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Original research paper

Ketorolac-dextran conjugates: Synthesis, *in vitro* and *in vivo* evaluation

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Ketorolac is a non-steroidal anti-inflammatory drug. Dextran conjugates of ketorolac (KD) were synthesized and characterized to improve ketorolac aqueous solubility and reduce gastrointestinal side effects. An N-acylimidazole derivative of ketorolac (KAI) was condensed with a model carrier polymer, dextran of different molecular masses (40000, 60000, 110000 and 200000). IR spectral data confirmed formation of ester bonding. Ketorolac contents were evaluated by UV-spectrophotometric analysis. The molecular mass was determined by measuring viscosity using the Mark-Howink-Sakurada equation. *In vitro* hydrolysis studies were performed in aqueous buffers (pH 1.2, 7.4, 9) and in 80% (*V/V*) human plasma (pH 7.4). At pH 9, a higher rate of ketorolac release from KD was observed as compared to aqueous buffer of pH 7.4 and 80% human plasma (pH 7.4), following first-order kinetics. *In vivo* biological screening in mice and rats indicated that conjugates retained analgesic and anti-inflammatory activities with significantly reduced ulcerogenicity compared to the parent drug.

Keywords: ketorolac-dextran conjugates, dextran carrier, NSAIDs

Ketorolac is a potent anti-inflammatory drug. Chemically, it is pyrrolizine carboxylic acid provided as a recemic mixture. This non-steroidal and non-narcotic drug is administered systemically (*via* oral and parenteral route) for the control of mild to moderate pain as well as of some postoperative and cancer pain (1). It inhibits the synthesis of prostaglandin by inhibiting the enzyme cyclooxygenase (COX). Administration of this non-selective COX inhibitor by oral route causes many gastrointestinal side effects, like nausea, vomiting, gastric irritation, peptic ulceration and bleeding that limit its clinical use (2). Gastrointestinal (GI) side effects produced by NSAIDs are either due to direct contact or indirect effect of the drug on the gastrointestinal mucus membrane. Acidic nature of NSAIDs, ion trapping and inhibition of cytoprotective prostaglandins are some of the reasons for the GI adverse effects (3–5). Literature reveals that conjugation of a

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polymeric carrier with drugs to form a polymeric or macromolecular prodrug might be one a useful approach to improve physicochemical properties and clinical acceptance of a drug (6–9). These can be obtained by conjugation of a drug to a biodegradable polymer by chemical linkage. The parent drug is then released *in vivo* through biotransformation.

The polysaccharide dextran can be used as a model carrier due to its physiological acceptance and excellent physicochemical characteristics such as high aqueous solubility with low toxicity, abundant hydroxyl groups for drug conjugation and availability in a wide range of molecular masses (10, 11). Larsen *et al*. (12, 13) reported many dextran conjugates of NSAIDs (diclofenac, ibuprofen, ketoprofen, naproxen, fenoprofen) for their improved physicochemical properties. Potential application of dextran-5-aminosalicylic acid (5-ASA) conjugates was suggested in the treatment of Crohn's disease (14). Flurbiprofen-dextran conjugates showed reduction in ulcerogenicity compared to flurbiprofen alone (15). Dextran conjugates of fluorouracil have been proposed for targeting and controlled release (16). Encouraging clinical outcomes were observed by Bue *et al.* (17); epidermal growth factor-dextran (EGF-dextran) could be effective as intravesical therapy, either conjugated with cystostatic drugs or labeled with suitable radionuclides. In the present work, dextran conjugates of ketorolac were synthesized and evaluated for their potential use as a polymeric prodrug for oral drug delivery. *In vivo* investigations in animals were performed to assess their pharmacological activities and gastrointestinal toxicity.

EXPERIMENTAL

Materials

Ketorolac tromethamine was a generous gift from Dr. Reddy's Lab. Ltd. (India). Dextran of different molecular masses and *N*,*N*′-carbonyldiimidazole (CDI) were purchased from Fluka Biochemika (Switzerland). Purity of the compounds was tested on Silica gel-G coated TLC plates (Merck, India) using iodine vapor as visualizing agent. Carrageenean was purchased from HiMedia Laboratories Pvt. Ltd. (India). Other chemicals were of synthetic grade.

Hydrochloric acid buffer (KCl + HCl, pH 1.2), phosphate buffer (KH_2PO_4 + NaOH, pH 7.4) and borrate buffer (KCl + H_3BO_3 + NaOH, pH 9) were used.

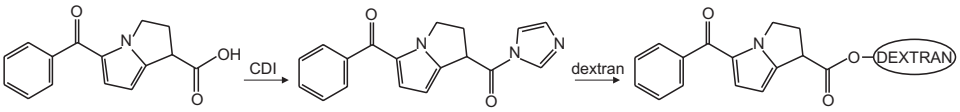
Preparation of free ketorolac

Free ketorolac was obtained from its tromethamine salt by precipitation and recrystallization. To a 5% (*m/V*) aqueous solution of ketorolac tromethamine, 1 mol L^{-1} HCl was added dropwise with stirring; solid ketorolac precipitated. This precipitate was then extracted with ethyl acetate and solvent was evaporated to dryness. Free ketorolac acid was obtained by recrystallization from a mixture of ethyl acetate/ether (1:1).

Melting point was measured in an open capillary tube and is uncorrected. The purity of ketorolac was achieved by thin layer chromatography (the mobile phase was made of chloroform/methanol, 10:1) and UV spectra were recorded on a double beam UV-Vis spectrophotometer-160 (Shimadzu, Japan).

Synthesis of ketorolac-dextran conjugates

To a solution of ketorolac (0.5 g, 1.96×10^{-3} mol, in dry DMSO, 4 mL), CDI was added (0.45 g, 2.78×10^{-3} mol) slowly in portions for 30 minutes maintaining the temperature of the reaction at 0 °C. A solution of dextran of different molecular masses (40000, 60000, 110000 and 200000) (1 g in dry DMSO, 15 mL) was added to the above mixture with stirring, maintaining the reaction at 10 °C for 30 min and then it continued for 3 days at room temperature in a dry area with occasional stirring. The conjugates were precipitated by addition of a methanol/diethyl ether (1:1) mixture 4–5 times remove unconjugated drug and then washed with acetone to obtain ketoralac-dextran conjugates KD1, KD2, KD3 and KD4 (Scheme 1).



ketorolac N-acylimidazole-ketorolac (KAI) ketorolac-dextran conjugate (KD)

Scheme 1

Characterization of ketorolac-dextran conjugates

Characterization data of KDs are presented in Table I. TLC on silica gel-G plates confirmed purity and absence of entrapped free drug in the conjugates. Chloroform/methanol (10:1) was used as mobile phase and iodine vapors were used for spot detection. The λ_{max} in borate buffer (pH 9) was found at 323.8 nm.

To confirm the conjugate formation, IR spectra of the samples were recorded on a Perkin-Elmer IR spectrophotometer (UK) in potassium bromide discs (Fig. 1).

Elemental analyses (C, H, N) were carried out on a Carlo-Erba model 1108 analyzer (Italy).

Table I. Characterization data of ketorolac and ketorolac-dextran conjugates

Conjugate	Drug content (%, *m/m*)[a]	Intrinsic viscosity dL g^{-1}	Molecular mass		Elemental analysis (%)					
			Calcd.[b]	Found	Calculated			Found		
					C	H	N	C	H	N
KD1	7	0.1911	43870	45705	47.62	5.54	0.55	47.44	5.68	0.58
KD2	10	0.2431	68445	71506	48.70	5.52	0.76	48.84	5.49	0.74
KD3	9	0.3300	123866	126170	48.35	5.33	0.69	48.74	5.16	0.65
KD4	6	0.4450	216395	219940	47.24	5.55	0.48	47.32	5.53	0.43

[a] Amount of parent drug in mg per 100 mg of drug-dextran conjugate.
[b] Calculated on the basis of drug content.

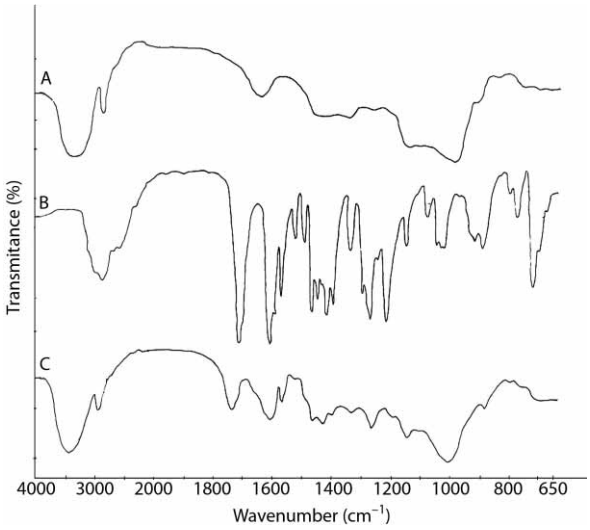


Fig. 1. IR spectra: (A) – dextran, (B) – ketorolac and (C)– ketorolac dextran conjugate.

Average molecular mass was evaluated by intrinsic viscosity measurements of the conjugates (2%, *m/V* aqueous solution) using a capillary viscometer (Ostwald, India) at 27 °C (single measurement at one concentration). Intrinsic viscosities were estimated by using Eq. (1). Average molecular masses were then calculated by the Mark-Howink-Sakurada equation (18) expressed as Eq. (2):

$$[\eta] = [\eta_{rel} - 1] \,/\, [c + 0.28\, c\, (\eta_{rel} - 1)] \quad (1)$$

$$\log [\eta] = \log K + \alpha \log M \quad (2)$$

where $[\eta]$ represents intrinsic viscosity, η_{rel} relative viscosity at concentration c (%, *m/V*), M is molecular mass, K and α are the constants.

Drug content

The ketorolac concentration (mg per 100 mg dextran conjugate) was estimated. Ketorolac-dextran conjugate (1 mg mL^{-1}) in aqueous borate buffer (pH 9) was maintained at 70 °C for 1 h. The solution was then left aside for 24 h at room temperature (r.t.) for complete hydrolysis. After neutralization with 1 mol L^{-1} HCl, the released ketorolac was extracted in chloroform and estimated by measuring the absorbance at 311 nm. Absence of ketorolac in aqueous layer was achieved by no absorption of conjugate or ketorolac in the aqueous layer at the characteristic wavelength.

In vitro *hydrolysis*

At pH 9. – Ten aliquots of 10 mg of KD were dissolved in 10 mL of borate buffer (pH 9) in test tubes. The reaction temperature was maintained at 37 ± 1 °C in a water bath. Test tubes were withdrawn at regular time intervals of 0.1, 0.2, 0.3, 0.4…to 1 h, cooled to r.t. and neutralized with 1 mol L^{-1} HCl. Ketorolac regenerated upon hydrolysis was extracted in chloroform and determined at 311 nm.

At pH 1.2, 7.4 and in 80% human plasma (pH 7.4). – Samples of KD (1 mg mL^{-1}) in aqueous medium (pH 1.2 or pH 7.4 or 80% human plasma, pH 7.4) were maintained at 37 ± 1 °C. Aliquots were withdrawn after fixed intervals of 0.5, 1, 2, and 3 h and the released parent drug was estimated using the same method as described for hydrolysis at pH 9.

Biological evaluation

In vivo animal studies were carried out according to the guidelines of IAEC (Institutional Animal Ethics Committee, M. G. M. Medical College, India). Wistar rats (150–200 g) and Swiss albino mice (20–25 g) of either sex were used for biological screening. Animals were acclimatized to laboratory conditions for at least 3 days before commencement of the experiments and were kept under a 12 h light/12 h dark cycle. Animals were fasted overnight prior to treatment and received free access to water during the experiment. Doses of KD1-KD4 were equimolar to the parent drug (ketorolac), which was calculated on the basis of their drug content in the conjugates. All the drugs were prepared in 2% gum acacia and administered orally (*p.o.* route).

Analgesic activity. – Acute analgesia produced by the drugs was assessed by the acetic acid induced writhing method in mice (19). Mice were divided into three groups ($n = 6$ in each group). The first group served as control (received an appropriate volume of 2% gum acacia only, *p.o.*). The second group was standard (ketorolac, 0.33 mg kg^{-1} body mass) and the third group, as the test group, received KD (KD1-KD4, 4.71, 3.30, 3.70 or 5.50 mg kg^{-1} body mass, respectively). Three hours after treatment, 0.6% (*V*/*V*) acetic acid solution (10 mL kg^{-1}) was injected to mice intraperitoneally. Total number of writhes, which was a parameter of chemically induced pain (*i.e.*, constriction of abdomen, turning of trunk and extension of hind legs), was counted for 20 min. The analgesic effect was expressed as percent reduction of writhes in comparison with the control.

Anti-inflammatory activity. – The carrageenean induced rat hind paw edema assay described by Winter *et al.* (20) was used to evaluate the acute anti-inflammatory activity of the conjugates. Rats were divided into control, standard and test groups of six animals each. Initial paw volumes of all animals were measured using a mercury plethysmometer before treatment. Control group was given no drug but received only 2% gum acacia orally in an appropriate volume. Standard group received ketorolac (0.33 mg kg^{-1} body mass, *p.o.*) while KD (KD1-KD4, 4.71, 3.30, 3.70 or 5.50 mg kg^{-1} body mass, respectively, *p.o.*) was administered to the test group. One hour after treatment, 0.1 mL of 1% (*m*/*V*) carrageenean solution in distilled water was injected into the plantar region of the left hind paw of each rat. The relative increase in paw volume was determined by measuring the paw volume after 3 h following the carrageenean administration. The percent inhibition of edema, as an indication of anti-inflammatory activity was compared with the controls.

Ulcerogenicity studies. – The ulcerogenic activity was assessed using the method adopted by Shanbhag *et al*. (21) in rats. Albino rats were randomly distributed into three groups ($n = 6$ in each group). Control received no drug while the standard group was given ketorolac (6.6 mg kg^{-1} body mass, *p.o.*) and the test group received KD1-KD4 (94.2, 66, 74 or 110 mg kg^{-1} body mass, respectively, *p.o.*) for 3 days. The animals were fasted 8 h pre-treatment and 4 h post-treatment. Food and water were available for the rest of the time. Four hours after the last dose, the animals were sacrified and the abdomen was opened. The stomach with 3 cm of duodenum was removed. The stomach was opened and washed with distilled water. The mucus was wiped off and the number of lesions was examined by means of a magnifying glass. The degree of mucosal damage and ulceration in each stomach was scored as + (strong irritation identified by redness and inflammation), ++ (ulcers < 0.5 mm), +++ (ulcers > 0.5 mm) and ++++ (perforation).

Data analysis

All the biological evaluation data are expressed as mean ± SEM and statistically evaluated by Student's *t*-test to determine the significance of the difference between the control group and the drug or conjugate treated group.

RESULTS AND DISCUSSION

An activated moiety *N*-acylimidazole of ketorolac (KAI) was synthesized by interaction of ketorolac with CDI, which then condensed with dextran of different molecular masses (40000, 60000, 110000 and 200000) to obtain KD1, KD2, KD3 and KD4 conjugates, respectively (Scheme 1). Due to the moisture sensitivity of CDI, the reaction was carried out under anhydrous conditions. Ester linkage formed in the conjugates was confirmed by the IR spectrum which revealed the presence of characteristic absorption bands at 3300 cm^{-1} (OH broad) and 1730 cm^{-1} (C=O ester) (Fig. 1). The amount of ketorolac in the conjugates was estimated after complete hydrolysis of the conjugates in borate buffer (pH 9) and indicated 7, 10, 9 and 6% (*m/m*) of drug in KD1, KD2, KD3 and KD4, respectively. These results show that varying the dextran molecular mass does not significantly influence the drug content. Chromatographic, spectral and average molecular mass data indicate that ketorolac is covalently bound to the dextran and support formation of ketorolac-dextran conjugates. Qualitative solubility analysis showed that ketorolac is poorly soluble in water and acidic medium (pH 1.2) while all its dextran conjugates (with estimated drug contents) were found to be soluble in water, acidic (pH 1.2) and basic media (pH 9).

In vitro chemical (aqueous medium: pH 1.2, 7.4 and 9) and enzymatic (80% human plasma pH 7.4) hydrolysis kinetics of KD were also studied at 37 ± 1 °C. Those studies indicated no hydrolysis at pH 1.2 for 3 h; hydrolysis of KD proceeds slowly at pH 7.4 and in 80% human plasma whereas relatively much faster hydrolysis was observed at pH 9. The half-lives are reported in Table II. Varying the molecular mass of dextran does not influence the hydrolysis *in vitro*. The fact that hydrolysis kinetics showed nearly similar half-lives for KD at pH 7.4 either water of human plasma (80% *m/m*) signifies that hydrolysis is not catalyzed by plasma enzymes. High stability of dextran conjugates in

Table II. In vitro *hydrolysis data of ketorolac-dextran conjugates*

Conjugate	$t_{½}$ (h)		
	pH 7.4	80% human plasma (pH 7.4)	pH 9
KD1	36.28	28.64	0.82
KD2	25.97	19.52	0.91
KD3	30.75	21.00	0.78
KD4	40.25	32.54	1.59

acidic pH was also suggested by Ahmad *et al.* (14) and Shrivastava *et al.* (15). The high susceptibility of dextran conjugates to hydrolysis in highly alkaline medium may be attributed to intramolecular catalysis by neighboring hydroxyl groups or it might be related to the basic character of the carbohydrate alkoxide ions. Lack of plasma enzyme-mediated hydrolysis is most likely due to steric hindrance by the dextran backbone (13).

Analgesic activity investigated by acetic acid-induced writhing in mice showed that conjugates reduce the number of writhes significantly in KD1-KD4 in comparison to the control group ($p < 0.05$). The standard drug ketorolac (0.33 mg kg^{-1} body mass, *p.o.*) showed 55% analgesic activity whereas ketorolac-dextran conjugates (equimolar doses to ketorolac, orally) showed analgesic activity ranging from 43 to 50%.

The percentage inhibition of carrageenean-induced rat hind paw edema three hours post-dosing of ketorolac (0.33 mg kg^{-1} body mass, *p.o.*) was found to be 57% while ketorolac-dextran conjugates (equimolar doses to ketorolac) showed 51, 50, 52 and 49% inhibition in KD1-KD4, respectively.

Table III. Analgesic and anti-inflammatory activities of ketorolac and ketorolac-dextran conjugates

Drug	Oral dose[a] (mg kg^{-1} b.m.)	Analgesic activity	Anti-inflammatory activity		
		Number of writhes[b]	Pain reduction (%)	Paw volume (mL)[b,c]	Inhibition of edema (%)
Control (2% gum acacia)	–	55 ± 2	–	0.90 ± 0.02	–
Ketorolac	0.33	25 ± 1[d]	55.6	0.39 ± 0.02[d]	57
KD1	4.71	29 ± 3[d]	47.3	0.44 ± 0.01[d]	51
KD2	3.30	30 ± 3[d]	45.5	0.45 ± 0.02[d]	50
KD3	3.70	27 ± 2[d]	51	0.43 ± 0.02[d]	52
KD4	5.50	31 ± 1[d]	44	0.46 ± 0.03[d]	49

[a] Dose equimolar to the parent drug calculated on the basis of drug contents.
[b] Mean ± SEM, $n = 6$.
[c] Change in paw volume 3 h after carrageenean injection.
[d] $p < 0.05$ *vs.* control.

Table IV. Ulcerogenic activity of ketorolac and ketorolac-dextran conjugates

Drug	Oral dose (mg kg^{-1} b.m.)[a]	Number of ulcers[b]	Degree of ulceration[c]
Control (2% gum acacia)	–	0	–
Ketorolac	6.60	14 ± 2	+, ++, +++, ++++
KD1	94.20	0.8 ± 0.5	+, +++
KD2	66.00	0	+
KD3	74.00	2 ± 0	+, ++, +++
KD4	110.00	0	+

[a] Dose equimolar to the parent drug calculated on the basis of drug contents.
[b] Ulcers > 0.5 mm; mean ± SEM, $n = 6$.
[c] + – strong irritation, ++ – ulcers < 0.5 mm, +++ – ulcers > 0.5 mm, ++++ – perforation.

Analgesic and anti-inflammatory activities are reported in Table III. The results of these biological evaluation indicate that conjugates have retained analgesic and anti-inflammatory activities comparable that of the parent drug.

Animals did not show any gross behavioral changes, sedation, morbidity or mortality for 72 h at the administered oral doses of ketorolac or its conjugates used for analgesic and anti-inflammatory activities. The ulcerogenic activity study indicated that all the ketorolac dextran conjugates showed remarkable decrease in ulcerogenic property compared to their parent drug; moreover, KD2 and KD4 showed only irritation without any ulceration. Results of ulcerogenicity studies indicate that dextran conjugates of ketorolac showed remarkably reduced ulcerogenicity compared to ketorolac itself (Table IV).

CONCLUSIONS

In conclusion, the present investigations suggest that the ketorolac-dextran conjugates can represent potentially useful polymeric conjugates for oral administration of ketorolac with improved water solubility and remarkably decreased gastro-intestinal side-effects while retaining comparable pharmacological activities of the parent drug.

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S A Ž E T A K

Ketorolak-dekstran konjugati: sinteza, *in vitro* i *in vivo* vrednovanje

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U radu je opisana sinteza konjugata dektrana i protuupalnog lijeka ketorolaka (KD). Konjugati su pripravljeni da bi se povećala topljivost ketorolaka u vodi i smanjile njegove nuspojave u gastrointestinanom traktu. Ketorak je prvo preveden u N-acilimidazolni derivat (KAI) koji je kondenziran s polimernim nosačem, dekstranom različitih molekulskih masa (40000, 60000, 110000 i 200000). IR-spektri potvrdili su nastajanje esterske veze. Udio ketorolaka u konjugatu određen je UV-spektrofotometrijskom analizom. Molekulske mase određene su mjerenjem viskoznosti koristeći Mark-Howink-Sakurada jednadžbu. Hidroliza *in vitro* praćena je u puferskim otopinama (pH 1,2, 7,4 i 9) i u 80% *V/V* humanoj plazmi (pH 7,4). Pri pH 9 primjećeno je značajno brže oslobađanje ketorolaka iz KD nego u puferskoj otopini pH 7,4 i krvnoj plazmi. Oslobađanje prati kinetiku prvog reda. *In vivo* biološka ispitivanja na miševima i štakorima ukazuju na to da konjugati imaju analgetsko i protuupalno djelovanje, a značajno smanjeno ulcerogeno djelovanje.

Ključne riječi: ketorolak-dekstran konjugati, dekstranski nosač, protuupalni nesteroidni lijekovi

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