REVIEWS

Peptides and Proteins as Drugs[1]

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Abstract: Relatively small modifications of clinically useful endogenous compounds have been shown to have therapeutically beneficial effects on their pharmacodynamic and pharmacokinetic properties. These effects include increased potency and effect selectivity, and prolonged duration of action. In addition, these modifications have resulted in compounds that can be administered orally where only parenteral administration was previously possible. One type of modification resulting in distinctive properties is exemplified by the hybrid interferons produced by the recombination of segments of genes coding for different molecular species. Chemical modification has also resulted in many examples of analogs of natural peptides that are more potent, more selective and more stable than the endogenous compounds. Conjugation to peptide or protein carriers is a third method used to selectively modify the properties of an endogenous compound. The carriers that have been used include synthetic polypeptides, endogenous proteins, toxins and monoclonal antibodies. The effect that covalent attachment to a carrier has on the properties of a ligand is highly dependent upon the carrier, the ligand and the linkage between them.

The human body produces a host of compounds specifically designed to interact with perfectly matched receptors to produce or regulate a variety of physiologically important responses. Where pathological processes create a disequilibrium between these endogenous agonists and their corresponding receptors, clinicians attempt to reestablish homeostasis through the administration of exogenous pharmacologic agents. A more rational approach to therapeutics would take advantage of the natural endogenous compounds in the treatment of disease states. However, the clinical usefulness of many important endogenous proteins and peptides and other endogenous compounds has been limited by their ubiquitous pharmacologic actions which may lead to undesirable side effects, and by their poor pharmacokinetic properties which result in prohibitively short durations of action and ineffective concentrations at the appropriate sites of action, thus limiting the use of convenient routes of administration. There has been increasing interest recently in the use of specifically modified analogs of these endogenous entities and/or the use of novel drug delivery systems to improve potency, increase effect selectivity, prolong duration of action and provide more convenient routes of administration. These techniques also provide a means by which pharmaceutical scientists may explore the mechanisms of action of these compounds, which leads to directed and selected modifications guided by prior knowledge instead of the frequently employed "shotgun" approach. Some of these goals have been achieved in the last few years by modifications of naturally occurring molecules using recombinant DNA techniques, chemical techniques and by the conjugation of the endogenous compounds or their analogs with synthetic peptides or naturally occurring proteins. These concepts will be briefly reviewed.

Recombinant DNA Techniques

Recombinant DNA techniques have been used to manipulate the properties of human leukocyte interferons, endogenous proteins that potentially may serve as antiviral and antineoplastic chemotherapeutic agents. Multiple molecular species of the alpha interferons are known (1, 2). Two of these alpha interferons have been designated LeIF-A and LeIF-D. The genes coding for these proteins have been cleaved with restriction endonucleases and the resulting gene fragments have been recombined to form hybrid genes which are expressed in *E. coli* (2). The hybrid interferons produced by this procedure (LeIF-AD and LeIF-DA) have properties unlike either parent interferon or mixtures of the parent interferons. The LeIF-AD hybrids have significantly greater antiviral activity than the parent interferons measured in cells from several different species and after challenge with several different viruses. The different hybrids exhibit specificity in activity with regard to cell species and virus; a relatively wide variation in the ratio of antiproliferative to antiviral activity on human cells has also been observed. Modified analogs of human interferon with unique properties have also been produced by complete gene synthesis (1); this technique permits the introduction of specific modifications. These results suggest that new, more potent or selective interferons and perhaps other proteins may be produced by recombinant DNA methods.

Chemical Modification

Chemical modification has also been used to produce analogs of endogenous peptides to improve one or more of their characteristics so that they may become viable therapeutic agents [for review, see reference (3)]. An excellent example is the work done on analogs of somatostatin. Endogenous somatostatin inhibits the secretion of several other hormones including insulin, glucagon, growth hormone and several intestinal peptides. It has been proposed as a potential therapeutic agent for the treatment of acromegaly excess growth hormone) and diabetes mellitus (excess glucagon). However, its use has been severely limited by its diverse effects on the secretion of many hormones and its short duration of

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action which requires frequent, if not continuous, parenteral administration (4). In 1982, an octapeptide analog of somatostatin was reported (4) to possess improved properties compared to the natural hormone. Tested *in vivo* in rats, this octapeptide analog is at least 70 times more potent than somatostatin in inhibiting the secretion of growth hormone when the compounds are administred by the i.v., i.m. and s.c. routes. The octapeptide analog also exhibits greater effect selectivity. It is 22 times more growth hormone-to-insulin selective than somatostatin and 3 times more growth hormone-to-glucagon selective in rats after i.m. administration. In addition, the analog had a much longer duration of action and was orally active. The structural modifications that lead to these improved properties include the deletion of six of somatostatin's 14 amino acids and substitution of D-amino acids for L-amino acids at 2 positions. It has been shown (5) that the single substitution of D-Trp for L-Trp at position 8 increases the potency eightfold, probably by increasing the molecule's stability to enzymic degradation (the 8-9 peptide bond is an important site of endopeptidase mediated cleavage). [Other somatostatin analogs are discussed in references (3, 6, 7)].

Roemer et al. (8) developed an analog of met-enkephalin that was more potent than the parent drug, had a longer duration of action and demonstrated analgesic activity when administered orally. Met-enkephalin itself produces a weak and short-lived analgesia even after intracerebral ventricular administration in mice. Its effect (in the tail-flick test) is maximal after 2 minutes, with a duration of 5 minutes (9). Intravenously administered met-enkephalin displays only transient effects even at high doses; analgesic activity is detectable only within the first 15 seconds after administration. In contrast, the analog's effect lasted at least 30 minutes after i.v. administration (8). The analog also had a much longer duration of action by the subcutaneous and oral routes. The substitution of D-Ala for glycine at position 2 may have increased the analog's stability to enzymic attack since the Tyr^1-Gly^2 bond is a site of rapid enzymatic degradation by aminopeptidase. *N*-Methylation at the Gly^3-Phe^4 bond may also prolong the analog's activity since this is the site of enzymic attack by enkephalinase. [Other enkephalin analogs are discussed in reference (3)].

In vivo increases in potency may result from enhanced affinity for the receptor, increased intrinsic activity, increased analog stability (allowing greater concentrations to reach the site of action), improved physico-chemical properties that permit increased access of the analog to its site of action (e. g. lipophilicity) or restriction of the molecule to a more active conformation (3). Increased effect selectivity probably results from these same properties: the analog may have a more restricted conformation permitting its interaction with a specific subset of receptors, or because of its altered physical properties, it may be better able to reach a sequestered population of receptors than the parent compound. In natural peptides of significant length (or proteins) there may be more than one amino acid sequence responsible for biological effects (3); in these cases elimination of superfluous message sequences also may increase the specificity of effect. A prolonged duration of action may result from increased resistance to enzymic degradation, increased lipophilicity with a concomitant increased distribution volume and prolonged half-life, or increased reabsorption by the renal tubules.

Other peptides and proteins whose characteristics have been manipulated by chemical modification (3) include thyrotropin releasing hormone (10), lutenizing hormone releasing hormone (10, 11), parathyroid hormone (12), glucagon (13), substance P (14), vasopressin (15), β-endorphin (16), growth hormone and prolactin (17), and corticotropin (ACTH) (18). An efficient analog development strategy (12, 19) involves characterization of the natural compound in various species so as to prioritize various molecular regions. Systematic comparison of analogs containing a variety of deletions will yield the minimum active sequence. Synthesis of analogs modified at single sites in these active sequences may clarify which amino acids are important for biological activity. Application of these steps should in the future lead to the predictable preparation of compounds with specifically altered properties.

Conjugation to Carriers

Another approach to the selective elimination of the undesirable properties of an endogenous compound while preserving its desirable characteristics involves conjugation to protein or peptide carriers. A naturally occurring example may be found in the pair met-enkephalin and β-endorphin. The 31 amino acid β-endorphin shares its amino terminal sequence with met-enkephalin, which may be considered to be the free or unconjugated drug. After i.v. administration, met-enkephalin has a weak and short-lived analgesic effect, while the conjugated or carrier-linked form, represented by β-endorphin, causes marked and relatively long-lived analgesia (3, 8) when it is administered by the same route. There are other examples of naturally occurring proteins that may be regarded as conjugates. These are multi-subunit proteins where the subunits have different roles in the protein's overall effect: one subunit mediates binding and the other is the pharmacologically active entity. Endogenous examples of this type of protein include the pituitary hormones: lutenizing hormone, follicle stimulating hormone and thyroid stimulating hormone, and the placental hormone, human chorionic gonadotropin. All four consist of one α and one β subunit. The α subunits are all very similar while the receptor specificity is directed by the β subunits.

This type of conjugation has been exploited in the design of new compounds that link a protein or its active subunit with the receptor binding subunit of another protein that has a different biological activity. The biological effect of the "ligand" subunit may thus be expressed after binding to the receptor for the "carrier" subunit. This technique may be useful for targeting purposes: increasing the effective concentration at the desired site of action and minimizing the concentration at other sites. An example of this kind of conjugate is the interferon-ricin toxin B (INF-RTB) chain conjugate described by Anderson and Vilcek (20). Ricin toxin is a dimeric protein toxin isolated from castor beans. Its two subunits are designated A and B. The B chain specifically interacts with galactose residues on sensitive cells to facilitate internalization of the toxic A chain which catalytically inactivates the 60S ribosomal subunit thereby causing inhibition of protein synthesis. The interferon-ricin B conjugate appears to possess interferon activity mediated through the receptor for the ricin B chain. The specific binding and the antiviral activity of the conjugate were inhibited in the presence of galactose (an inhibitor of ricin B chain binding) (20). Antiserum to interferon neutralized the antiviral activity of both interferon and the conjugate. Since breakdown of filtered interferon in the kidney after reabsorption into the renal tubular cells is an important route of interferon loss (1), increasing molecular size through conjugation may also prolong the duration of action by preventing filtration or

providing resistance to degradation. A conjugate between the ricin toxin B chain and insulin has also been shown to express insulin's biological activity through binding to the ricin receptor (21).

Converse experiments linking the toxic ricin A chain to the receptor binding portion of other proteins have also been described. These conjugates exhibit receptor specificities that reflect the binding properties of the protein to which the A chains are conjugated. Epidermal growth factor (EGF) is one of the proteins used as the cell-targeting entity (22). This is a small (6000 MW, 53 amino acid) protein that stimulates the growth and differentiation of epidermal tissues during embryogenesis and enhances cell growth. The ricin A-EGF conjugate and ricin itself had similar potencies in the inhibition of protein synthesis. Ricin A alone was 4 orders of magnitude less potent. Antiricin A inhibited the activity of both ricin and the conjugate, though lactate (an inhibitor of ricin B binding) and antiricin B only affected ricin's activity. In addition, EGF itself blocked the toxicity of the conjugate, strongly suggesting that the conjugate's activity was mediated through EGF receptors. The receptor binding subunit (β) of human chorionic gonadotropin (23) has also been linked to the toxic ricin A chain. Many other proteins have been used as carriers for ligands including tobacco mosaic virus (24), albumin (25), transferrin (26), α2-macroglobulin (27), insulin (28) and concanavalin A (29, 30). Synthetic polypeptides have also been used as carriers for ligands such as muramyl dipeptide (31). The conjugation in these cases is often multiple and results in increased potency, increased receptor affinities, enhanced resistance to enzymic degradation and prolonged durations of action. Cell surface receptor aggregation, cooperative effects or an increased effective local concentration of the ligand at the receptor may play a role in the improved properties of these multiple-ligand conjugates (3). Sequestration of the large conjugate at the receptor site may also be important. In the case of the antitumor agent *N*-phosphonoacetyl-L-aspartic acid linked to peptides (32) it has been postulated that the peptide provides the entrée into the cell for the antitumor drug.

Recently this method has been applied to monoclonal antibodies as carriers for toxic protein subunits or chemotherapeutic agents. These conjugates may have wide potential application in therapeutics or as diagnostic aids (33–37). The toxic ricin A chain has been conjugated to a monoclonal anti-carcinoembryonic antigen (CEA) antibody (38, 39). This conjugate was strongly cytotoxic *in vitro* to CEA-producing tumor cells and demonstrated cytotoxic effects *in vivo* on free tumor cells, but it failed to affect tumor growth for established solid tumors. The ricin A chain and pokeweed antiviral protein have been conjugated to anti-Thy 1.1 and anti-Thy 1.2 monoclonal antibodies (40–42); these conjugates selectively kill leukemia cells containing the Thy 1.1 antigen *in vitro* and *in vivo* in mice. One study (41) suggests that the formation of anti-toxin antibodies may limit the ability of a conjugate to be used for the treatment of relapsing disease. Human tumor cells have been shown to be killed specifically *in vitro* by anti-transferrin receptor monoclonal antibodies covalently linked to the toxic subunits of ricin or diphtheria toxin (43). A conjugate of ricin A with antibody to human lambda and kappa chains killed cells from the human lymphoblastoid B-cell line, Daudi, but was harmless to colony-forming cells in normal human marrow (44). Human alpha interferon coupled to monoclonal antibodies directed against Epstein-Barr virus membrane antigen has been shown *in vitro* to retain its antiviral and antiproliferative effects (45). The conjugate acted selectively on those cells containing the target antigen, and its activity appeared to be increased compared to the unconjugated compound. A methotrexate-albumin-monoclonal antibody conjugate showed selective toxicity toward a human osteogenic sarcoma cell line *in vitro* (46). A macromomycin/anti-HLA monoclonal antibody conjugate was shown to be specifically cytotoxic *in vitro* to leukemia cells expressing HLA (47). Chlorambucil/anti-carcinoembryonic antigen conjugates were cytotoxic *in vitro* at much lower concentrations than the free drug against human colon carcinoma cells that produce CEA (48). Similar results were obtained *in vitro* and *in vivo* in mice with daunomycin linked to a variety of anti-tumor antibodies (49). Conjugates of intact ricin and anti-T cell monoclonal antibodies have been suggested as potential agents to prevent graft-versus-host disease in man (34, 37, 44, 50–52). In addition, it has been suggested that conjugates with ricin A chain alone are not as effective in cell killing as conjugates with intact ricin. One group (53) has suggested that the Fab′ fragment of an antibody is sufficient to act as the carrier for a cytotoxin (diphtheria toxin A chain).

Monoclonal antibodies alone may be used therapeutically for their neutralizing, opsonizing or cytotoxic actions (33, 43, 54–60); in addition, the Fab fragments of antibodies have been shown to facilitate the removal of drugs (digoxin) in overdose patients (33). Monoclonal antibodies have also been used as diagnostic aids in tumor identification (59, 61, 62) and as carriers for radioisotopes for the treatment (63, 64) or imaging of primary tumors, metastases, thrombi or infarcts (33, 64–68).

Model Studies with Drug-Carrier Conjugates

In general, little is known about the carrier characteristics that are crucial for the selective modification of a ligand's properties. As noted above, the conjugation may be multiple and uncontrolled, and peptide carriers may be polydisperse and poorly characterized. In addition, it is often unclear whether the carrier modifies the free ligand's pharmacokinetic behavior, or if an actual change in the pharmacodynamic effects of the drug is achieved. We have collaborated in a systematic investigation of the carrier and linkage properties that are important to the optimization of a ligand's pharmacological effects for a series of new drugs that are models for compounds such as isoproterenol, propranolol, practolol and histamine conjugated to monodisperse peptide and protein carriers (69, 70). These studies illustrate a method for probing the molecular environment of the receptor-ligand interaction to determine its crucial characteristics; the results of these studies should provide us with the ability to predict the results of future modifications and lead to the rational design of drug conjugates. The carriers used have been varied systematically with respect to the number of drug molecules conjugated to each carrier, the spacing between the ligands, the hydrophobicity of the carrier and the carrier's molecular size (71, 72). It was found that each carrier and linkage had to be optimized individually for the particular ligand so as to preserve the desirable pharmacologic properties and critically modify other properties.

The general structures for some of the compounds investigated are shown in Figure 1. These compounds are models for the attachment of the ligand to a peptide or protein through a *p*-aminophenylalanine residue. Table I shows the effect of varying the linkage group length (73). For the isoproterenol and propranolol series, maximum activity occurs when the spacer group is 4 carbons long (n=4); while for histamine, the

maximum occurs when n=3. Table II shows the effect of changing the substituent (73–77). The *p*-trifluoromethyl derivative is the most active compound in the isoproterenol and histamine series, through no increase is seen for the same derivative in the β-blocker series. In addition, in the *ortho* position this substituent decreases activity. Further pharmacological studies have focused on the compounds shown in Figure 2, where isoproterenol is used for comparison. Compounds **119** and **143** are models of isoproterenol conjugated to a peptide or protein and **149** is a dipeptide conjugate. Studies have shown that compounds **143** and **149** are more potent *in vitro* in the stimulation of cAMP accumulation in S49 mouse lymphoma cells and *in vivo* in the reduction of blood pressure in anesthetized rats than isoproterenol (69). A prolonged effect with these compounds was observed on contractile force in isolated guinea pig atria compared to free drug (isoproterenol), and there appeared to be a separation of chronotropic and inotropic effects for these compounds (69). Preliminary studies in dogs indicate that compounds **143** and **149** administered intravenously exhibit positive inotropic effects that persist for 80 to 100 minutes (69, 74).

Pharmacokinetic and metabolic studies on these compounds have been initiated. High specific activity tritium derivatives of **119, 143** and **149** have been synthesized by reductive amination with carrier free tritium gas (78) to establish the metabolic pathways and plasma elimination kinetics of the intact molecules and their relevant metabolites to attempt to correlate their kinetic parameters with the established pharmacodynamic profiles.

The numerous studies cited above illustrate that drug attached to a pharmacologically inert carrier may retain its useful pharmacological properties and acquire more desirable characteristics such as increased selectivity of effect, prolonged duration of action and increased flexibility with respect to routes of administration (oral bioavailability). It is apparent that structural changes in the inert carrier portion of a conjugate relatively far from the pharmacologically active portion of the ligand result in distinct improvements in the free drug's pharmacological properties (69). For compounds of this type,

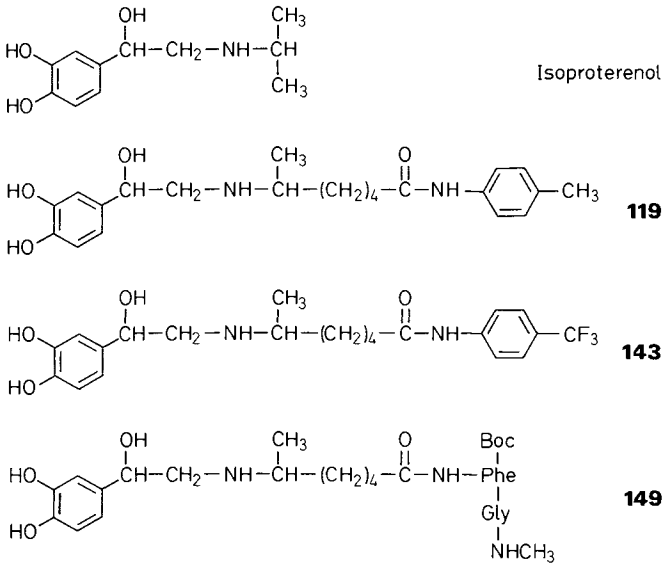


Fig. 1 General structures of conjugate model compounds.

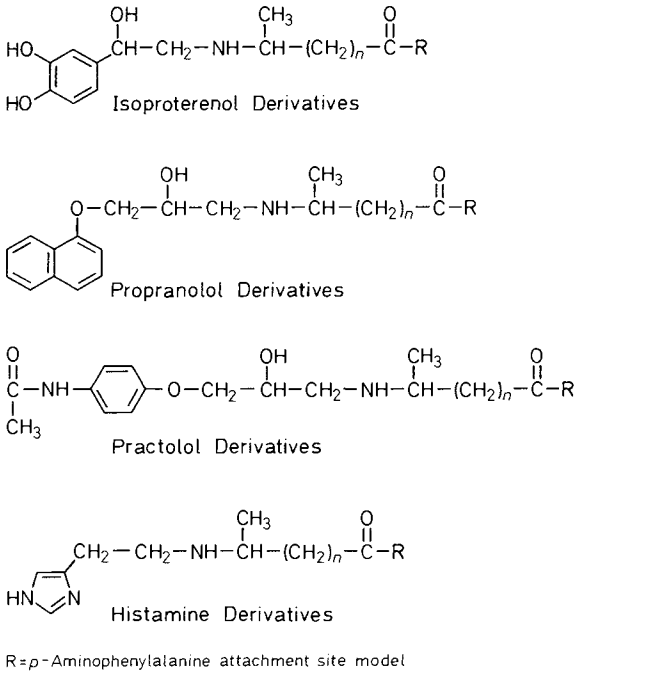
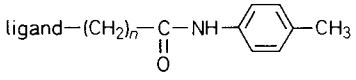


Fig. 2 Structures of models of isoproterenol conjugated to peptide or protein carriers.

Table I. Comparative potencies of similar isoproterenol, β-blocker and histamine derivatives: variations in length of linkage group.

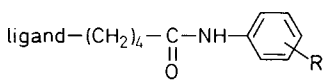


Ligand[a,b]

n	Isoproterenol	Propranolol	Practolol	Histamine
5	1.1×10^{-1}	1.4×10^{-2}	–	0.2×10^{1}
4	5.4×10^{1}	4.5×10^{2}	2.1×10^{1}	0.3×10^{1}
3	3.1×10^{-1}	6.8×10^{-5}	Inactive	3.0×10^{3}
2	6.4×10^{-1}	6.8×10^{-5}	–	–

[a] Potency of derivative is expressed relative to the parent compound as measured by cAMP accumulation in S49 mouse lymphoma cells (isoproterenol, propranolol and practolol) and in a murine T cell clone (histamine) (see reference 73).
[b] Refer to Figure 1 for ligand structures.

Table II. Comparative potencies of similar isoproterenol, β-blocker and histamine derivatives: effect of substituents.



Ligand[a,b]–

R	Isoproterenol	Propranolol	Practolol	Histamine
p-CH_3	5.4×10^{1}	4.5×10^{2}	2.1×10^{1}	0.3×10^{1}
p-CF_3	9.5×10^{1}	0.9×10^{1}	0.6×10^{1}	4.2×10^{4}
o-CF_3	2.6×10^{-4}	1.3×10^{-2}	–	Inactive

[a] Potency relative to isoproterenol, propranolol and practolol measured by cAMP accumulation in S49 mouse lymphoma cells and for histamine in a murine T cell clone (see references 73–77).
[b] Refer to Figure 1 for ligand structures.

however, tertiary structure and the effect of numerous optically active centers must also be considered.

Conjugation to the appropriate carrier (e. g. monoclonal antibodies) may provide the means for the specific delivery of a drug to specific tissues or sets of cells and open new avenues of approach to the treatment of disease states.

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Sympathomimetic Bronchodilators: Increased Selectivity with Lung-Specific Prodrugs

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Abstract: The development of selective bronchodilator β-adrenoceptor agonists is reviewed with emphasis on a *pharmacodynamic approach,* which is directed to drugs with high specificity for the β_2-adrenoceptor, and on a *pharmacokinetic approach* in which known β-adrenoceptor agonists are converted to prodrugs with selectivity for the lung. The pharmacodynamic approach has produced drugs that display high specificity for the β_2-adrenoceptor but still suffer from side-effects including tremor and palpitations. This is due to the fact that the β_2-adrenoceptors present in skeletal muscle and blood vessel are indistinguishable from those in the airways. On the other hand, the prodrug pharmacokinetic approach offers a promising way to obtain selectively acting bronchodilators with significantly fewer side-effects.

For more than ten years selective β_2-adrenoceptor (β_2-AR) agonists have been valuable drugs in the treatment of bronchial asthma. The development of these drugs is an example of the effective utilization of the principle of pharmacologic selectivity and receptor specific drug design. The history of this field began with the α-, β_1- and β_2-AR agonist epinephrine which was introduced at the turn of this century and proceeded via the β_1- and β_2-AR-agonist isoproterenol discovered in the fifties to the almost pure β_2-AR agonists we have today. This effort has resulted in the availability of safer drugs that provide improved and more convenient therapy. Increased safety of the newer β_2-AR agonists is mainly due to elimination of the undesirable β_1-AR cardiac stimulating activity found in many of the older drugs. The side-effects generally encountered with the β_2-AR agonists are tremor, palpitations and nervousness. Tremor usually is the dose-limiting factor with the newer drugs, whereas with the unselective β-AR agonists the more dangerous tachycardia is dose-limiting.

This review will focus on the search for more selectively acting β_2-AR bronchodilators, i. e. for compounds with a significantly increased therapeutic efficacy. The research in this field has progressed along the following two principal paths:

i) The pharmacodynamic approach in which the aim was to design agonists with increased selectivity for the β_2-AR.

ii) The pharmacokinetic approach in which known, therapeutically effective β_2-AR agonists are used as parent compounds in the design of lung-specific prodrugs.

This review will discuss the principles of drug design in this field and will use representative drugs to illustrate different approaches that attempt to optimize the therapeutic efficacy of candidate drugs through consideration of both pharmacodynamic and pharmacokinetic principles.

The β-Adrenoceptor

In 1948 Ahlquist (1) introduced a classification which described adrenergic receptors as α- and β-AR. A further refinement of this classification was made by Lands in 1967 (2). He proposed a subclassification of the β-AR into the β_1-AR and the β_2-AR with the suggestion that the β-AR of an organ could be classified as belonging to either of the subgroups. The variety of effects mediated through β-AR is given in Table I, with those effects relevant to the treatment of asthma printed in bold faced type. In 1972 Carlsson et al. (3) further refined Lands' theory by presenting evidence for the co-existence of both β_1- and β_2-AR in the same organ. Both receptors

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