EXHIBIT B

PART 4 OF 5

agents, most commonly hormones, proteins or nucleic acids, whose use has become economically advantageous after the advent of the recombinant DNA technology. Despite their potency, these compounds (and mostly proteins and nucleic acids) suffer of intrinsic chemical instability, poor bioavailability and pharmacokinetics that hamper their use as therapeutic agents. PEGylation, namely the chemical modification by covalent attachment of polyethylene glycol, was thus suggested as a strategy to overcome some of these drawbacks.

PEGylation is a well-established technology in the field of biopharmaceutical formulation. It is commonly used to improve either the stability, solubility, bioavailabilty, and immunological properties of bioactive compounds and it is mostly useful in the case of proteins, due to the intrinsic instability, poor bioavailability and antigenic properties of this class of bioactive compounds. Indeed, PEGylation of proteins for pharmaceutical applications has been studied for the past 30 years. As a result, several PEGylated proteins having different therapeutic applications are now available in the market and more are about to come.

Protein PEGylation was initially developed to improve the properties of enzymes. Pioneering studies carried out in the 1970s[1,2] demonstrated that several proteins could be covalently modified with PEG without loosing their bioactivity, while their stability was implemented. Since then, protein PEGylation has been extended to other classes of bioactive proteins, among which also anticancer agents.

As a general rule, PEGylated proteins are less immunogenic and antigenic as compared to their nonmodified parents and display longer body permanence upon administration. PEGylation, however, is often accompanied with diminished protein bioactivity which may depend on the number and location of the PEG chains covalently attached to the protein and the molecular weight and geometry of the polymer (e.g. linear *versus* branched). It turns out that optimization of the coupling strategy and protocols is necessary for each protein under analysis. Covalent attachment of bulky PEG chains can be deleterious for protein activity either because amino acids that are fundamental for substrate recognition may be directly involved in the coupling or because of the polymer steric hindrance in proximity of the recognition surface. The issue of steric hindrance is relevant for those proteins that act upon interaction with large substrates, namely cytokines or antibodies whose activity requires the recognition of membrane receptors or antigens located on the cell surface. Several years of

experience have demonstrated that, in this case, site directed or chemically tailored PEGylation is necessary to preserve biological activity. On the other hand, enzymes that act on low molecular weight substrates are less sensitive to inactivation and random modification has often been successfully applied in this case. A more thorough discussion on strategy, potentials and limits of PEGylation is being reported in Chap. 4 of this book and in recent reviews from F.M. Veronese and G. Pasut, published in *Drug Discovery Today* and *Expert Opinion in Therapeutic Patents*.

Presently, two PEGylated proteins — PEG-granulocyte colony stimulating factor (PEG-GCSF or PEG-filgrastim or Neulasta®) and PEG-L-asparaginase (Oncaspar®) — are FDA approved specifically in cancer therapy. Other PEGylated proteins have FDA approval for therapeutic applications, other than cancer, but are undergoing clinical trials to extend their use to certain tumors. Among these are two PEG-Interferons (PEG-IFN), PEG-IFNα-2b or (PEG-Intron®) and PEG-IFNα-2a (PEGASYS®), presently FDA approved only for the treatment of chronic hepatitis C. Other PEG-proteins are in earlier stages of investigation, among which several PEG-cytokines (namely PEG-IFN1, PEG- r-human megakariocyte growth and development factor -PEG-r-Hu-MGDF-, PEG-interleukines, and PEG-TNFα), PEG-enzymes (namely PEG-adenosin deiminase, PEG-methioninase, and PEG-uricase) and PEGylated antibodies against cancer epitopes. This chapter will focus only on PEGylated enzymes and antibodies having potential or established anticancer applications. Some of these products are already marketed, while others are under preclinical or phases I–III clinical trials. Other PEG-proteins that are important in cancer therapy, belonging to the cytokine class, are not described here and are extensively reviewed in Chap. 6.

2. Enzymes and PEG-Enzymes in Cancer Therapy

Several enzymes have proven to be useful in the field of cancer therapy by acting through different mechanisms.[3] Metabolite depleting enzymes, such as asparaginase, methioninase, and arginin-deiminase, induce cancer cell death by depleting the environment of essential amino acids for which tumor cells only are auxotrophic. The enzyme chondroitinase AC is able to prevent cell proliferation by removing cell surface chondroitin sulfate proteoglycans that are fundamental for tumor growth, neovascularzation, and metastasis.[4] Other enzyme-related proteins that are being studied in

cancer therapy are the hybrid molecules belonging to the antibody directed enzyme prodrug therapy (ADEPT) family, which are composed of an enzymatic and a site-directing moiety linked together. Enzymes are also investigated to reduce or prevent certain chemotherapy side effects. This is the case of uricase, an enzyme that degrades the poorly soluble uric acid whose build-up (that causes gouty arthritis and chronic renal disease) is induced by chemotherapy.[5,6]

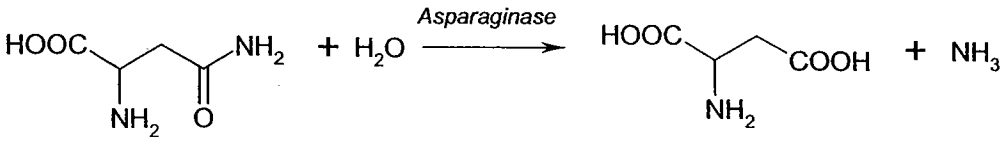
Some of these compounds are already available in the clinic in their native form and, in most cases, PEGylation has been investigated to improve their pharmacological properties. PEGylated asparaginase is already FDA approved (Oncaspar®), whereas PEG-argininedeiminase, PEG-methioninase and PEG-uricase are still under preclinical or clinical evaluation. To our knowledge, PEGylation of chondroitinase and ADEPT proteins has not yet been reported in the literature.

2.1. *Metabolite depleting enzymes*

Novel strategies for tumor treatment take advantage of different metabolic requirements expressed by tumor cells with respect to healthy tissues. For example, a tumor selective target with high therapeutic potential may be the elevated requirement of a selected metabolite of tumor cells relative to normal cells. Examples are the inability of hematological tumor cells to grow in the absence of asparagine, the dependence of several solid tumors on high level of methionine and the auxothropy for arginine displayed by some melanomas and hepatocellular carcinomas. On the basis of these metabolic differences, enzymes that are able to reduce plasma levels of these metabolites namely asparaginase, methioninase and arginine deiminase, are studied as therapeutic agents in cancer therapy. All of these enzymes have bacterial origin and their clinical use is hampered by poor pharmacokinetics and high risks of immunogenicity. PEGylation has thus become the strategy of choice to solve these problems and allow the therapeutic use of these enzymes.

2.1.1. *PEG-asparaginase (PEG-ASNase)*

The enzyme asparaginase catalyzes the hydrolysis of asparagine to aspartic acid and ammonia. The resulting depletion in asparagine is fatal to leukemic lymphoblasts and certain other tumor cells that, by lacking or having very low levels of asparagine synthetase, are unable to synthesize



Scheme 1. Conversion of asparagine to aspartic acid and ammonia by asparaginase.

asparagine *de novo* and rely on asparagine supplied in the serum for survival.

The potential for asparaginase treatment stemmed from the early observation of Kidd in 1953,[7] who described an activity in guinea pig sera that caused regression of transplanted lymphomas in mice and rats. This observation was later related to presence of asparaginase activity in the guinea pig serum.[8] An asparaginase form was later isolated from *E. coli* which exhibited antitumor activity similar to that found in guinea pig sera[9,10] and provided a practical source of the enzyme for later preclinical and clinical investigations.

Preclinical studies in the late 1950s and early 1960s showed that the enzyme was effective against several tumors. The majority of susceptible tumors were of lymphoid origin, and T-cell lineage was found to be more sensitive than the B-cell one. Several clinical trials demonstrated the efficacy of asparaginase in the treatment of many tumors and FDA approval for the native enzyme was granted in 1978. Since then, asparaginase has become an important chemotherapeutic agent in the treatment of acute lymphoblastic leukemia (ALL) and other lymphoid malignancies. It has demonstrated effectiveness in induction, and in subsequent phases of various multiagent chemotherapeutic regimens. Because this enzyme is generally not myelosuppressive and is not cross-resistant with other antineoplastic agents, it is easily added to combination chemotherapy protocols. New therapeutic indications, as for the treatment of myeloma or solid tumors, are presently being evaluated in clinical trials.[11,12]

Two enzyme forms are presently available in the clinic, one is isolated from *E. coli* (marketed commercially by Merck & Co. as Elspar®), has a molecular weight of 138 000–141 000 Da and is composed of four identical subunits with an active site on each. The second one is isolated from *Erwinia chrysanthemi* (available as *Erwinia* L-asparaginase from Ogden BioServices Pharmaceutical Repository in the United States), it is also a tetramer and has a molecular weight of 138 000 Da. Both enzymes have high activity and

stability. They differ in isoelectric point and plasma permanence and lack antigenic cross-reactivity. Both native preparations are approved for use in the therapy of patients in the front line and at relapse.

The major limitation to the use of native asparaginases is clinical hypersensitivity, which develops in 3–78% of patients treated with native forms of the enzyme. Either acute allergic reactions or silent hypersensitivities occur in a significant proportion of patients exposed to multiple courses of asparaginase and the antibodies developed often account for treatment failures. In fact, a clear correlation between anti-L-asparaginase antibody titer and clearance of L-asparaginase (ASNase) was demonstrated.

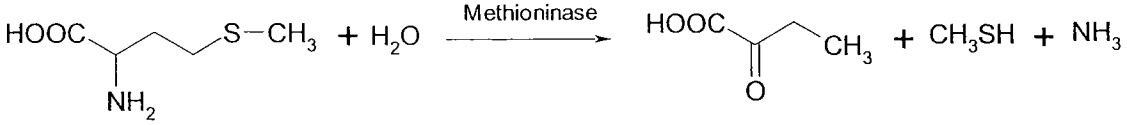
PEGylation of ASNase was thus investigated as a means to reduce the hypersensitivity problem. The first PEG conjugate of *E. coli*-derived L-asparaginase was already developed in 1979.[13] Asparaginase from *E. coli* was chemically modified with PEG of 5000 MW and the conjugate was shown to cause tumor regression in transplanted mice with less immunogenicity than the native *E. coli*[14–16] form. PEGylation also improves the enzyme chemical stability and its resistance to plasma proteases[17] PEG-asparaginase was entered into clinical trials already in 1984 and has since been administered to thousands of patients with ALL.[18] The PEG-conjugated enzyme is less immunogenic than either of the two native products and can be administered safely to most patients with allergic reactions to *E. coli* or *Erwinia* asparaginases. The longer serum $t_{1/2}$ of PEG-asparaginase allows for a longer interval between doses. PEG-asparaginase was developed by Enzon and was FDA approved in 1994 for use in combination chemotherapy for the treatment of patients with ALL who are hypersensitive to native forms of the enzyme. It is now available commercially from Rhone-Poulenc Rorer as Oncaspar®. The product has similar toxicological profile as the non-modified enzyme. However, PEGylation improves both the immunological and pharmacokinetic properties of the enzyme. The mean serum $t_{1/2}$ of PEG-ASNase is about 15 days as opposed to the 24 hr of the nonmodified *E. coli* enzyme and to the 10 hr of the *Erwinia* form. The longer serum $t_{1/2}$ of PEG-asparaginase allows for a longer interval between administration doses. The rate of total clearance of PEG-asparaginase was found to be 17-fold lower than that of the unmodified enzyme, whereas the volume of distribution was similar for the two preparations. The duration of asparagine serum depletion upon parenteral administration correlated with the serum $t_{1/2}$ of the different

enzyme forms. Already early studies showed that ASNase levels were undetectable immediately following the 1-hr infusion of PEG-asparaginase and remained low during the 14-day interval between doses. Interestingly, a recent pharmaco-economic study[19] demonstrated that despite the higher pharmacy cost of PEG-asparaginase *versus* the unmodified enzymes, the overall cost of the treatment with the PEGylated product is similar to the one with the unmodified enzymes.

Since FDA's approval in 1994, drug monitoring has been performed by several phase IV clinical studies and detailed recent reviews are available in the literature.[20,21] Recent studies have been carried out on the rational basis that immunological, pharmacokinetic, and pharmacodynamic factors have a considerable impact on the efficacy of asparaginase therapy. Therefore, investigation is now aimed at defining the optimum dose and dosing schedule of the different asparaginase preparations that are used in the clinic[22] or at correlating antibody levels with pharmacological response.[23] Additional pharmacokinetic studies of old and new asparaginases will improve general understanding of the reasons behind treatment success or failure and allow for the development of more rational dosing schedules in individual patients.

2.1.2. *PEG-methioninase (PEG-METase)*

Methionine dependence is a metabolic defect seen only in cancer cells and precludes cells from growing in a methionine-depleted medium. Methioninase (methionine-α-deamino-γ-mercaptomethane-lyase, METase), an enzyme isolated from *Pseudomonas putida* (Hori, 1996), tranforms L-methionine into α-ketobutirrate, methanethiol and ammonia and is thus able to induce methionine depletion in the medium. METase is a pyridoxal-L-phosphate-dependent enzyme, it is a homotetramer, each subunit being composed of 398 amino acids. The enzyme was efficiently cloned and purified from a highly expressing *E. coli* system and its potentiality as



Scheme 2. Conversion of methionine to α-chetobuttirate, methanthiol and ammonia by methioninase.

an antitumor agent, alone or in combination with other chemotherapeutic agents, was demonstrated in a number of solid tumors, both *in vitro*[24] and in *in vivo* models. Phase I clinical trials have also been carried out and demonstrated the low toxicity of this protein in cancer patients.[25,26] Nevertheless, studies performed in balbC mice demonstrated the immunogenic properties of the recombinant enzyme.[27] This fact, together with its relatively short body permanence upon i.v. administration ($t_{1/2}$ = 80–120 min), justified the search for PEGylated derivatives with improved pharmacodynamic properties.

A number of studies carried out by the group of Hoffman demonstrated the superiority of PEGylated forms of this enzyme by a combination of *in vitro* and *in vivo* experiments. The enzyme has been PEGylated with methoxy-PEG of 5000 MW, activated either as succinimidylpropionate[28] or as succinimidylglutarate,[27] at different PEG/methioninase molar ratios. Even if partial loss of enzymatic activity was observed after PEG coupling, the PEGylated enzyme had similar cytotoxic effect on *in vitro* cultured tumor cells[28] and superior properties in *in vivo* experiments, namely a more efficient and longer-lasting serum methionine depletion and significant lower immnunogenicity.[27,29] More precisely, serum half life upon i.v. administration in mice increased from 2 hr for the native protein to 12, 18, and 30 hr for PEGylated forms having, respectively, 4, 6, and 8 PEG molecules attached to each subunit (Fig. 1). Besides, the duration of methionine depletion in serum upon a single i.v. administration of these same compounds was increased from 4 hr (registered for the native enzyme) to 24, 48, and 72 hr respectively. Antibodies (IgG and IgM) against METase were raised by all the enzyme forms, upon a strong immunization protocol carried out in the presence of Freund's adjuvant. Nevertheless, the PEGylated enzymes were significantly less immunogenic as compared to the native enzyme, and the IgG titer raised by the highly PEGylated enzyme was four orders of magnitude less than the one raised by the native enzyme. Less PEGylated samples displayed intermediate immunogenic properties, but in any case, were significantly better than the native enzymes.

A detailed investigation on the pharmacokinetics, antigenicity and toxicity of native and PEGylated-r-METase was recently carried out in mice and primate models.[30,31,29] Native METase and a PEGylated form of the enzyme, modified with PEG-succinimidylglutarate and containing 3–7 molecules of PEG per protein were analyzed. Native r-METase has a biological half life

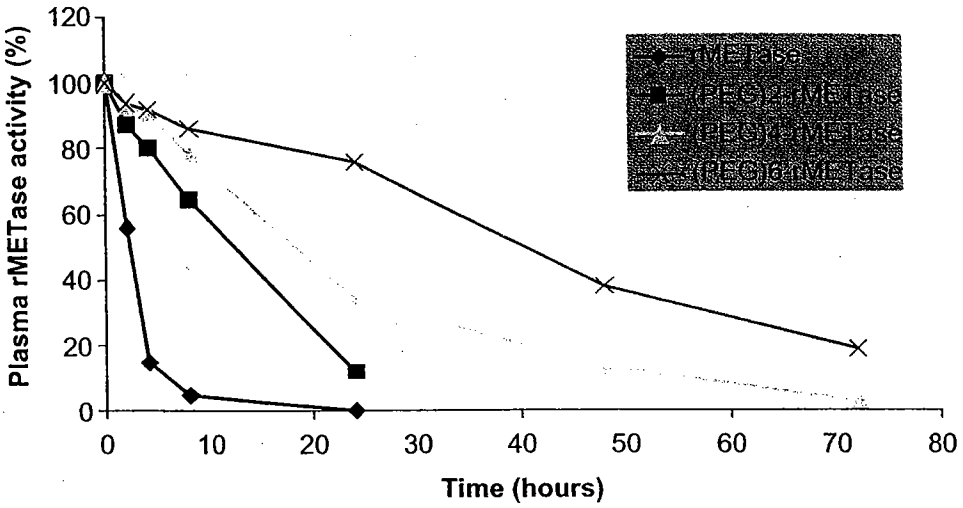


Fig. 1 Plasma rMETase enzyme activity after i.v. injection of native or PEGylated rMETase in mice. Mice received 80 units of the native rMETase or of the indicated PEGylated form.[27,29]

of 2.5 hr and repeated administrations of the enzyme to primates resulted in severe anaphylactic reactions.[30] Upon PEGylation, the plasma $t_{1/2}$ of the enzyme in its "apo" form increased 36-fold while the active holo form displayed a $t_{1/2}$ of 143 hr only. It was found that the limited circulating half-life of the active holo-PEG-rMETase is due to rapid *in vivo* dissociation of its cofactor pyridoxal-5′-phosphate (PLP).[30] A combination of PEG-rMETase treatment with PLP infusion using an osmotic pump was thus suggested for an effective therapy with a reduced number of protein injections. Some antibodies against anti-PEG-rMETase were produced upon repeated challenges in the primate model. However, the level of such antibodies was 100–1000 fold less than the one elicited by the native enzyme and the antibodies were unable to neutralize the activity of the enzyme so that each challenging dose was effective in depleting serum methionine levels.

2.1.3. *PEG-arginine deiminase (PEG-ADI)*

Arginine is one of the nonessential amino acids for humans. Normal cells synthesize it from citrulline using the enzymes argininosuccinatesyntase (ASS) and argininosuccinate lyase. It was found that some cancer cells do not express ASS[32] and are thus unable to synthesize arginine from its precursor. Therefore, it was suggested that an arginine degrading enzyme could be effective against tumors that are auxotrophic for arginine.

independently of PEG MW, shape and of the linker used. Modification of 10–12 lysines/enzyme molecule leads to products having about 50% specific activity as the nonmodified enzyme. Two formulations obtained with PEG of either 5000 or 20 000 Da MW, both with 10–12 PEG chains/enzyme and displaying similar specific acivity *in vitro*, were compared in *in vivo* experiments. Pharmacokinetic studies (Table 1) revealed a superiority of the 20 000 Da MW adduct, independently of the route of adiministration and animal sex.[40] The 20 000 Da MW (ADI-PEG20 000) adduct was compared to native ADI in terms of pharmacokinetics and antitumor efficacy in cancer animal models. A single i.m. administration of ADI-PEG20 000 (5 IU/mouse) was able to lower arginine serum levels below the detection limit for 1 week, as opposed to unmodified ADI that induced a 50% reduction of arginine serum levels which lasted for 1 day only. The PEG adduct showed a half life of about 7 days as opposed to the 5 hr of the native enzyme. Weekly i.m. administrations of PEG-ADI (5 IU/dose, for total 2 weeks) greatly increased the survival rate of animal models growing ADI sensitive tumors, as opposed to the administration of native ADI on a twice-a-week schedule which was found to be ineffective. These promising results opened the way to phases I and II clinical trials for the PEG20 000 adduct in humans with metastatic melanoma and HCC.[41] Nineteen patients with advanced or inoperable HCC were treated. An optimal biological dose (OBD) of 160 IU/m^2 to be administered on a weekly base, which was able to reduce the serum arginine levels below detection limits for more than 7 days, was assessed. Out of the 19 patients treated, 16 showed partial to complete response to ADI-PEG20 000 treatment; two patients showed a complete response, seven patients had partial response, seven had stable disease and only three patients showed progressive disease. No significant side effects were observed and the drug was well tolerated. Mild immunogenicity was registered, serum antibodies against ADI-PEG20 000 increased with the duration of treatment. However, none of the plasma samples obtained from any of the patients inhibited the *in vitro* enzymatic activity of ADI-PEG2000 and no patient developed evidence of systemic or local allergic response to ADI-PEG20 000 injections. These results are very promising and further clinical investigations are presently underway.

2.1.4. *PEG-uricase (PEG-UK)*

Another enzyme that finds application in cancer treatment is uricase (urate oxidase, EC 1.7.3.3.). This enzyme catalyzes the oxidation of uric acid to

renal failure in patients with hematologic malignancy with a high tumor burden and patients at risk or a rapid tumor lysis or shrinkage at initiation of chemotherapy.

This enzyme is a tetramer with identical subunits of 34 kDa, where two dimers are superimposed face-to-face to form a tunnel-shaped protein. It has four active sites which are located in a pocket open toward the exterior of the protein. Uricase is present in most mammals but it is lacking in humans because of a nonsense mutation in exon 2. Since humans do not make uricase, all forms of uricases are highly antigenic, and multiple administrations of native uricase have resulted in allergic reactions, anaphylaxis, and even death. As for other proteins, PEGylation has been suggested to overcome these problems.

PEGylation of uricase from different sources has been described in the literature. More precisely, PEGylation has been performed on uricases from *Candida Utilis*,[43–49] from *Arthrobacter protoformiae*,[50] from *Bacillus fastidiosus*[51] and from porcine source.[44,52] It was recently shown that uricase from *C. utilis* has preferable biochemical features compared with uricase from other organisms, including *A. flavus* (rasburicase), hog liver, and *A. protoformiae*. These preferable features include the highest affinity for uric acid and greatest catalytic rate at physiologic pH.

The first PEGylation of uricase from *C. utilis* was described more than 20 years ago by Nishimura.[43,46] The enzyme was modified with a PEG of 5000 Da MW activated as 4,6-dichloro-s-triazine. 43 or 36% of the total 120 amino groups in the enzyme were coupled to the polymer and the modified uricases retained 15 and 45% of the original enzymatic activity, respectively. In both cases, complete loss of the binding ability toward antiuricase serum from rabbit was achieved. The conjugates displayed less immunogenicity and improved plasma residence but, unfortunately, antibodies to the conjugate were raised that recognized to the linker used.[43,46] The same enzyme was later modified with PEG 5000 activated as succinimidyl ester. Modification of 50–70% of the primary amines successfully masked the protein immunogenicity in both mice and humans.[44,45]

Urate oxidase from *C. utilis* is now expressed in *E. coli*. PEGylation of this recombinant enzyme was carried out with succinimidyl succimide activated PEGs having 5000 or 20 000 Da MW[49] and with linear 5000 and branched 10 000 Da PEGs containing a terminal amino acid (nor-leucine or lysine) activated as succinimidyl ester.[47,48] In the work of Bomalaski

and colleagues,[49] the 20 000 Da MW conjugate displayed best performance in terms of specific activity and improved plasma residence. PEG was coupled to approximately 66% of the available primary amines and the product retained about 86% of the original activity as compared to 50% residual activity of the 5000 Da adduct. The 20 000 Da MW conjugate was less immunogenic than the 5000 MW and less than other PEG-uricases previously administered to humans. The 20 kDa adduct was administered i.m. in an experimental mouse system and 87% of the drug was recovered in the plasma (Fig. 2). Plasma $t_{1/2}$ was about 3 days. A phase-1 clinical trial was performed for the 20 kDa adduct in patients with hyperuricemia and gout and demonstrated safety and efficacy.[53] A single dose cohort escalation conjugate was performed: plasma residence was maintained for 2–8 days, nontoxicity and no immunogenicity were recorded.[49]

The work carried out by Padua University research group[47,48] compared uricase conjugates obtained with different polymers, namely linear (5000 Da MW) and branched (10 000 Da MW, PEG_2) PEGs, PVP, and PAcM. 40–47% of the available primary amines were modified. Active site protection was achieved during the coupling reaction by adding uric acid in the reaction mixture. Residual activity in the conjugates varied between 20 and 80%. Extensive pharmacokinetic[47] and immunogenic evaluation[48]

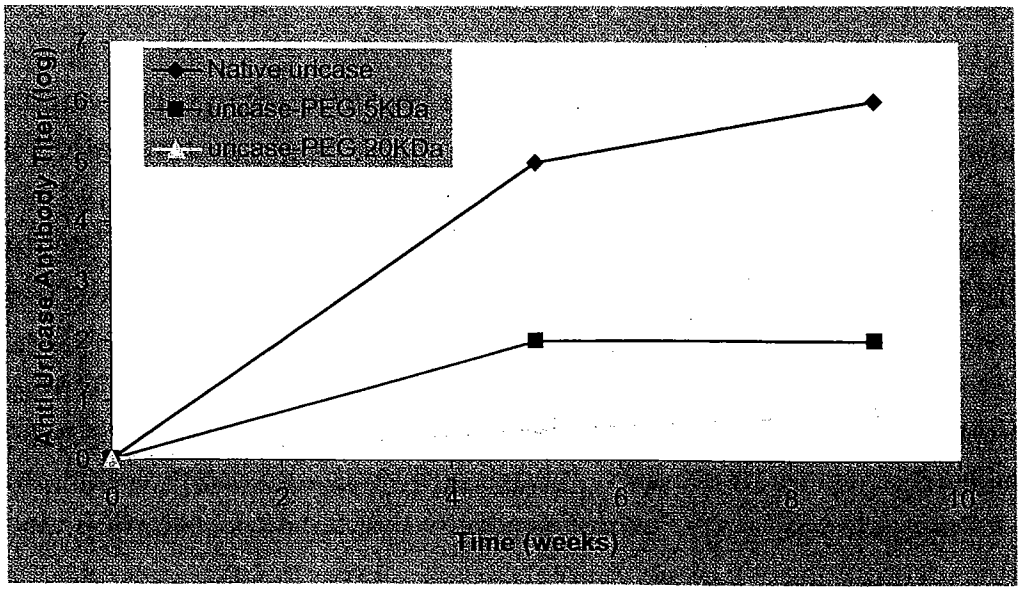


Fig. 2 Immunogenicity of native urate oxidase, uricase-PEG 20 kDa and uricase-PEG 5 kDa in mice. CD-1 mice were injected i.m. once a week for 8 weeks with 80 IU/m^2 of either native or PEG conjugated uricase.[49]

were carried out. Native uricase displayed an elimination $t_{1/2}$ of 6.5 hr and PEGylation improved significantly this parameter as the PEG_2 conjugate showed a $t_{1/2}$ of 43 hr while the linear PEG conjugate displayed a $t_{1/2}$ of 56 hr. Differences in tissue distribution were observed between the two conjugates even if in both cases spleen and liver accumulation were preferential. It is, however, noteworthy that the dose administered in this study is well above the therapeutic one.

PEGylation decreased but did not totally eliminate the protein immunogenicity. The PEG_2 conjugate was the less immunogenic. Interestingly, antibodies against the conjugated polymer were observed after the second injection.

Uricase from *A. protoformiae* was also modified with PEG of 5000 Da MW and produced by Enzon.[50] About 60% of the proteins primary amines were modified leading to a product devoid of immunogenicity in humans. The conjugate was tested in a single patient that had undergone chemotherapy and had shown exfoliative reaction to alloperidol. PEG-uricase was administered i.m. and uricase activity reached the plasma peak within 24 hr and persisting for about 5 days. No antibodies to either uricase or PEG-uricase developed over a three-week period, during which four doses of the drug had been administered.

Uricase from porcine source has also been PEGylated.[44,52] It was recently shown that increased immunogenicity and accelerated clearance of uricase from this source were due to the presence of large protein aggregates that are undetectable by UV absorbance under normal analytical conditions, but that can be detected by static or dynamic light scattering. The removal of such very large aggregates prior to conjugation with PEG was found to decrease significantly both the immunogenicity of the native protein and the accelerated clearance of the resultant PEG-uricase conjugates. By using a uricase preparation that is devoid of these large aggregates, a lower degree of modification with PEG was found to be necessary to reduce the protein immunogenicity. Lower degree of modification corresponded to a higher bioactivity in the conjugate. Indeed, modification of 12 primary amines with PEG 10 000 led to a product which was devoid of immunogenicity and maintained 75% of the original activity.[52]

Puricase®, a PEGylated form of a uricase from porcine source is now being developed by Savient Pharmaceuticals and recently passed phase-1 clinical investigation. A phase-2 clinical study, an open-label, randomized,

multicenter dose-ranging trial involving 41 patients, has recently been concluded. This study compared three dosage levels and two dosage regimens over a twelve-week period. The results are presently being evaluated and will probably be released by mid 2005. Press release from Savient pharmaceuticals Inc. is optimistic and state that data are acceptable for proceeding to more extensive patient exposure in a phase-3 development program.

3. PEG-Antibodies

In cancer therapy, antibodies or antibody fragments are most commonly studied as drug targeting agents. In some cases, antibodies and antibody fragments have been developed to have anticancer properties by themselves. Among these, recent examples are the antibodies directed toward the cytokines (e.g. IGF, EGF, and ErbB) receptors,[54–56] or growth factors (e.g. VEGF)[57] that have a direct effect on tumor cell growth by interacting with the receptor-mediated cell proliferation signalling pathway.

This field of investigation comprehends either monoclonal murine or humanized entire antibody molecules, as well as smaller fragments, namely the products of enzymatic degradation Fab′ and $(Fab')_2$ and, more recently, the engineered scFv fragments that, directly expressed in *E. coli* systems, are economically advantageous (Fig. 3).[58–63] Full-length antibodies are

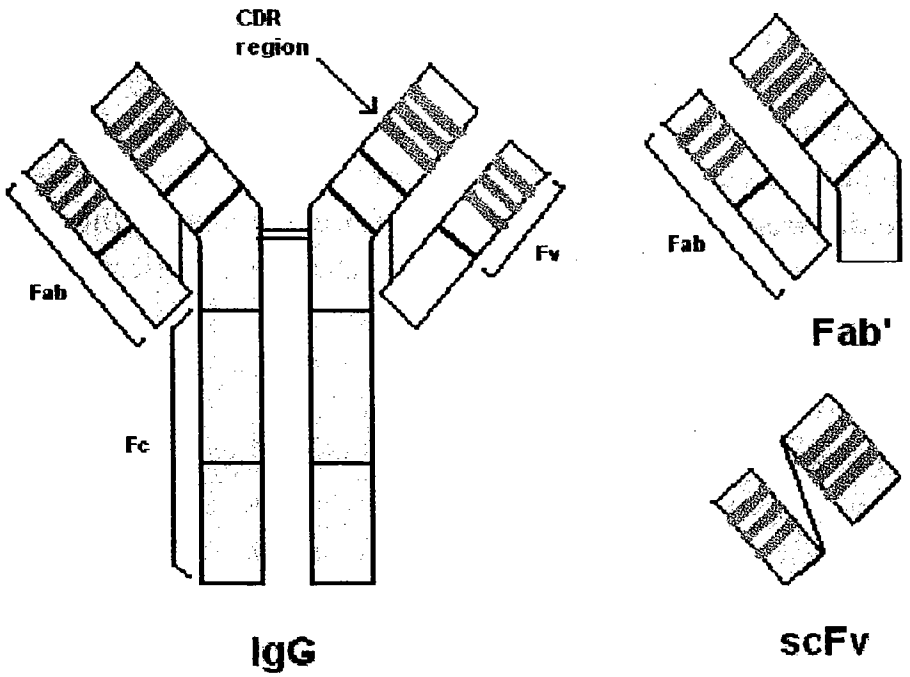


Fig. 3 Schematic cartoon of an IgG antibody and its fragments

characterized by a long plasma permanence and in the early days, when humanized antibodies were not yet available, rationale for PEGylation was only to diminish the high immunogenic properties of these xenoproteins. Humanized antibodies do not have immunogenic problems and, in principle, PEGylation is not needed. On the other hand, Fab′, $(Fab')_2$ and scFv fragments have very short body permanence upon parenteral administration and PEGylation is necessary to improve this parameter (Table 2). Several studies demonstrated the usefulness of PEGylation to improve plasma residence of Fab′, $(Fab')_2$ and ScFv fragments.[58,64–67] Plasma residence increases with PEG molecular weight and with the number of polymer molecule linked per antibody. However, it appears that it is enough to add one large (40 kDa) MW PEG molecule to restore similar body residence as the one displayed by the entire IgG.[67] Most commonly, when tumor-bearing animal models are injected with PEGylated tumor-epitope-targeting antibodies or antibody fragments, tumor : blood ratio decreases as a consequence of improved plasma residence. However, PEGylated products do show advantages over the nonmodified products, namely an improved tumor uptake,[58,67] probably as a consequence of the polymer induced EPR effect, and minor overall tissue distribution that corresponds also to diminished renal and liver toxicity.[68,69] Despite the positive impact exerted by PEGylation on the pharmacokinetics parameters, several studies performed on both entire antibody molecules and Fab′ fragments also demonstrated that random PEGylation, that is carried out by using amine-directed PEG reagents, has a negative impact on antigen binding properties. Even a small number of PEG molecules/protein may be sufficient to decrease significantly the antigen-binding properties.[67] As expected, loss of binding increases with the number of PEG molecules attached and their

Table 2 Pharmacokinetic and antigen-binding data for site specific Fab′-PEG conjugates injected in rats.

Compounds	$t_{1/2}\alpha$ (h)	$t_{1/2}\beta$ (h)	AUC (0−∞)	AUC (% IgG)	Antigen binding (% Fab′)
IgG	5.80	104	8791	100	—
Fab′	0.33	22.7	329	3.7	100
Fab′-PEG (1 × 25 kDa)	4.97	30.5	2261	26	107
Fab′-PEG (1 × 40 kDa)	5.60	45.8	4465	51	102
Fab′-PEG (2 × 25 kDa)	9.05	49.1	6999	80	100

molecular weight, consistent with both a steric effect and the direct modification of lysines involved in antigen recognition. Site directed modification has thus become a strategy of choice. A general approach for targeted PEGylation relies on the use of PEG-maleimide for the selective modification of engineered cysteine-bearing antibody fragments. By introducing, by genetic manipulation, the target cysteine on a location far away from the antigen binding site, monoPEGylation of either Fab'[58] or ScFv[61] fragments has been achieved without loss of antigen-binding ability.

These conjugation strategies have been investigated independently of the therapeutic application of the PEGylated antibody. In the specific field of anticancer therapeutics, several studies on PEGylated antibodies have been performed, but no product so far has entered clinical trials. Examples of cancer epitopes that were chosen as targets for PEGylated antibodies are the A33 and CEA antigens and the $ErbB_2$ receptor. The $ErbB_2$ receptor is a molecule that is overexpressed in epithelial cells and in tumors of epithelial origins.[70] Murine monoclonal antibodies and their pepsin digested Fab' fragments have been modified either with PEG5000 maleimide or with branched PEG20 000NHS (PEG_2). PEG-maleimide was conjugated to sulphydryl groups located at the hinge region, which were obtained upon partial reduction of the molecule with dithiotreitol (DTT); modification with PEG_2 was randomly targeted towards the amine residues. In the case of the maleimide 5000 Da MW conjugate, modification lead to loss of antigen binding ability, whereas PEG_2 adducts maintained reactivity towards their target, as shown by both ELISA and cell culture tests. Besides, experiments carried out in animal tumor models demonstrated that PEG_2 adducts were effective in preventing tumor growth and were more efficacious than the unmodified antibodies. On the contrary thiol modified derivatives were less effective.

The A33 antigen[66] is a cell surface glycoprotein expressed on normal gastrointestinal epithelium and on 95% of primary metastatic colon cancers, while being absent in most other normal tissues. Humanized α-A33 antibodies, randomly modified with linear 5000 and 20 000 Da PEGs, retained more than 50% of their antigen binding ability and were less immunogenic than the unmodified antibody. Even if the tumor/blood ratio did not improve upon PEGylation, tumor uptake of the modified antibodies was still efficacious.[64]

The CEA antigen is the first oncorelated surface antigen discovered. It is expressed on the surface of many carcinomas and it is extensively

investigated as a target for radio-immuno-therapy. The monoclonal α-CEA antibody A5B7 and its Fab′ and $F(ab')_2$ fragments have been PEGylated with PEG of 5000 Da MW and their pharmacokinetics were compared with the unmodified forms in the LS174T colonic xenograft in nude mice[71,72] PEGylation of the intact antibody had little effect on biodistribution, although tumor localization was slightly reduced. In contrast, modification of $F(ab')_2$ and Fab′ A5B7 significantly prolonged plasma half-life and increased antibody accumulation in the tumor but reduced tissue to blood ratios. Prior to modification, Fab′ A5B7, that has a MW of 50 kDa, was cleared more rapidly from the circulation than the larger $F(ab')_2$ (MW 10 kDa), but after PEG attachment their biodistributions converged, while the tumor to blood ratios were reduced and resembled that of the intact antibody.

Despite these early investigations, next generation of PEGylated tumor-antigen targeting molecules will probably rely on the recombinant Single chain Fv antibody fragments (ScFv). Not only these product are economically advantageous as compared to entire antibody molecules, but also optimization of their target binding ability is possible by the combination of recent antibody engineering tools and phage display. The generic approach described by Yang for the obtainment of engineered thiol bearing ScFvs suitable for site specific PEGylation[61] has been recently applied to target cancer epitopes.[62,63] Four free-cysteine containing ScFv fragments (Sc-Fv-c) targeting the cancer epitope MUC-1 were obtained by recombinant technology. The proteins were PEGylated using either mono- or bifunctional, maleimide- or *o*-pyridyl-disulfide-, thiol reactive PEGs having a molecular weight ranging from 2 to 40 kDa. The yield of modification varied depending on the polymer molecular weight, functionality (mono- or bivalent) and the reactive group. Monofunctional low molecular weight maleimide derivatives displayed better yields of modification. The most relevant result is that all PEG derivatives maintained the same antigen binding properties as the nonmodified scFv-cs, holding promises for future applications in cancer immunotherapy.

4. Conclusions

After almost 40 years of experimentation, PEGylation has become a well-established technology in the field of biopharmaceutical formulation. The remarkable advantages of PEGylated proteins over their nonmodified

parents will likely lead to further expansion of this technology to more and more bioactive compounds. Lessons from the past have shown that PEGylation of a bioactive protein has often followed the initial clinical approval of the native compound. Indeed, the number of protein pharmaceutics in clinical trials — among which almost half are indicated for cancer treatment — is increasing dramatically every year[73] and PEGylation will be useful any time poor pharmacokinetics and immunogenic problems will occur. The costs related to the development of PEGylated products is also likely to diminish, mostly thanks to the wide expertise in protein PEGylation acquired in recent years that provided general clues for faster coupling strategy optimization.

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PEGylated Proteins in Immunotherapy of Cancer

James F. Eliason

Abstract

The half-lives of proteins *in vivo* can be increased by covalently attaching the polymer polyethylene glycol (PEG) to them. This can significantly decrease their rate of clearance from plasma. The clearance rate is inversely proportional to the total molecular weight of the construct. This approach has been used to design cytokine constructs that can be administered once a week, rather than on a daily or every other day schedule. Two cytokines for which this approach appears to be successful are PEG-interferon-α_{2a} (PEG-IFN-α_{2a}; Pegasys™) and PEG-granulocyte colony-stimulating factor (PEG-G-CSF; SD/01). Both use large MW PEG (20–40 kDa) to give sufficiently long duration *in vivo*. With PEG-G-CSF conjugates, the *in vivo* efficacy is directly proportional to molecular weight, whereas the *in vitro* activity is inversely proportional, suggestion that overall duration of contact is more important than affinity of the interaction. Conjugates of a number of other cytokines have been prepared, but until recently, few have used the large molecular weight polymers. As this approach is likely to be taken in the future to make new PEG-cytokine constructs, thorough *in vivo* pharmacokinetic studies will be essential for their development and clinical use.

1. Introduction

Historically, the treatment of cancer has involved a variety of cytoreductive therapies, including radiation therapy and chemotherapy. In the initial phases of treatment, these therapies often result in major reductions in tumor burden. However, with the exception of children with certain types of cancers, there has been relatively little impact on survival of cancer patients. Even high-dose therapy requiring hematopoietic stem cell support to counteract lethality to this system can provide long-term survival for 15–20% of patients with metastatic breast cancer, at best. Other approaches are clearly needed to improve the cure rate for advanced adult cancers.

Immunotherapy represents another approach, which has been around for many years but has recently received renewed attention, as a means to eliminate residual disease following cytoreductive therapy. A number of trials in the past provided evidence that this approach can result in dramatic tumor reduction in a small subset of patients with certain types of tumors. However, these studies were usually performed in patients with bulky disease, where this approach is least likely to work. Combinations with conventional therapies and new approaches to stimulating and/or modifying the immune system are now being explored for treatment of cancer.

Cytokines are regulatory proteins, produced by cells, which can stimulate proliferation and/or activation of (1) neighboring cells in a paracrine fashion, (2) the producing cells themselves in an autocrine fashion, or (3) distant cells in a hormonal fashion. They are important components of immune responses, regulating the activities of both the specific immune system, consisting of various lymphocyte subsets, and the nonspecific immune system, composed of myeloid cells such as granulocytes, and macrophages. The latter cell types are not always considered in conjunction with immunotherapy but are important in combating microbial infections in patients receiving cytotoxic therapies that can drastically reduce myeloid cell numbers. A number of cytokines have been tested in the clinical and several are approved for use. The most important of these are the interferon-α's of which three (IFN-α_{2a}, IFN-α_{2b}, and IFN-consensus) are registered[1] and granulocyte colony-stimulating factor (G-CSF).[2]

One of the practical problems with clinical use of cytokines is that they have to be injected intravenously or subcutaneously. In addition, they have

short half-lives in the body, whereas their activities are usually optimal with continuous exposure. Therefore, they have to be administered frequently, usually on a daily basis.

Removal of proteins from circulation is usually through clearance in the kidney. Increasing the molecular weight of proteins by attachment of PEG can significantly reduce their renal clearance, presumably by increasing the size of the conjugates in relation to the pore sizes of the renal vascular bed.[3] A study using radiolabeled PEG molecules (not conjugated to proteins), which had different molecular weights demonstrated that the area under the plasma concentration time curves (AUCs) increased with increasing molecular weight.[4] Another mechanism relevant for removing cytokines from circulation is receptor-mediated endocytosis.[5,6] The effects of PEG on this mechanism are not clear, but if PEGylation significantly decreases the binding affinity of conjugates to their receptors, then clearance will also be decreased. A number of different linkage chemistries have been used for preparing PEG conjugates and these can, in some cases, affect the activity and pharmacokinetics.[7,8]

The pharmacokinetics of PEGylated proteins can also be influenced by the route of their administration. Compared to intravenous or intraperitoneal injection, administration subcutaneously, or intramuscularly may delay the release of proteins into circulation. There is no evidence, however, that PEGylation affects the distribution of circulating cytokines in the body, particularly with respect to retention in tumors.

A further problem is that some of the recombinant proteins can be immunogenic, so resistance can build up due to development of neutralizing antibodies. This has been a problem with IFN-α.[1] One approach being applied to overcome these problems is conjugation of the proteins with the polymer polyethylene glycol (PEG), which may hide the antigenic peptide sequences from the immune system. One of the best examples of decreased immunogenicity of PEGylated proteins is the enzyme L-asparaginase, a treatment for acute lymphocytic leukemia. Two versions of this protein have been isolated, one from Escherichia coli and the other from Erwinia Carotovora. Because of their xenobiotic origins, hypersensitivity develops in up to 50% of patients.[9] The PEGylated *E. coli* protein (pegaspargase) has multiple 5 kDa MW chains attached. This conjugate has been registered for use in patients who had reactions to the un-PEGylated protein,[10] demonstrating that PEG can decrease antigenicity of proteins even in patients

who are sensitive to the unmodified protein. In addition, this modification increases the plasma half-life from 20 hr for the unmodified protein to 357 hr for the conjugate.[11]

This article will summarize the current status of PEGylated cytokines that may be beneficial for cancer patients.

2. PEGylated Interferon-α

Interferon-α was the first cytokine produced by recombinant DNA technology to be registered for cancer therapy.[1] A type-I interferon that is produced by fibroblasts was first identified as an antiviral protein. Three species of interferon-α have been registered for treatment of hepatitis B and C virus (HBV and HCV) infections. Two, IFN-α_{2a} and IFN-α_{2b} are naturally occurring forms and the other, consensus IFN (IFN-con1), is a mutein that was designed by using the most frequently observed amino acids found in several of the natural IFN-α subtypes. This usage as an antiviral therapy can also be considered as a cancer prevention therapy because chronic infections with HBV and HCV are important causative factors in hepatocellular carcinoma.

Interferon-α has demonstrated clinical activity against a number of fully developed cancers including hairy cell leukemia, chronic myelogenous leukemia (CML), B- and T-cell lymphomas, myeloma, Kaposi's sarcoma, melanoma, and renal cell carcinoma.[12] In combination with retinoids, IFN-α is also active against squamous cell carcinoma of the skin[13] and cervix.[14]

The first PEGylated IFN to be tested in the clinic was prepared from IFN-α_{2a} by attaching 5 kDa PEG in a 1:1 molar ratio via a urea linkage.[15] This modification increased the half-life of the conjugate only 2-fold compared to the unconjugated cytokine because of its relatively small increase in overall molecular weight and did not provide sufficient improvement to justify further development.[16] Following the experience with high molecular weight conjugates of G-CSF, described in the following section, a new PEG-IFN-α_{2a} conjugate with branched 40 kDa PEG was prepared. This molecule has a 51 hr half-life in rat compared to 0.7 hr for the unmodified protein. This increased half-life resulted in greater antitumor activity against a human renal cell model in athymic nude mice.[17] Several recent reports have demonstrated that this drug, which has the name Pegasys, given once a week is more effective than unmodified IFN-α_{2a} given three times a week