).

EGCG-dependent stimulation of COLO 205 cell viability was reversed by co-treatment with TNF-α, similarly to LR modulators (Fig. 3B, P<0.05). We concluded that EGCG effect is mediated by LR, and that TNF-α could be holding up EGCG activity, though this cytokine given alone stimulated cell survival (Fig. 2B, P<0.01). Administration of IMP, the aSMAse inhibitor revealed that even then, viability of COLO 205 cells was to some extent hampered by unknown factors (Fig. 2B, P<0.01). The TNF-α-induced effect was LR-dependent as MßCD co-treatment lessen the NaB-dependent aid to TNF-α cytotoxicity (Fig. 3B, P<0.01).

The use of volatile fatty acids such as butyrate (sodium salt) is a powerful instrument in the eradication of colon tumors (41). In our study, this low-molecular weight substance (depending on concentration, 2-5 mM) was able to eliminate 60-80% of tumor cells (Figs. 2B and 3B, P<0.01). Furthermore, TNF-α, previously shown to be negligible in cell deletion appeared cytotoxic in the presence of NaB (Figs. 2B and 3B, P<0.05). Earlier we reported that in contrast to NaB, CHX sensitized COLO 205 cells to TNF-α-dependent extrinsic apoptosis, where resistance relied upon anti-apoptotic FLIP protein (31). The finding that CHX blocked cytotoxicity of NaB points to *de novo* synthesis of proapoptotic proteins, degradation of anti-apoptotic proteins or both as crucial factors in the death promoting effect of NaB. None of the NaB-induced effects was dependent on LR, because neither MßCD nor NY, could reverse NaB cytotoxicity. Similarly, IMP, an inhibitor of aSMAse, could not inhibit NaB-induced cytotoxicity of TNF-α, although it markedly reduced the effect of NaB or TNF-α given alone (Fig. 2B, P<0.05). It indicates, that IMP and lack of ceramides significantly stimulated viability of COLO 205 cells. Actually, IMP stimulated cell viability in parallel to a marked reduction in the expression of TNF-R1 (Fig. 5B).

To our knowledge, this study gives the first evidence that TNF-R1 receptors are clustered in COLO 205 cells without addition of the cognate ligand. We also provide compelling evidence that acid sphingomyelinase (aSMAse) inhibitor imipramine (IMP) blocks the appearance of TNF-R1. Additionally, the study indicates that sodium butyrate (NaB) toxicity against COLO 205 cells acts independently of the lipid rafts, but its action is inhibited by non-specific

translation and transcription inhibitors. These results indicate, that *de novo* protein synthesis, rather than post-transcriptional modifications play important role in NaB-enhanced cell death. In fact, NaB is known as a histone deacetylase inhibitor, which by epigenetic correction of gene activity triggers differentiation and/or apoptosis of gut epithelia (42,43). Immunoblotted proteins separated by SDS-PAGE confirmed that NaB with the exception of cIAP-1 elevated pro- (Bax, caspase-3) and lowered the expression of antiapoptotic proteins (Bcl-2, XIAP, survivin, Fig. 5C). Furthermore, procaspase-3 has been converted to caspase-3 whenever NaB was given alone or together with TNF-α (Fig. 5C). Bid expression was decreased at this same time. This study confirms our recent study (17) that NaB induces intrinsic apoptosis and facilitates TNF-α-dependent extrinsic apoptosis. Metabolic activity of COLO 205 cells descended in concert with the reduced level of procaspase-3 and was counterbalanced by increased levels of caspase-3 (Fig. 5C). Moreover, the expression of TRADD adaptor protein was elevated if NaB and TNF-α were used together. This result indicate that NaB administration might be considered as an important determinant of the TRADD protein expression. The latter is essential for the assembly of signalosome at TNF-R1. It was actually NaB that sensitized COLO 205 adenocarcinoma cells to TNF-α rather than vice versa.

Selected anti-cancer drugs (CIS and CLA) applied in moderate concentrations apparently did not diminish viability of COLO 205 cells (Fig. 2C, $P>0.05$), whereas high, toxic concentrations were successful merely by 24 h of treatment (data not shown). These results might raise a question whether these cytostatics are of any use in the elimination of colorectal cancers. However, Gong *et al* (28) showed that e.g. paclitaxel (taxan) used sequentially with TRAIL could efficiently induce cell death in xenografts and cultured COLO 205 cells. Paclitaxel elevated the expression of TRAIL surface receptors by post-translational mechanism that could not be circumvented either by protein synthesis or transcription inhibitors (28). To verify whether the tested compounds can sensitize colorectal cancers to death ligand cytotoxicity, additional studies are required.

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