Emerging Drugs – Potential for Misuse in Sport and Doping Control Detection Strategies

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Abstract: Preventive doping research includes the development of methods for the detection of new or emerging drugs to be implemented in routine screening analysis. Candidates with great potential for misuse in elite sports include selective androgen receptor modulators, growth hormone secretagogues, hypoxia-inducible factor stabilizers and erythropoietin mimetics.

Key Words: Doping, sport, mass spectrometry, SARMs, EPO, HIF, growth hormone, secretagogues.

INTRODUCTION

Enhancing physical performance by means of legal as well as illegal drugs has been a temptation to athletes ever since the inception of sport competitions. Development of pharmaceutical products for the treatment of serious diseases and the contemporary search for effective anti-ageing drugs provide a reliable pipeline of new therapeutics. This might be subject to misuse in order to provide the winning edge to athletes, especially in professional sports. Doping control laboratories are continuously confronted with new therapeutic agents that possess great potential for being misused in elite sports. Hence, the list of prohibited substances as established by the World Anti-Doping Agency (WADA) as well as the corresponding doping control strategies have become highly dynamic, and frequent updates and improved analytical assays are essential [1-3]. Here, one of the most important features of the prohibited list is the fact that it includes not only defined drugs but also entire classes of compounds in a chemical as well as pharmacological manner. Hence, compounds or therapeutics with structures or effects similar to banned analogs are prohibited too. An important task of so-called "preventive doping research" is to establish detection methods for new, emerging drugs that might be candidates for abuse in elite sports due to their clinical and pharmacological properties. Analytical procedures need to be implemented in routine screening as soon as new compounds are available, preferably before they enter the pharmaceutical market.

Drug testing laboratories concentrate not only on traditional performance enhancing drugs such as stimulants and anabolic androgenic steroids but also on recently developed therapeutics, including those which have yet to obtain regulatory approval. Peptide hormones identical to or modified from natural analogues as well as new low-molecular weight drugs have extended the portfolio of compounds possessing great potential for misuse in sports. Hence, new assays or extended routine screening procedures with conventional approaches are required to enforce sports drug testing activities.

NEW DRUGS WITH ANABOLIC PROPERTIES

Selective Androgen Receptor Modulators (SARMs)

Endogenous androgens such as the primordial matter testosterone (Fig. (**1**)) [4-6] are vital to male development [7] and secondary male characteristics such as muscle mass, bone mass and body composition [8]. Testosterone in particular has beneficial effects on physical performance [9], and numerous structural analogues have been synthesized in order to produce even more potent compounds. Synthetic analogues of testosterone have been developed and clinically employed for the treatment of anemia and other debilitating diseases [10], and more recently also for treatment of hypogonadal conditions, delayed puberty, as well as the regulation of male fertility [11, 12]. However, serious side effects associated with steroid replacement therapies including hepatic toxicity, decreased levels of HDL cholesterol, negative influences on prostate and cardiovascular systems, and gynecomastia based on the lack of tissue selectivity [13] have led to the development of numerous non-steroidal drug candidates referred to as SARMs (reviewed in [14]).

There are a minimum of 5 classes of SARMs as illustrated in Fig. (**1**), where representatives of each group are presented [15]. The categories include a) propionanilides, b) tricyclic quinolines, c) tetrahydroquinolines, d) bicyclic modulators, and e) prolyl urea derivatives, all of which have demonstrated *in vivo* and/or *in vitro* androgen receptor (AR) agonist activities with significantly reduced side effects. The AR is a nuclear hormone receptor that controls numerous physiological functions, not only the initiation of male sexual development and differentiation, but also protein anabolism and erythrocyte production. Various studies were conducted to characterize the AR structure and ligand binding domains [16, 17] using dihydrotestosterone (DHT, Fig. (**1**)) or methyltrenbolone (methyltrienolone) as model ligands. Four amino acid residues of the AR, namely Gln 711, Arg 752, Asn 705 and Thr 877, were identified to play key roles in ligand binding affinities and resulting activation or inhibition.

Steroid binding to the AR is predominantly supported by H-bonding of Arg 752 to the 3-keto function of DHT or T, which stabilizes the initial AR-steroid binding despite its comparably weak interaction [18]. Stronger H-bonds are established between the 17β-OH functions of DHT and T to Asn 705 and Thr 877 [19], and a considerable decrease in receptor binding affinity is observed when the 3-keto/17β-OH composition of steroids is destroyed. However, the 17β-OH residue is more important than the 3-keto functionality, presumably due to the stronger nature of the H-bonding. In addition, hydrophobicity and steric bulk of ligands have also been studied, demonstrating that spacious substituents such as alkyl residues at C-6 or C-7, C-11 or C-12 as well as C-17 contribute to an increased receptor binding affinity [16]. Based on these findings, model SARMs such as propionanilides and tricyclic quinolines [20-23] or bicyclic modulators [24] were optimized in terms of favorable receptor-ligand interactions. Compounds of utmost efficiency with improved oral bioavailability, androgen receptor specificity combined with tissue selectivity, and the lack of steroid-therapy related side effects have been developed [13-15]. Detailed receptor binding studies were conducted with propionanilides such as compound S-4 (Fig. (**1**)), the functional groups of which resemble all important features of DHT, promoting a high binding affinity to the AR. The nitro residue and the chiral hydroxyl function of S-4 mimic the 3-keto- and 17β-OH-group of DHT, respectively, establishing interactions with the earlier identified amino acid residues Gln 711, Arg 752, and Asn 705 [22]. Analogously, receptor binding studies with a bicyclic modulator (BMS-564929, Fig. (**1**)) were

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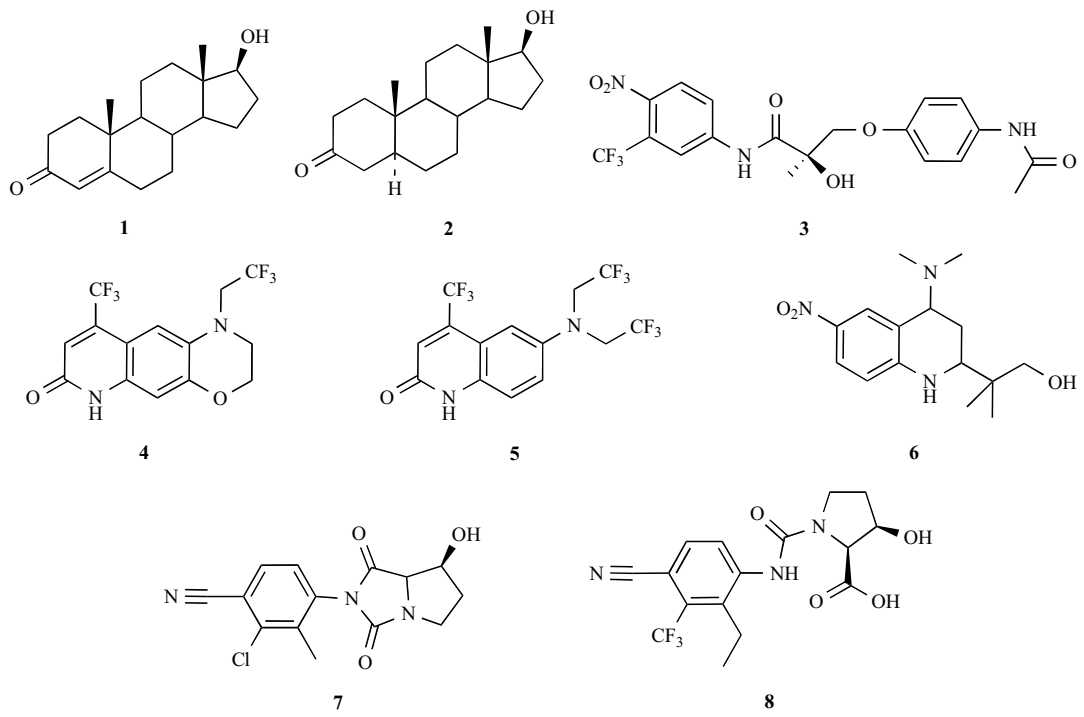

Fig. (1). Chemical structures of anabolic agents: testosterone (**1**, mol wt = 288), dihydrotestosterone (**2**, mol wt = 290), propionanilide derived SARM S-4 (**3**, mol wt = 441), quinoline SARMs (**4**, mol wt = 352; LG-2226, **5**, mol wt = 392), tetrahydroquinoline SARM S-40503 (**6**, mol wt = 293), bicyclic hydantoin SARM BMS-564929 (**7**, mol wt = 305), prolyl urea SARM (**8**, mol wt = 371).

recently published where ligand-receptor interactions were also observed at amino acids Arg 752, and Asn 705 [24].

Numerous SARMs are currently undergoing pre-clinical or clinical trials and have proven to act as full agonists in anabolic target tissues (e.g. muscle and bone) [25]. Their potential for clinical utility is widespread and promising in particular for the treatment of hypogonadism, osteoporosis, muscle wasting, and numerous symptoms associated with androgen decline in ageing males (e.g. sexual dysfunction, altered cognition, etc.). In contrast to anabolic-androgenic steroids they demonstrated only partial agonist activity in androgenic tissues such as prostate and seminal vesicles; hence, they have been immediately considered relevant for doping controls as they possess an enormous potential for misuse in elite sports.

Doping Control Detection Strategies

Although SARMs belong pharmacologically to the class of anabolic agents as categorized by the World Anti-Doping Agency (WADA) [26], their physicochemical properties are considerably different from commonly screened steroidal drugs and compounds. In sports drug testing, steroids are preferably measured using gas chromatographic-mass spectrometric (GC-MS) assays [1, 3, 27], but SARMs have demonstrated a distinguished suitability for liquid chromatography and electrospray ionization (tandem) mass spectrometry (LC-ESI-MS(/MS) [28]. In addition, the chemical diversity of SARMs requires a comprehensive screening and identification tool for drug testing laboratories.

A first approach to determine propionanilide-derived SARMs (e.g. S-4, Fig. (**1**)) and also potential metabolites, was recently published using four metabolically stable model compounds [29]. Target analytes were isolated from urine specimens using common solid-phase extraction (SPE) techniques [30] followed by negative ionization LC-MS/MS analysis, which employed simultaneous MS experiments. SARMs of known composition and structure were measured using targeted multiple reaction monitoring (MRM) for utmost sensitivity, while precursor ion scanning on product ions generated from common core structures was utilized to cover a broad variety of possible derivatives. As known from the structure-activity-relationship studies (*vide supra*), distinct functional groups of SARMs are essential for sufficient receptor binding affinities leading to agonistic activities. Product ions resulting from substituted anilide residues were considered for the precursor ion scanning approach enabling the detection of numerous additional propionanilide-derived SARMs and also potential metabolites, some of which have recently been elucidated [31]. The detection limits for the selected SARMs ranged from 1ng/mL to 50ng/mL depending on the detection method, i.e. whether MRM or precursor ion scanning was employed. Although none of the presented SARMs are commercially available, sports drug testing laboratories should implement these emerging drugs into regular screening protocols. Several doping cases have demonstrated that therapeutics are surreptitiously used in sports even without full clinical approval [32-36].

Growth Hormone Secretagogues (GHS)

As early as 1977, the first synthesis of GH releasing peptides [37] initiated a new field of research aiming the elucidation of mechanisms underlying the GH generation, release and mode of action, as well as the possibility of artificially stimulating GH secretion. Based on the knowledge of the direct relation of growth, ageing and anabolism with the GH / insulin-like growth factor-1 (IGF-1) axis [38], numerous studies were conducted on the synthesis of compounds able to trigger the release of GH in animals and humans. Momany and Bowers [39-44] prepared peptide analogs of Leu- and Met-enkephalins that were capable of stimulating isolated rat brain cells to secrete GH. These syntheses yielded, amongst others, the GH releasing peptide 6 (GHRP-6, Table **1**) that has become a reference compound for comparison with new candidates of

Table 1. Primary Structures of Peptides with Growth Hormone Secretagogue Activity

Compound	Sequence
GHRP-1	Ala-His-D-βNal-Ala-Trp-D-Phe-Lys-NH_2
GHRP-2	D-Ala-D-βNal-Ala-Trp-D-Phe-Lys-NH_2
GHRP-6	His-D-Trp-Ala-Trp-D-Phe-Lys-NH_2
Hexarelin	His-D-Mrp-Ala-Trp-D-Phe-Lys-NH_2
Ipamorelin	Aib-His-D-2-Nal-D-Phe-Lys-NH_2
Alexamorelin	Ala-His-D-Mrp-Ala-Trp-D-Phe-Lys-NH_2

Non-standard abbreviation:
Aib = aminoisobutyric acid, Nal = naphthylalanine, Mrp 2-methyltryptophane

GHS. Due to the potential clinical benefit of such compounds, in particular for the treatment of GH deficient children and adults as well as their properties to improve body composition in elderly subjects, GHS have received considerable attention. Smith *et al*. reported the synthesis of the first non-peptidyl GHS in 1993 [45], which subsequently enabled the identification and characterization of the endogenous receptor (GHS-R) for synthetic GHS as well as the corresponding signal transduction pathway [46, 47]. The receptor's structural elucidation revealed a novel G-protein-coupled species [48] with 7 transmembrane domains encoded by GHS-R-Ia mRNA. A second mRNA (GHS-R-Ib) variant was discovered encoding a protein comprising only 5 transmembrane domains, the function of which is currently not clear [49]. The knowledge of the GHS-R structure allowed the identification of its natural endogenous agonist, Ghrelin, in 1999 [50] and a comprehensive review on the composition and activation of the Ghrelin receptor has recently been published [49].

Numerous GHS drug candidates have been prepared during the last decade [37, 47, 51-62], and most compounds demonstrated efficacy after intravenous, intranasal as well as oral administration in humans [63] although none of these has yet obtained clinical approval. A selection of potent non-peptidyl growth hormone secretagogues is depicted in Fig. (**2**), which contains six chemical classes namely benzolactame derivatives, quinazolinones, 4-spiropiperidines, capromorelin derivatives, tetrahydroisoquinoline 1-carboxamides and oxinole derivatives.

Based on molecular modeling and computational design strategies using peptidyl and non-peptidyl model compounds, six pharmacophoric sites were assigned to GHRP-6 [64]. Most of these sites, which include two aromatic ring structures, a proton acceptor residue (e.g. a keto function) and a protonated amine adjacent to a small hydrophobic group, are common in peptidyl and non-peptidyl GHS and share a binding domain in the GHS-R. Contacts to the different transmembrane domains 2, 3, 5 and 6 were identified, and essential amino acid residues were characterized as Asp 99, Cys 116, Glu 124, Met 213, Ser 217, and His 280 [65, 66].

Several GHS drug candidates with potential use for treatment of a variety of pathologies have advanced to clinical trials [67]. Beneficial effects of GHS-based therapies are expected in the treatment of GH deficiencies in children and adults assuming a dysfunctional hypothalamo-pituitary unit. The treatment of catabolic states and age-related frailty is another promising application. Rejuvenation of the GH/IGF-1 axis by GHS has been described where 70-90 years-old subjects produced serum IGF-1 levels and GH pulse amplitudes that are typically observed in healthy persons younger than 30 years. Improvements of body composition, strength and bone density were reported, and increased levels of IGF-1 were sustained for more than 12 months [52, 68]. Additional possible benefits have been summarized in a recent review, including positive effects on metabolism (resulting in an increased lean body mass and reduced fat deposition), on skeletal muscle tissue, on the ageing brain, as well as the ageing immune system. These results are likely to encourage athletes to (mis)use this emerging class of drugs and improve their physical constitution and resulting athletic performance.

Doping Control Detection Strategies

In addition to specifically designed GHS, growth hormone secretagogue activity has been described for many drugs [69] including cerebral-selective acetylcholine esterase inhibitors commonly

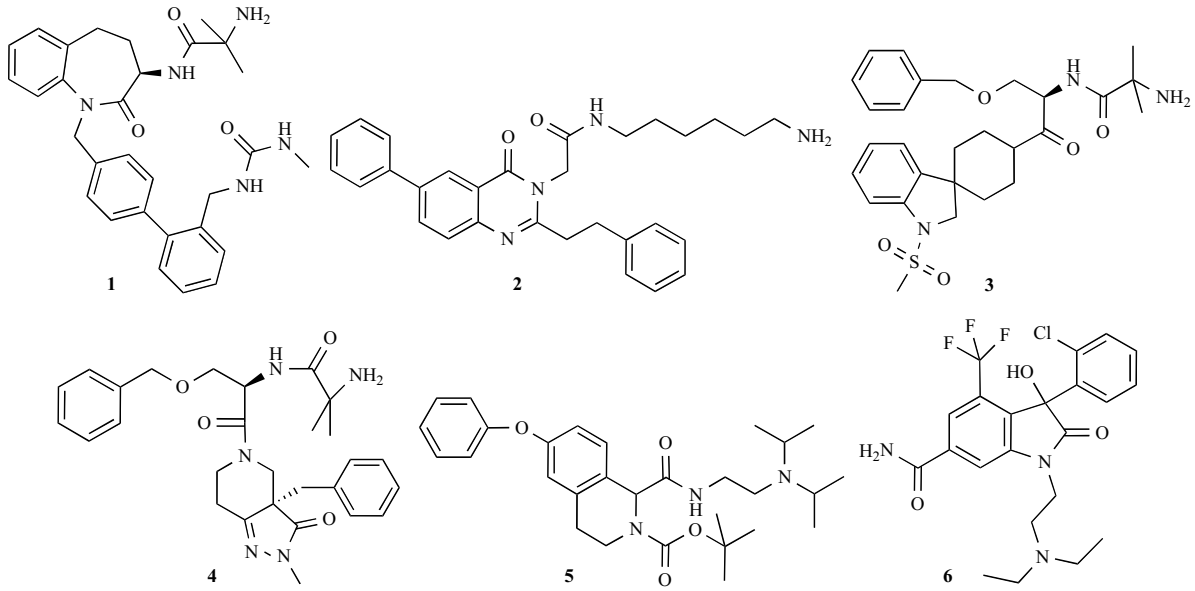

Fig. (2). Chemical structures of growth hormone secretagogues: benzolactame GHS L-739,943 (**1**, mol wt = 513), quinazolinone GHS Compound 21 (**2**, mol wt = 482), 4-spiropiperidine GHS MK-0677 (**3**, mol wt = 429), capromorelin GHS CP-424391 (**4**, mol wt = 505), tetrahydroisoquinoline 1-carboxamide GHS (**5**, mol wt = 495), oxinole derivative GHS SM-130686 (**6**, mol wt = 469).

employed in the treatment of Alzheimer's disease [70, 71]. Hence, GHS as well as other drugs resulting in an increase of serum GH and IGF-1 are targets for sports drug testing. An initial approach to determine clinically approved therapeutics for Alzheimer's disease as well as an analog to the oxindole-derived GHS SM-130686 (Fig. (**2**)) has been reported recently [72]. The drugs rivastigmine, donepezil, and galantamine as well as major metabolites resulting from desmethylation plus a constitutional isomer of SM-130686 were determined in human urine using liquid-liquid extraction followed by conventional LC-MS/MS analysis. Detection limits of 15-20 ng/mL were accomplished using MRM experiments for target analytes; however, due to the enormous variety of drug candidates belonging to this class of GHS, new comprehensive screening procedures must be developed based on either LC-MS/MS or GC-MS techniques.

NEW DRUGS STIMULATING ERYTHROPOIESIS

The glycoprotein erythropoietin (EPO, mol wt = 30.4 kDa) is a member of the cytokine family and a key factor of erythropoiesis, the production of red blood cells. Human EPO is composed of 165 amino acid residues and four glycosidic side chains, three of which are linked to asparagines 24, 38 and 83, and another is connected to serine 126. EPO is primarily produced by peritubular fibroblast like cells of the kidneys, with tissue hypoxia being the main stimulus leading to EPO biosynthesis and secretion into circulation. The complex mechanisms underlying erythropoiesis have been reviewed by Jelkmann [73], with numerous factors required for the release of EPO from kidneys and, consequently, proliferation of erythrocytic progenitors. The most important regulators and cellular oxygen sensors are the hypoxia-inducible transcription factors (HIF), in particular the HIF-1α subunit. In normoxia, prolyl-hydroxylases cause hydroxylations at residues 402 and 564 in the oxygen-dependent degradation domains of HIF-1α, resulting in a von-Hippel-Lindau protein/ubiquitin ligase complex mediated proteasomal degradation [74, 75]. Additionally, asparaginyl hydroxylation impedes the binding to transcriptional coactivators. In contrast, hypoxic conditions allow the formation of an active transcription complex composed of HIF-1α, HIF-1β and p300/CREB that triggers endogenous EPO production.

Owing to an increasing number of patients suffering from anaemia, numerous therapeutic approaches have been implemented over the last 20 years. With the advent of biotechnological options for the production of recombinant human proteins, synthetic EPO has been frequently employed; however, due to a short half-life and the necessity to inject EPO formulations several times per week, numerous drugs and drug candidates have been developed that are either EPO derivatives capable of mimicking its presence at respective receptors with prolonged bioavailability or agents stimulating endogenous EPO production by stabilizing the HIF-1α subunit [76].

Erythropoietin Mimetic Agents

The survival of EPO circulating in the blood stream is directly dependent on its extent of glycosylation and receptor binding. Although the metabolic fate and clearance of EPO is not entirely understood, there is evidence for its elimination by internalization during EPO receptor activation, and a minor proportion is renally excreted [77].

A hyperglycosylated derivative termed Darbepoetin alpha was developed in 2001 [78] that comprises a modified amino acid sequence (Ala30Asn, His32Thr, Pro87Val, Trp88Asn and Pro90Thr) and two additional glycosylation sites introduced by site-directed mutagenesis at the new Asn residues 30 and 88. The resulting average molecular weight of Darbepoetin alpha is 37.1 kDa, and its elimination half-life (approx. 50 h) increased by a factor of 2.5 compared to conventional Epoetins alpha and beta after subcutaneous administration (approx. 20-25 h) [79, 80]. Due to the prolonged biological activity applications of Darbepoetin alpha were required only once or twice every fortnight.

An extension of the half-life of EPO to more than 130 h was accomplished by attachment of a 30 kDa methoxy-polyethylene glycol polymer chain yielding the so-called *continuous erythropoietin receptor activator* (CERA, mol wt = 60 kDa (approx.)) [81]. The covalent linkage between EPO and the polymeric structure consists of amide bonds utilizing a succinimidyl butanoic acid linker that is predominantly connected to the lysine residues 45 or 52. Dosing frequencies of once per month have become feasible, which are of considerable interest for applications involving patients suffering from chronic kidney diseases. A comprehensive overview on the development of new drugs for the treatment of anaemia has recently been published by MacDougall [82].

An entirely synthetic analogue to human EPO was described by Kochendoerfer *et al*. in 2003. Synthetic erythropoiesis protein (SEP) was assembled by sequential conjugation of peptide segments [83]. The resulting protein core bears 166 amino acid residues with a backbone differing from human EPO at 7 positions, and two oxime-linked precision polymers substituting the four naturally occurring carbohydrate moieties [2]. The haematopoietic activity of the resulting 51 kDa molecule was compared to that of recombinant EPO, demonstrating an increased potential in animal experiments.

In addition to modified proteins closely related to EPO, mimetic agents have been clinically investigated based either on peptidyl- or non-peptidyl structures. All these compounds have been designed to dimerize the EPO receptor and, thus, imitate an EPO presence. Agents mimicking the action of EPO offer the option of an alternative route of administration, i.e. the avoidance of subcutaneous injection or infusions. Peptides 20 amino acids in length have demonstrated considerable *in vitro* and *in vivo* potency (e.g. the erythropoietin mimetic peptide 1, EMP1, Fig. (**3a**)), in particular after covalent dimerization as described by Wrighton *et al*. [84, 85]; however, limited bioavailability caused by a short half-life required further modifications, yielding a fusion protein generated from EMP1. The enlarged EPO mimetic agent possessed increased *in vivo* activity, but the protein structure of the new product precluded oral administration [76].

A new product, Hematide, bearing a dimeric peptidyl structure with intramolecular cyclization and an approximate molecular weight of 4872 Da has been described recently to efficiently activate the EPO receptor [86, 87]. Its conjugation to polyethylene glycol provided the required circulation half-life in rats and monkeys. This compound is a promising alternative to biotechnologically derived recombinant EPO therapeutics, due to its lack of cross-reaction with anti-EPO antibodies *in vitro*.

Development of non-peptidyl EPO mimetics has been previously reported. These compounds, which exhibited at least *in vitro* activity, were based on different structures such as *N*,*N*-disubstituted amino acids [88], dimeric iminodiacetic diamides [89], or bisprenylindoles derivatives [90] (Fig. (**3b**)). All of these compounds provided proof in principle for the possibility to develop orally active erythropoietic drugs. To date, no EPO mimetic therapeutic has been fully disclosed or entered the pharmaceutical market.

Doping Control Detection Strategies

EPO has been a serious issue for sports drug testing due to the difficult task of differentiating the endogenously generated peptide hormone and recombinantly produced therapeutic agents. Its misuse by athletes has been reported numerous times. The beneficial effects of EPO including an increased number of erythrocytes have made the drug particularly attractive for athletes. Hence, the need for reliable detection strategies was omnipresent, and procedures enabling the identification of recombinant human EPO preparations, including the modified derivative Darbepoetin alpha, have

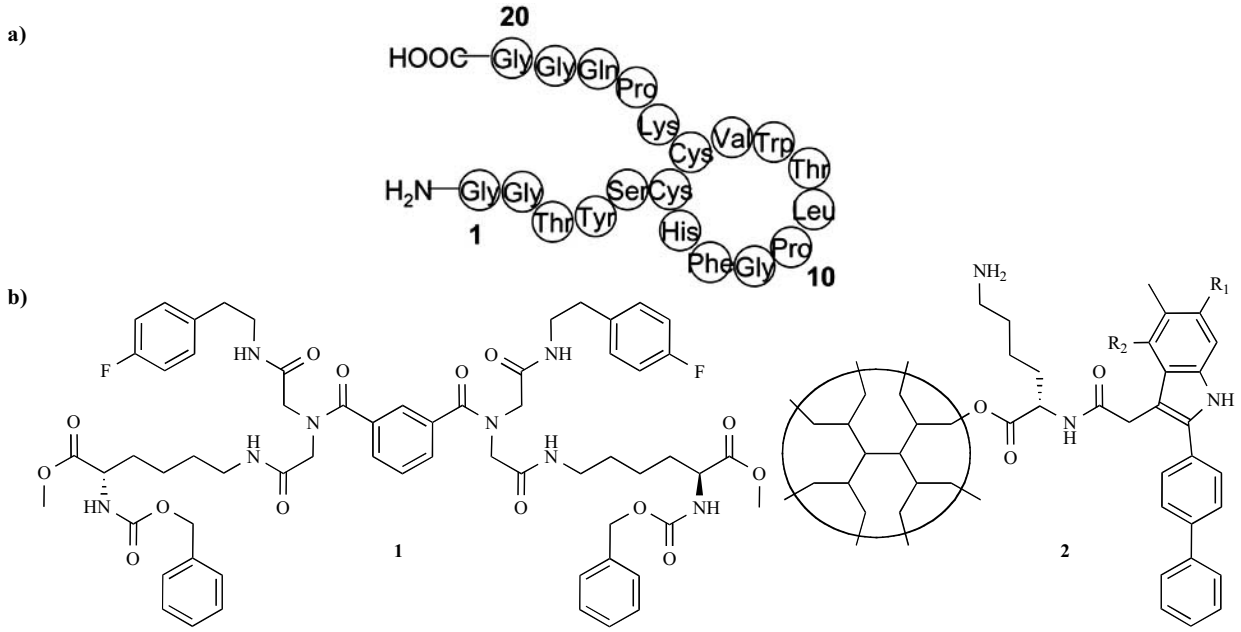

Fig. (3). a) Primary structure of EMP-1; b) Chemical structures of non-peptidyl EPO mimetic agents A6B10C4 (**1**, mol wt = 1191) and compound 5 (**2**, R1 = Cl, R2 = H and vice versa). The latter is composed of eight side chains each weighing 518 Da, which are connected by a central core (polyamidoamino-octa-4-hydroxymethylbenzamide) of approx. 2 kDa. The core is schematically illustrated and one side chain attached exemplarily.

been developed based on the strategies described by Lasne *et al*. [91-93] and summarized in recent reviews [2, 94]. Here, isoelectric focusing followed by immunoblotting is employed as chromatographic-mass spectrometric assays have not yet demonstrated sufficient sensitivity and selectivity for this category of analytes.

The synthetic analogs CERA, SEP as well as the peptidyl- and non-peptidyl derived EPO mimetics have yet to be released, and details on the composition of advanced compounds such as Hematide have not been disclosed. Detection assays using LC-MS/MS approaches have been reported recently [95]; however, it remains to be seen whether conventional doping control approaches (such as isoelectric focusing combined with immunoblotting techniques) or LC-MS/MS procedures will be capable of routinely identifying these new classes of compounds.

Hypoxia-Inducible Factor-1α (HIF-1α) Stabilizers

Novel alternatives to EPO and its mimetic agents include hypoxia-inducible factor (HIF) stabilizers, which are currently in clinical studies for the treatment of anemia. The progress in understanding adaptation of humans to hypoxia at the molecular level has led to the development of a new class of small molecules which up-regulate endogenous EPO production by mimicking hypoxia to the transcription factor HIF-1 and its degrading enzymes. Hypoxia is the physiological trigger that activates HIF, the 1α-subunit of which responds to changes in oxygen tension. HIF-1 is a transcription factor that binds specifically in hypoxia to hypoxia-responsive elements in the promoter or enhancer of hypoxia-inducible genes such as EPO. Under normoxia, the half-life of HIF-1α is less than 5 min since hydroxylases modify the protein at different positions and "mark" it for degradation [96, 97]. As a consequence, the artificial stabilization of HIF-1 by orally bioavailable therapeutics enables the treatment of anemia by stimulating erythropoiesis and, thus, increasing the number of red blood cells and hemoglobin concentration.

Doping Control Detection Strategies

The primary strategy to enhance HIF-1 stability is based on the inhibition of prolyl hydroxylase activities. Hence, a whole series of potential inhibitors has been prepared including compounds such as acyl sulfonamides [98], 5-amide substituted pyridine-2-carboxylic acids [99], N-oxaloglycine derivatives [100], hydroxyanthraquinones [101], 2-oxoglutarate analogues [102], and 3,4-dihydroxybenzoates

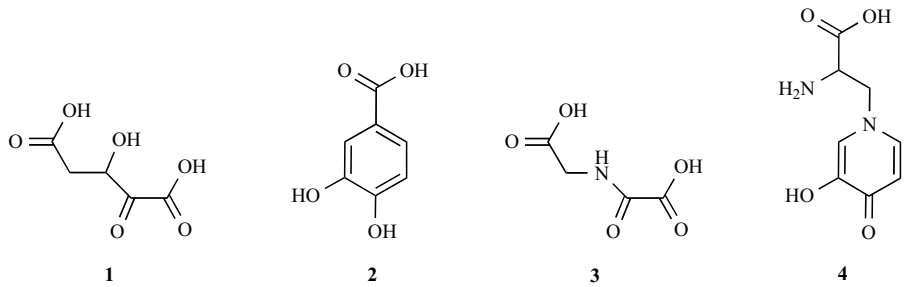

Fig. (4). Chemical structures of prolyl hydroxylase inhibitors: 2-oxoglutarate (**1**, mol wt = 162), 3,4-dihydroxybenzoate (**2**, mol wt = 154), oxalyl glycine (**3**, mol wt = 147), L-mimosine (**4**, mol wt = 198).

[103]. One drug candidate, FG-2216, has recently entered phase-II clinical trials [104] but its structure has yet to be disclosed. Due to the diverse chemistry of prolyl hydroxylase inhibiting substances (Fig. (**4**)), determining an exact structure is difficult. Since all candidates are of low molecular weight with highly polar functional residues, new detection assays based on LC-MS/MS techniques are likely to be established in the future for compounds such as FG-2216.

CONCLUSION

The dynamically developing pharmaceutical market continuously provides new drugs and drug candidates that possess great clinical utility with the potential for misuse in sports. Therefore, following clinical trials for new therapeutics is an important field of preventive doping research. Detection strategies need to be frequently updated to allow clean and fair sports competitions.

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