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# PEG-uricase in the management of treatment-resistant gout and hyperuricemia ☆

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## Abstract

Hyperuricemia results from an imbalance between the rates of production and excretion of uric acid. Longstanding hyperuricemia can lead to gout, which is characterized by the deposition of monosodium urate monohydrate crystals in the joints and periarticular structures. Because such deposits are resolved very slowly by lowering plasma urate with available drugs or other measures, the symptoms of gout may become chronic. Persistent hyperuricemia may also increase the risk of renal and cardiovascular diseases. Unlike most mammals, humans lack the enzyme uricase (urate oxidase) that catalyzes the oxidation of uric acid to a more soluble product. This review describes the development of a poly(ethylene glycol) (PEG) conjugate of recombinant porcine-like uricase with which a substantial and persistent reduction of plasma urate concentrations has been demonstrated in a Phase 2 clinical trial. Two ongoing Phase 3 clinical trials include systematic assessments of gout symptoms, tophus resolution and quality of life, in addition to the primary endpoint of reduced plasma urate concentration.
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*Keywords:* Poly(ethylene glycol); Urate oxidase; Therapeutic enzyme; Gout; Hyperuricemia

## Contents

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## 1. Introduction

Gout is characterized by persistent hyperuricemia, which results in the deposition of monosodium urate monohydrate (MSU) crystals in the joints and periarticular structures and of uric acid calculi in the urinary tract ("kidney stones") [1]. While gout has been recognized for several millennia [2], its increasing incidence during the past few decades [3,4] and the growing recognition of the renal and cardiovascular consequences of persistent hyperuricemia [5–9] have enhanced the urgency of developing more effective treatments. For a significant number of patients with gout and other conditions in which hyperuricemia is poorly controlled with available drugs, polymer-coupled uricase and other drugs in development offer new hope of improved clinical outcomes.

Unlike nearly all other mammals, humans and higher apes lack the enzyme uricase (urate oxidase, E.C. 1.7.3.3), which catalyzes the oxidation of uric acid to allantoin, a more soluble product that is readily excreted in the urine [10]. The lack of uricase in humans results in plasma uric acid concentrations that are much higher than in most mammals. When these concentrations exceed the solubility limit of about 7 mg/dL at physiological pH, uric acid may nucleate to form crystals in tissues and joints [11]. Shedding of MSU crystals into the synovial cavity induces acute inflammatory responses known clinically as gout flares. The persistence of crystals in the synovial fluid and synovial membrane induces chronic inflammation. In addition to gout, another condition associated with hyperuricemia is tumor lysis syndrome, in which the induction of effective chemotherapy for hematologic cancers such as leukemia and lymphoma may result in dramatic elevations of plasma uric acid [12–15]. Hyperuricemia also occurs with high frequency during the months and years following organ transplantation, when renal function and hence the excretion of uric acid are compromised by immunosuppressive agents, such as cyclosporine A, used to prevent rejection of the transplanted organ [16–19] and by diuretic drugs administered after kidney transplantation.

A major contribution to the management of gout and other consequences of hyperuricemia was the introduction in the 1960s of allopurinol, a precursor of oxypurinol that is a potent inhibitor of xanthine oxidase, which catalyzes the last steps in the metabolism of purines to uric acid [20,21]. Intractable gout can result from allergic reactions to allopurinol, some of which are life-threatening or fatal [22–25] or from an inadequate response to the highest tolerated doses of the available drugs. Patients suffering from treatment-resistant gout often have painful gout flares and/or tophi (inflamed deposits of MSU crystals), as do patients whose serum uric acid levels are lowered to just below 6 mg/dL [26]. Resolution of tophi occurs when sub-saturating serum urate levels are maintained for long periods, but this rarely happens in response to the in-

hibition of uric acid synthesis by allopurinol [27]. More rapid resolution of tophi is observed when serum uric acid levels are lowered further by the administration of more effective urate-lowering therapy, e.g., uricosuric agents with or without allopurinol [28].

During the past seven decades, there have been numerous attempts to manage treatment-resistant gout by the administration of uricase extracted from various sources or expressed in recombinant organisms. In the early 1940s, Oppenheimer and colleagues injected uricase into hens, cocks and one patient [29,30]. London and Hudson [31] were also among the pioneers who administered purified uricase to two human patients. Recognition of the immunogenicity of uricase in humans led to efforts to prepare poly(ethylene glycol) conjugates of uricase (PEG-uricase) [32–38] and to the first clinical study of PEG-uricase in five patients with advanced solid tumors [39]. The first U.S. patent claiming "water-soluble non-immunogenic polypeptide compositions," which issued in 1979, described the synthesis of PEG-uricase as Example I [32]. Nevertheless, it has taken another quarter century to overcome the challenges presented by the development of a form of PEG-uricase that fulfills the requirements for a useful drug:

1. sufficient reduction of the immunogenicity of this non-human enzyme to permit repeated dosing;
2. retention of sufficient enzymatic activity to be effective at a reasonable dose, e.g., retention of at least 75% of the intrinsic activity in the conjugate;
3. sufficient solubility under physiological conditions to enable reasonable bioavailability;
4. reproducible and cost-effective synthesis of a conjugate with adequate stability during storage and shipping and *in vivo*; and
5. a sufficiently long half-life in patients to permit a convenient schedule of administration, e.g., once or twice a month.

All of the above objectives appear to have been fulfilled by a PEGylated form of recombinant mammalian uricase that was developed jointly by scientists from Mountain View Pharmaceuticals, Inc. and Duke University [40–43]. Savient Pharmaceuticals, Inc. (East Brunswick, NJ) holds a worldwide exclusive license to this form of PEG-uricase, for which the trademark Puricase® was registered by Mountain View Pharmaceuticals, Inc. Savient expects to complete its Phase 3 clinical trials of Puricase® in North America during 2007.

## 2. Preclinical development of PEGylated recombinant mammalian uricase

The collaborative development of PEGylated recombinant mammalian uricase by scientists at Duke University and

Mountain View Pharmaceuticals, Inc. (MVP) began during 1995, with support during 1996–1998 from the U.S. National Institutes of Health. Drs. Michael S. Hershfield and Susan J. Kelly of Duke constructed various recombinant mammalian uricases, among which the selected amino acid sequences resembled that of porcine uricase most closely, but contained several residues from the baboon sequence [42]. These sequences are referred to in this review as "porcine-like" sequences. During 1996 through 1999, the scientists at MVP designed a PEGylation strategy for these recombinant uricases that fulfilled the objectives outlined in the Introduction [40].

## 2.1. Coupling chemistry and molecular weight of PEG

Optimization of the PEG conjugates involved covalent coupling of porcine-like uricases to various numbers of strands of methoxyPEGs of various molecular weights: 5 kDa, 10 kDa, 20 kDa and 30 kDa. Except as indicated to the contrary (as in Section 4, below), the abbreviation PEG denotes methoxyPEG. The selected coupling chemistry utilized *p*-nitrophenylcarbonate derivatives of the PEGs to form stable urethane bonds between the polymer and the solvent-accessible amino groups on the protein. This activation chemistry was initially developed by Wilchek and Miron for immobilization of enzymes and affinity ligands [44,45]. The same investigators and their colleagues [46] and others later used *p*-nitrophenylcarbonate derivatives as PEGylation reagents [47–49]. Williams et al. [40] demonstrated that coupling a relatively small number of strands of 5-kDa PEG led to marked reduction of the catalytic activity of uricases extracted from various sources or having various amino acid sequences expressed in recombinant organisms. The tested sequences included those of *Aspergillus flavus*, *Candida utilis*, soybean and several porcine-like uricases. The number of strands of 5-kDa PEG that inactivated the enzyme by >50% was smaller than the number needed to confer a long half-life in rodents or to suppress the binding of anti-uricase antibodies *in vitro*. Similar results had been reported by earlier investigators who had attempted to prepare 5-kDa PEG conjugates of uricases from various sources [33,35,36,39,50,51].

In contrast with the results obtained with 5-kDa PEG, Williams et al. [40] and Sherman et al. [41] found that conjugates of porcine-like uricase could be prepared with a sufficient number of strands of 10-kDa PEG to achieve very long half-lives in laboratory animals and to suppress their antigenicity and immunogenicity, while retaining approximately 90% of the uricolytic activity of the unmodified enzyme. In addition, the 10-kDa PEG conjugates of porcine-like uricases were readily soluble at physiological pH, in contrast with the unmodified enzyme that was soluble only at a high pH (e.g., pH 10 to 11). Among three conjugates containing the same total mass of PEG (60 kDa per subunit), the conjugate with six strands of 10-kDa PEG per subunit had a significantly longer half-life in mice than those with three strands of 20-kDa PEG or two strands of 30-kDa PEG per subunit. On the basis of such experiments, conjugates synthesized with 10-kDa PEG were selected for further development.

## 2.2. Number of strands of PEG

Although the amino acid sequences of each subunit of the tested porcine-like uricases contain an amino-terminal amino group and a total of 28 or 29 lysine residues [42] that could theoretically be sites of PEG attachment, only about 12 of the amino groups on each subunit are accessible on the surface of the native tetrameric structure, based on the molecular model described in Section 2.6. Furthermore, a few of the solvent-accessible lysine residues are sufficiently close to each other that if PEG is coupled to one of them, steric hindrance is expected to inhibit the coupling of another strand of PEG to the neighboring residue. In a series of experiments, Williams et al. found that a maximal average of about 11 strands of 10-kDa PEG could be coupled per subunit of porcine-like uricase without disrupting the tetrameric structure and inactivating the enzyme. In all of these experiments, the number of strands of PEG per subunit of uricase was determined by size-exclusion chromatography with refractive index (RI) and ultraviolet absorption (UV) detectors in series. Since both the PEG and the protein components of the conjugates contribute to the RI signal, but only the protein contributes to the UV signal, the mean ratio of PEG to protein in each peak of the chromatogram can be calculated from the ratio of these signals, as described by Kunitani et al. [52]. As discussed in Section 2.5 below, the conjugate selected for clinical development contains $9\pm1$ strands of PEG per subunit [53].

## 2.3. Proof of principle in a mouse model of severe gout

Various conjugates of porcine-like uricase containing 10-kDa, 20-kDa or 30-kDa PEG were tested in laboratory animals by the scientists at Duke and MVP. The most dramatic demonstration of the urate-lowering potency of a conjugate containing six strands of 10-kDa PEG per subunit was obtained by comparing microscopic magnetic resonance images (microscopic MRI) of the kidneys of normal mice, uricase knockout mice (in which the uricase gene had been inactivated [54]) and uricase knockout mice that had been treated with PEG-uricase for ten weeks at intervals of five to seven days. While the renal architecture (visualized by microscopic MRI) and all of the tested renal functions were grossly abnormal in the untreated uricase knockout mice, the structure and function of the kidneys were nearly normal in the PEG-uricase-treated knockout mice. These results were published by Kelly et al. [43] and were included in the patent of Williams et al. [40]. An elegant application of the microscopic MRI technique as applied to intact normal mice and uricase knockout mice was later published by Maronpot et al. [55].

## 2.4. Effects of aggregated uricase on the pharmacokinetics and immunogenicity of PEG-uricase conjugates

Additional studies of PEGylated porcine-like uricase demonstrated that the immunogenicity of the conjugates could be reduced below the level conferred by PEGylation alone if the conjugates were prepared from uricase from which all traces of

aggregated protein detectable by light scattering measurements had been removed [41]. Ion-exchange chromatography was used to fractionate preparations of porcine-like uricase into pools of three size ranges: tetramers of the uricase subunit (with a total molecular weight, $M_r$, of about 140 kDa), octamers (with a total $M_r$ of about 280 kDa) and large aggregates (with a total $M_r$ exceeding 300 kDa). Fractions from the ion-exchange column were characterized by size-exclusion chromatography in which the eluate was monitored both by absorption of ultraviolet light and by light scattering. The large aggregates were detectable only by the latter technique, in which the signal is proportional to the product of the size of the scattering particle times its weight concentration. Pools of fractions containing predominantly tetramers or octamers, with or without large aggregates, were then coupled to an average of six strands of 10-kDa PEG per subunit.

The pharmacokinetics and immunogenicity of the resultant conjugates were tested after weekly injections into mice for six weeks. Serum was prepared from blood drawn 24 h after each injection. Residual uricase activity in the serum was assayed spectrophotometrically and anti-uricase antibodies were measured by an enzyme-linked immunosorbent assay (ELISA). The results revealed that conjugates prepared from uricase containing traces of large aggregates were cleared significantly more rapidly than those prepared from aggregate-free tetrameric or octameric uricase and that anti-uricase antibodies were detectable only in mice injected with conjugates prepared from uricase containing large aggregates [41]. These studies clearly indicated that even extensive PEGylation, e.g., with an average of 24 strands of 10-kDa PEG per tetramer, cannot completely block the immunogenicity in mice of porcine-like uricase that contains even a small proportion of large aggregates.

## 2.5. Optimization of the extent of PEGylation based on antigenicity

Using aggregate-free porcine-like uricase, conjugates were synthesized with various numbers of strands of 10-kDa PEG and tested for their ability to bind to anti-uricase antibodies raised in mice or in rabbits (see [56]). On the basis of the studies with murine antiserum, a conjugate containing an average of $9\pm1$ strands of 10-kDa PEG per subunit was selected for clinical development, as reported by Baraf et al. [53]. Under the conditions tested, the antigenicity of the latter conjugate was only 0.1% of that of non-PEGylated porcine-like uricase, or about 10-fold lower than that of a conjugate with six strands of 10-kDa PEG per subunit.

## 2.6. Molecular model of the selected conjugate

A molecular model of tetrameric uricase coupled to 36 strands of 10-kDa PEG is shown in Fig. 1D. The models of the enzyme itself, shown in Fig. 1A–C, were generated using the program RasMol [57] and the coordinates of the crystal structure of uricase from *A. flavus* [58] (protein data base, PDB 1uox). To generate the structure shown in Fig. 1D, the lysine residues of the *A. flavus* sequence were replaced by the

lysine residues of porcine-like uricase within the RasMol model. Molecular models of each of the 36 strands of 10-kDa PEG that are coupled to the most accessible lysine residues were generated using a program adapted from the program Add_PEG described by Lee et al. [59]. In the space-filling model of the conjugate (Fig. 1D), the carbon and oxygen atoms of the polymer strands are shown at the same scale as those of the uricase.

## 2.7. Final stages of preclinical development

The PEG-uricase developed jointly by the scientists at Duke and MVP was licensed to Bio-Technology General Corporation (BTG) in 1998. Scientists from BTG's subsidiary in Israel undertook further optimization of the amino acid sequence of the recombinant uricase, primarily involving modifications of the amino-terminal or carboxyl-terminal sequences [60,61], scale-up of the recombinant protein expression and purification, scale-up of the PEGylation process, and the preclinical toxicologic and pharmacologic studies required for regulatory approval to conduct studies in patients. The collaboration among scientists at BTG-Israel, Mountain View Pharmaceuticals, Inc. and Duke University was supported by a grant from the U.S.–Israel Binational Industrial Research and Development Foundation (BIRD Foundation). In 2003, BTG Corporation changed its name to Savient Pharmaceuticals, Inc. (Savient).

## 3. Clinical studies

### 3.1. Phase 1

In the first reported open-label Phase 1 trial of PEG-uricase (Puricase®) sponsored by Savient, 13 treatment-failure gout patients, including 11 with tophaceous gout, received single subcutaneous (s.c.) injections of PEG-uricase containing 4, 8, 12 or 24 mg of protein [62]. By day 7 after injection, the plasma uric acid concentration had decreased from a mean of about 11 mg/dL to a mean of about 3 mg/dL. Adverse reactions included injection-site reactions in three patients and gout flares in six patients, as are often observed in the early phase of effective anti-hyperuricemic therapy with other drugs, e.g., febuxostat [63]. The accelerated clearance of the PEG-uricase in five of the 13 patients, the slow release from the injection site of this large conjugate (with a total molecular weight of about 500 kDa) and injection-site reactions contributed to the decision to terminate the trial of s.c. administration and to conduct a Phase 1 study in which the drug was administered intravenously (i.v.). It should be noted that in monitoring uric acid levels in PEG-uricase-treated patients, measurements are performed on plasma, rather than serum, since the PEG-uricase present in the blood would metabolize the uric acid during clotting to form serum, resulting in spuriously low concentrations of uric acid.

In a second Phase 1 study, 24 treatment-failure gout patients, including 16 with tophaceous gout, received PEG-uricase as a single i.v. dose of 0.5, 1, 2, 4, 8 or 12 mg of protein (4 patients per dosage level) [64,65]. At the three highest dosage levels, plasma uric acid decreased from about 11 mg/dL to normal
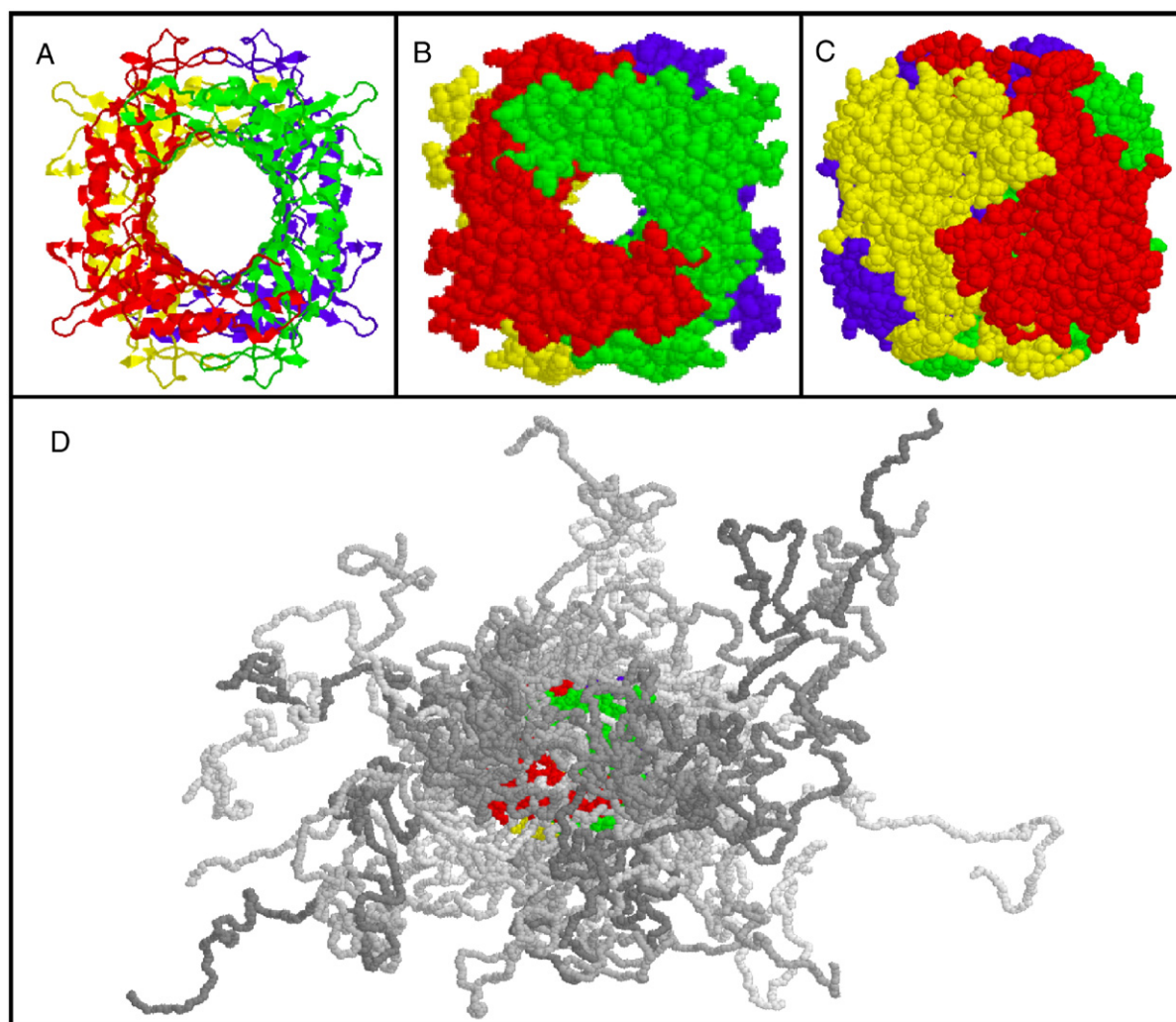
Fig. 1. Molecular models of uricase tetramer (A–C) and of PEG-uricase (Puricase®) containing 36 strands of 10-kDa PEG per uricase tetramer (D). A. Cartoon model of uricase tetramer, based on the crystal structure of *A. flavus* uricase [58]. Each subunit is shown in a different color (red, blue, green or yellow). B. Space-filling model of *A. flavus* uricase tetramer, showing the tunnel. C. Space-filling model of *A. flavus* uricase tetramer, rotated around the vertical axis so that the tunnel is not visible. D. Space-filling model of uricase tetramer, in the same orientation as in B, to which nine strands of 10-kDa PEG are attached per uricase subunit. The structures of the PEG strands (shown in various shades of gray) were generated using a program adapted from that of Lee et al. [59]. The scale of D is about half that of A–C.

levels (generally defined as ≤ 6 mg/dL) within 24 h and reached a minimum of <2 mg/dL. The half-lives of uricase activity of the conjugate in plasma ranged from about 6 to14 days. There were no severe or serious adverse events, although gout flares were reported in 14 subjects, as are often observed in the early phase of effective anti-hyperuricemic therapy with other drugs, as discussed above. The safety, tolerability and apparent efficacy of PEG-uricase in this single-dose study supported the approval to conduct Phase 2 studies involving repeated administration of this drug.

### 3.2. Phase 2

Phase 2 dose-ranging studies were performed during 2004 and 2005, for which preliminary results have been published and presented at several conferences [60,61,66–68]. Four dosing regimens of PEG-uricase (Puricase®) were compared during a treatment period of 12 weeks: 4 mg every two weeks; 8 mg every two or four weeks, and 12 mg every four weeks. Some of the results are summarized in Table 1 [66,67].

In order to document the effects (if any) of 12 weeks of treatment with Puricase® on the resolution of tophi, one of the clinical investigators in the Phase 2 study, Dr. Herbert S.B. Baraf, took photographs of the hands of two pre-selected patients with inflamed tophi at the joints of their fingers. Despite the relatively short duration of the Phase 2 study, the photographs taken after treatment for 12 weeks revealed dramatic improvements in both patients, who had been diagnosed with gout for about one year and about 25 years, respectively. These impressive, but preliminary results have been presented at several scientific meetings [53,69] and they provide further evidence that very low plasma urate levels correlate with the rapid disappearance of tophaceous deposits [28].

The effects of PEG-uricase in the Phase 1 and Phase 2 studies sponsored by Savient are compared with some previous clinical studies of uricase or PEG-uricase in Table 2.

Table 1
Reduction in Plasma Uric Acid (PUA) levels in a Phase 2 trial of puricase®

| | Dosing cohort | | | |
|---|---|---|---|---|
| | 4 mg/ 2 weeks | 8 mg/ 2 weeks | 8 mg/ 4 weeks | 12 mg/ 4 weeks |
| Patients treated | 7 | 8 | 13 | 13 |
| Patients completed | 4 | 8 | 8 | 6 |
| PUA pre-treatment mean mg/dL (s.d.) | 7.56 (2.24) | 9.09 (1.73) | 9.08 (3.10) | 8.47 (2.74) |
| PUA during 12 weeks, mean (s.d.) | 4.20 (2.47) | 1.42 (2.06) | 2.57 (1.67) | 2.60 (3.08) |
| Mean reduction | 44% | 84% | 72% | 69% |

The following conclusions can be drawn from the Phase 2 clinical trial [53,66–69].

1) Multiple dose administration of PEG-uricase led to substantial and sustained lowering of plasma uric acid (PUA) levels in all treatment groups.
2) The most effective dose was 8 mg every two weeks, which lowered the mean PUA to less than 2 mg/dL throughout the dosing interval.
3) In two patients whose affected hands were photographed, there was a marked reduction in tophi by the end of the 12-week treatment with PEG-uricase. The reduction in tophi appeared to be more extensive than that observed in a patient treated with weekly infusions of rasburicase for one year [72].
4) Gout flare was the most common adverse event, but the incidence decreased during the 12-week treatment period.
5) Infusion reactions were clinically similar to those reported with other biological agents, e.g., with intravenous immunoglobulin [73].
6) Detection of antibodies against PEG-uricase was not predictive of accelerated clearance of the drug or of adverse events.

7) The terminal elimination half-lives of PEG-uricase in these patients ranged from five to 17 days, which are much longer than that of rasburicase, which was reported to be $21 \pm 12$ h [15].
8) The data from the Phase 2 trial supported the continued development of PEG-uricase as an anti-hyperuricemic agent for patients with treatment-failure gout.

### 3.3. Phase 3

Patients with treatment-failure gout were recruited in the United States, Canada and Mexico for two Phase 3 clinical trials of PEG-uricase starting in May 2006 and enrollment was completed in March 2007 (see press releases at www.savientpharma.com). The six-month, double-blind Phase 3 trials include patients receiving placebo at two-week intervals, patients receiving 8 mg of PEG-uricase at two-week intervals and patients receiving alternating infusions of 8 mg of PEG-uricase and placebo at two-week intervals (see www.clinical-trials.gov). While the primary endpoint is reduced plasma urate concentration, these trials include assessments of gout symptoms, tophus resolution and quality of life. At the end of the six-month treatment period, all patients are given the option of continuing treatment in an open-label study, in which each patient and his/her physician can select treatment with 8 mg of PEG-uricase at either two-week or four-week intervals. To date, nearly 100% of those patients who have completed the blinded phase of the study have elected to continue (or, for placebo recipients, to start) receiving treatment with 8 mg of PEG-uricase for an additional one or two years.

## 4. Anti-PEG antibodies

In a family of patent applications first published in 2004, Martinez et al. [56] showed that rabbits immunized with

Table 2
Effects of uricase and PEG-uricase administration on uric acid in humans

| Uricase or PEG-uricase conjugate | Dose administered | Effect on serum or plasma uric acid | References |
|---|---|---|---|
| Suspension of uricase, presumably porcine, based on the reference to Mahler [70] | 104 IU/mL or 95 mcg/mL × 5 or 10 mL = c. 0.5–1 mg per i.v. injection | Decreased c. 1 mg/dL for c. 1 h each time, i.e., by <16% | London and Hudson [31]; Mahler [70] |
| PEG-modified *Candida utilis* uricase from Enzon, Inc. | 120 IU/m² by i.v. infusion, repeated after 30 days [a] | Decreased to undetectable level for <48 h | Davis et al. [39] |
| PEG-modified *Arthrobacter protoformiae* uricase from Enzon, Inc. | 1 IU/kg, then after 30 h start 2 IU/kg each 5–6 days by i.m. injection, three times [a] | Decreased c. 70% for <5 days after first dose; smaller effect after each subsequent dose | Chua et al. [71] |
| PEG-modified porcine-like uricase by Savient Pharmaceuticals, Inc. | 8 mg by s.c. injection, once | Decreased to <6 mg/dL after 2 days and remained low for 10–21 days | Ganson et al. [62] |
| PEG-modified porcine-like uricase by Savient Pharmaceuticals, Inc. | 8 mg by i.v. infusion, once | Decreased to <6 mg/dL (>90% decrease) within 1 day and remained <6 mg/dL for 21 days | Sundy et al. [64]; Sundy et al. [65] |
| PEG-modified porcine-like uricase by Savient Pharmaceuticals, Inc. | 8 mg by i.v. infusion, every 2 weeks for 12 weeks | Decreased to <6 mg/dL within 1 day and remained <6 mg/dL for 92% of a 12-week observation period | Sundy et al. [66,67]; Hartman and Mendelovitz [60,61] |
| PEG-modified porcine-like uricase by Savient Pharmaceuticals, Inc. | 8 mg by i.v. infusion, every 4 weeks for 12 weeks | Decreased to <6 mg/dL within 1 day and remained <6 mg/dL for 86% of a 12-week observation period | Sundy et al. [66,67]; Hartman and Mendelovitz [60,61] |

[a] 120 IU/m² corresponds to about 2 IU/kg, based on an assumed body weight of about 75 kg and an assumed surface area of about 1.3 m². Since the specific activity of the PEG-uricase of Davis et al. [39] was reported to be 0.47 IU/mg protein, the administration of 120 IU/m² corresponds to a total dose of >300 mg protein.

recombinant mammalian uricase coupled to an average of about two strands of 10-kDa methoxyPEG (mPEG) per subunit produced antibodies against both the enzyme and the polymer component. It should be noted that the conjugate used for these immunizations was known to contain an insufficient number of strands of PEG per subunit to inhibit the binding of the conjugate to anti-uricase antibodies. Such inhibition had been determined to require about six to nine strands of PEG per subunit, as described in Section 2.5 above.

Competitive binding experiments were performed with various polymers against the mixture of anti-PEG-uricase antibodies in the sera of rabbits immunized with the "two-stranded" conjugate (eight strands of mPEG per uricase tetramer). Experiments in which anti-polymer antibodies were measured were performed on assay plates coated with mPEG conjugates of an unrelated protein. The results showed that 5-kDa methoxyPEG bound to the anti-polymer antibodies with 100-fold higher affinity than PEGs of any length tested that lacked methoxyl groups at their termini, e.g., dihydroxyPEGs. Among PEGs of the same length containing 0, 1 or 2 methoxyl groups, the affinities for the anti-polymer antibodies increased with the number of methoxyl groups. The ensemble of results published in the patent application of Martinez et al. [56] was unexpected in the context of nearly three decades of publications on PEGylated drugs in which the polymer component, which was virtually always methoxyPEG, was described as "non-antigenic" and/or "non-immunogenic."

In view of the preceding observation of anti-mPEG antibody formation in rabbits, the observations of Ganson et al. [62] and of Sundy et al. [65] on the formation of anti-PEG antibodies in some patients in both the first and second Phase 1 clinical trials of PEG-uricase, in which the drug was given by s.c. and i.v. routes, respectively, was not unexpected, since the PEG-uricase conjugates used in all clinical studies to date have been synthesized with methoxyPEG. It has not been shown that the formation of anti-mPEG antibodies is treatment limiting with respect to either the neutralization of uricase activity or accelerated clearance, mediated by antibodies, that could preclude a convenient dosage regimen. If the ongoing Phase 3 clinical trials of PEG-uricase during six months of treatment show that anti-PEG antibodies diminish the utility of the drug for a substantial percentage of patients, then it is reasonable to expect that a next-generation form of PEG-uricase might be developed in which uricase is coupled to PEG lacking a methoxyl or other alkoxyl group at the terminus remote from the protein-coupling function.

## 5. Induction vs. maintenance therapy with PEG-uricase

As in many chemotherapy protocols, e.g. as reported by Hainsworth et al. [74], it is possible that a relatively high dose of PEG-uricase will be needed to induce rapid resolution of tophi or other signs and symptoms of severe gout, while either lower and/or less frequent doses of PEG-uricase will be sufficient for maintenance therapy [11]. Once the accumulation of uric acid deposits in the joints and tissues of patients who have been treated suboptimally for years has been depleted by an initial course of PEG-uricase treatment, it is plausible that less fre-

quent dosing or administration of a lower dose of PEG-uricase might be sufficient to prevent the renewed accumulation of such deposits. This hypothesis is consistent with the observations of Perez-Ruiz et al. [75] that the time between the withdrawal of urate-lowering drugs and the reappearance of flares and other symptoms of gout is dependent on the average serum urate levels during the treatment period.

## 6. Alternative drugs for treatment-resistant gout

While the focus of this review is the development and early clinical trials of PEGylated recombinant mammalian uricase, several classes of alternative urate-lowering drugs should be mentioned. Drugs that are currently marketed or in clinical trials for the treatment of chronic hyperuricemia include: 1) uricosuric drugs, such as probenecid, sulfinpyrazone and benzbromarone (marketed in Japan); 2) uricostatic drugs, which are xanthine oxidase inhibitors such as allopurinol, oxypurinol and febuxostat; and 3) uricolytic drugs, such as extracted and recombinant uricases and PEGylated non-mammalian uricases [1,76,77].

As an alternative to the classical urate-lowering drugs, febuxostat, a non-purine inhibitor of xanthine oxidase, has been evaluated in large Phase 3 clinical trials, some of which have been completed and reported. These trials were designed to compare the safety and efficacy of various doses of febuxostat with the most commonly prescribed dose of allopurinol (300 mg/day). They showed that this dose of allopurinol was less effective than expected in double-blind, parallel clinical trials, while febuxostat at either 80 or 120 mg/day was more effective in reducing serum urate to <6 mg/dL [1,27,63,78]. Additional Phase 3 clinical trials of febuxostat at 40 or 80 mg/ day are in progress (see www.clinicaltrials.gov).

Non-recombinant uricases that have been studied include *A. flavus* uricase (Uricozyme®, Sanofi-Synthelabo, Paris, France); methoxyPEG-modified *C. utilis* uricase and PEG-modified uricase isolated from *Arthrobacter protoformiae* [39,71,79]. Uricozyme was used mainly for prevention and treatment of malignancy-associated hyperuricemia and tumor lysis syndrome, but its use was associated with a significant risk of serious allergic or anaphylactic reactions [80]. Despite their success in lowering serum uric acid levels, the earlier forms of uricase had short circulating half-lives and inherent immunogenicity. Recombinant non-mammalian uricases include non-PEGylated recombinant *A. flavus* uricase (rasburicase) and recombinant *C. utilis* uricase conjugated to 20-kDa mPEG (urate oxidase-PEG 20) [81–84]. There have been extensive clinical trials of rasburicase for short-term treatment of tumor lysis syndrome [13–15,85,86], but only anecdotal reports about the use of rasburicase as compassionate treatment in chronic or treatment-resistant tophaceous gout [72]. In contrast with the results obtained with PEG-uricase treatment within 12 weeks (see Section 3.2 above), rasburicase had to be given at a high dose every week for one year to normalize serum urate levels and to reduce (but not eliminate) the subcutaneous tophi [72]. Rasburicase has been approved only for use in tumor lysis syndrome in the European Union and the United States, where it carries a "black box" warning mandated by the Food and Drug

Administration regarding the risks of analphylaxis, methemoglobinemia, interference with uric acid measurements and, in patients with glucose-6-phosphate dehydrogenase deficiency, hemolysis [87].

## 7. Conclusions

Converting an enzyme that is totally foreign to the human body into a clinically useful drug presents formidable obstacles. While the covalent attachment of poly(ethylene glycol) (PEGylation) may appear to be an obvious approach, most of the PEGylated proteins that are currently in clinical use have amino acid sequences that are identical or nearly identical to those of a natural human protein. Nevertheless, it has been possible to mask the immunogenicity of porcine-like uricase and to achieve a half-life in the range of one to two weeks in patients, while conserving most of the intrinsic enzymatic activity. Because the substrate of this enzyme (uric acid) is small, it is able to diffuse through a web of polymer molecules to reach the active sites. Optimization of the PEGylation strategy was based on molecular modeling, assays of enzymatic activity under various conditions, pharmacokinetic studies in laboratory animals, assays of the antigenicity (binding of anti-uricase antibodies *in vitro*) and immunogenicity studies of various conjugates in animals. The bioavailability of the selected conjugate after subcutaneous injection is diminished by its very large size, with a total molecular weight of about 500 kDa (140 kDa of protein plus about 360 kDa of PEG). Conversely, its large size confers sufficient stability and long half-life after intravenous infusion that dosing at intervals of two weeks or four weeks leads to profound and sustained reduction of uric acid levels in patients with long histories of hyperuricemia. Photographs of the hands of two patients in a Phase 2 clinical trial have provided anecdotal evidence for the rapid resolution of inflamed and disfiguring deposits of uric acid in the joints.

If this PEG-uricase is found to be safe and effective in the ongoing Phase 3 clinical trials, it will fulfill an unmet medical need for patients with chronic gout and other conditions in which hyperuricemia has not responded adequately to available treatments.

## Acknowledgments

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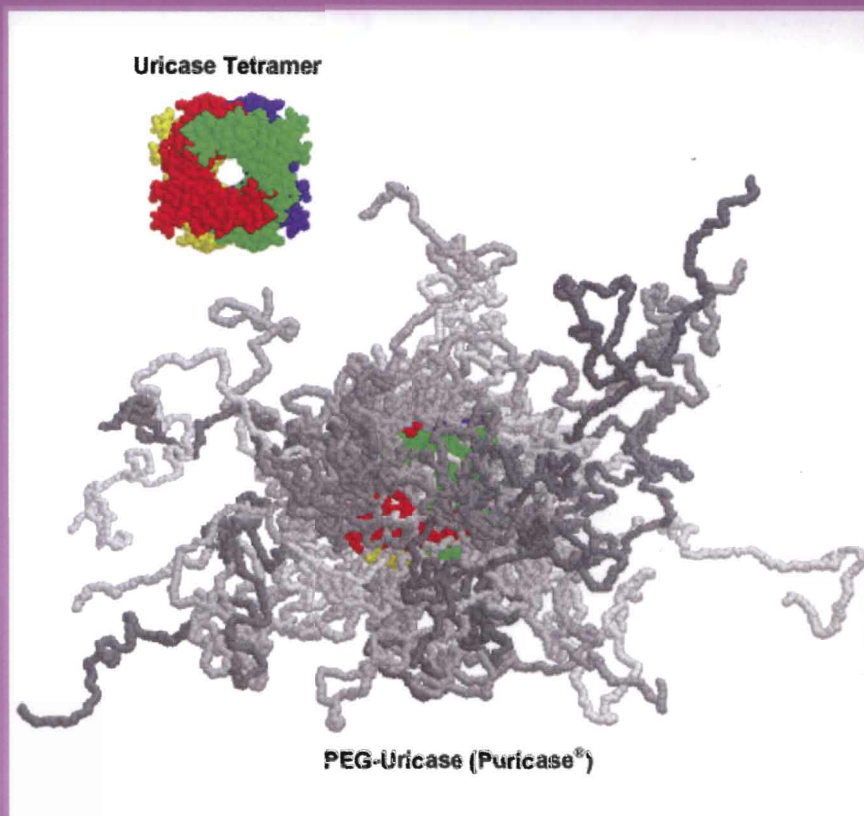
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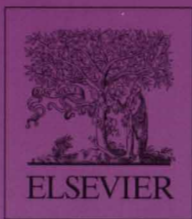
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