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Carbonyl reduction of naltrexone and dolasetron by oxidoreductases isolated from human liver cytosol

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Abstract

The opioid receptor antagonist naltrexone and the antiemetic 5-HT_3 receptor antagonist dolasetron are ketonic drugs that are efficiently reduced to their corresponding alcohols in-vivo. These experiments aimed at characterizing the role in these reactions of individual oxidoreductases present in human liver cytosol. Aldo-keto reductases (AKRs) and carbonyl reductase (CR, EC 1.1.1.184) purified from human liver cytosol were incubated with varying substrate concentrations and 6β-naltrexol or reduced dolasetron were analysed by HPLC. AKR1C1, AKR1C2, and AKR1C4 were able to reduce both substrates. On the basis of k_{cat}/K_m values, AKR1C4 was nearly 1000-fold more efficient in reducing naltrexone than was AKR1C1, while AKR1C2 was of intermediate efficiency. Substrate inhibition was observed on incubating AKR1C2 or AKR1C4 with naltrexone. In contrast, dolasetron was also a substrate of CR. AKR1C1 and AKR1C4 were the most efficient enzymes in producing reduced dolasetron. We concluded that the efficient reduction of naltrexone by AKR1C4 probably causes the high 6β-naltrexol/naltrexone ratio in man. The rapid disappearance from human plasma of dolasetron given intravenously and its virtual absence after oral dosage are explained by its liability to reduction by several enzymes, including CR which shows widespread expression in human tissues.

Introduction

Carbonyl reduction is the most common Phase I metabolic reaction in the majority of xenobiotic aldehydes and ketones. The secondary alcohols resulting from reduction of drug ketones often retain pharmacological activity, but they differ from the original compounds with regard to kinetic behaviour (Felsted & Bachur 1980). The opioid receptor antagonist naltrexone and the antiemetic dolasetron are examples of drugs in which carbonyl reduction governs total kinetics.

Due to the potential usefulness of naltrexone for prevention of relapse in opiate and alcohol dependence, its kinetics have been studied following single and repeated oral doses. The major compound in human plasma and urine is 6β-naltrexol (Figure 1) in free and conjugated form, which is already produced by first-pass metabolism (see review by Gonzalez & Brogden1988); the isomeric 6α-naltrexol could not be detected (Dayton & Inturrisi 1976). Reported kinetic data vary markedly among subjects as well as among groups, but mostly 6β-naltrexol was found to have a longer half-life than naltrexone (Gonzalez & Brogden 1988). 6β-Naltrexol is a potent μ-opioid receptor antagonist in-vitro (Porter et al 2002), but it is much less active than naltrexone in rodents in-vivo (Rukstalis et al 2000). A contribution to clinical effects in patients has been deduced from relationships between plasma 6β-naltrexol levels and subjective symptoms or outcome criteria (McCaul et al 2000). Naltrexone reduction to 6β-naltrexol is catalysed by human liver cytosol with apparent K_m values of 0.017–0.053 mM (Porter et al 2000). However, in a study of the individual cytosolic aldo-keto reductases AKR1C1, AKR1C2 and AKR1C4, K_m values of 0.19–1.6 mM were found (Ohara et al 1995). These AKR enzymes were previously designated as dihydrodiol dehydrogenases 1, 2 and 4 (DD1, 2 and 4); however, due to their high affinity for NADPH, they function preferentially as reductases in cells (Penning et al 2004) and participate in the production and inactivation of sex hormones (Penning et al 2000).

Dolasetron belongs to the group of serotonin (5-HT_3) receptor antagonists used as adjuvants in cancer chemo- or radiotherapy due to their potent antiemetic effect (Perez

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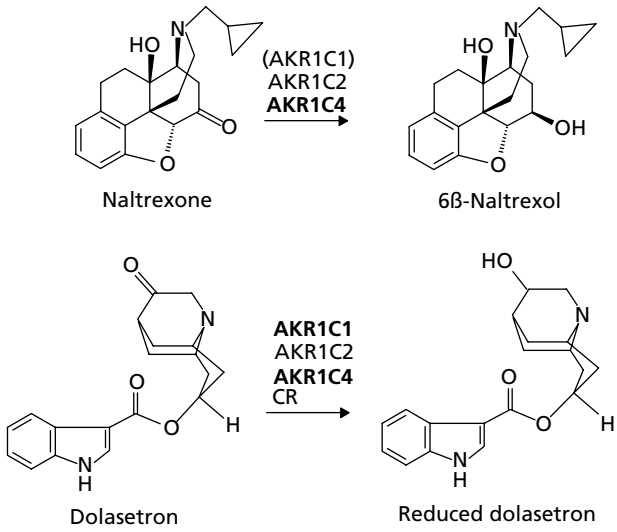

Figure 1 Structural formula of naltrexone and dolasetron and of the alcohols formed from them by enzymatic reduction. The relative efficiency of enzymes is indicated by names in bold letters, in normal letters or in parentheses.

1998). It is even more efficiently reduced in man in-vivo than is naltrexone, such that the original compound is hardly detectable in plasma after oral administration to healthy subjects, while after intravenous infusion it mostly becomes undetectable within 2 h (Boxenbaum et al 1992, 1993; Stubbs et al 1997). The chiral alcohol resulting from carbonyl reduction, reduced dolasetron or hydrodolasetron (Figure 1), proved to be about 40 times more potent than dolasetron as a 5-HT_3 receptor antagonist, such that dolasetron may be regarded as a prodrug (Boeijinga et al 1992). In healthy subjects given dolasetron orally or intravenously, the elimination half-life of reduced dolasetron was around 7–8 h (Boxenbaum et al 1992, 1993; Stubbs et al 1997). Enantiomer analysis revealed stereoselective reduction with 95% (*R*)-enantiomer in reduced dolasetron excreted in human urine. Incubation of dolasetron with human whole blood resulted in rapid reduction to (*R*)- and (*S*)-enantiomer in an 88:12 ratio (Dow & Berg 1995). Apart from this observation, the biochemistry of dolasetron reduction has apparently not been studied. These experiments were designed to clarify the role of individual oxidoreductases in naltrexone and dolasetron reduction.

Materials and Methods

Materials

The following enzymes were purified from human liver cytosol to electrophoretic homogeneity by gel filtration, ion exchange chromatography and chromatofocusing: AKR1C1, AKR1C2, AKR1C4 and the carbonyl reductase (CR) (EC 1.1.1.184) isoforms I and II (Breyer-Pfaff & Nill 2000). Naltrexone hydrochloride and 6β-naltrexol (free base) were kindly provided by DuPont Pharma (Bad Homburg, Germany) and dolasetron mesilate hydrate by Hoechst Marion Roussel (Bad Soden, Germany). Reduced dolasetron was prepared (Dow & Berg 1995) by reacting a solution of dolasetron free base in ethanol with an excess of sodium borohydride for 2 h at room temperature. The product was distributed between water and ethyl acetate and the residue of the organic layer crystallized from 1-propanol/toluene. It gave a single peak in HPLC (see below) and its identity was confirmed by electron impact mass spectrometry (SSQ 7000 from Finnigan MAT, Bremen, Germany), which resulted in the expected mole peak at m/z 326.

Enzyme assay

Enzymatic reduction tests were performed at 37°C in a total volume of 360 μL containing 100 mM Tris-HCl buffer adjusted to pH 7.4 at 37°C, 8 mM $MgCl_2$, 25 mM KCl, 0.2 mM $NADP^+$, 2 mM glucose-6-phosphate, 1 U mL^{-1} glucose-6-phosphate dehydrogenase and 0.3–15 μg of enzyme. After 5 min, the reaction was started by the addition of substrate and it was stopped after a further 10 or 20 min by 40 μL of 2 N perchloric acid. The vials were cooled in ice and centrifuged and the supernatant was analysed by HPLC.

HPLC analysis

For 6β-naltrexol measurement, 0.2 mL was applied to a 4 × 5 mm clean-up column with C_{18}-silica gel (Polygosil 40–60 μm; Macherey-Nagel, Düren, Germany) by pumping 100 mM sodium phosphate buffer pH 7.5 at 1.5 mL min^{-1} for 2 min. The analytes were transferred to the analytical column (Prodigy 5 μm ODS (3) 100 Å, 250 × 4.6 mm; Phenomenex, Hösbach, Germany) by running the eluent (25 mM sodium phosphate pH 7.0–methanol (40:60 v/v), 1.2 mL min^{-1}) for 1 min through the clean-up column in the reverse direction. The clean-up column was reconditioned by pumping 100 mM sodium phosphate pH 7.5 for 2.5 min. The eluate from the analytical column was monitored at 280 nm, data being registered by the MT2 integration program (Kontron Instruments, München, Germany). 6β-Naltrexol was eluted at 10.5 min and naltrexone at 15.5 min. Peak heights of 6β-naltrexol were evaluated with the aid of a calibration curve from standards with 1–10 μM 6β-naltrexol.

Reduced dolasetron was chromatographed on the same analytical column using 25 mM sodium phosphate pH 7.0–methanol (43:57 v/v) 1.2 mL min^{-1} as the eluent, but with a different clean-up column (20 × 4.6 mm GROM-Sil 120 ODS-HE, 11 μm) run with 25 mM sodium phosphate pH 7.0 at 1.5 mL min^{-1}. Peaks monitored at 280 nm were eluted at 10 min (reduced dolasetron) and 13.5 min (dolasetron).

Calculations

Kinetic parameters were calculated according to the Michaelis–Menten equation by non-linear least-squares regression analysis (Sigma Plot 2000; Statistical Solutions Ltd, Cork, Ireland). All kinetics included had r^2 values of at least 0.99 with 4 or more degrees of freedom. Values of k_{cat} were based on M_r 36 000 for AKR

enzymes and 33 000 for CR. Intrinsic clearances (also designated as enzymatic efficiencies) were calculated as k_{cat}/K_m. In measurements with CR and dolasetron, the relationship between substrate concentration and reaction rate was not clearly hyperbolic. As a measure of enzymatic efficiency, (reaction rate)/(substrate concentration) was evaluated, since the Michaelis–Menten equation predicts this to be constant in the substrate concentration range sufficiently below K_m. For substrate inhibition, a formula for non-competitive inhibition was used.

Results

On incubation of human liver cytosolic enzymes with naltrexone or dolasetron, one product each was formed according to HPLC analysis. In the case of naltrexone, this had the same retention time as 6β-naltrexol; when dolasetron was the substrate, the (*R*)- and (*S*)-enantiomers of reduced dolasetron can be assumed to be formed, which are not separated by achiral HPLC. The activity of the individual enzymes towards the two substrates varied considerably (Table 1, Figure 1). AKR1C4 efficiently reduced both substrates, but naltrexone produced substrate inhibition (Figure 2B). AKR1C1 exhibited very little activity towards naltrexone, but a high activity towards dolasetron. AKR1C2 reduced dolasetron with high affinity, but at a low reaction rate; it was inhibited by high naltrexone concentrations (Figure 2A). Naltrexone was not a substrate of CR I, whereas dolasetron was reduced by CR I and CR II at similar rates; saturation was not observed at concentrations achievable without substrate precipitation. Estimation of enzymatic efficiency from the ratio of reduction rate and substrate concentration gave values comparable with that for AKR1C2 (Table 1).

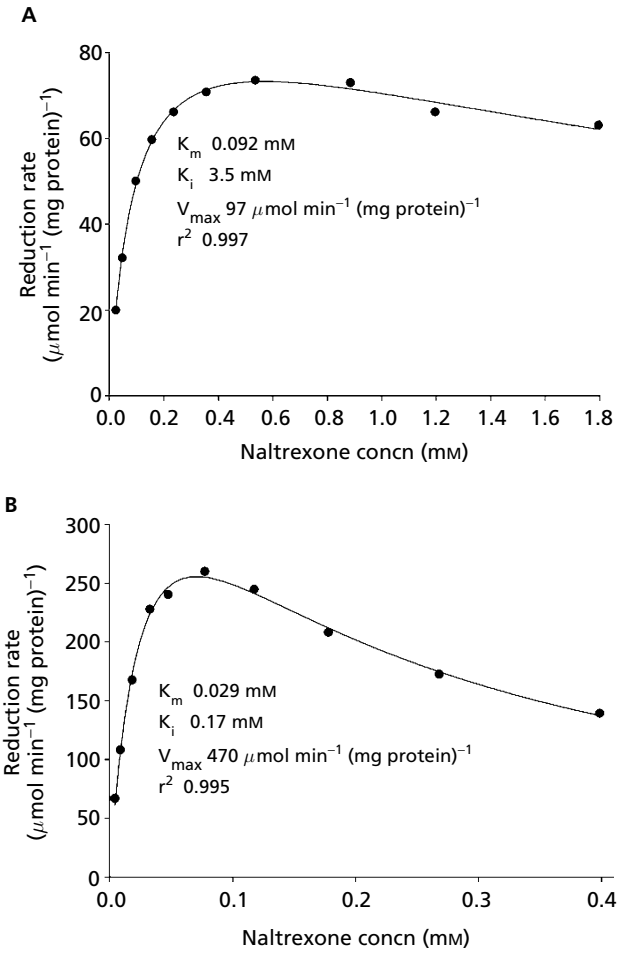

Figure 2 Representative examples of kinetics of naltrexone reduction by AKR1C2 (A) and AKR1C4 (B) showing substrate inhibition.

Discussion

These results are in accordance with those of Ohara et al (1995) in as much as naltrexone is not reduced by CR, but is a substrate of the AKR enzymes. However, with regard to the kinetic data, marked discrepancies became apparent (Table 2). Intrinsic clearances differed nearly 1000-fold among AKR1C1, AKR1C2 and AKR1C4 in this

Table 1 Kinetic parameters for carbonyl reduction of naltrexone and dolasetron by aldo-keto reductases (AKR) and carbonyl reductase (CR)

Substrate	Enzyme	n	K_m (mM)	k_{cat} (min^{-1})	k_{cat}/K_m (mL μmol^{-1} min^{-1})	K_i (mM)
Naltrexone	AKR1C1	3	1.4 ± 0.1	1.14 ± 0.04	0.84 ± 0.08	
	AKR1C2	3	0.13 ± 0.06	4.4 ± 1.2	34 ± 3	2.6 ± 1.2
	AKR1C4	2	0.037; 0.029	27; 17	740; 580	0.16; 0.17
	CR I	1	N.D.*			
Dolasetron	AKR1C1	4	0.064 ± 0.09	7.1 ± 0.8	110 ± 4	
	AKR1C2	5	0.030 ± 0.002	1.0 ± 0.2	35 ± 10	
	AKR1C4	4	0.22 ± 0.13	19 ± 11	90 ± 41	
	CR I	1	no sat.		26**	
	CR II	3	no sat.		32 ± 9**	

Substrates were incubated with enzymes purified from human liver cytosol and an NADPH-generating system for 10 or 20 min at 37°C. Following $HClO_4$ addition, alcoholic products were analysed by HPLC in supernatants. *N.D., no product detected **Ratio (reaction rate)/(substrate concentration) between 20 and 120 μM dolasetron with reaction rate expressed as (nmol product) (nmol enzyme)$^{-1}$ min^{-1}.

Table 2 Comparison of mean kinetic parameters reported for carbonyl reduction of naltrexone by individual AKR enzymes from human liver or by liver cytosol

Enzyme or material (Ref.)[a]	Incubation conditions	Assay procedure	K_m (mM)	V_{max}/K_m (μL min^{-1} (mg protein)$^{-1}$)	K_i (mM)
AKR1C1					
(1)	pH 6.0, 25°C	Optical test	0.21	595	
(2)	pH 7.4, 37°C	HPLC	1.4	23	
AKR1C2					
(1)	pH 6.0, 25°C	Optical test	1.6	110	
(2)	pH 7.4, 37°C	HPLC	0.13	950	2.6
AKR1C4					
(1)	pH 6.0, 25°C	Optical test	0.19	332	
(3)	pH 6.0, 25°C	Optical test	0.12	440	
(2)	pH 7.4, 37°C	HPLC	0.033	19 000	0.16
Cytosol					
(4)	pH 7.4, 37°C	HPLC	0.034	17[b]	

[a]Data are taken from (1) Ohara et al (1995); (2) this investigation; (3) Kume et al (1999); (4) Porter et al (2000). [b]Data refer to total cytosolic proteins, whereas all others refer to purified proteins.

investigation, whereas data reported by Ohara et al (1995) and in a later publication by the same group (Kume et al 1999) were in the same order of magnitude. In addition, substrate inhibition was not observed previously. There were distinct differences in procedure with regard to pH, temperature and analytical technique (Table 2). Better agreement was found with the data of Porter et al (2000) who used HPLC analysis for measuring 6β-naltrexol production in human liver cytosol. They observed apparent K_m values for naltrexone of 17–53 μM, which are in the range of those found now for AKR1C4 (Table 2). Enzyme quantities obtained on purification from liver cytosol (Breyer-Pfaff & Nill 2000) allow the conclusion that the AKRs are present at comparable concentrations. Therefore naltrexone reduction in liver cytosol should be governed by AKR1C4 and this is reflected in the low K_m value found by Porter et al (2000). The substrate inhibition is not expected to influence in-vivo kinetics because the K_i value of 0.16 mM is at least 100-fold higher than the maximal naltrexone concentrations measured in chronically treated patients (500 ng mL^{-1}, Ferrari et al 1998).

Dolasetron was reduced with higher efficiency by AKR1C1 and AKR1C4 than by AKR1C2 and CR, but the difference was only 3–4 fold. CR, a member of the short-chain dehydrogenases/reductases (SDR) (Jörnvall et al 1995; Oppermann et al 2003), acts on a number of other drug ketones including haloperidol, but these usually are poor AKR substrates (Ohara et al 1995). When the carbonyl reduction of haloperidol was reinvestigated with purified enzymes, the efficiencies of AKR1C1 and AKR1C2 proved to be more than 100-fold lower than those of CR I and II (Breyer-Pfaff & Nill 2000).

Large differences exist in ketone/alcohol concentration ratios in human plasma for drug ketones that are substrates of the enzymes studied. The mean ratio for haloperidol was between 1 and 3 depending on ethnic group (Lam et al 1995). The drug is mainly reduced by CR, while back-oxidation is catalysed by CYP3A4 (Kudo & Ishizaki 1999).

Data are contradictory for naltrexone, since following a single oral dose, the AUC of the alcohol usually exceeded that of the ketone 2.5–5 fold in healthy subjects (Wall et al 1981) and in post-addict patients (Verebey et al 1976; Ferrari et al 1998); this corresponds to ketone/alcohol ratios of 0.2–0.4. However, in the largest study with healthy subjects (Meyer et al 1984), mean ketone/alcohol AUC ratios were between 0.03 and 0.04 at three dose levels. On the other hand, a minority of chronically treated patients exhibited higher naltrexone and lower 6β-naltrexol levels than the main group and a mean ketone/alcohol ratio of 1.3 (Ferrari et al 1998). Since these patients were positive for hepatitis C virus infection, it is conceivable that impairment of hepatic function comprises a reduced activity of cytosolic AKR enzymes. In view of the pharmacological activity of 6β-naltrexol (see Introduction), interindividual differences in carbonyl reduction capacity may not influence clinical effects to a significant extent. 6β-Naltrexol failed to be oxidized to naltrexone on incubation with human liver microsomes and NADPH (Porter et al 2000), and reducing enzymes seem to be confined to the AKR1C subfamily. According to the expression patterns, substrates of AKR1C4 are metabolized nearly exclusively in liver, those of AKR1C2 also in lung and those of AKR1C1 mainly in liver, lung and kidney (O'Connor et al 1999; Penning et al 2000).

For dolasetron the participation of CR in enzymatic reduction led to an extremely rapid disappearance of the parent drug and ketone/alcohol ratios close to zero from 1 h after intravenous infusion as well as throughout after oral dosing. Moderate or severe hepatic impairment allowed a small fraction of oral dolasetron to escape first-pass carbonyl reduction (Stubbs et al 1997). The occurrence of CR in the majority of human tissues (Wirth & Wermuth 1992) enables them to participate in

dolasetron reduction; this has for instance been demonstrated for whole blood (Dow & Berg 1995). On the other hand, reoxidation of the alcohol apparently does not take place. Though reduced dolasetron is more active than dolasetron as a 5-HT_3 receptor antagonist (Boeijinga et al 1992), it may be rational to use the latter as a prodrug, because as a more lipophilic compound it will be absorbed more readily.

An additional substrate efficiently reduced by CR is daunorubicin (Wermuth 1981; Ohara et al 1995). On intravenous infusion to leukaemic patients its plasma levels decrease rapidly and its reduced metabolite daunorubicinol attains 2- to 4-fold higher concentrations (Galettis et al 1994). With metyrapone, another good CR substrate (Ohara et al 1995), data on in-vivo kinetics are available for a single subject only; they reveal a large excess of the two metyrapol enantiomers over the parent drug in plasma as well as in urine (Chiarotto & Wainer 1995). Interestingly, carbonyl reduction has not been described as a biotransformation reaction in ondansetron, another ketonic 5-HT_3 receptor antagonist (Roila & Del Favero 1995). Since the crystal structure of CR has not been elucidated, structure–function considerations, as have been deduced for steroids as substrates of AKR enzymes (Penning et al 2004), are not available.

In conclusion, carbonyl reduction of naltrexone is primarily due to hepatic AKR1C4, which exhibits high affinity and efficiency. Together with the apparent absence of a back-oxidation mechanism, this may explain the excess of plasma 6β-naltrexol over naltrexone in the majority of subjects. Dolasetron differs from other drugs in being reduced by AKR enzymes and the SDR enzyme CR with comparable efficiency. The widespread expression of CR in human tissues and the lack of back-oxidation are probably the reasons why following oral administration of dolasetron, the active reduced metabolite is nearly exclusively present in human plasma. Ketonic drugs vary widely with regard to their propensity to undergo enzymatic carbonyl reduction, but data to explain this on the basis of structure–function relationships are lacking.

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