EXHIBIT B

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in patients with chronic hepatitis C infections.[18,19] Results of a phase I trial in patients with renal cell carcinoma demonstrated that there were no unique toxicities not seen with unmodified IFNs.[20]

A second PEGylated interferon, IFN-$\alpha_{2b}$, has also been developed. It is a conjugate with a 1:1 molar ratio of 12 kDa PEG.[21] The pharmacokinetics and pharmacodynamics of this molecule indicate that it has a shorter half-life than Pegasys as predicted by the lower overall molecular weight.[22] It has activity against CML, but has not been demonstrated to be statistically noninferior to nonPEGylated IFN-$\alpha_{2b}$.[23] This conjugate also has activity to decrease platelet numbers in essential thrombocythemia.[24]

The use of either PEGylated IFN-$\alpha$ in combination of ribavirin for treatment of HCV reduces the relative risk (RR) for remaining infected by 17% with nonPEGylated interferon plus ribavirin.[25] The combination treatment with the PEGylated proteins also results in a more cost effective approach to treating hepatitis C than the combination with unconjugated IFN-$\alpha$.

## 3. PEGylated G-CSF

Granulocyte colony-stimulating factor is a member of the hematopoietic growth factor family that increases the numbers of neutrophilic granulocytes.[2] As such, G-CSF is an important supportive agent for cancer cytotoxic therapy. Although it appears to have no direct or indirect activity on cancer cells, its activity helps alleviate the neutrophilia associated with standard chemotherapy thereby allowing patients to tolerate higher doses of cytotoxic therapy. The so-called dose-dense chemotherapies, which use higher dosages than standard chemotherapy because of hematopoietic growth factor support, have shown promise in treatment of hematological and solid tumors, especially breast cancer.[26] In addition, G-CSF induces migration of hematopoietic stem cells from bone marrow into peripheral blood, making it possible to collect large numbers of stem cells by leukapheresis. This is a much more tolerable source of hematopoietic stem cells from donors, especially in the case of allogeneic transplants, than drawing bone marrow aspirates; although the same is true for obtaining these stem cells from patients who receive them back as autologous stem cell rescue.

Three different molecular forms of recombinant G-CSFs are registered for clinical use in various countries. Filgrastim is produced in *E. coli* and has an N-terminal methionine. Lenograstim is produced in Chinese hamster ovary cells and is therefore glycosylated.[27] Nartograstim is a mutein

produced in *E. coli* with five amino acids substitutions in the N-terminal portion that increase its specific activity.[28]

A systematic approach to design a PEG-G-CSF that could be given once a week was undertaken. The goal was to make a molecule that would have pharmacokinetic properties that fit standard cancer chemotherapy practices that are usually designed on weekly schedules.

A number of different conjugates with one to three PEG chains of 5, 10, or 20 kDa per molecule of nartograstim were prepared. These conjugates were tested for their capacity to stimulate proliferation of G-CSF dependent cells *in vitro* and to increase granulocyte numbers in mice.[29] The activity *in vitro* was inversely proportional to the amount of PEG attached. The constructs having the highest the total molecular weight had the lowest the activity on cell proliferation in culture. This result suggests that the larger bulkier constructs may not bind as well to the G-CSF receptors. Interestingly, the *in vivo* activity was directly proportional to the total molecular weight of the constructs. Those molecules with the highest molecular weight had the highest activity in mice.[29] Similar results were evident in studies with cynomolgus monkeys where the higher molecular weight constructs had increased activities.[30] These results indicate that the duration of interaction between PEG-G-CSF and its receptors is more important than the affinity of this interaction.

A once weekly PEG-filgrastim (SD/01) has recently been produced based on the findings with PEG-nartograstim describe above.[31] This molecule has a single PEG chain of 20 kDa molecular weight attached to the N-terminus of filgrastim.[32] In mice, SD/01 causes a similar prolongation of neutrophilia in mice following a single injection and has been shown to reduce the mortality in an experimental murine model of invasive *Candida* albicans infection.[33] In clinical trials, a single administration of SD/01 results in prolonged elevation of neutrophils in patients receiving chemotherapy[34] as well as in normal volunteers.[31] No dose limiting toxicities were seen. In the clinical studies, it was also demonstrated that the peripheral blood stem cell levels were elevated in the patients and volunteers to a comparable extent as with filgrastim injected daily at the same cumulative dose. A trial using a fixed dose of pegfilgrastim demonstrated equivalence to daily filgrastim and significantly reduced incidence of febrile neutropenia than found with filgrastim.[35]

## 4. Other PEGylated Hematopoietic Growth Factors

A number of other hematopoietic growth factors have been PEGylated, but most published studies describe work in preclinical models. One factor that has similar activity to G-CSF is granulocyte macrophage colony-stimulating factor (GM-CSF). It has not received the same wide spread use as G-CSF because it is more toxic. The increased toxicity is due to the fact that it is, as the name implies, less lineage specific and stimulates macrophages as well as granulocytes. However, this additional activity may provide a rationale for its use in immunotherapy as an adjuvant for cancer vaccines. For vaccines to be effective, it is important to have the antigens presented in the proper manner to the immune effector cells. Dendritic cells, which are the most effective antigen presenting cells, can be expanded *in vitro* by GM-CSF.[36] A number of studies in animals have demonstrated that the high GM-CSF levels greatly enhance the antitumor effects of cancer vaccines.[37,38] In a murine study, PEG-GM-CSF has been shown to expand a subpopulation of dendritic cells *in vivo*,[39] so could be an active adjuvant for cancer vaccines.

Another factor with clinical activity is megakaryocyte growth and development factor (MGDF). This is a truncated form of thrombopoietin. MGDF has been shown to enhance platelet recovery in mice following bone marrow transplantation and the PEGylated form has a single 20 kDa PEG chain attached to the amino terminus.[40] This modification helps stabilize the tertiary structure of the truncated protein and gives it approximately 3-fold more active than the unmodified version.[41] The half life *in vivo* is short compared to that of PEG-filgrastim described above, so that that daily dosing is required. Two phase I trials have been reported, one in cancer patients receiving moderately myelosuppressive chemotherapy[42] and the other in breast cancer patients receiving high-dose therapy with autologous peripheral blood stem cell support.[43] Both studies showed that the drug was well tolerated, even at the maximum doses tested of 5 and 10 μg/kg/d, respectively. A recent report,[44] demonstrated PEG-MGDF enhances platelet numbers in cancer patients receiving chemotherapy. Unfortunately, this molecule has not had a significant effect on platelet levels in patients receiving high dose therapy for leukemia or in those receiving stem cell support after chemotherapy.[40]

PEG-MGDF as a single agent mobilizes stem cells, but did not significantly enhance mobilization above that achieved by chemotherapy plus filgrastim in these patients.

Interleukin-6 (IL-6) is another thrombopoietic factor that has been PEGylated.[45] Because its toxicity is greater than that of PEG-MGDF, it seems unlikely that it will play a significant role in clinical practice.

## 5. PEGylated Lymphokines

Another set of cytokines that have been used in cancer treatment trials are called lymphokines because they are produced by lymphocytes and often act on lymphocytes. One, Interleukin-2 (IL-2), formerly known as T-cell growth factor has been used in a number of clinical studies, but it has not become part of standard clinical practice because of its high toxicity. This toxicity has limited its use primarily to major academic medical centers where there is more experience dealing with these toxicities. A phase I clinical trial demonstrated that a PEGylated IL-2 conjugate with 10–20-fold increased half-life had similar toxicities to those of the unmodified protein.[46] Direct injection of PEG-IL-2 into locoregional recurrent head and neck tumors appears to be less toxic than systemic administration, resulting in only temporary local swelling and redness. The response rate was not sufficient to justify additional studies. Interestingly, the one tumor that responded was small.[47] Low dose PEG-IL-2 has been used in patients with HIV with relatively low toxicity and objective stimulation of the immune system.[48,49] A potential therapeutic use for this molecule in cancer may be in minimal residual disease settings, or in combination with anticancer vaccines.

Tumor necrosis factor-$\alpha$ (TNF-$\alpha$), as the name implies, has direct cytotoxic effects on tumor cells. Initial studies in mice suggested that the human factor had little effect on normal cells. However, this was due to lack of species cross-reactivity for one type of TNF receptor, but not another.[50] In fact, TNF-$\alpha$ has considerable toxicity in patients. Preclinical studies with PEG-TNF-$\alpha$ conjugates prepared with different molecular weight PEG chains demonstrated that there was an optimal molecular size of 100–110 kDa for *in vitro* activity in mice.[51]

PEGylation of IFN-$\gamma$, a type II interferon produced by lymphocytes, has been tested in animals and is reported to have a lower affinity for its receptor but higher *in vivo* activity in rats,[52] which is similar to situation

with PEG-G-CSF. This IFN has not found wide clinical use because its toxicities are similar to those of IL-2 in relation to potential therapeutic benefit.

## 6. PEGylated Cytokine Inhibitors

In addition to PEGylation of cytokines for therapeutic use, there are also efforts to develop PEGylated protein inhibitors of cytokine action. Because cytokines have powerful effects on the immune system, such as those described above for IL-2 and TNF, inhibition of their activity may be beneficial for indications such as toxic shock and tumor cachexia as well as chronic conditions like arthritis, asthma, Crohn's disease and autoimmune encephalomyelitis. These inhibitors are of two types: (1) soluble cytokine receptors, some of which are produced by cells and appear to be physiological inhibitors; (2) antibodies to the cytokines or to their receptors.

Soluble receptors against TNF, IL-1, IL-4, IL-5, and IFN- $\gamma$ have been studied in preclinical models and in some cases clinical trials.[53] A recombinant TNF receptor hybrid with the Fc portion of human immunoglobulin has been registered for use in rheumatoid arthritis. PEGylated TNF receptor constructs have been reported to be active in a variety of models[54,55] and might be useful for this indication.

Antibody reagents can be directed against the cytokine or the receptor itself. A PEGylated antibody against IL-8 has been prepared with potential application in adult respiratory distress syndrome.[55] An important anticancer antibody, trastuzamab (Herceptin™), is directed against the oncogene ERB-B2 (also called Her-2/Neu), a member of the epidermal growth factor receptor family that is overexpressed in about 30% of breast cancers as well as a number of other tumor types. Treatment of patients having ERB-B2 positive tumors with the humanized antibody trastuzamab leads to regression of the tumors, presumably because the antibody blocks the cell growth stimulus from the receptor and causes the cells to undergo programmed cell death (apoptosis). A PEGylated construct of Herceptin has been tested in animal models and has prolonged activity compared to the unmodified antibody.[56]

## 7. Conclusions and Outlook

After decades of repeated cycles of promises and failures, immunotherapy has finally lead to effective treatments for cancer. At this time, these have

primarily come from antibody therapeutics such as trastuzamab and rituximab (Rituxan™), which is directed against the CD20 antigen expressed on some B-cell nonHodgkin's lymphomas.[57] The protein drugs that have the largest sales in the cancer market, however, are for support of standard toxic therapies, namely the hematopoietic growth factors.[58] Studies over the years with hematopoietic cells in culture have shown that the continuous presence of the protein is necessary for proliferation, differentiation, and even survival of these cells.[59–61] These factors all have short half-lives and thus, exogenous injection has limited effectiveness.

PEGylation can significantly extend the plasma half-lives these proteins, not only allowing less frequent administration, but also enhancing their efficacy. Until recently, most of the cytokine constructs had relatively small amounts of PEG attached so the increases in half-life were modest. Modifications of interferon and G-CSF with high molecular weight PEG have shown that such constructs can provide therapeutic benefits. One reason this approach was not taken previously was that constructs were initially selected on the basis of maintaining high activity in various *in vitro* assays. This can lead to selection against the most active forms *in vivo*. Therefore, following the experience with PEG-G-CSFs, it is likely that higher molecular weight constructs with decreased *in vitro* activities will be appearing in the future.

As our knowledge of the immune system increases, our ability to develop effective immunotherapies, including vaccines is enhanced. Hopefully the future will provide a range of agents that will help the body fight cancers as they form.

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