Bile acids as constituents for dental composites: *in vitro* cytotoxicity of (meth)acrylate and other ester derivatives of bile acids

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Methacrylic derivatives of bile acids have been synthesized for use as monomers in dental composites. Polymeric dental materials are known to leach cytotoxic unreacted monomers and degradation products. In this study, the *in vitro* cytotoxicity of bile acids and their derivatives towards 3T3 fibroblasts has been evaluated by colorimetric MTT assay and compared with that of the common dental monomers BisGMA, UDMA and TEGDMA. In general, the bile acids and their derivatives induced mitochondrial dysfunction at similar or higher concentrations than the commercial dental monomers. Certain monomers did not influence MTT response over their entire range of solubility.

Keywords: bile acids; crosslinking; cytotoxicity; dental polymers; dental monomers

1. INTRODUCTION

Bile acids and their derivatives are currently used for biomedical (Hofmann 1995) and supramolecular applications (Virtanen & Kolehmainen 2004). In particular, polymeric biomaterials with main- or side-chain cholic or lithocholic acid groups (Zhu & Nichifor 2002) have attracted significant attention because their (bio)degradation is expected to lead to the release of endogenous compounds. In fact, the *in vitro* exposure of primary human and pig cells (normal human dermal fibroblasts, pig fibroblasts, pig smooth muscle cells and pig aortic endothelial cells) to polyanhydride implant materials containing main-chain lithocholic acid groups has been shown to adversely affect neither the rate of proliferation nor the morphology of the cells versus unexposed controls (Gouin *et al.* 2000). These results have prompted efforts towards improving the physical and toxicological properties of polymeric biomaterials by incorporation of bile acids into the structure of these materials (Gautrot & Zhu 2006).

Crosslinking methacrylate monomers derived from bile acids have been proposed as monomers for composite dental fillings in an effort to improve their physical properties and potentially reduce the toxicity of leachates (unreacted monomers and (bio)degradation products; Hu *et al.* 2005a; Gauthier 2007). The objective of this study is to evaluate the *in vitro* cytotoxicity of cholic acid (4), chenodeoxycholic acid (14), deoxycholic acid (17) and lithocholic acid (20) as well as their simple ester (5, 8, 15, 18 and 21) and (meth)acrylate (6, 7, 9–13, 16, 19 and 22) derivatives. The structures of all compounds are listed in figure 1. These compounds correspond either exactly to the monomers proposed for use in dental composites or to their hypothesized degradation products. This study places particular emphasis on comparing the cytotoxicity of these compounds to the common dental monomers 2,2-bis(4-(2-hydroxy-3-methacryloxypropoxy)phenyl)propane (BisGMA, 1) and 1,6-bis(methacryloyloxy-2-ethoxycarbamylamo)-2,4,4-trimethylhexane (UDMA, 2) which are known for their hydrophobicity and *in vitro* cytotoxicity (Yoshii 1997; Geurtsen *et al.* 1998). Another common dental monomer, triethylene glycol dimethacrylate (TEGDMA, 3), was also tested in order to allow for better comparison with results from the literature. A previously unreported tetra-acrylate derivative of cholic acid (13) was also prepared and evaluated in order to better establish structure–cytotoxicity relationships.

2. EXPERIMENTAL PART

UDMA, TEGDMA, cholic acid (98%), chenodeoxycholic acid, deoxycholic acid, lithocholic acid, ethylene glycol, triethylamine, sodium dodecyl sulphate (SDS; electrophoresis grade), 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H tetrazolium bromide (MTT), dimethyl sulfoxide (DMSO) and concentrated HCl were obtained from Sigma Aldrich (Milwaukee) and used...
as received. BisGMA was purchased from Polysciences (Warrington, Pennsylvania) and purified by silica column chromatography (100 g silica per 1 g BisGMA; ethyl acetate: hexane (1/1 v/v) as eluent). Acryloyl and methacryloyl chloride (Aldrich) were distilled immediately prior to use. All organic solvents were used as received except for dichloromethane which was dried using a column solvent purification system.

Compounds 5–12, 15, 16, 18, 19, 21 and 22 were synthesized as described previously (Hu et al. 2005b; Gauthier 2007). Compound 13 was prepared in 50% yield in exactly the same fashion as 12, while replacing methacryloyl chloride with acryloyl chloride. $^1$H NMR (400.26 MHz in CDCl$_3$; 13): $\delta$ (p.p.m.) = 0.77 (s, 18-CH$_3$); 0.82 (d, 21-CH$_3$); 0.97 (s, 19-CH$_3$); 4.35 (m, COOCH$_2$CH$_2$OCO); 4.64 (m, 3\textalpha-CH); 5.05 (m, 7\textalpha-CH); 5.21 (m, 12\textalpha-CH); 5.75–6.5 (m, =CH). The full $^1$H NMR spectrum for this compound is available in the electronic supplementary material. The purity of all substances as well as the octanol–water partition

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M, methacrylate group; A, acrylate group

Figure 1. The chemical structures of the compounds examined in this study.
coefficients ($\log(K_{OW})$) for previously unreported compounds were evaluated by high-performance liquid chromatography (HPLC) as described elsewhere (Gauthier 2007). The substances were considered pure when they eluted as a single peak in all solvent systems examined.

Cytotoxicity was evaluated by MTT assay using 3T3 fibroblasts purchased from ATCC (CCL92). A total of $10^4$ cells per well were plated into 96-well microplates and pre-cultured for 24 h in 100 ml Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10 vol% foetal bovine serum, 100 U ml$^{-1}$ penicillin G and 100 µl ml$^{-1}$ streptomycin. Monolayers of exponentially growing 3T3 fibroblasts were then incubated in the 96-well plate with the individual molecule to be tested for 24 h. These molecules were initially dissolved in DMSO which served as carrier for distribution into the wells. The concentration of DMSO was always 1 vol% of the total volume of culture medium. Cells treated with 1 vol% DMSO served as controls. The cells were then incubated for 48 h and MTT (10 mg ml$^{-1}$ in PBS) was added to each well. After further 3 h of incubation, SDS (100 µl of a 10% (w/v) solution in 0.01 N hydrochloric acid) was added to each well to dissolve the reduced MTT. The absorbance at 570 nm was read 24 h after the addition of the SDS solution using a Safire$^2$ microplate reader from Tecan, US. Cell viability at each concentration and for each analyte was measured nine times (i.e. three times each on three different microplates). The maximum solubility of the analytes in the culture medium was established by examining the individual wells of the microplate at the end of the 24 h incubation.

![Figure 2](image1.png)

**Figure 2.** Dose-response profiles obtained for (a) the commercial monomers, (b) bile acids and (c) bile acid derivatives (methyl or ethylene glycol esters).

![Figure 3](image2.png)

**Figure 3.** Dose-response profiles obtained for (a) mono-methacrylate, (b) di-methacrylate, (c) tri- and tetra-(meth)acrylate bile acid derivatives.
period with a transmission microscope. The IC₅₀ value was obtained from the dose–response curves shown in figures 2 and 3. Statistical comparison of IC₅₀ values was performed by one-way ANOVA followed by pairwise comparison of means using Dunnett’s T3 post hoc test (data was not equivariant).

3. RESULTS AND DISCUSSION

The concentration-dependent effect of each analyte on the viability of 3T3 fibroblasts was assessed by MTT assay. This cell line and cytotoxicity assay were selected based on their extensive precedence for evaluating the cytotoxicity of dental monomers (Geurtsen et al. 1998; Al-Hiyasat et al. 2003). The results of these assays are presented in table 1 alongside the octanol–water partition coefficients (useful parameter for comparing compound hydrophobicity) of each analyte obtained from a previous report (Gauthier 2007) (compound 13 reported for the first time in this study). Dose–response profiles were measured for each analyte (figures 2 and 3) up to 10 mM or up to their saturation concentration in the culture medium.

For the commercial monomers BisGMA, UDMA and TEGDMA, IC₅₀ values (concentration which inhibited 50% cell growth relative to the controls) were consistent with values found in the literature (Geurtsen et al. 1998; Stanislawski et al. 2003). The mechanism by which these compounds provoke cell death remains unclear yet previous reports indicate that BisGMA and UDMA cause cell death via necrosis (disruption of cell membrane), while TEGDMA causes cell death via apoptosis (depletion of intracellular glutathione) as well as by necrosis (Fujisawa et al. 1988; Reichl et al. 2006).

3.1. Bile acids and their esters

The cytotoxicity of bile acids has been studied extensively in the past given the relationship between abnormally high bile acid concentrations in hepatic tissues and cholestatic liver diseases (Rolo et al. 2000, 2004; Ferreira et al. 2005). These studies have focused on establishing the mechanism by which bile acids induce hepatocyte death. The issue of extrahepatic bile acid circulation (i.e. in plasma) has also been studied with particular emphasis on assessing the cytotoxicity of unconjugated bile acids towards fibroblast cultures (Ceryak et al. 1998). These cell cultures differ from hepatocytes in that they do not possess bile acid-activated death receptors (such as the Fas receptor) and therefore constitute a better model for assessing the cytotoxicity of molecules designed to be used in proximity to dental pulp (odontoblasts) or mucosa (fibroblasts). The cytotoxicity of the primary (4 and 14) and secondary bile acids (17 and 20) correlated well with the expected hydrophobicity of these compounds as previously reported (Ceryak et al. 1998). This is an indication that cellular uptake of the bile acids may arise from passive diffusion. The water–octanol partition coefficient, a term which relates to the hydrophobic/hydrophilic balance of a molecule, measured for
lithocholic acid was anomalously low, possibly due to the inaccuracies related to the HPLC method when used for assessing the hydrophobicity of weak acids when the pH of the mobile phase is not controlled (Lucangioli et al. 2001). Unfortunately, literature values for the octanol–water partition coefficient for lithocholic are unavailable, even in those studies which assess the effects of conjugation with amino acids or the pH of the mobile phase on the measured hydrophobicity (Heuman 1989; Roda et al. 1990). The concentration-dependent reduction of cell viability for these compounds (figure 2b) has been attributed to apoptosis and/or necrosis (depending on bile acid concentration) resulting from oxidative stress and mitochondrial dysfunction. This is thought to arise from the induction of the mitochondrial permeability transition (MPT) by bile acids (Sokol et al. 2005), though the exact mechanism by which they induce the formation of the permeability transition pore (PTP) remains unclear (Rolo et al. 2001). The ethylene glycol esters of cholic acid, chenodeoxycholic acid and deoxycholic acid (8, 15 and 18) follow a trend similar to that of the free acids but were more cytotoxic (figure 2c). Cholic acid with a methyl ester on position 24 was more cytotoxic than its ethylene glycol analogue (8). The greater cytotoxicity of these bile acid esters versus their free acid analogues may result from their greater hydrophobicity which would favour diffusive cellular uptake. The ethylene glycol ester of lithocholic acid had an unexpectedly low \( \log(K_{OW}) \) and high IC\(_{50} \) when compared with the other bile acid esters. The dose–response profile for this compound also shows a more gradual decrease of cell viability with concentration when compared with the other bile acid esters, possibly indicative of a different mechanism of cell lysis or to a less pronounced induction of the MPT for this compound.

3.2. (Meth)acrylate derivatives of bile acids

The cholic acid derivative with a single methacrylate group on position 3 (compound 6) was as cytotoxic as its methyl ester precursor 5, despite being more hydrophobic. The decrease of cell viability with concentration for 6 (figure 3a) was also less abrupt than for 5 (figure 2c). Contrarily, the mono-methacrylate 9 was more cytotoxic than its precursor, ethylene glycol ester 8, explainable by its greater hydrophobicity. These seemingly contradictory trends are an indication that the mechanism of cell lysis caused by bile acids is relatively structure specific and modifications made at different positions on these compounds may influence cytotoxicity in different manners. In this case, it appears as though hydrophobic modifications made to position 3 on cholic acid may help to reduce cytotoxicity, while similar modifications made to position 24 may increase cytotoxicity, independently of hydrophobicity. The existence of a relationship between the nature of the group on position 24 and cytotoxicity has been previously shown in the literature (Rolo et al. 2000).

Of all di-methacrylate derivatives of bile acids, 10 was the only one to induce a reduction of cell viability over its range of solubility (figure 3b). This indicates that simultaneous modification of positions 3 and 24, as well as the number of hydroxyl groups on the steroid backbone influence the compound’s susceptibility to induce mitochondrial dysfunction. The insolubility of 22 in DMSO (carrier) prevented the evaluation of its cytotoxicity. The tri- and tetra-methacrylate derivatives of cholic acid (7, 11 and 12) also exhibited no cytotoxic response over their range of solubility, even though the great hydrophobicity of these compounds may lead to their accumulation within the cell membrane, a phenomenon which can be further studied by NMR spectroscopy (Engelmann et al. 2001).

The tetra-acrylate derivative of cholic acid (13) was more hydrophilic than its methacrylate analogue (12) and was the most cytotoxic substance tested. It has been suggested that acrylates are more susceptible to reaction with glutathione (via Michael’s addition) than methacrylates and deplete the intracellular content of this tri-peptide, thus increasing the cell’s susceptibility to death via formation of reactive oxygen species (Stanislawski et al. 2003; Schultz et al. 2005). The cytotoxicity and hydrophobicity of 13 strongly suggest that the more hydrophobic di- (with the exception of 10), tri- and tetra-methacrylate compounds must also be taken up by the cells, but that neither cell metabolism nor membrane integrity is affected over their range of solubility.

4. CONCLUSION

Our results indicate that di-, tri- and tetra-methacrylate derivatives of bile acids are less cytotoxic than the commercial dental monomers BisGMA and UDMA. In fact, aside from 10, these monomers did not affect cell viability over their entire range of solubility. This indicates that the possible extraction of these compounds from a dental composite by a continuous flow of saliva in vivo may be less likely to provoke localized cytotoxic or inflammatory responses when compared with BisGMA or UDMA. The mono-methacrylates and bile acid esters, which are potential intermediates of the (bio)degradation of the multi-methacrylates, had comparable cytotoxicity to BisGMA or UDMA, but the ultimate degradation products of these derivatives are natural bile acids, which were all significantly less cytotoxic than BisGMA or UDMA. These results justify further development of polymeric materials based on bile acid derivatives for biomedical applications and warrant a more extensive evaluation of the mechanism underlying cell lysis and the exact nature of the biodegradation compounds produced for these molecules. This can be accomplished by evaluating cell membrane stability (Issa et al. 2004), monitoring the production of reactive oxygen species (Stanislawski et al. 2003), investigating the influence of these compounds on cell/mitochondrial metabolism (Sokol et al. 2005) by mass spectrometry and NMR spectroscopy (Engelmann et al. 2001).

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