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(57) Abstract :

Anecortave (3), 17a, 21-Dihydroxy-pregna-4, 9(11)-diene-3,20-dione, is a synthetic Steroid having angiostatic activity. Folkman et al observed enhancement of angiostatic activity of a number of Steroids in the presence of heparin or its fragments. The present invention relates to investigation of the effect of heparin and other glycosaminoglycans (GAGs) and PEGylated GAGs conjugated to Anecortave on the enhancement of the angiostatic activity of Anecortave. Accordingly, this patent describes the methods of preparation of novel conjugates of Anecortave with glycosaminoglycans and PEGylated glycosaminoglycans and reports their angiostatic activities determined by Chick Chorio Allantoin Membrane Assay (CAM ASSAY). Besides, syntheses of anecortave and functionalized anecortave, starting from hydrocortisone 21-acetate, suitable for covalently linking it to GAGs and PEGylated GAGs, are also disclosed. These conjugates significantly enhanced the angiostatic activity of anecortave and also improved its solubility in water, facilitating their clinical use through oral, parenteral, Ophthalmic, topical and other routes of administration. These conjugates are also expected to enhance other pharmacological activities of anecortave such as antitumor activity. Besides, these conjugates may serve as good carriers of anecortave in pharmaceutical dosage forms, possibly with its sustained release. In addition, PEGylated derivative of anecortave, a water soluble derivative of anecortave, was synthesized and its angiostatic activity determined and compared with those of the conjugates described herein.

No. of Pages : 35 No. of Claims : 22

We Claim:

- 1) Synthesis of anecortave conjugates with glycosaminoglycans (GAGs) and PEGylated GAGs which exhibit enhanced angiostatic activity and also possibly the other pharmacological activities of anecortave.
- 2) GAGs, as in claim 1, include both sulfated and non-sulfated GAGs.
- 3) GAGs as in claim 1 include those having different molecular weights.
- 4) GAGs as in, claim 1, include heparin and low and ultra-low molecular weight heparins prepared by various known methods.
- 5) PEGylated GAGs, as in claim 1, include those derivatives having mPEG group attached to some of the carboxyl groups of GAGs.
- 6) mPEG, as in claim 5, is mono methyl ether of polyethylene glycol and includes those having various molecular weights, preferably, that having an average mol. weight of 5000 Daltons, but not limited to it.
- 7) Methods for preparing conjugates, as in claim 1, employing quaternary ammonium salts of GAGs and PEGylated GAGs and functionalized anecortave.
- 8) Methods for preparing functionalized anecortave, as in claim 7, to facilitate the synthesis of the conjugates, as in claim 1, creating a suitable linker for covalently connecting anecortave to GAGs and PEGylated GAGs.
- 9) Methods for preparing functionalized anecortave, as in claim 8, where in base may be an organic base, such as pyridine or triethylamine, to prepare anecortave 21-chloro acetate.
- 10) Methods for preparing quaternary ammonium salts of GAGs and PEGylated GAGs to facilitate synthesis of conjugates, as in claim 1.
- 11) Quaternary ammonium salts of GAGs and PEGylated GAGs, as in claim 9, are tetraalkyl quaternary ammonium salts of carboxylic acid groups of GAGs and PEGylated GAGS.
- 12) Functionalized anecortave, as in claim 7, is haloalkanoyl esters of anecortave, wherein halogen atom may be chlorine, bromine, or iodine and may be present on any of the carbon atoms of the alkyl group.

13) Linker, connecting GAGs and PEGylated GAGs to anecortave, as in claim 8, is an alkyl group with an ester function at 21-hydroxyl of anecortave at one end and an ester function at the carboxylic group of GAGs or PEGylated GAGs at the other end.

14) A water soluble derivative of anecortave, namely, PEGylated anecortave, wherein anecortave is covalently linked to mPEG, useful for the development of appropriate pharmaceutical formulations to deliver anecortave.

15) Method for preparing PEGylated anecortave as in claim 13.

16) Water soluble PEGylated anecortave, as in claim 14 and 15, synthesized by condensing functionalized anecortave with mPEG acid quat.salt (22), which is salt of carboxymethylated mPEG.

17) Water soluble PEGylated anecortave, as in claim 14, exhibits enhanced angiostatic activity and also anticipated to have other pharmacological activities of anecortave enhanced.

18) Water soluble PEGylated anecortave, as in claim 14, is expected to facilitate development of sustained release pharmaceutical formulation of anecortave.

19) Conjugates of anecortave, as in claim 1, enhanced angiostatic activity of anecortave in comparison with the activities of water soluble PEGylated anecortave and plain microsuspension/solution of anecortave and are also anticipated to enhance other pharmacological activities of anecortave.

20) Conjugates of anecortave with PEGylated GAGs, as in claim 1, also exhibited enhanced angistatic activity of anecortave and are anticipated to exhibit similar enhancement of other pharmacological activities of anecortave. Besides, these conjugates, by virtue of PEGylation, apart from having improved water solubilities, are also expected to act like a depot, exhibiting sustained release of anecortave in pharmaceutical formulations.

21) Use of all these conjugates in parenteral, ophthalmic, oral and topical formulations to treat diseases caused by uncontrolled angiogenesis, such as, age-related macular degeneration, cancer, psoriasis, etc.

22) Use of these conjugates as active ingredients in pharmaceutical formulations for imparting other pharmacological activities attributed to anecortave.

Date: Aug 02, 2018

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SYNTHESIS AND ANGIOSTATIC ACTIVITIES OF CONJUGATES OF ANECORTAVE WITH GLYCOSAMINOGLYCANS

FIELD OF INVENTION

The invention relates to synthesis of conjugates of a molecule having angiostatic activity, more specifically, anecortave, a synthetic steroid, with glycosaminoglycans (GAGs) and PEGylated GAGs. GAGs include sulfated and non-sulfated glycosaminoglycans; they also include those with different molecular weights. Besides, synthesis of anecortave and functionalized anecortave, suitable for linking anecortave to GAGs and PEGylated GAGs, is also disclosed. In addition, synthesis of PEGylated anecortave, a water-soluble derivative of anecortave, and its angiostatic activity are reported.

BACKGROUND OF THE INVENTION

Angiogenesis, the new blood vessel formation, under normal conditions is associated with growth, female reproductive cycle, and wound healing and is very well regulated. However, angiogenesis, if unregulated, may lead to many neovascular diseases, including age-related macular degeneration, solid tumor growth, diabetic neuropathy, rheumatoid arthritis and psoriasis etc. Folkman et al. observed enhancement of angiostatic activity of a number of steroids in the presence of heparin or its fragments. They reported that heparin or its fragments, with or without anticoagulant activity, enhanced the angiostatic activity of steroids [Judah Folkman et al, *Science*, 221 (4612), 719-725 (1983), Rosa Crum et al, (*Ibid*, 230 (4732), 1375-1378 (1985), J.M.Braugher et.al. U.S.Pat.4,771.042 (1988), and P.A.Aristoff et.al. U.S.Pat.4,975,537 (1990)]. Their study involved use of a mixture of heparin and one of a number of angiostatic steroids; these angiostatic steroids include anecortave (3) (17 α ,21-Dihydroxy-pregna-4,9(11)-diene-3,20-dione). Later, Thorpe et al reported synthesis of conjugates of a non-anticoagulant heparin, covalently linked to angiostatic steroids through adipic dihydrazide, forming hydrazones at C-3 of the

steroid and hydrazide at carboxylic group in heparin molecule; these linkages were expected to be acid-labile [Philip E. Thorpe et al, *Cancer research*, 53, 3000-3007 (1993)]. Similarly, conjugates of heparin derivatives linked to the carbonyl of steroids at C-20 were also synthesized. These conjugates were reported to exhibit anti-tumor and angiostatic activities [Elaine J.Derbyshire et al, *Biochimica et Biophysica Acta*, 1310, 86-96 (1996)].

A.F.Clark et al screened a large number of steroids having angiostatic activity for the treatment and prevention of ocular hypertension and neovascularization and among them, were 17 α ,21-Dihydroxy-pregna-4,9(11)-diene-3,20-dione, and 17 α ,21-Dihydroxy-pregna-4,9(11)-diene-3,20-dione 21-acetate, also known by the trivial names, anecortave and anecortave acetate, respectively [A.F.Clark U.S.Pat.No. 5,770,592 (1998); also see J.M.Braugher et.al. U.S.Pat.No.4,771,042 (1988) and P.A.Aristoff et.al. U.S.Pat.No.4,975,537 (1990)]. Alcon developed a depot formulation of anecortave 21-acetate, as a sterile injectable suspension and got it approved in Australia under the trade name RETAANE for the treatment of subfoveal choroidal neovascularization (CNV) due to exudative age-related macular degeneration and their applications for its approval in Europe and U.S. were under review. However, it was later reported that Retaane was withdrawn from the market.

PRESENT INVENTION

Definitions

The names "Anecortave" (3) and "Anecortave acetate" (2), herein, refer to molecules having the chemical structures: "17 α ,21-Dihydroxy-pregna-4,9(11)-diene-3,20-dione (CAS:10184-70-0)" and "17 α ,21-Dihydroxy-pregna-4,9(11)-diene-3,20-dione 21-acetate (CAS: 7753-60-8)" respectively [Refer: National Center for Advancing Transnational Sciences, N.I.H, U.S.Department of Health & Human Services (NCATS) under the name Anecortave]. The term 'Glycosaminoglycan', herein abbreviated as GAG, is intended to include complex polysaccharides, made up

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of sulfated and non-sulfated derivatives of repeating disaccharide units comprising of uronic acid and amino sugar moieties and the repeating disaccharide units may be structurally same or different. These also include polysaccharides consisting of homogeneous polysaccharide chains having same repeating units and those consisting of a heterogenous mixture of polymeric chains having different disaccharide units. Some of the structures of disaccharides of different GAGs are given in Chart II and, in the case of heterogeneous GAGs, the structures given therein represent only one of the major disaccharides and structures of the other disaccharides are not shown. Chondroitin, used in this study is derived from bovine cartilage, mainly consisting of chondroitin A and smaller amounts of chondroitin C as present in bovine cartilage.

The term 'conjugate', used herein, represents molecular entities formed by covalently linking a glycosaminoglycan (GAG) or a PEGylated GAG molecule to the angiostatic molecule, anecortave. These conjugates may be formed by randomly linking the anecortave molecules to the polymeric chains of GAGs or PEGylated GAGs. Thus, they may be heterogeneous mixtures of polymeric chains of GAGs or PEGylated GAGs linked randomly to multiple anecortave molecules and the structures shown in the charts represent only one such possibility.

The term 'angiostatic' molecule refers to one having activity to inhibit angiogenesis. The term 'linker' refers to the chain connecting GAGs and PEGylated GAGs to anecortave to yield the required conjugates. The term 'mPEG' refers to polyethyleneglycol monomethyl ether. The term mPEG acid (21) represents carboxymethylated derivative of mPEG. In the present invention, mPEG molecule is linked to the other molecules being studied, as and where required, to improve their solubility and also, presumably, to increase the biological life time of the conjugates and this chemical process is termed as PEGylation and herein used in that sense. "Quaternary ammonium salts", abbreviated herein as 'quat.salts, represent tetraalkyl ammonium salts. In the present invention, these salts of GAGs, PEGylated GAGs or mPEG acid (21) are employed to link them to the functionalized anecortave.

GENERAL METHODS

A solution of anecortave, for CAM assay, was prepared using a mixture of mPEG (preferably with average mol. weight of about 550 Daltons, but not limited to it) and Tween 80 (4:1) as solvent. Concentration of the solution was 10mg/ml which was appropriately diluted with water to the required concentration for testing angiostatic activity and the diluted solution is likely to be a colloidal solution or microsuspension.

FUNCTIONALIZATION OF ANECORTAVE

Anecortave (3) was functionalized to facilitate its conjugation with GAGs and PEGylated GAGs and also for PEGylation of anecortave employing mPEG acid (21). The method involves acylating the 21-hydroxyl group of anecortave with haloalkanoyl chloride (halogen may be chlorine, bromine or iodine and may be located on any of the carbon atoms in the alkyl chain), preferably, but not limited to, 2-chloroacetyl chloride in the presence of an organic base, yielding anecortave 21-chloroacetate (4) which is suitable to react with quat.salts of GAGs or PEGylated GAGs and also quat.salt (22) of mPEG acid (21). Synthesis of anecortave (3), starting from hydrocortisone 21-acetate (1), is shown in chart I and the conversion of anecortave to the desired functionalized anecortave (4), is shown in chart II. The latter (1) was mesylated in the presence of a base and the resulting mesylate converted to anecortave 21-acetate (2). Alkaline hydrolysis of the latter (2) afforded anecortave (3) which was treated with haloalkanoyl halide to give the desired functionalized anecortave (4). Alternatively, 21-haloacyl ester function was introduced at an early stage of the synthesis of the functionalized anecortave, starting from hydrocortisone (5) through 21-haloacylated hydrocortisone (6), followed by mesylation and elimination to yield the functionalized anecortave (4), as shown in chart-II.

FUNCTIONALIZATION OF mPEG

In order to attach mPEG to carboxylate groups of GAGs (PEGylation of carboxylates), mPEG was functionalized by tosylating its hydroxyl group and the resulting tosylate is suitable to react with quat.salts of GAGs to yield PEGylated GAGs. For PEGylating anecortave, functionalized anecortave was treated with quat.salt (22) of mPEG acid (Chart VIII) and mPEG acid (21) was prepared by carboxymethylation of mPEG (mPEG having average mol. weight of 5000 Daltons was preferably used, but not limited to it)-(Chart.VIII).

Estimation of anecortave component of the conjugates

Anecortave component of the conjugates was estimated by hydrolysis of the conjugate, isolation, and determination of free anecortave by HPLC.

Characterization of Conjugates

Linkage between GAGs or PEGylated GAGs and anecortave in the resulting conjugates was evident from the IR and UV spectral data:

IR spectra: 1700-1800 cm^{-1}

UV spectra: Characteristic absorptions at 236 nm and 254 nm.

Spectral data of PEGylated GAGs and PEGylated anecortave complied with expected UV and IR spectral absorptions.

DETAILED DISCUSSION OF PRESENT INVENTION

Anecortave (3) is a synthetic steroid having angiostatic activity. Alcon, employing anecortave 21-acetate, developed an injectable suspension, under the trade name, RETAANE for the treatment of age-related macular degeneration. However, Alcon, later, discontinued marketing this product. The main problem in the development of an injectable formulation of anecortave is its lack of solubility in

water which compels formulators to employ sterile micronized form of anecortave for formulating it as a suspension and, therefore, a soluble derivative of anecortave is desirable. It is known that heparin in combination with angiostatic steroid either as a mixture with heparin or as a conjugate covalently linked to heparin enhances the angiostatic activity of the steroid. With these findings in view, the present invention was directed towards synthesis of water-soluble conjugates of angiostatic compound, anecortave (3) with both sulfated and non-sulfated glycosaminoglycans (GAGs) (Example: heparin, chondroitin, dermatan, hyaluronic acid and low mol.weight and ultra-low mol. weight heparins, but not limiting to them) and PEGylated GAGs.

These conjugates were synthesized with the view to:

1. Enhance angiostatic activity of anecortave
2. Increase the solubility of anecortave in water as a conjugate
3. Facilitating sustained release of anecortave in pharmaceutical dosage forms
4. Function as an effective carrier of anecortave for the development of pharmaceutical dosage forms

The study also aims at investigating the effect of solubilization of anecortave on the angiostatic activity. This was done by derivatizing anecortave suitably. PEGylation of anecortave was considered as suitable approach to achieve this. Besides PEGylated anecortave may serve as a prodrug useful in the preparation of sustained release pharmaceutical formulations.

The aim of the present invention is also to design such a linkage, between anecortave and GAGs and PEGylated derivatives or mPEG (used for PEGylation), which alters the structural features of anecortave molecule to a minimum extent and the resulting conjugates may be easily cleaved under normal biological conditions to release anecortave. Thus, functionalization of anecortave was carried out at its 21-hydroxyl group and the resulting derivatives are linked to the carboxyl groups of GAGs, PEGylated GAGs and quat.salt (22) of mPEG acid (21). Accordingly, 21-

hydroxyl of anecortave is acylated with 2-haloacyl halide to yield haloalkanoates (4). Condensation of the latter (4) with quaternary ammonium salts of GAGs, PEGylated GAGs and mPEG acid (21) yielded the corresponding esters of anecortave. Alternatively, functionalized anecortave was obtained by incorporating haloacyl ester functional group at C-21 at the early stage of the synthesis starting from hydrocortisone (5) by a novel approach as shown in Chart II.

Another aspect of the present invention involves the preparation of conjugates of anecortave with partially PEGylated glycosaminoglycan. PEGylation is generally believed to protect a drug from internalization into the cells, thereby, making the PEGylated drug relatively more stable and also giving longer circulation time in the biological system. Besides, it is also expected to improve solubility of resulting conjugates in water, especially, in view of the fact that attaching varying number of lipophilic anecortave molecules to carboxyl groups of GAGs tends to reduce solubility of the resulting conjugates in water.

Yet another aspect of the invention is to employ glycosaminoglycans with different degrees of sulfation and without sulfation, with varying molecular weights, and with or without anti-coagulant activity, so as to test if such changes have any effect on the biological activity being studied.

First embodiment of the present invention involves appropriate derivatization of anecortave to facilitate linking GAGs and PEGylated GAGs to it to yield the proposed conjugates.

Accordingly, synthesis of anecortave starting from hydrocortisone 21-acetate (1) was carried out, as shown in Chart I. By similar approach, as described in chart-II, the 21-haloacyl esters of anecortave, the functionalized anecortave esters, suitable for the syntheses of proposed conjugates, were synthesized as a part of the embodiment. Thus, anecortave is derivatized at 21-hydroxyl group by treating it with haloacyl halide wherein the halide may be present on any of the carbon atoms of alkyl chain.

Accordingly, in one example, preferably, but not limited to it, anecortave was converted to anecortave 21-chloroacetate (4) using 2-chloroacetyl chloride (chart-II). These derivatives are suitable to react with quaternary ammonium salts of GAGs and PEGylated GAGs yielding the proposed conjugates of anecortave. The resulting conjugates are expected to enhance the angiostatic activity of anecortave.

Chart I

Synthesis of Anecortave from Hydrocortisone 21-acetate:

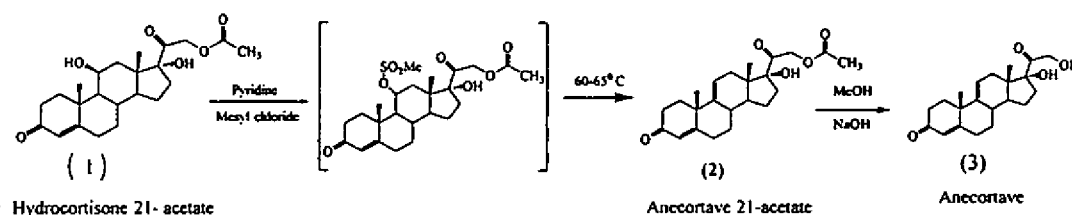
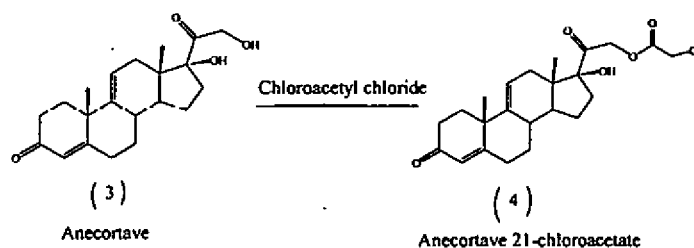


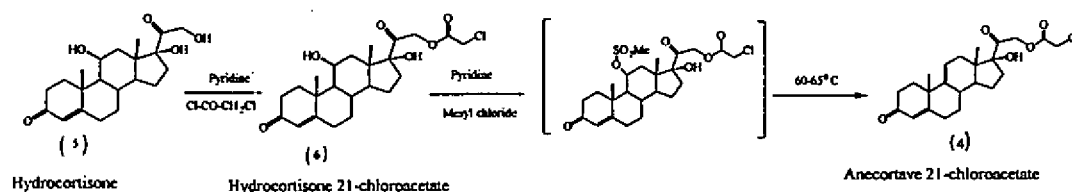
Chart II

Synthesis of functionalization of Anecortave:

1. From anecortave to haloacyl anecortave:



2. From Hydrocortisone to functionalized Anecortave:

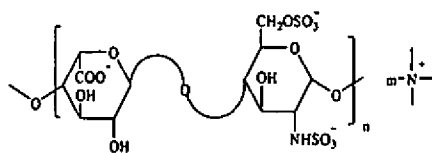


Another embodiment of the present invention is to employ quaternary ammonium salts of GAGs and PEGylated GAGs for the synthesis of anecortave conjugates. These salts were synthesized using cetrimide (Hexadecyltrimethylammonium bromide) or Adogen464 [methyltrialkyl (C_8-C_{10}) ammonium bromide], but not limited to them. Chart III illustrates the structures of various quat.salts of GAGs. As shown in it, structure (7) represents quaternary ammonium salts of heparins, including, but not limited to, heparin (7a), low molecular weight (7b & 7c) and ultra-low molecular weight heparins (7d), obtained by conventional methods, such as nitrous acid depolymerization, alkaline depolymerization of the benzyl ester derivative of heparin or radical catalyzed depolymerization with hydrogen peroxide and a cupric salt.

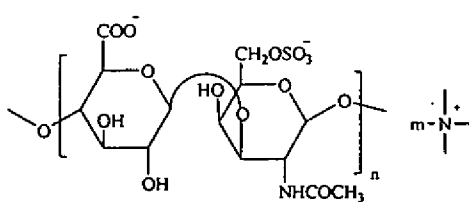
Structures (8), (9) and (10) represent quaternary ammonium salts of chondroitin, dermatan and hyaluronic acid respectively. Structures (7), (8), (9) and (10) represent only one of the main building blocks and structures of other disaccharide building blocks with some variations are not shown here; the numbers 'n' and 'm' vary depending on molecular weights and degree of sulfation, respectively. The quat.salt (22) of mPEG acid ((21)) was specifically employed for PEGylation of anecortave.

Chart III

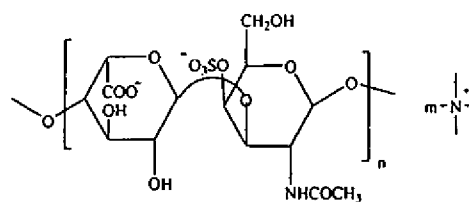
Quaternary salts of GAGs:



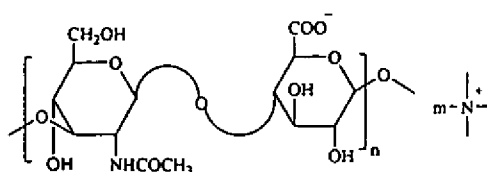
Quaternary salt of heparins (7) [(7a), (7b), (7c) and (7d)]



Quaternary salt of chondroitin (8)



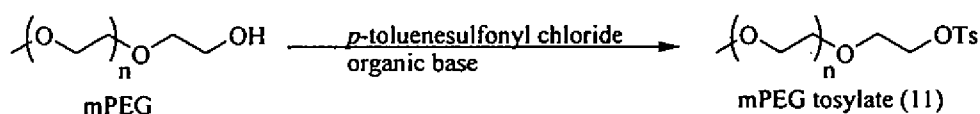
Quaternary salt of dermatan (9)



Quaternary salt of hyaluronic acid (10)

Chart IV

Synthesis of mPEG tosylate (11):

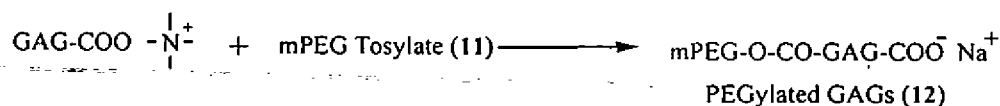


mPEG Tosylate (11)

Yet another embodiment of the present invention is to prepare conjugates of GAGs partially esterified with mPEG. To accomplish this, the free hydroxyl group of mPEG was tosylated with *p*-toluenesulfonyl chloride in pyridine as shown in chart IV and the resulting mPEG tosylate (11) was employed for partial PEGylation of GAGs as shown in chart V.

Chart V

Partial PEGylation of GAGs:



No. PEGYLATED GAGS

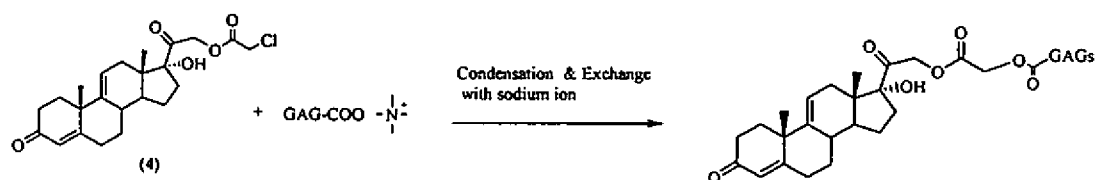
- 12a: PEGylated heparin
- 12b: PEGylated chondroitin
- 12c: PEGylated dermatan
- 12d: PEGylated hyaluronate

Yet another embodiment of the present invention is reaction of the quaternary ammonium salts of GAGs (7, 8, 9 and 10) and PEGylated GAGs (12a), (12b), (12c), (12d) with functionalized anecortave (4) to yield the novel conjugates of anecortave with GAGs and PEGylated GAGs which are expected to enhance the angiostatic and other pharmacological activities of anecortave (Chart VI and VII).

Thus, the quaternary ammonium salts of GAGs (7), (8), (9) and (10) were treated with haloacylated anecortave (4) to yield novel conjugates of (13), (14), (15) and (16).

Chart VI

Conjugation of Anecortave with GAGs:

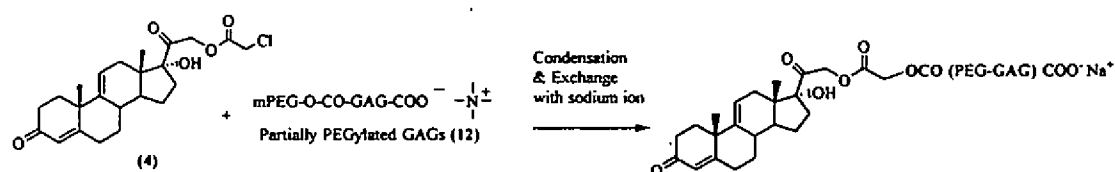


No.	CONJUGATED GAGS
13:	Heparin
14:	Ultra-low mol.weight heparin
15:	Low mol.weight heparin (Dalteparin)
16:	Low mol.weight heparin (Enoxaparin)

Reaction of mPEG tosylate (11) with quaternary ammonium salt of GAGs yielded PEGylated GAGs (12a), (12b), (12c) and (12d) as shown in chart V. Degree of PEGylation was controlled by using appropriate quantities of mPEG tosylate (11), preferably, to esterify about 10 to 15% of carboxylic groups of GAG, but not limited to that. The PEGylated GAGs (12a), (12b), (12c) and (12d), thus obtained, had, for example, around 85 to 90 % free carboxyl groups available for conjugation with anecortave. Thus, the quaternary ammonium salts of PEGylated GAGs (12a), (12b), (12c) and (12d) were treated with haloacylated anecortave (4) to yield novel conjugates of (17), (18), (19) and (20).

Chart VII

Conjugation of Anecortave with PEGylated GAGs:



Conjugates of Anecortave with PEGylated GAGs

No.	<u>CONJUGATES OF PEGYLATED GAGS</u>
17:	PEGylated heparin (12a)
18:	PEGylated chondroitin (12b)
19:	PEGylated dermatan (12c)
20:	PEGylated hyaluronate (12d)

The novel conjugates of PEGylated GAGs, described above, were synthesized

with the view to significantly increase the water solubility of the conjugates and also to increase their stability in biological environment, thereby, facilitating its longer life in the biological system; this is in addition to the main aim of synthesizing the conjugates to enhance the angiostatic and other pharmacological activities of anecortave.

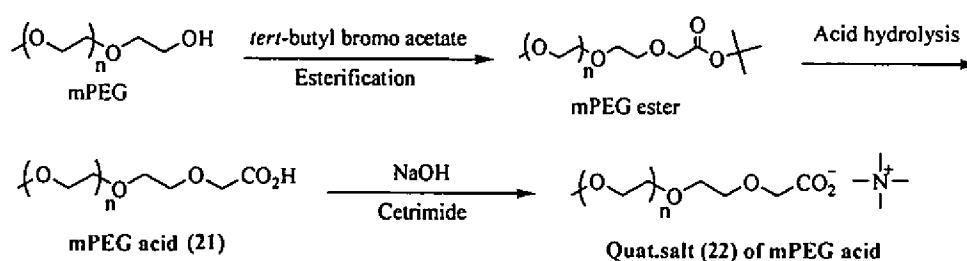
Thus, conjugation with PEGylated GAGs is aimed at:

- Enhancing angiostatic and other pharmacological activities of anecortave,
- Increasing aqueous solubility of anecortave as a conjugate,
- Facilitating sustained release of anecortave in pharmaceutical dosage forms,
- Functioning as an effective carrier of anecortave for pharmaceutical formulations.

Another embodiment of this invention is to synthesize a water-soluble derivative of anecortave, which can be used for the development of appropriate pharmaceutical formulations, possibly, for sustained drug delivery. This was accomplished by PEGylating anecortave. To this end, mPEG was carboxymethylated to yield mPEG acid (21) and its quat.salt (22) was treated with functionalized anecortave (4) to yield PEGylated anecortave (23), as shown in Chart VIII.

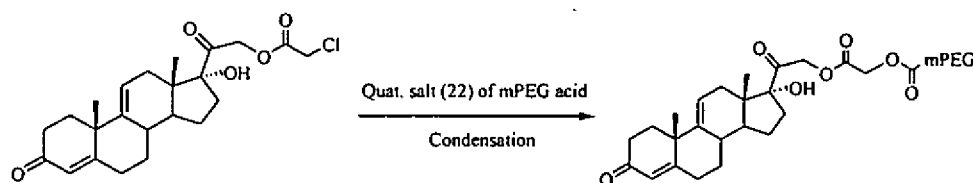
Chart VIII

Synthesis of mPEG acid (21) and its Quat.salt (22):



mPEG acid (21) and its Quat.salt (22)

PEGylation of Anecortave:



PEGylated Anecortave (23)

EXPERIMENTAL

EXAMPLE 1

Synthesis of anecortave 21-acetate (2):

To a solution of hydrocortisone 21-acetate (1) (46.5 g.) in a mixture of DMF (232.5 ml.) and pyridine (40.6 ml.), was added methanesulfonyl chloride (20 ml.) at 0°C during 15 min. and the mixture stirred at 80-85°C for 4 hr. The reaction mixture, cooled to room temperature, was diluted with methanol (1 L.) and stirred at 0°C for 1 hr. to obtain a solid which was filtered, washed with cold methanol (50 ml.) and dried to yield 45 g. of anecortave 21-acetate (2).

Analytical data: Melting point: 236°C; $[\alpha]_D$ (c 1% chloroform) +122° [Lit. m.p.236-237°C and $[\alpha]_D$: (c 1% chloroform) +117° (J. FRIED and E.F. SABO, *J.Am.Chem.Soc.*75, 2273 (1953)] IR (KBr): 1738 cm^{-1} ; PMR spectrum (DMSO) $\{\delta$ (ppm): 2.18 (OCO-CH₃, 3H, singlet), 5.75 (-CO-CH=, 1H, singlet), 8.01 (*tert* C-OH, 1H, singlet), 4.86 & 5.07 (-CO-CH₂-O-, 2H, doublet), 5.56 (CH₂-CH=, 1H, doublet), 2.88 (CH₂-CH=, 2H, doublet), 1.34 (-CH₃, 3H, singlet), 0.65 (-CH₃, 3H, singlet); Molecular weight and Molecular formula: 386.488 and C₂₃H₃₀O₅.

EXAMPLE 2

Synthesis of anecortave (3):

To a solution of anecortave 21-acetate (2) (38 g.) in methanol (380 ml.), was added NaOH solution (10.18 g. in 63.3 ml. water), the mixture stirred at 25-30°C for

3 hr. The reaction mixture was cooled to 0°C, diluted with cold water (570 ml.) and its pH adjusted to 3.0 with aqueous HCl to obtain a solid which was filtered and washed with cold water (38 ml.) and dried to get yield 22 g. of anecortave (3).

Analytical data: m.p. 242°C ; [Lit. m.p.242-243°C <https://steraloids.com/4-9-11-pregnadien-17-21-diol-3-20-dione>]; [α]D (c 1% DMSO) +92.012° [Lit.: [α]D (c, 0.32 dioxan) +103 ° (*The Dictionary of organic compounds 26.1(1).*)], IR (KBr): 1710 cm⁻¹; PMR spectrum (DMSO) [δ (ppm): 5.658 (-CO-CH=, 1H, singlet), 4.49 & 4.55 (-CO-CH₂-OH, 2H, doublet), 4.70 (CO-CH₂-OH, 1H, triplet), 2.23-2.35 (CH₂-CH=, 2H, multiplet), 5.50 (CH₂-CH=, 1H, doublet), 1.29 (-CH₃, 3H, singlet), 0.477 (-CH₃, 3H, singlet); UV data: Optical density (absorbance) at 236 nm wavelength of the conjugate is 0.42 at 0.01 mg/ml concentration in chloroform.

EXAMPLE 3

Synthesis of anecortave 21-chloroacetate (4):

To a solution of anecortave (3) (21g.) in pyridine (440ml.), was added chloroacetic anhydride (25 ml.) at 0°C and the mixture stirred at 0°C for 7 hr. The reaction mixture was cooled to 0°C and diluted with cold water (840 ml.) to afford a solid which was filtered, washed with cold water (20ml) and dried to yield 23g. of anecortave 21-chloroacetate (4).

Analytical data: m.p. 219°-222° C; IR (KBr): 1759 cm⁻¹; PMR spectrum (DMSO) [δ (ppm): 5.66 (-CO-CH=, 1H, singlet), 4.93 & 5.14 (-CO-CH₂-O-, 2H, doublet), 4.52 (O-CO-CH₂-Cl, 2H, singlet), 2.89 (CH₂-CH=, 2H, multiplet), 5.53 (CH₂-CH=, 1H, multiplet), 7.95 (OH, 1H,broad singlet), 2.24-2.35 (CO-CH₂-CH₂, 2H,multiplet), 1.31 (-CH₃, 3H, singlet), 0.49 (-CH₃, 3H, singlet); [α]D (c 1%, chloroform) +104.9, Molecular weight and Molecular formula (HRMS): 421.17763 and C₂₃H₃₀O₅Cl; UV data: Optical density (absorbance) at 237 nm wavelength of the conjugate is 0.423 at 0.015 mg/ml concentration in chloroform.

EXAMPLE 4

Synthesis of hydrocortisone 21-chloroacetate (6):

To a solution of hydrocortisone (5) (45 g.) in a mixture of dichloromethane (225 ml.) and pyridine (43.2 ml.), at 0°C was added chloroacetyl chloride (14.4 ml) during 30 min. and the mixture further stirred at 0°C for 4 hr. The reaction mixture was concentrated to dryness under vacuum, the residue diluted with cold water (250 ml.) and stirred for 1 hr. at 0°C to get a solid which was filtered, washed with cold water (50 ml) and dried to yield 32g. of hydrocortisone 21-chloroacetate (6).

Analytical data: Melting point: 230°-234°C; IR (KBr): 1763 cm^{-1} ; PMR spectrum (DMSO) [δ (ppm): 5.56 (-CO-CH=, 1H, singlet), 4.84 & 5.20 (-CO-CH₂-O-, 2H, doublet), 4.53 (O-CO-CH₂-Cl, 2H, singlet), 1.23-1.31 & 1.44-1.51 (CH₂-CH-OH, 2H, multiplet), 5.48 (CH₂-CH-OH, 1H, singlet), 2.33-2.45 (CO-CH₂-CH₂, 2H, multiplet), 1.37 (-CH₃, 3H, singlet), 0.77 (-CH₃, 3H, singlet); [α]_D (c 1% 1,4-dioxane)+147.26°; Molecular weight and Molecular formula (HRMS): 439.187 and C₂₃H₃₂O₆Cl.

EXAMPLE 5

Synthesis of anecortave 21-chloroacetate (4):

To a solution of hydrocortisone 21-chloroacetate (6) (28g.) in a mixture of DMF (280 ml.) and pyridine (56 ml.), at 0°C was added methanesulfonyl chloride (28 ml.) during 15 min and stirred at 60-65°C for 4 hr. The reaction mixture was cooled to 0°C, diluted with cold water (4 lit) and stirred at 0°C for 1hr to obtain a solid which was filtered, washed with cold water (30 ml.) and dried to yield 46g. of anecortave 21-chloroacetate (4).

Analytical data: m.p. 219°-222°C; IR (KBr): 1759 cm^{-1} ; PMR spectrum (DMSO) [δ (ppm): 5.66 (-CO-CH=, 1H, singlet), 4.93 & 5.14 (-CO-CH₂-O-, 2H, doublet), 4.52 (O-CO-CH₂, 2H, singlet), 2.89 (CH₂-CH=, 2H, multiplet), 5.53 (CH₂-CH=, 1H,

multiplet), 7.95 (OH, 1H, broad singlet), 2.24-2.35 (CO-CH₂-CH₂, 2H, multiplet), 1.31 (-CH₃, 3H, singlet), 0.49 (-CH₃, 3H, singlet); [α]_D (c 1% chloroform) +104; Molecular weight and Molecular formula (HRMS): 421.17763 and C₂₃H₃₀O₅Cl; UV data: Optical density (absorbance) at 237 nm wavelength of the conjugate is 0.423 at 0.015 mg/ml concentration in chloroform.

EXAMPLE 6

Synthesis of mPEG acid (21) and its quat.salt (22):

To a solution of mPEG (50g.) in toluene (500ml.) was added potassium *tert*-butoxide (2.5g.) followed by *tert*-butyl bromoacetate (5ml.) and stirred for 24 hrs. at room temperature under N₂ atm. Addition of methyl *tert*-butyl ether (2.5 L) to the reaction mixture precipitated the product which was filtered, washed with the same solvent and dried to yield mPEG ester (48g.).

- a) **mPEG acid (21):** mPEG ester (20g.) was dissolved in a mixture of trifluoroacetic acid (100ml.), dichloromethane (200ml.) and water (0.25ml.) and stirred for 4 hr. The reaction mixture, after distilling out all the solvent, was diluted methyl *tert*-butyl ether (200ml.) and the precipitate obtained was filtered and dried to yield mPEG acid (21) (19g.)
- b) **mPEG acid quat. Salt (22):** mPEG acid (21) (5g.) was dissolved in water (20ml) and its pH adjusted to 7.0 using 10% aqueous sodium hydroxide. After distilling out all the solvent from the reaction mixture, a solution of cetrimide (0.5g.) in toluene (50ml.) was added to the residue. Toluene was distilled out of the reaction mixture to yield crude quat.salt (22) of mPEG acid which was used as such in the next step.

EXAMPLE 7

Synthesis of mPEG tosylate (11):

mPEG (100g.) was dissolved in toluene (1L) and 200 ml. of the solvent distilled out to remove water; potassium *tert*-butoxide (6gms) was added to the resulting solution and stirred at 40⁰c for 2hr. A solution of *p*-toulenesulfonyl chloride (19g.) in toluene (20ml.) was added to the reaction mixture, stirred for 35hr. at 40⁰c under N₂ atm., cooled to room temperature, filtered, and the solvent from the filtrate distilled out. The residue was dissolved in dichloromethane (100ml.), the product, precipitated by adding methyl *tert*-butyl ether (1L), filtered, washed with the same solvent and dried to yield mPEG tosylate (11) (90g.).

EXAMPLE 8

Conjugation of anecortave with heparin, Low mol.weight and Ultra low mol.weight heparins:

To a solution of quat.salt of heparin (3g.) (0.00178moles) in dichloromethane (36 ml) was added anecortave 21-chloroacetate (1.8gms). The mixture was heated under reflux for 48hr., concentrated to 24 ml., cooled to room temperature and treated with a methanolic solution of sodium bromide (12.4g. in 20ml.) to yield a gummy product. The solid was separated by decanting the liquid and washing the residue with methanol (60ml.) and then acetone (100ml.) to yield 1.5g. of crude heparin conjugates of anecortave. The latter was dissolved in 30ml. of water and filtered through celite to remove unchanged anecoretave derivative; the filtrate was concentrated and residue dried to yield 1.4g. of heparin conjugate of anecortave (13).

Similarly, the anecortave conjugates of low molecular weight heparin (Dalteparin, Enoxaparin) and ultra-low molecular weight heparins (15, 16 & 14) respectively were prepared.

Analytical data:

Heparin conjugates with anecortave (13):

IR (KBr): Broad peak from 1720–1800 cm^{-1} (The peak due to carbonyl ester); UV data: Optical density (absorbance) at 236nm wavelength of the conjugate is 0.4945 in water.

Ultra-low molecular weight heparin conjugates with anecortave (14):

IR (KBr): Broad peak from 1730 –1800 cm^{-1} (the peak due to carbonyl ester); UV data: Optical density (absorbance) at 236nm wavelength of the conjugate is 0.4437 in water.

Low molecular weight heparin (dalteparin) conjugates with anecortave (15):

IR (KBr): Broad peak at 1720–1800 cm^{-1} (the peak due to carbonyl ester); UV data: Optical density (absorbance) at 236nm wavelength of the conjugate is 0.5138 in water.

Low molecular weight heparin (Enoxaparin) conjugates with anecortave (16):

IR (KBr): Broad peak at 1720–1800 cm^{-1} (the peak due to carbonyl ester); UV data: Optical density (absorbance) at 236nm wavelength of the conjugate is 0.4941 in water.

EXAMPLE 9

PEGylation of GAGs:

To a solution of quat.salt of GAGs in DMF/THF, a solution of mPEG tosylate (11) in a mixture THF and dichloromethane, was added and heated at 50 to 70°C for 6 to 10 hr. After evaporation of solvent, the reaction mixture was triturated with acetone to yield a residue containing the PEGylated GAGs (12a), (12b), (12c) and (12d). The products were used as such in the reaction with anecortave haloalkanoate to yield the corresponding conjugate of anecortave.

By the general procedure, described above, quat.salt of heparin, chondroitin,

dermatan and hyaluronate were PEGylated separately using appropriate proportions of mPEG tosylate (11).

EXAMPLE 10

Conjugation of Anecortave with PEGylated GAGs:

Conjugation was carried out by condensing quat.salt of mPEGylated GAGs with anecortave 21-chloroacetate (4). The reaction was carried out in the same solvent, used for PEGylation of the GAG, at 45-50°C for about 48hrs. After evaporating the solvent almost completely (under vac. if necessary), the product was dissolved in a 3:2 mixture of dichloromethane and methanol containing sodium bromide and stirred for 1hr to yield sodium salt of the conjugate. The solid obtained was filtered, washed with acetone and dried to obtain the conjugate. The latter was dissolved in water and filtered through celite to remove unchanged anecortave derivative; the filtrate was concentrated and the residue dried to obtain conjugate of anecortave.

EXAMPLE 11

By the general procedure, described in Example 11, quat.salt of PEGylated heparin (3gm) (12a) was condensed with anecortave 21-chloroacetate (1.8 g.) (4) and the product obtained was dissolved in a 3:2 mixture (50ml.) of dichloromethane and methanol containing sodium bromide (1.2g.) to yield the corresponding conjugates as sodium salt. The latter was dissolved in 30ml of water and filtered through celite to remove unchanged anecortave derivative and the filtrate concentrated and dried to yield 1.4g. of PEGylated heparin conjugate of anecortave (17).

Similarly, anecortave conjugates of PEGylated chondroitin, dermatan and hyaluronate (18, 19 & 20) (sodium salts) were prepared using appropriate proportions of anecortave 21-chloroacetate).

Analytical data:

Anecortave conjugate of PEGylated heparin (17):

IR (KBr): broad peak at 1700–1800 cm^{-1} (peak due to carbonyl ester) **UV data:** Optical density (absorbance) of the conjugate is 0.6376 with water at 236nm wavelength.

Anecortave conjugate of PEGylated chondroitin (18):

IR (KBr): broad peak at 1700–1760 cm^{-1} (peak due to carbonyl ester); **UV data:** Optical density (absorbance) of the conjugate is 0.752 with water at 236nm wavelength.

Anecortave conjugate with PEGylated dermatan (19):

IR (KBr): broad peak at 1720–1760 cm^{-1} (peak due to carbonyl ester); **UV data:** Optical density (absorbance) of the conjugate is 0.74 with water at 236nm wavelength.

Anecortave conjugate with PEGylated hyaluronate (20):

IR (KBr): broad peak at 1710–1800 cm^{-1} (peak due to carbonyl ester); **UV data:** Optical density (absorbance) of the conjugate is 0.868 with water at 236nm wavelength.

EXAMPLE 12

PEGylation of Anecortave (23):

To a mixture of mPEG acid quat.salt (5g. 0.001 moles) in dichloromethane (50ml) was added anecortave 21-chloroacetate (4) (3g.) and stirred at 40–45°C for 40hrs under N_2 atm. After evaporating solvent from the reaction mixture, acetone (100ml) was added to the residue and filtered through celite. After distilling out solvent from the filtrate, the residue was dissolved in water and filtered through celite. Water was distilled out of the filtrate and the residue washed with methyl *tert*-butyl ether and dried to yield 4g. of crude PEGylated anecortave (23) which was purified by chromatography using silica to remove unchanged starting material to

yield 3.5g. of pure PEGylated anecortave (23).

Analytical data:

IR (KBr): broad peak at $1720-1780\text{ cm}^{-1}$ (the peak due to carbonyl ester); **UV data:** Optical density (absorbance) of the conjugate is 0.202 with water at 236nm wavelength.

EXAMPLE 13

Preparation of Anecortave solution for CAM testing:

Anecortave solution for CAM assay was prepared by dissolving anecortave (500mg) in a mixture containing sodium dihydrogen phosphate monohydrate (5.84mg), disodium hydrogen phosphate dihydrate (10.176mg), polyvinyl alcohol (100mg), benzyl alcohol (90mg), polysorbate 80 (50mg). Concentration anecortave in the resulting mixture (24) was 10mg/ml. This was appropriately diluted with water to the required concentration for testing angiostatic activity.

EXAMPLE 14

Preparation of a solution of a mixture of heparin and anecortave for CAM testing:

A mixture of anecortave (50 mg) and heparin (50 mg) was dissolved in a mixture containing mPEG 550 (5ml), Tween 80 (3ml) and water (2ml). Concentration of anecortave (25) in the solution was 5mg /ml and 1 μ l of this was used for testing of angiostatic activity.

EXAMPLE 15

Preparation of a solution of a mixture of hyaluronic acid and anecortave for CAM testing:

A mixture of anecortave (50 mg) and hyaluronic acid (50) was dissolved in a mixture containing mPEG 550 (5ml.), Tween 80 (3ml.) and water (2ml.). Concentration of anecortave (26) in the solution was 5mg /ml and 1 μ l of this was

used for testing of angiostatic activity.

Estimation of Anecortave component of the Conjugates

The conjugate sample was hydrolyzed using aqueous sodium hydroxide and the reaction mixture neutralized with acid to the required pH and was analyzed by HPLC, after diluting it with suitable mobile phase to the required pH and concentration.

Anecortave, isolated after hydrolysis as described above, was estimated by HPLC using: instrument (SHIMADZU series LC-2010A) equipped with a vacuum degasser, auto injector, photo diode array (PDA) fitted with base deactivated end-capped ODS, (250mm X 4.6mm; 5 μ m) column, a mixture of tetrahydrofuran (72%) and water (28%) as mobile, phase at a flow rate of 1.0 ml / min, UV detection at 254nm, column oven at 45°C and injection volume of 20 μ l.

CAM ASSAY

Materials and Methods

Preparation of Test Substance: Test samples were submitted in air tight glass vials and were stored at 4°C, light controlled environment.

Test samples: Each sample solution with 100 μ g/ μ l concentration was prepared in PBS and sterilized by passing through a syringe filter (0.22 μ m). Solution of Staurosporine with 2 μ g/ μ l concentration was prepared in PBS containing 0.1% DMSO. Solution of hVEGF (SIGMA) with 50ng/ μ l concentration was prepared in sterile PBS.

Grafting: Gelatin sponges (Abogel) was cut in approximately 2mm³ pieces and loaded with 2 μ l of 1:1 mixture of test substance solution and VEGF solution. The graft was placed on the CAM.

Eggs: Fertile Hen eggs were procured from hatchery and were cleaned and

decontaminated using alcohol. 1ml of albumin was removed using a syringe and incubated for 8 days. Grafts were placed on developing CAMs and further incubated to day 12. On day 12 CAMs were fixed with formaldehyde and dissected.

Imaging: Fixed CAMs were imaged under constant illumination and magnification under a stereo microscope fitted with a digital camera (CANON).

Image analysis: Images were analyzed on MS power point keeping the image size constant. A ring was drawn around the graft and the size was kept constant. Blood vessels crossing the ring were counted for each test group.

Statistical Analysis: Data was analyzed on MS Excel 2007.

REPORT OF ANALYSIS

Drawings:

Figure-1 & Figure-2: Angiogenesis inhibition of test compounds with reference to staurosporine.

Company	Gland Pharma Ltd. 6-3-865/1/2, flat No. 201, Greenland Apts, Ameerpet, Hyderabad -500016 INDIA
Activity	Chick Chorio Allantoin Membrane (CAM) Assay
Experimental Groups	Fourteen
Date of Report Generation	October 20, 2014 ; July 15, 2016
Internal Standard	Staurosporine (27)

CAM surfaces were grafted with gelatine sponges impregnated with 100 µg of test substance and 50ng VEGF dissolved in PBS. Staurosporine 2µg was used as a positive control. Untreated CAM received only VEGF and PBS. Error bars represent SEM, n=5. P values for all the treated groups were calculated by comparing with the untreated group. *p<0.05, **p<0.01, ***p< 0.001.

Table-I: Table-I significant comparative blood vessel score results for untreated (UT), anecortave (24), compound (23), compound (13), compound (14) and compound (15) against staurosporine (27).

Blood Vessel score							
	UT	Staurosporine (27)	Anecortave (24)	23	13	14	15
1	26	3	7	13	5	13	8
2	27	3	8	10	10	11	7
3	25	3	7	14	6	14	7
4	27	2	6	12	6	8	7
5	25	3	13	10	5	14	8
Avg.	26.0	2.8	8.2	11.8	6.4	12	7.4
Stdev	1.0	0.4	2.8	1.8	2.1	2.5	0.5
SEM	0.4	0.2	1.2	0.8	0.9	1.1	0.2
Pvalue		<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

Table-II: Table-II significant blood vessel score results for compound (16), compound (17), compound (18), compound (19) and compound (20).

Blood Vessel score					
	16	17	18	19	20
1	13	9	13	13	8
2	15	3	15	10	10
3	12	11	16	14	15
4	12	4	14	11	10
5	16	6	10	11	8
Avg.	13.6	6.6	13.6	11.8	10.2
Stdev	1.8	3.4	2.3	1.6	2.9
SEM	0.8	1.5	1.0	0.7	1.3
p value	<0.001	<0.001	<0.001	<0.001	<0.001

Table III: Table-III significant comparative blood vessel score results for untreated (UT), compound (25) and compound (26) against staurosporine (27).

Blood Vessel score				
	UT	Staurosporine (27)	25	26
1	12	6	0	7
2	11	1	0	9
3	10	0	6	8
4	12	0	0	4
5	10	0	0	7
Avg.	11	1.4	1.2	7

Table IV: CAM ASSAY ANALYTICAL DATA

S. N o.	Compound number	GAGs linked to anecortave (4)	Anecortave/ 100µg conjugate	Inhibition/ 100µg conjugate	Inhibition/1µg anecortave linked in the conjugate	Inhibition enhancement	
						compared to anecortave solution #X	compared to PEGylated anecortave *Y
1	13	with heparin	16 µg	75.38 %	4.71 %	6.92	0.47
2	14	with Ultralow mol.weight heparin	5 µg	53.84 %	10.77 %	15.83	1.08
3	15	with low mol.weight heparin	3.6 µg	71.53%	19.87 %	29.22	2.0
4	16	with low mol.weight heparin	31 µg	47.69 %	1.54 %	2.26	0.15
5	17	with PEGylated heparin	8.12 µg	74.61 %	9.18 %	13.5	0.92
6	18	with PEGylated chondroitin	2.4µg	47.69 %	19.87 %	29.22	2.0
7	19	with PEGylated dermatan	3.24 µg	54.61 %	16.85 %	24.78	1.69
8	20	with PEGylated hyaluronate	5 µg	60.77 %	12.15 %	17.86	1.22
9	25	Heparin & anecortave physical mixture	50 µg	89.09 %	1.78 %	2.61	0.18
10	26	Hyaluronic acid & anecortave physical mixture	50 µg	36.36 %	0.72 %	1.06	0.07

1) Staurosporine (27) Inhibition Percentage: 45% per 1µg

2) Concentration of anecortave stock solution (24): 10 mg/ ml; quantity equivalent to 100µg was taken for testing. Percentage inhibition by anecortave solution: 0.68 % per 1µg of anecortave present in (24).

3) Percentage of inhibition by PEGylated anecortave (23): 9.93% per 1 μ g of anecortave present in (23).

X: Inhibition enhancement: Inhibition by conjugate/Inhibition by anecortave solution

*Y: Inhibition Enhancement: Inhibition by conjugate/Inhibition by PEGylated anecortave

§: This is microsuspension/solution of the components of the mixture

RESULTS AND DISCUSSION

Table IV summarizes comparative angiostatic activities of the conjugates of the invention. For comparison, all activities were calculated per 1 μ g of anecortave attached to GAGs or PEGylated GAGs. Microsuspension/solution of anecortave (24) (Formation of microsuspension is a possibility when the stock solutions of anecortave in a mixture of mPEG550 and Tween 80 is diluted with water to the required concentration for determining the activity) exhibited angiostatic activity of 0.68 % per 1 μ g and standard staurosporine inhibited up to 45.0% per 1 μ g. PEGylated anecortave, a soluble derivative of anecortave showed angiostatic activity of 9.93 % per 1 μ g of anecortave attached to PEG. This amounts to 15 times increase in the activity compared to that of anecortave microsuspension/solution and is significant enhancement of the activity. Besides, PEGylated anecortave is likely to facilitate development of sustained release pharmaceutical formulation of anecortave.

All the conjugates, described herein, exhibited significant enhancement of angiostatic activities in comparison with that of microscopic suspension/solution of anecortave (24) in mPEG550 and Tween 80. Similar microsuspension/solution of a mixture of anecortave and a GAG (25 & 26) did not exhibit notable difference in activity. A strikingly significant observation is that activity does not proportionately increase with amount of anecortave covalently attached in the conjugates; besides, higher load of anecortave apparently tends to decrease the activity, if calculated per μ gm, more so in comparison with PEGylated anecortave. It is not clear if anecortave inhibits angiogenesis after getting detached from the conjugate or the conjugate itself binds to the receptor, facilitated by the affinity of GAGs for the receptors. This,

however, indicates that all the anecortave molecules attached to the conjugates apparently do not bind to the relevant receptors simultaneously. If the activity is due to free anecortave molecule cleaved from the conjugate, all anecortave molecules may not be cleaved and released at the same time and the activity depends on the kinetics of the cleavage and release of anecortave molecule. It is important to note that activity per μgm of some of the conjugates containing larger proportion of anecortave recorded lower activity per μgm , especially in comparison with PEGylated anecortave. In both the cases, sustained activity over longer period is logically anticipated. Another significant and unexpected observation of the present invention is that sulfation of GAG in the conjugates does not seem to be an essential structural feature to enhance the angiostatic activity of anecortave. Supporting this inference is the observation that anecortave conjugated to PEGylated hyaluronic acid, a non-sulfated GAG, exhibited significantly high activity (29% per $1\mu\text{g}$ of anecortave attached to the conjugate). Another notable observation is that molecular weight of heparin used for conjugation does not make significant difference in enhancing the activity, as indicated by the activities exhibited by the conjugates with low and ultra-low molecular weight heparins in comparison with the those of other conjugates.

PEGylation of GAGs was included in the synthesis of conjugates purposefully to improve solubility of the conjugates in water, especially, in view of the possibility that attaching more number of lipophilic anecortave molecules to GAGs decreases the solubility of resulting conjugate and increase the stability of the conjugates in biological environment facilitating a sustainable in vivo angiostatic activity.

A specific observation and advantage of the invention is that the conjugates of GAGs lacking (conjugates of hyaluronic acid) or having low anticoagulant property (conjugates of dermatan) also exhibit equally appreciable enhancement of angiostatic activity. This is extremely important since such conjugates will not have bleeding propensities when administered clinically. Besides, they are logically more suitable as carriers in the formulations, such as, parenteral and ophthalmic preparations and ointments and creams for topical applications for treating diseases like, age-related

macular degeneration, cancer, psoriasis, and other problems associated with uncontrolled angiogenesis. Besides, all the conjugates are soluble in water facilitating their use in parenteral dosage forms. These conjugates are likely to be stable in the gastrointestinal track environment and so suitable for oral administration to deliver anccortave.

ABSTRACT:

Anecortave (3), 17 α , 21-Dihydroxy-pregna-4, 9(11)-diene-3,20-dione, is a synthetic steroid having angiostatic activity. Folkman et al observed **enhancement of angiostatic activity of a number of steroids in the presence of heparin or its fragments.** The present invention relates to **investigation of the effect of heparin and other glycosaminoglycans (GAGs) and PEGylated GAGs conjugated to Anecortave on the enhancement of the angiostatic activity of Anecortave.** Accordingly, this patent describes the methods of preparation of **novel conjugates of Anecortave with glycosaminoglycans and PEGylated glycosaminoglycans** and reports their angiostatic activities determined by Chick Chorio Allantoin Membrane Assay (CAM ASSAY). Besides, syntheses of anecortave and functionalized anecortave, starting from hydrocortisone21-acetate, suitable for covalently linking it to GAGs and PEGylated GAGs, are also disclosed. These conjugates significantly enhanced the angiostatic activity of anecortave and also improved its solubility in water, facilitating their clinical use through oral, parenteral, ophthalmic, topical and other routes of administration. These conjugates are also expected to enhance other pharmacological activities of anecortave such as antitumor activity. Besides, these conjugates may serve as good carriers of anecortave in pharmaceutical dosage forms, possibly with its sustained release. In addition, PEGylated derivative of anecortave, a water soluble derivative of anecortave, was synthesized and its angiostatic activity determined and compared with those of the conjugates described herein.

Figure-1:

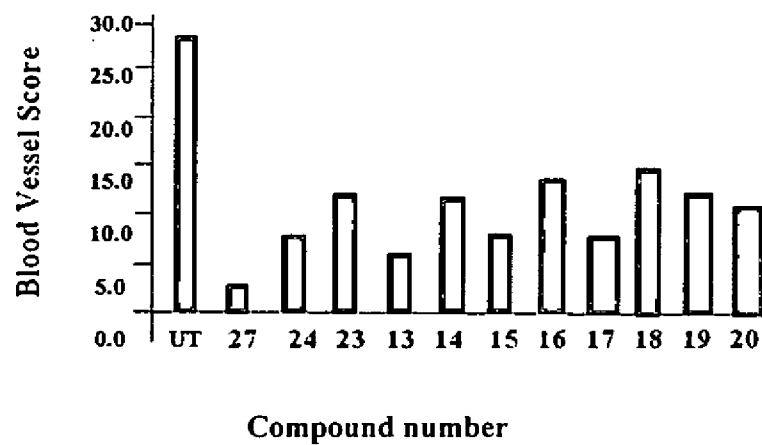
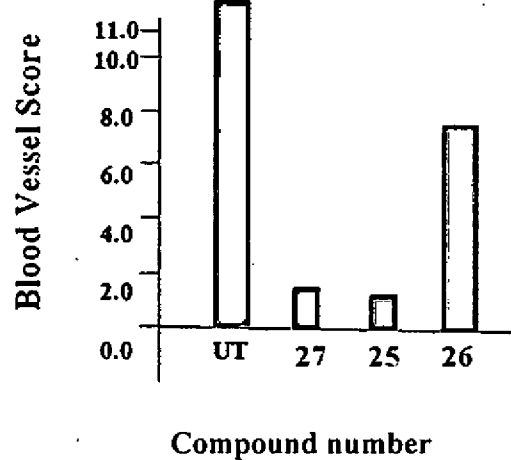


Figure-2:



Signature: _____

Name: Dr. Dr.C.S.VENKATESAN

Gland Pharma Ltd