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Topological aspects of oligomeric UDP-glucuronosyltransferases in endoplasmic reticulum membranes: advances and open questions

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Running title: Topological aspects of oligomeric UGTs

Abbreviations: BaP, benzo[a]pyrene; ER, endoplasmic reticulum; 3-MC, 3-methylcholanthrene; ROS, reactive oxygen species; T4, thyroxine; UDPGA, UDP-glucuronic acid; UGT, UDP-glucuronosyltransferase

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Abstract

UDP-glucuronosyltransferases (UGTs) represent major Phase II enzymes involved in detoxification of endo- and xenobiotics, including many drugs. The intraluminal orientation of the active site of UGTs in endoplasmic reticulum membranes necessitates a number of transporters in these membranes, for example, for UDP-glucuronic acid and glucuronides, the latter being insufficiently characterized. In addition, accumulating evidence suggests that UGTs are functional as homo- and hetero-dimers in monoglucuronide formation. They may form tetramers in diglucuronide formation. UGT oligomers probably serve to stabilize UGT monomers and fine-tune UGT activity. Glucuronide disposition may also be influenced by endoplasmic reticulum-localized β-glucuronidase, possibly involved in hydrolysis of hormone and drug glucuronides in target cells. The present commentary reviews recent advances and addresses open questions. Resolution of these questions may help to understand many problems of glucuronide synthesis and disposition in vivo, for example, under-prediction of the in vivo clearance of drugs mostly eliminated by glucuronidation by in vitro enzyme kinetic parameters of UGTs.

Key words: UDP-glucuronosyltransferases; UGT topology in endoplasmic reticulum membranes; UGT oligomers; diglucuronide formation; β-glucuronidase.

1. Introduction

Glucuronidation represents a major Phase II biotransformation reaction. It is catalyzed by two evolutionary conserved UGT enzyme families, each including at least 8 enzymes which are adaptively regulated in a tissue-specific manner [1-5]. The review by Geoffrey Dutton [1] represents an invaluable source of the earlier literature. UGTs are involved in detoxification of endobiotics including bilirubin, thyroxine, steroids, and xenobiotics including phytochemicals, carcinogenic polycyclic hydrocarbons and a variety of prescribed drugs from all therapeutic classes. The intraluminal orientation of the active site of UGTs and of β-glucuronidase in ER membranes necessitates a number of transporters in these membranes. Following transport of glucuronides between ER lumen and cytosol, conjugate transporters in the plasma membrane are required to prevent the accumulation of polar conjugates in cells [6-8]. Recently, accumulating evidence suggests that UGTs are functional in ER membranes as dimeric complexes, and may form tetramers in diglucuronide formation. The present commentary reviews recent advances and addresses open questions about the UGT topology in ER membranes and their oligomeric nature. Glucuronide disposition may be influenced by ER-localized β-glucuronidase, possibly involved in futile cycling of glucuronides and hydrolysis of hormone and drug glucuronides in target cells. Resolution of these questions may help to understand many problems of glucuronide synthesis and disposition in vivo, for example underprediction of the in vivo clearance of drugs mostly eliminated by glucuronidation by in vitro enzyme kinetic parameters of UGTs.

2. Topology of UGTs in ER membranes

A number of advances have recently been made on the structure and topology of UGTs and β-glucuronidase in ER membranes (Fig. 1). The schematic representation should be viewed as an extension of the previously published hypothetical model of UGT dimers [4]. UGTs are integral ER membrane proteins with a transmembrane segment and a short cytoplasmic tail. They are known to be active at the luminal side of the ER [3,4]. This topology necessitates recently identified nucleotide sugar transporters (NSTs) to carry the cofactor UDP-glucuronic acid (UDPGA) to the ER lumen [9]. They act as antiporters requiring countertransport of UDP-N-acetylglucosamine [10]. The reason for luminal orientation of UGTs is unknown but

may be due to evolutionary constraints since many glycosyltransferase reactions including those involved in glycoprotein synthesis occur in the ER lumen. UGT monomers consist of the N-terminal aglycone substrate-binding half of the protein and the UDP-glucuronic acid-binding C-terminus, the latter containing one transmembrane sequence [2-4]. The crystal structure of the UDPGA-binding C-terminal half of UGT2B7 has recently been determined at 1.8-Å resolution. Mutants at residues predicted to interact with UDPGA exhibited impaired catalytic activity, and mutants at predicted aglycone binding sites abrogated UGT activity [11]. In addition, an internal signal sequence has been identified embedding part of the N-terminal half of UGTs in the ER membrane, a feature which may facilitate access of lipophilic aglycones to the active site [12].

UGTs conjugate glucuronic acid to functional groups of a variety of aglycones mostly hydroxyl, amino or carboxyl groups. With hydroxyl groups (XOH) they catalyze the following reversible reaction: XOH + UDPGA ⇆ XOGA + UDP. Recent extensive kinetic analysis in microsomal preparations in vitro suggested an ordered bi bi mechanism in which the aglycone substrate may act as an inhibitor by binding to the enzyme-UDP complex and thus depleting the active enzyme pool [13]. However, the reversible reaction may only rarely occur in the intact cell since the two products of the UGT reaction are rapidly removed: (i) UDP is rapidly hydrolyzed to UMP by nucleoside diphosphatase in the hepatic ER lumen [14]. Rapid hydrolysis of UDP in microsomes may explain the lack of UDP-dependent inhibition of UGT reactions in liver microsomes in contrast to preparations from expressed UGTs [15]. (ii) Glucuronides appear to be rapidly translocated to the cytosol. Evidence for multiple ER-localized organic anion transporters (AT_{ER}) has been obtained which - in contrast to plasma membrane-localized ATP-dependent glucuronide transporters - do not need ATP but transport organic anions through ER membranes by facilitated diffusion [16,17]. Some of these transporters may also be involved in transport of hormone glucuronides such as thyroxine glucuronide from the cytoplasm to ER-localized β-glucuronidase (see chapter 5.3).

3. Monoglucuronide formation by UGT dimers

Accumulating evidence suggests that UGTs mostly operate as dimeric complexes. Using mutants and chimeric constructs Meech and Mackenzie demonstrated that

oligomerization of two inactive mutants could yield an active unit [18]. Nearest neighbor crosslinking studies followed by gel filtration provided evidence that UGTs form dimers in microsomes [19,20]. Using a variety of techniques evidence was obtained that oligomers may function as homo- [20,21] or hetero-oligomers [21-25]. For example, in live cells intermolecular interactions among UGT1A proteins was demonstrated by fluorescently tagged UGT1A proteins and homo- and heterodimerization by co-immunoprecipitation analysis [21]. Cotranslational insertion of UGTs into the membrane appears to be a requirement for oligomerization [21]. UGTs as oligomeric enzymes were critically discussed [23]. Functional implications of dimer formation were studied in several ways: for example, co-expression of an inactive mutant of UGT1A6 (the only enzyme catalyzing serotonin glucuronidation [26]) and of UGT1A4 restored UGT1A6-mediated serotonin glucuronidation, suggesting tight interaction between the two recombinant enzymes. Interestingly, these dimers did not share substrate binding sites [23,24]. Heterodimerization has been shown to up- or down-regulate UGT activity [25]. In addition, heterodimers may include recently identified inhibitory UGTs [27]. However, more work is needed to characterize these homo- and hetero-oligomers and their functional implications.

4. Diglucuronide formation by UGT tetramers

Some UGT dimers may form tetramers as suggested by radiation target analysis, a method which has been conceptually and experimentally established by Ellis Kempner, and frequently applied to determine the functional molecular mass of membrane proteins [28-30]. Studies of bilirubin glucuronidation suggested that diglucuronide formation may be carried out by UGT tetramers [31]. Expressed human UGT1A1 efficiently converts the two bilirubin monoglucuronides (at either the C8 or C12 propionic acid group) to the diglucuronide [32], and bilirubin is known to be mainly secreted in human bile as the diglucuronide.

Radiation inactivation analysis was also carried out in studies of 3,6-quinol monoglucuronide (MG) and diglucuronide (DG) formation of BaP and the chemically more stable chrysene (Fig.2). We got interested in these reactions because of strong induction of these UGT activities in hepatic microsomes from 3-MC-treated rats [33,34]. Induction factors were 10- and 40-fold for BaP-3,6-quinol MG and DG

formation, and 7- and 310-fold for chrysene MG and DG formation, respectively. 3-MC-inducible UGT1A7 efficiently converted the studied quinol monoglucuronides to their diglucuronides [35], as suggested by much lower MG/DG ratios compared to microsomes from untreated controls (Table 1). However, enzyme induction alone may not explain the efficiency of DG formation. For example, 3-MC treatment of rats enhanced chrysene-3,6-DG formation about 310-fold in liver microsomes, suggesting - in addition to induction of UGT isoforms- an influence of topological features of UGT quaternary structure, discussed subsequently. Glucuronidation of quinols is toxicologically relevant since it probably prevents quinone-quinol redox cycles and associated oxidative stress [33-37]. BaP quinol diglucuronides represent major conjugates in bile and urine after administration of BaP [38,39].

Radiation inactivation analysis was carried out using the described quinol MG and DG reactions [40]. For monoglucuronide formation of BaP- and chrysene-3,6-quinol target sizes were 118 ± 33 and 109 ± 21 kDa, respectively (in agreement with the existence of dimers) whereas for diglucuronide formation target sizes were 218 ± 24 and 192 ± 34 kDa, respectively, suggesting the formation of tetramers. It should be noted that in early enzyme purification studies, gel filtration in presence of detergents revealed peaks at the position of tetrameric UGT complexes [41,42]. In diglucuronide formation some dimers may loosely interact in ER membranes to form tetramers (Fig. 1), findings which, however, need further validation. Loose interaction may explain why tetramers have not been detected in cross-linking studies [20]. Nevertheless, as discussed previously [23], high molecular weight bands possibly corresponding to tetramers can be seen by close inspection of the cross-linking studies of Gosh et al. [20 (Fig. 3, lane 3 and 4)]. Tetramers may generate a compartment between two dimers in which monoglucuronides reach high levels to facilitate diglucuronide formation, as evidenced in studies on BaP-3,6-quinol diglucuronide formation: while a high K_M value (>70 µM) was detected when the diglucuronide was formed from synthesized monoglucuronides, a much lower K_M of 10-20 µM was determined when starting the reaction from the quinol [43,44]. However, it is acknowledged that the results could also reflect the ease of access of quinols versus quinol glucuronide across the ER membrane. Radiation target analysis of bilirubin glucuronidation using human microsomes and expressed UGT1A1 may be useful to substantiate formation of UGT tetramers.

5. In vitro-in vivo comparison of glucuronide formation

To guide future studies on synthesis and hydrolysis of glucuronides in ER membranes, the relevance of UGT activity is emphasized (i) by correlation studies using UGT polymorphism UGT1A1*28, and (ii) by comparative studies between in vitro kinetic parameters of UGTs and in vivo clearance of drugs mostly eliminated by glucuronidation.

5.1. Bilirubin UGT polymorphism UGT1A1*28

Allelic variants often clearly demonstrate the relevance of proteins. Functional UGT polymorphisms are increasingly identified, and are updated in the UGT web site at http://som.flinders.edu.au/FUSA/ClinPharm/UGT. It is assumed that further analysis of bilirubin mono- and diglucuronide formation may help to answer open questions on UGT oligomers. Therefore, in vivo findings using the frequent bilirubin UGT polymorphism UGT1A1*28 are highlighted.

Early clinical studies of inherited hyperbilirubinemias identified the rare and fatal Crigler-Najjar syndromes I and II and the frequent mild form of Gilbert's syndrome. Frequent occurrence of allelic variants is often due to 'balanced polymorphism' [45], i.e. balancing blood bilirubin between high neurotoxic levels and low levels acting as powerful antioxidant [46, for references]. Bilirubin is the final product of heme catabolism, as heme oxygenase cleaves the heme ring to form the water-soluble biliverdin, which is reduced by biliverdin reductase to bilirubin (Fig. 3). Why should mammals have evolved the potentially toxic and insoluble bilirubin? Recently, it was established that bilirubin is oxidized by reactive oxygen species (ROS) to biliverdin which is efficiently reduced back to bilirubin. This amplification cycle establishes a physiologic function of bilirubin as powerful antioxidant [47]. Epidemiologic evidence indicates that homozygous UGT1A1*28 allele carriers with high serum bilirubin exhibit a strong association with lower risk of cardiovascular disease [48]. Bilirubin and bilirubin monoglucuronides produced in extrahepatic tissues are efficiently taken up into hepatocytes by OATP1B1 and converted to bilirubin diglucuronide by UGT1A1.

Gilbert's syndrome in Caucasians is mostly due to an additional TA repeat in the TATA box of UGT1A1 (a promoter polymorphism termed UGT1A1*28), leading to reduced UGT1A1 expression and significantly decreased UGT activity. Liver

homogenate bilirubin UGT activity in Caucasian carriers of the UGT1A1*28 genotype was only 48% (ranges 27-75; n=6) of the UGT1A1*1 reference genotype (ranges 47-170; n=17) [62]. The frequency of homozygous carriers of UGT1A1*28 in Caucasians is approximately 10% and the allele frequency 30 to 40% [46]. Correlation studies between populations expressing UGT1A1*1 and UGT1A1*28 proteins are often used to associate UGT1A1 activity with in vivo responses. Examples include (i) adverse side effects of irinotecan. The topoisomerase inhibitor irinotecan has been approved for standard therapy of colorectal cancer. Its active metabolite SN-38 is mostly metabolized by UGT1A1 and 1A7 [49]. Poor glucuronidators, i.e., carriers of the promoter variant UGT1A1*28 have been suggested to be at higher risk to develop unwanted side effects such as diarrhea [50]. (ii) A strong correlation was observed between UGT1A1-mediated glucuronidation of SN-38 and of T4 [51]; there was a significant trend of decreasing T4 glucuronide levels and expression of UGT1A1*28. Despite low levels of T4 glucuronide in serum, the role of T4 glucuronidation in vivo is supported by alteration of thyroid hormone homeostasis in antiepileptic drug-treated patients [52]. (iii) UGTs such as UGT1A1 and UGT2B7 appear to be involved in homeostasis and further metabobolism of estradiol in endometrium [53]. Estradiol is metabolized to genotoxic 4-hydroxy- or antiproliferative 2-hydroxy-catechol estrogen. Interestingly, reducing the excretion of 2-hydroxyestradiol in carriers of the UGT1A1*28 allele has been suggested to be responsible for decreased endometrial cancer risk [54]. These three examples may serve to highlight the relevance of in vitro microsomal UGT activity for a variety of in vivo functions.

5.2. Comparative studies between in vitro enzyme kinetic parameters of UGTs and in vivo drug clearance

Comparison of in vitro intrinsic clearance of drugs (represented by the enzyme kinetic parameter V_{max}/K_M) with their hepatic clearance in vivo represents a challenging exercise**.** In the case of drugs mainly excreted by glucuronidation, it was found that the in vitro intrinsic clearance under-predicts in vivo hepatic clearance [4,55,56]. Many factors have been suggested to be responsible for this discrepancy. In addition to general factors such as the nutritional state, the following liver microsomal UGT assay conditions may be relevant: (i) In the case of the antiretroviral drug zidovudine (mainly eliminated via glucuronidation by UGT2B7 [57]) inclusion of the physiologic carbonate buffer or Williams E medium increased V_{max} and reduced K_M in liver

microsomes, thereby increasing the intrinsic clearance in vitro [58]. Determination of UGT activity in physiologic buffer may better preserve the topology of UGTs and associated proteins in the ER. (ii) Fatty acid-free human serum albumin and bovine serum albumin reduce the K_M values for UGT2B7 substrates (but not UGT1A1 and UGT1A6 activities) by sequestering inhibitory long-chain fatty acids released by incubations of human liver microsomes and cell-expressed UGTs. This observation has been termed 'albumin effect' [59]. (iii) As mentioned in chapter 2, rapid hydrolysis of UDP in the ER may explain the lack of product inhibition by UDP [15]. In addition, rapid translocation of glucuronides from ER into the cytoplasm may further decrease product inhibition in vivo. (iiii) Atypical enzyme kinetics, i.e. non-Michaelis-Menten kinetics, is frequently observed in studies of drug glucuronidation and often interpreted by evoking a two site model; in the light of UGT oligomers these sites could be present on separate molecules within the same oligomeric complex [23]. Atypical enzyme kinetics leading to autoactivation could in part be responsible for paradoxical effects observed with the over the counter analgesic drug paracetamol (acetaminophen) [60]. Its metabolism has been intensely investigated, in particular due to its hepatotoxicity in suicidal overdose. Interestingly, paracetamol is a high-affinity substrate for UGT1A6 [60,61]. However, it appears to be mainly conjugated in liver by UGT1A9 (and also by UGT1A1) [60]. Of course, the presently unknown relative abundance of UGT isoforms may also play a role. In conclusion, understanding oligomeric UGTs and the influence of associated proteins may be necessary for the development of generalizable models for the in vitro-in vivo comparison of drug glucuronidation.

5.3. Role of ER-localized β-glucuronidase in glucuronide disposition

Glucuronide disposition may be influenced by ER-localized β-glucuronidase, possibly involved in futile cycling of glucuronides and hydrolysis of conjugated hormones in target cells. Murine β-glucuronidase is derived from a single gene but is located in two subcellular sites, the ER and lysosomes. On the way from synthesis in the rough ER to lysosomes part of the enzyme is retained in the lumen of the ER by associating with the carboxylesterase egasyn which contains an ER retention sequence [63-65]. ER-localized β-glucuronidase has been shown to form tetramers (Fig. 1). Its in vivo activity has been demonstrated in the hydrolysis of bilirubin monoglucuronides [66]. The activity of β-glucuronidase in the lumen of the ER may lead to futile cycling of

glucuronides [67], which, however, appears to be limited since ß-glucuronidase activity is low. ER-resident β-glucuronidase may also be responsible for hydrolysis of hormone and drug glucuronides in cells, examplified by the hydrolysis of T4 glucuronide. T4 is widely used for treatment of hypothyroidism. It is mainly deiodinized to T3 (>70%) but is also metabolized by conjugation. T4 sulfate is very unstable because sulfation accelerates inner ring deiodination by approximately 200-fold [51]. T4 glucuronidation (carried out by UGT1A1 and 1A3) leads to a stable T4 glucuronide which may serve as a mechanism of delivery of T4 into intracellular compartments, as discussed in chapter 5.1 [51,52]. However, the postulated uptake of circulating T4 glucuronide into target cells and its hydrolysis by cellular β-glucuronidases needs to be further studied. In contrast, lysosomal glucuronidase is involved in the degradation of glycosaminoglycans, synthesized in the Golgi apparatus. Transit of lysosomal glucuronidase from the ER to lysosomes has been reviewed [63].

Hydrolysis of glucuronides is also important for enterohepatic and entero-enteric recirculation of many important drugs including SN-38 (the metabolite of irinotecan (mentioned in chapter 5.1) and in the action of the cholesterol-lowering drug ezetimide which is mainly conjugated by UGT1A1 [68]. It has been suggested that ezetimibe glucuronide represents a storage form for long lasting recycling of the active ezetimibe to the intestinal absorption compartment via the systemic circulation. However, it is unknown to what extent glucuronide hydrolysis contributes to the clearance of drugs mainly eliminated by glucuronidation.

6. Conclusions

UDP-glucuronosyltransferases (UGTs) represent major Phase II biotransformation enzymes involved in detoxification of endobiotics such as bilirubin, thyroxin and steroid hormones and xenobiotics including a variety of phytochemicals, environmental pollutants such as benzo[a]pyrene, and many drugs. UGTs represent integral membrane proteins. Accumulating evidence suggests that UGTs operate in ER membranes mostly as homo- and heterodimeric complexes which may fine-tune UGT activity and stabilize the enzyme. Evidence includes recombinant technology [18], cross-linking studies [20], co-immunoprecipitation, and fluorescence resonance technology in live cells [22]. The results of radiation target analysis suggest that tetramers are involved in diglucuronide formation, a finding which, however, needs

further validation. Orientation of the active site toward the intraluminal space of the ER necessitates a number of transporters (i) for the cofactor UDP-glucuronic acid synthesized in the cytosol, and (ii) for glucuronides between the ER lumen and the cytosol, the latter being insufficiently characterized. Glucuronide hydrolysis by ER-localized β-glucuronidase may also require ER-localized glucuronide transporters involved in transport of glucuronides from cytoplasm to ER lumen. Many advances have been made in recent years on the topology and quaternary structure of oligomeric UGTs in ER membranes; but a lot of open questions are remaining. It is hoped that resolution of these questions may help to understand many problems of glucuronide formation and disposition in vivo, for example, under-estimation of in vivo clearance of drugs mainly eliminated by glucuronidation by in vitro enzyme kinetic parameters of UGTs.

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Figure legends

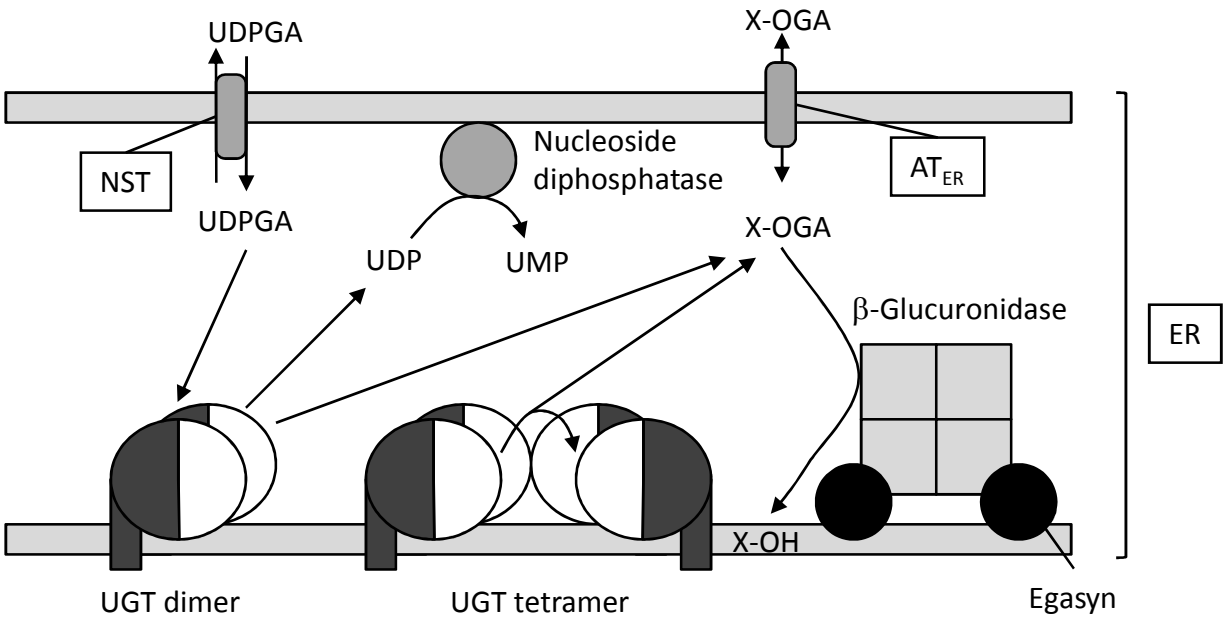
Fig. 1. Schematic model of ER-localized UGT oligomers, β-glucuronidase and functionally associated transporters. UGTs consist of the N-terminal aglycone (X-OH)-binding half of the monomer and the UDPGA-binding C-terminus with transmembrane segment and cytoplasmic tail (dark). Accumulating evidence suggests that UGTs are functional as dimers in monoglucuronide formation. Two dimers may interact to form a tetramer in diglucuronide formation (see text). Tetrameric β-glucuronidase retained in ER membranes by the carboxylesterase egasyn [63] may be involved in futile cycling of glucuronides and the hydrolysis of hormone glucuronides in target cells (discussed in chapter 5.3). The luminal orientation of UGTs and β-glucuronidase requires the action of additional proteins such as nucleotide sugar transporters (NSTs), transporting the cofactor UDPGA to the lumen of the ER, and multiple organic anion transporters in ER membranes (AT_{ER}) transporting glucuronides to the cytosol or back into the ER lumen. It is tempting to speculate that glucuronide transporters may be localized in the proximity of UGTs to prevent accumulation of glucuronides in the lumen.

Fig.2. (A) Structures of benzo[a]pyrene (BaP)- and chrysene-3,6-quinols. (B) Role of glucuronidation in detoxification of BaP quinones (Q). QOH, quinols; QOGA and $QO(GA)_2$, quinol monoglucuronides and diglucuronide, respectively; NQO1, NAD(P)H quinone oxidoreductase-1, which bypasses the semiquinone step; GSTs, glutathione S-transferases.

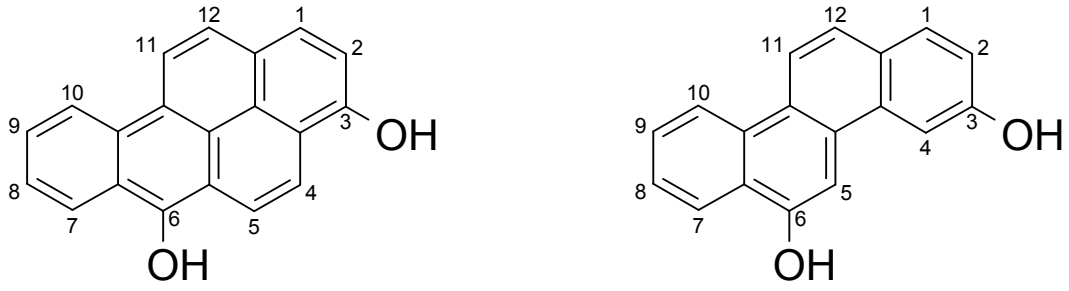
Fig.3. Scheme of heme catabolism to bilirubin and its diglucuronide in hepatocytes and the physiologic role of bilirubin as antioxidant. Bilirubin can be oxidized by ROS to biliverdin which is efficiently reduced back to bilirubin by biliverdin reductase. This magnifying cycle represents the basis for the role of bilirubin as antioxidant [47].

Table 1. Formation of moglucuronides (MG) and diglucuronides (DG) of benzo[a]pyrene (BaP)- and chrysene-3,6-quinol. For calculation of the MG/DG ratio the sum of the two MGs are used. Data for BaP-3,6-quinol UGT activity in microsomes and expressed UGT1A6 were taken from [33] and [34], respectively; data for chrysene-3,6-quinol UGT activity in liver microsomes of untreated controls and 3-MC-treated rats and expressed UGT1A6 were taken from [34] and for expressed UGT1A7 from [35]. MG/DG ratios are operationally used as inverse parameters for the efficiency of diglucuronide formation.

Substrate	UGT activity (nmol/min/mg protein)			
	Liver microsomes			
	Control	3-MC	rUGT1A6	rUGT1A7
BaP-3,6-quinol				
MG	5.8	60	1.5	0.75
DG	0.17	6.4	0.09	0.09
MG/DG	34	9	17	8
Chrysene-3,6-quinol				
MG	2.8	20.8	0.03	0.057
DG	0.02	6.2	-	0.033
MG/DG	140	3	-	2



A



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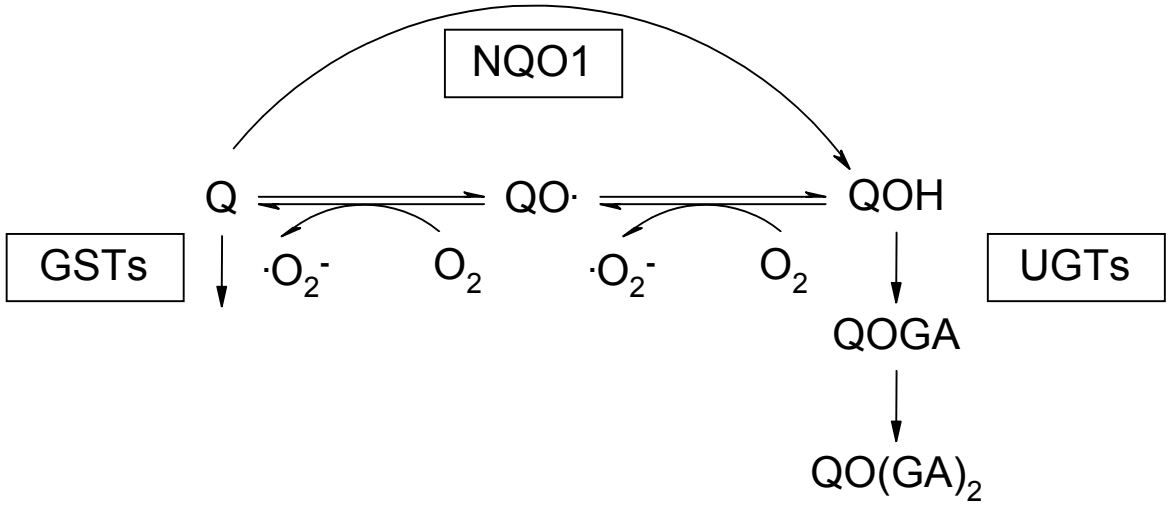
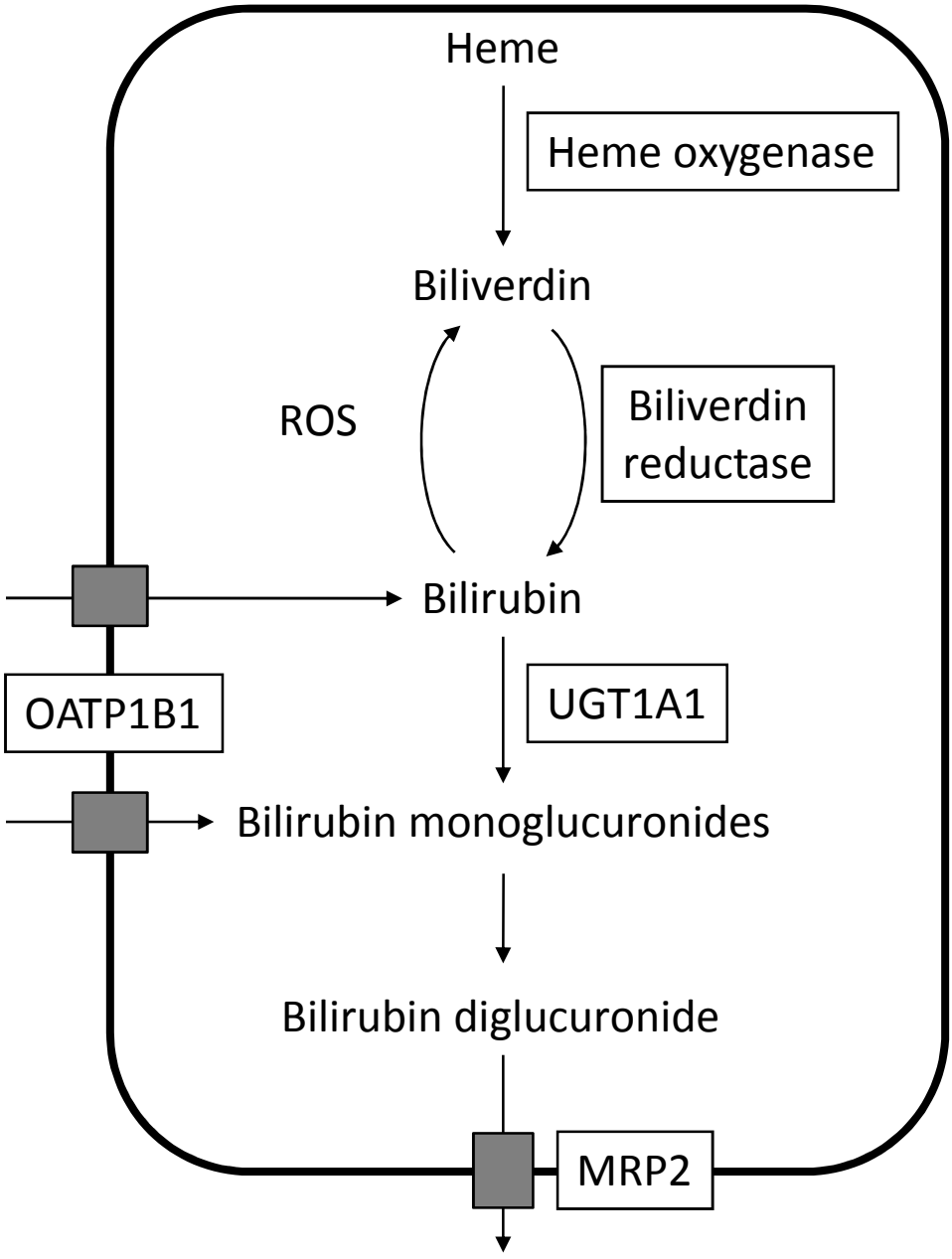
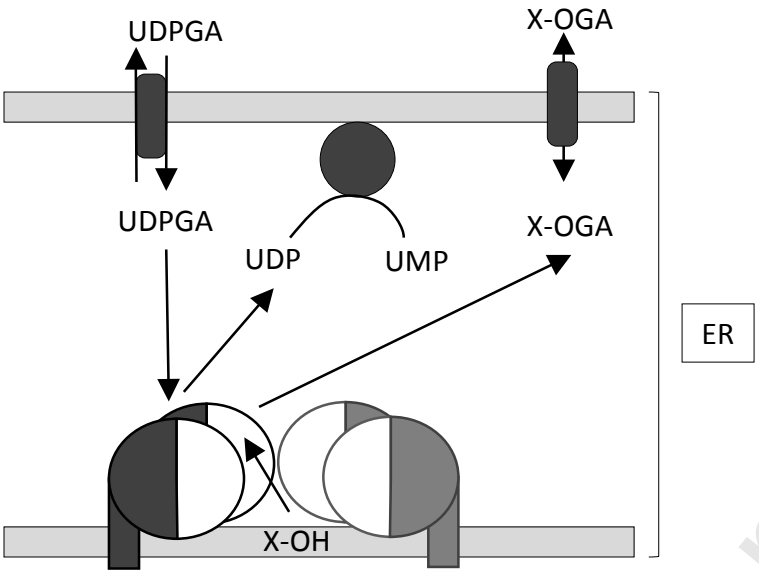




UDP glucuronosyltransferases are functional as oligomers. The intraluminal orientation of the active site in endoplasmic reticulum membranes necessitates a number of transporters for the cofactor UDP-glucuronic acid and glucuronides.



UGT dimer / teramer