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Special Lecture

The Chemical and Biological Route from Podophyllotoxin Glucoside to Etoposide: Ninth Cain Memorial Award Lecture[1]

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The course of events which led to etoposide is an example of a development which started from a long-known natural compound and ended in a new chemical structure with a new mechanism of action and increased medical utility. The path to this new structure and its activity was, as is often the case in such developments, not a straightforward one, but very tortuous. Although the finally successful, semisynthetic glucoside does not deviate much from a compound occurring in nature (the condensation of an aldehyde group to the glucose unit makes all the difference), many intermediate steps and detours were necessary to arrive there; nearly 600 derivatives had to be prepared and tested in a period of about 20 years. Retrospectively and heuristically, perhaps the most interesting aspects of the whole story are, on the one hand, that aldehyde condensation products of demethylepipodophyllotoxin glucoside were synthesized somehow by serendipity, and, on the other hand, that this chemical alteration entails a dramatic increase in potency, a radical change in mechanism of action, and a quantum step in therapeutic utility.

History

Podophyllum emodi Wall., which grows in the Himalayan region, and the American *Podophyllum peltatum* L. (may apple, mandrake) are old medicinal plants. They belong to the family of the *Berberidaceae* and were used by the natives of both continents as cathartics and anthelminthics. Renewed interest in the podophyllum plant was generated in the 1940s when Kaplan (1) demonstrated the curative effect of podophyllin, an alcoholic extract of the *Podophyllum* rhizomes, in condylomata acuminata. Podophyllotoxin, the main constituent of podophyllin, had already been described by Podwyssotzki (2) in 1880. Podophyllotoxin and its naturally occurring derivatives do not contain nitrogen; they are therefore not alkaloids. Its correct structure was proposed by Hartwell and Schrecker (3). These investigators studied podophyllin extensively and isolated a number of podophyllotoxin derivatives.

All these substances (Fig. 1) belong to the class of lignans, natural products containing the 2,3-dibenzylbutane skeleton. A comprehensive review of earlier knowledge about the biological effects and the chemistry of *Podophyllum* has been presented in 1954 by Kelly and Hartwell (4) and subsequently by Hartwell and Schrecker (5). Later work, particularly that performed at Sandoz, Ltd., Basel, Switzerland, and which resulted in the development of etoposide, has been reviewed recently (6).

In the early 1950s, chemists in the pharmaceutical research department of Sandoz, Ltd. reasoned that *Podophyllum* lignans might be present in the plant as glycosides. It was hoped that, in analogy to cardiac glycosides, they would exhibit pharmacological properties superior to those of the aglycones. Based on experience with *Digitalis* glycosides, *Podophyllum* roots were extracted by procedures which would preserve glycosides, and indeed it was possible to isolate podophyllotoxin glucoside and its 4′-demethyl derivative as well as the glucosides of α- and β-peltatin (7–12). Although the glucosides were less hydrophobic and less toxic than the aglucones, their cytostatic activity was reduced at least as much as the toxicity.

In our attempts to find more useful drugs, large series of derivatives of both glucosides and aglucones were prepared. Based on their chemical and biological properties, aldehyde condensation products of *Podophyllum* glucosides on the one hand and derivatives of podophyllinic acid hydrazides on the other hand were of particular interest. Two preparations were selected as potential anticancer agents for extensive testing *in vitro*, in animals, and in humans, namely SP-G, the condensation product of the (crude) *Podophyllum* glucoside fraction with benzaldehyde, and SP-I, podophyllinic acid ethyl hydrazide. Based on favorable clinical results, both preparations were commercialized in 1963 under the experimental designations SP-G (later to be called Proresid oral) and SP-I (later Proresid i.v.). However, the search for still better compounds in the *Podophyllum* series continued. Most of these endeavors did not lead to useful products and will not be mentioned here.

In early 1962, analysis of the cytostatic potency of SP-G by means of a novel assay using nonadhering cultured cells (13) revealed that the activity of the known constituents could not fully account for the effects of the mixture. It was therefore concluded that small quantities of unknown, highly active by-products must be present. We had also observed that SP-G produced a significant increase in the life span of mice inoculated with leukemia L1210, an effect not seen with the hitherto isolated components.[2] We then invested quite some time to find out whether the known constituents of SP-G would potentiate each other. This was, however, not the case. Then, intensive work on the chemical side on SP-G was again taken up and a combined chemical and pharmacological search for new cytostatic principles in SP-G and in the crude glucoside fraction of *Podophyllum* species was started. This resulted in the identification of a number of new podophyllotoxin derivatives in these preparations, all occurring in very small amounts. After more than 2 years of chemical and biological endeavors, a compound was found in SP-G which was not only quite potent as an inhibitor of cell proliferation *in vitro* but was also able to considerably prolong the survival time of leukemic mice at low doses. This "antileukemic" factor, originally designated as benzylidene lignan P (the previous lignans had received the letters

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[2] H. Stähelin, unpublished results.

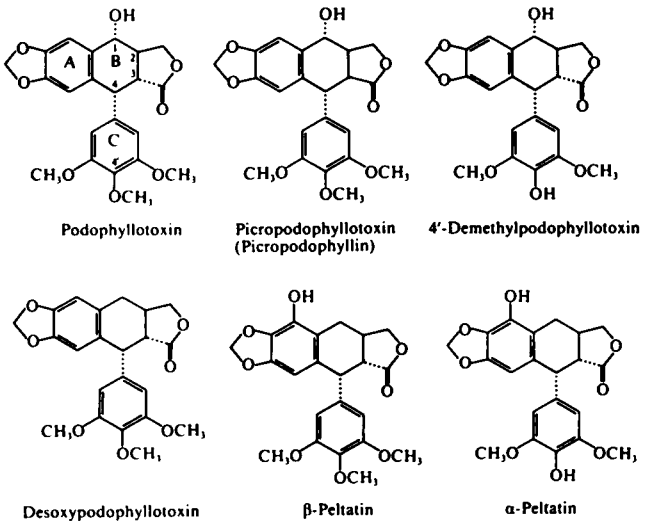

Fig. 1. *Podophyllum* lignans known in the early 1950s.

A-Q), was then soon identified as DEPBG.[3,4] When the effect of benzylidene lignan P was investigated in cultures of chick embryo fibroblasts (for method see Refs. 14 and 15), it immediately became clear that we were dealing with a different mechanisms of action; instead of producing an arrest of mitosis in metaphase, as all previous *Podophyllum* compounds had done, the new derivative obviously prevented proliferating cells from entering mitosis and thus reduced the mitotic index to almost zero. After elaboration of a synthesis for DEPG (16, 17), a large number of other aldehydes were then condensed to this glucoside and the products were analyzed as to biological effects. Two of them were selected for further development, the condensation products with thiophene aldehyde and acetaldehyde, representing teniposide (VM-26, Vumon) and etoposide (VP-16, VePesid), respectively. Results with these two compounds in clinical studies with cancer patients arranged by Sandoz were rather encouraging, but by the mid-1970s, cancer chemotherapy was no longer among the priorities in the pharmaceutical division of the company. VM and VP were licensed out to the United States company Bristol-Myers in 1978, after Sandoz had commercialized VM in some countries. Bristol-Myers successfully continued development of the two epi Ps and introduced etoposide in the United States market in 1983. Some steps of the chromology of *Podophyllum* research are listed in Table 1.

Table 1 *Short chronology of Podophyllum drugs*

1820	Podophyllin is included in the United States Pharmacopoiea
1861	Bentley mentions local antitumor effects of podophyllin
1880	Podwyssotzki isolates podophyllotoxin
1942	Kaplan describes effects of podophyllin in benign tumors, condylomata acuminata; the drug disappears from the United States Pharmacopoiea
1946	King and Sullivan report mechanism of action of podophyllin: stop of cell division in metaphase of mitosis
1951	Hartwell and Schrecker determine the correct structure of podophyllotoxin; beginning of clinical trials with systemic administration of some *Podophyllum* compounds
1954	Discovery of glucosides of podophyllotoxin and peltatins in the *Podophyllum* plant by the Sandoz chemists Renz, von Wartburg, and coworkers
1956–1959	Condensation of lignan glucosides with aldehydes by von Wartburg and coworkers; preparation of SP-G by Angliker and coworkers; synthesis of SP-I by Rutschmann and Renz; pharmacological testing by Stähelin and Emmenegger
1962	Biological analysis by Stähelin suggests the presence of very small amounts of until then unknown, highly active compounds in SP-G with *in vitro* and *in vivo* antitumor effects
1963	First commercialization of *Podophyllum* drugs (SP-G and SP-I) for systemic cancer treatment
1963–1965	Isolation of the "antileukemia" factor in SP-G by close chemical and biological collaboration (Keller, Kuhn, von Wartburg, Stähelin) and characterization as demethylepipodophyllotoxin benzylidene glucoside (DEPBG)
1965	Stähelin establishes a mechanism for DEPBG which is new for *Podophyllum* compounds: inhibition of entry of cells into mitosis; synthesis and first biological testing of teniposide
1966	Synthesis and first biological testing of etoposide
1967	Kuhn, Keller, and von Wartburg elaborate a stereoselective synthesis of demethylepipodophyllotoxin glucoside, suitable for large scale production; start of clinical trials of teniposide
1971	Start of clinical trials of etoposide
1974	Loike *et al.* report DNA fragmentation by teniposide and etoposide
1976	Commercialization of teniposide as Vumon in some countries
1978	Sandoz hands over further development of teniposide and etoposide to Bristol-Myers
1982	Long *et al.* find interaction of etoposide with the enzyme topoisomerase II
1983	Approval by the Food and Drug Administration of etoposide as VePesid for testicular cancer

Podophyllotoxin *versus* Podophyllotoxin Glucoside

In the early 1950s, when work in this field began at Sandoz, the cancer chemotherapy armamentarium was very restricted (alkylating agents and folic acid antagonists; 6-mercaptopurine just appearing), and any drug with some cytostatic activity and acceptable toxicity had to be considered as potentially interesting. It therefore seemed worthwhile to pursue the idea, originating from experience with *Digitalis* glycosides in the treatment of heart failure, that the pharmacological properties of (natural) compounds may improve when they are glycosylated. *Podophyllum* had at that time become of interest in the treatment of neoplasias (4). It was hoped that some of the then known constituents of podophyllin (an alcoholic extract of *Podophyllum* rhizomes with P as the main component) would occur in the plant as genuine glycosides which could be less toxic and more water soluble. Based on our experience with cardiac glycosides from *Digitalis* we extracted fresh rhizomes of the Indian *Podophyllum* species using special procedures to inhibit enzymatic degradation. And indeed, we obtained a mixture of glycosides which could be separated by partition chromatography into the main component PG (7, 8) and its 4'-demethyl derivative (9). Both glucosides were also isolated from the American *P. peltatum* which, in addition, contained the glucosides of α- and β-peltatin (10–12) (Fig. 2).

As expected, the glucosides were less toxic and more water soluble than the aglucones, which confirmed part of the original hypothesis. As deduced from the microscopic aspect of treated Ehrlich ascites tumor cells and chick embryo fibroblasts (14, 15), the mechanism of aglucones and glucosides is the same: they produce c-mitoses (colchicine-mitoses, arrested in metaphase with clumped chromosomes) and must thus be regarded as spindle poisons which inhibit the polymerization of tubulin to microtubules, a process required for the formation of the mitotic spindle. That podophyllin exhibits the same mechanism of action as does colchicine had already been shown in 1946 (18).

[3] The abbreviations used are: P, podophyllotoxin; D, 4'-demethyl-; E, epi-; G, β-D-glucoside; B, benzylidene (these in combinations); VM 26, teniposide; VP 16, etoposide; i.c., intracerebral.

[4] C. Keller, M. Kuhn, and A. von Wartburg, unpublished results.

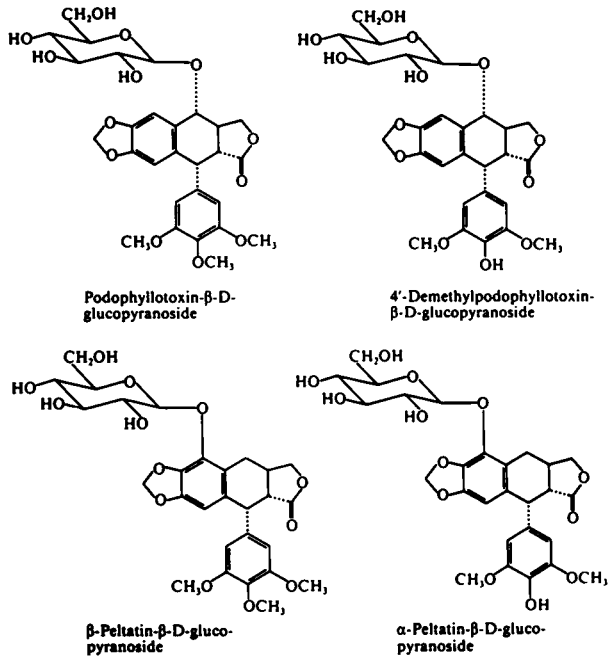

Fig. 2. Major *Podophyllum* glucosides isolated from the rhizomes of the Indian and American plant.

Table 2 *Cytostatic and toxic activity of podophyllotoxin (P), its glucoside (PG), the benzylidene derivative (PBG), of SP-G 827 and SP-I 77*

	IC_{50} P-815 (μg/ml)[a]	Sarcoma (% of tumor inhibition)[b]	L1210 (% ILS)	LD_{50} mouse (mg/kg)
P	0.005	29	35	35
PG	6	40	7	297
PBG	3	NS	5	240
SP-G	0.5	47	65	214
SP-I	0.5	46	17	283

[a] IC_{50}, concentration inhibiting by 50% proliferation of P-815 mastocytoma cells *in vitro*; ILS, increase in life span of mice inoculated s.c. with 10^6 L1210 cells with daily treatment at doses resulting in maximal ILS; LD_{50}, 50% lethal dose, single parenteral administration.

[b] Inhibition of growth of mouse sarcomas 37 and 180 by 8 days of treatment with maximal tolerated doses; NS, not significant.

The finding of reduced toxicity of the glucosides generated considerable enthusiasm; however, upon further investigation they turned out to be much less effective in inhibiting cell proliferation. PG was found to be about 1000 times less potent than podophyllotoxin in producing mitotic arrest in fibroblast cultures or in inhibiting the proliferation of P-815 mastocytoma cells *in vitro*. In addition, the glucoside was inactive in mouse leukemia L1210 at the highest tolerated dose, while podophyllotoxin increased the survival time of leukemic mice to a significant degree (Table 2). Another negative finding was that, in rats, the [^{14}C-labeled (19)] glucoside is very poorly absorbed from the gastrointestinal tract (15, 20).

The hopes that genuine glucosides of podophyllotoxin or of its early known derivatives would constitute useful antitumor drugs, had, due to the mentioned results, to be dropped. We therefore embarked on a more extensive program for chemically modified podophyllotoxin derivatives, glucosides as well as aglucones.

Condensation Products with Aldehydes: SP-G and SP-I

One of the more interesting series of derivatives were the aldehyde condensation products of *Podophyllum* glucosides

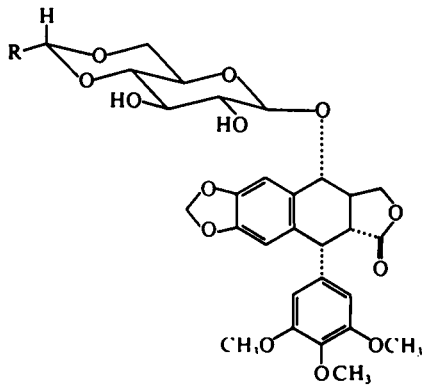

Fig. 3. Cyclic acetals of podophyllotoxin glucoside.

representing cyclic acetals (Fig. 3).

Attachment of a benzaldehyde to the glucose of PG (Fig. 3, R = phenyl) (20) does not change much the cytostatic properties of the glucoside (Table 2), but it makes the molecule resistant to glucosidases and less water soluble. Probably connected to these changes is a much better oral bioavailability compared to the glucoside (15, 20). PBG was investigated in more depth. Tests in a few patients did show positive effects; these could not, however, be attributed unequivocally to the *Podophyllum* drug since chemotherapy had been combined with X-ray treatment (21).

Condensation with benzaldehyde was carried out not only with pure PG but also with a nonpurified extract of roots of the Indian *Podophyllum* plant containing all glycosidic compounds. The main constituent of this preparation, called SP-G, was PBG (Fig. 3, R = phenyl), but it also contained smaller amounts of other *Podophyllum* lignans and of chemically unrelated natural compounds. SP-G turned out to be more potent *in vitro* than the pure PBG and also to have a good efficacy in leukemia L1210, which PBG does not have (Table 2). The mechanisms of action, as deduced from fibroblast culture experiments, was primarily that of a spindle poison (14); at higher concentrations, a certain inhibition of the entry of cells into mitosis could be observed. In rats and mice, SP-G is able to inhibit the immune response to foreign erythrocytes quite substantially (22) and to suppress the symptoms of Freund adjuvant arthritis in rats.[5]

After clinical testing in a large number of cancer patients, the preparation was introduced into the market for p.o. administration under the experimental designation SPG 827. Favorable aspects of the drug were its comparatively low bone marrow toxicity and that it could be given p.o., which made home treatment possible. Clinical results with this preparation (which was often combined with SP-I, see below) were recorded in several hundred publications. A short summary is given elsewhere (6).

Another structural subclass of *Podophyllum* lignans which we investigated quite extensively are the derivatives with an open lactone ring. Among the more than 200 compounds of this type which we studied, derivatives of podophyllinic acid hydrazides (23) seemed, due to their pharmacological properties, to be of particular interest. A synthetic approach to the free (2,3-*trans*)podophyllinic acid, unknown at that time, was therefore sought. All attempts to prepare podophyllinic acid by alkaline cleavage of the lactone ring of podophyllotoxin, undertaken in several laboratories, had failed thus far; epimerization

[5] D. Wiesinger, unpublished results.

and formation of the isomeric (2,3-*cis*)picropodophyllinic acid proved to be kinetically the predominant reaction (Fig. 4).

Using a different approach, namely a transesterification reaction with methanol and $ZnCl_2$ as catalyst, we obtained a mixture of unchanged podophyllotoxin, an isomeric 1,3-lactone, named neopodophyllotoxin, and podophyllinic acid methyl ester. With neopodophyllotoxin, base-catalyzed epimerization did not occur due to steric hindrance; thus ring opening with bases led to the desired (2,3-*trans*)podophyllinic acid (Fig. 5) (24, 25). To our disappointment, podophyllinic acid turned out to have only marginal cytostatic potency.

One hydrazide derivative of podophyllinic acid was selected for more in-depth analysis, namely the ethyl hydrazide. While SP-G had the disadvantage of not being a single chemical entity and could, due to low water solubility, hardly be given parenterally, podophyllinic acid ethyl hydrazide (later called SP-I 77 or just SP-I) did not exhibit these weak points. An injectable aqueous solution could be prepared with the help of some ethanol. In cell cultures, the cytostatic potency of SP-I is the same as that of SP-G, and in some solid mouse and rat tumors (sarcoma 37 and Walker carcinosarcoma, respectively) it exhibits an efficacy similar to that of SP-G (14) (Table 2). Its mechanisms of action is that of a pure spindle poison, like that of podophyllotoxin. After appropriate toxicological evaluation, which was uneventful, SP-I was tested clinically, often in combination with SP-G, and then introduced into the market. Although objective, positive effects were found in a significant percentage of cancer patients, and in many cases a remarkable improvement of the general condition of the treated subjects was observed, the long-term results with both forms of Proresid did not achieve the level obtained with some of the newer and more aggressive anticancer drugs which became available at that time. Their clinical use therefore gradually decreased.

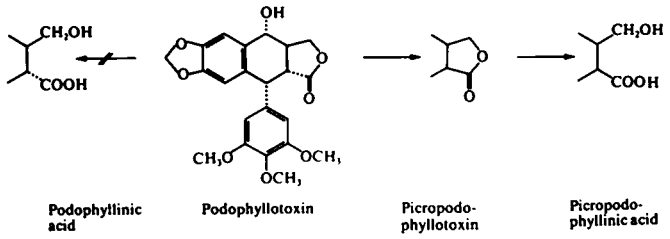

Fig. 4. Reaction of podophyllotoxin with bases leading to picropodophyllinic acid.

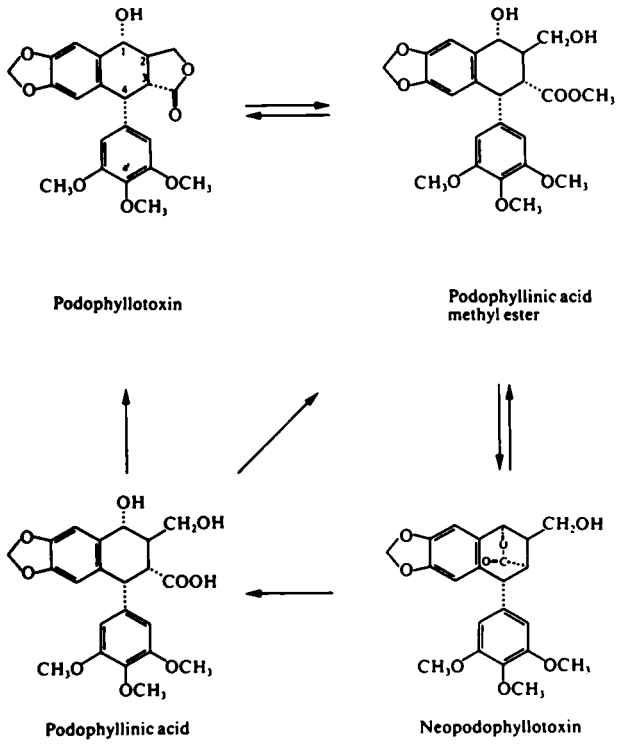

Fig. 5. Synthesis of 2,3-*trans*-podophyllinic acid via neopodophyllotoxin.

Demethylepipodophyllotoxin Aglucones, Glucosides, and Their Aldehyde Condensation Products

As mentioned under "History," after it had been found that the activity of the then known constituents of SP-G could not explain the biological effects of that mixture, further chemical and biological work resulted in the identification of small amounts of additional podophyllotoxin derivatives present in SP-G. Systematic chromatographic separation procedures led first to the isolation of benzylidene derivatives of the following minor products present in the plant: podorhizol glucoside; 4′-demethyldeoxypodophyllotoxin glucoside; and deoxypodophyllinic acid 1-β-D-glucopyranosyl ester (6). All three components were also encountered as free, genuine glucosides in *Podophyllum* species (26–28). They displayed cytostatic activity *in vitro* but lacked, as far as tested, significant antileukemic effect *in vivo* (6). Their unusual structural features suggested that they may act as intermediates of *Podophyllum* lignan biosynthesis (Fig. 6).

While these latter constituents of SP-G contributed to the *in vitro* cytostatic effects of the mixture, they apparently were not responsible for the activity in mouse leukemia L1210. Since, in the 1960s, L1210 was considered one of the most important animal models for human malignancies (29), particular importance was attached to results of this test.

After an extended collaborative effort, we could trace down the long-sought "antileukemia factor" of SP-G and elucidate its structure as DEPBG (Fig. 7). Important structural features of the new component consist of the presence of a phenolic

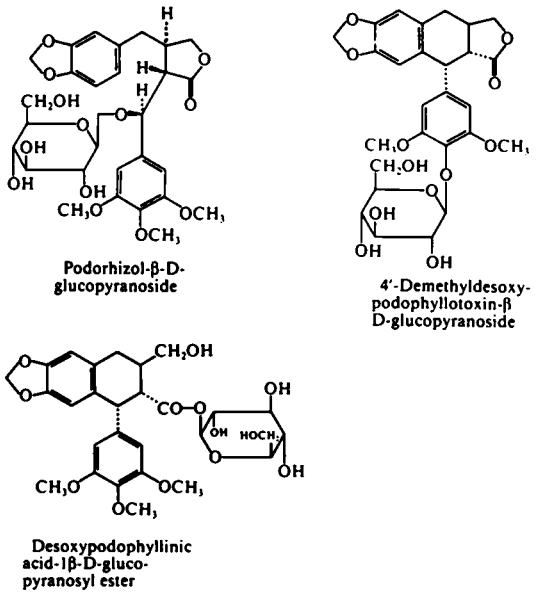

Fig. 6. Minor *Podophyllum* glucosides.

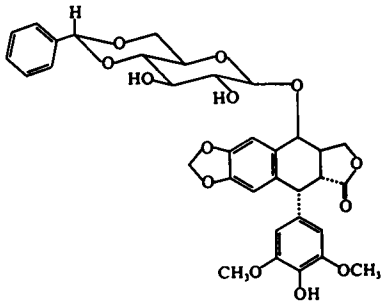

Fig. 7. 4′-Demethylepipodophyllotoxin benzylidene glucoside, the "antileukemia factor" in SP-G.

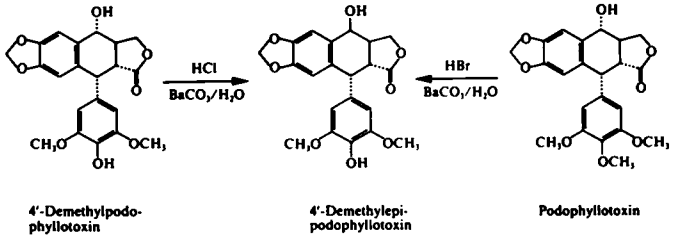

Fig. 8. Synthesis of 4′-demethylepipodophyllotoxin.

hydroxyl group at C-4′ and, more striking, of the 1-epi configuration. Its close chemical relationship to other minor components in SP-G and the scarce content explain the difficulties encountered in the course of the isolation.

The novel SP-G constituent, to which its effect in L1210 was apparently due, was considered of high interest. Extensive analyses of *P. emodi* showed that DEPG (the parent free glucoside without aldehyde residue) occurs in the plant only in very small amounts. The poor availability of the glucoside and its benzylidene derivative made it necessary to elaborate a synthesis in order to perform derivatization and more extensive pharmacological testing.

The synthesis presented three major problems: (*a*) to prepare large amounts of DEP; (*b*) to elaborate a new specific glycosidation method; and (*c*) to remove the protecting groups of the resulting glucoside, considering its sensitivity towards bases and acids. In a first step, the easily accessible podophyllotoxin could be converted to the 4′-demethyl derivative by selective cleavage of the 4′-methoxy group with HBr, followed by hydrolysis and epimerization of the 1-bromo intermediate (30) (Fig. 8). For the glucosidation reaction, classical methods such as the Koenigs-Knorr procedure could not be applied satisfactorily in this case [this method, however, enabled us to achieve the first total synthesis of PG (31)]. The problem in the 1-epi series could be solved by treatment of DEP (protected as benzyloxycarbonyl derivative) with pure tetraacetyl-β-D-glucose in presence of BF_3 etherate at low temperature to yield the tetraacetate of 4′-benzyloxycarbonyl-DEPG (17). It is remarkable that the same procedure furnished, due to stereochemical reasons, the identical tetraacetylglucoside when 4′-benzyl-oxycarbonyl-DP (C-1 = αOH) was used instead of the 1-epi compound (C-1 = βOH). The new glycosidation reaction proceeds in a highly specific way with respect to the glycosidic linkage and leads exclusively to β-glycosides of 1-epi derivatives. Moreover, the method is not restricted to glucose; other hexoses, *e.g.*, β-D-galactose, are also suitable sugar residues (17, 32). The last problem, the removal of the protecting groups, could be overcome by submitting the tetraacetylglucoside to zinc acetate-catalyzed methanolysis (31). In the final step, the deacetylated intermediate was hydrogenolyzed with H_2/palladium to furnish the required DEPG in crystallized form (17) (Fig. 9). With synthetic material at our disposal, we could prepare large series of 1-epi derivatives.

DEP, the aglucone, was found to be quite potent as an inhibitor of cell proliferation *in vitro* and to exhibit a spindle poison type of mechanisms; in leukemia L1210, it proved inactive (Table 3). Some of its 1-*O*-acyl derivatives, particularly substituted carbamates, are of interest because they arrest cell division by a different mechanisms, namely by inhibiting the entry of cells into mitosis and thus reducing the mitotic index (6). At the same time, these derivatives, *e.g.*, the 1-*p*-chlorophenylcarbamoyl DEP, significantly increase the life span of L1210 leukemic mice, and there is an inverse correlation between efficacy in L1210 mice and the mitotic index. This suggests that the new cytostatic mechanisms is responsible for the improved oncostatic activity. No correlation exists between cytostatic potency *in vitro* and the effect in L1210 (Table 3). More recently, other derivatives of DEP with nitrogen-containing substituents in position 1 have been synthesized which show good antitumor activity (33) or inhibit topoisomerase II (34). It will be interesting to see how the antitumor activity of these

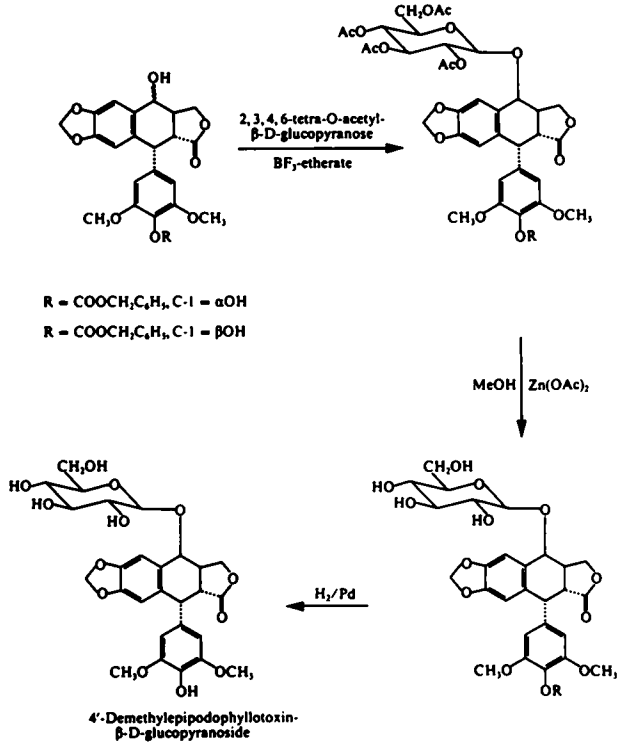

Fig. 9. Synthesis of 4′-demethylepipodophyllotoxin glucoside.

Table 3 *Cytostatic activity of 1-O-acyl derivatives of 4′-demethylepipodophyllotoxin (DEP) aglucone*

	IC_{50}[a] P-815 (μg/ml)	RMI	L1210 (% ILS)
DEP	0.06	10	7
Acyl residue			
Benzoyl	0.5	10	NT
Furoyl	0.04	10	0
Carbamoyl	0.2	17	9
Phenylcarbamoyl	0.08	3	68
p-Chlorophenylcarbamoyl	0.4	0.2	74

[a] IC_{50}, 50% inhibitory concentration; RMI, relative mitotic index: the number of mitotic figures per 1000 cells in treated fibroblast cultures, divided by the same number in controls; for explanation of other column, see Table 2; ILS, increase in life span; NT, not tested.

aglucones compares with that of etoposide upon more extensive testing in animals and perhaps in humans.

Because DEPBG had shown impressive effects in leukemia L1210, our attention was focused not so much on aglucone derivatives but on compounds derived from its glucoside and, more specifically, on condensation products with aldehydes and ketones. Free DEPG exhibits a comparatively low cytostatic potency *in vitro* with an ID_{50} of >1 μg/ml (Table 4), which is the case for all podophyllotoxin derivatives with a free glucose attached. Its mechanisms of action is that of a spindle poison, and it increases the survival time of mice inoculated with L1210 to a low but significant degree.

Numerous aldehydes (besides benzaldehyde) and ketones were condensed to the glucose moiety of DEPG (35). As mentioned above, the condensation product with benzaldehyde isolated from SP-G was highly potent as an inhibitor of cell proliferation *in vitro* and, using a suboptimal treatment schedule, nearly doubled the survival time of leukemic mice at low doses. What was at least as interesting was the new mechanism. The comparatively minor chemical alteration of condensing an aldehyde to DEPG not only brings about an increase of cytostatic potency of the order of up to 1000-fold (depending on the aldehyde) but also confers on the molecule a new mechanism of action. While all previous *Podophyllum* compounds were spindle poisons and produced, in our fibroblast cultures, a considerable increase of the mitotic index, mitotic figures were practically absent in tissue cultures treated with DEPBG.[2] This observation was so surprising that doubts arose (at a time when the structure of the then new compound was not exactly known) whether we were dealing with a podophyllotoxin derivative or a completely different structure. However, this issue was soon resolved by elucidation of the structure of this component of SP-G. It may be pointed out that the tissue and cell cultures which we used were essential for discovering the presence of DEPBG in SP-G and for the recognition of its new mechanisms of action. The P-815 mastocytoma cell culture assay (13) is a rapid and sensitive method for determining the cytostatic potency of compounds in a reproducible way. Our primary fibroblast cultures were obtained by explanting pieces of blood vessel walls of chick embryos and fixing them to the supporting coverglass with coagulated plasma (14, 15); this technique provides a sheet of proliferating cells from which, in contrast to ordinary monolayer cultures, cells do not easily detach when in mitosis or when damaged and therefore enable a reliable count of normal or (accumulated or diminished) abnormal mitoses.

The nature of the aldehyde condensed to DEPG is of some importance for the biological activity of the molecule (Table 4) (35). Cytostatic potency *in vitro* (*i.e.*, inhibition of proliferation of P-815 mastocytoma cells) and increase in life span in L1210 leukemic mice vary independently with the aldehyde. Two of the aldehyde derivatives were then selected for further development, the condensation product of DEPG with thiophene aldehyde which then received the code designation VM 26, and the condensation product with acetaldehyde, later designated VP 16–213 or VP 16 (Fig. 10). They will be dealt with in more detail below. Noteworthy among the other derivatives are those containing an amino group in the aldehyde moiety and exhibiting a much increased water solubility compared to VM and VP. Their potency as to inhibition of cell proliferation *in vitro* is rather low while they produce, upon parenteral administration to leukemic mice, a dramatic increase in survival time of the animals (see, *e.g.*, the dimethylaminopropylidene derivative in Table 4). One of these water soluble derivatives was studied in more detail in animal models and found to be somewhat inferior to etoposide (36).

Table 4 *Cytostatic activity of DEPG and some of its aldehyde condensation products*

	IC_{50} P-815 (μg/ml)[a]	L1210 ILS (%)
DEPG	4	34
Aldehyde residue		
H	0.06	56
CH_3 (etoposide)	0.05	167
C_2H_5	0.009	97
C_5H_{11}	0.009	65
CH_3COCH_2	0.1	109
$(CH_3)_2NCH_2CH_2$	2	317
C_6H_5 (DEPBG)	0.007	97
o-ClC_6H_4	0.004	24
1-Naphthyl	0.01	95
2-Thiophene (teniposide)	0.005	121

[a] For explanation of columns and abbreviations, see Table 2.

Teniposide and Etoposide

Among the aldehyde condensation products of DEPG, the thenylidene derivative (teniposide, VM 26, original code designation 15–426, commercial name Vumon) was first selected for in depth evaluation in view of possible clinical testing, based, among other things, on its high cytostatic potency *in vitro* and its effect in mouse leukemia L1210 and other animal tumor models. A summary of preclinical results was given in 1969 (37) and a more detailed analysis was presented in 1970 (38), almost simultaneously with the publication of the first results of clinical studies which had been initiated primarily by the group of O. Selawry (39, 40) (clinical testing of VM had started within 2 years after synthesis!). VP was selected for development somewhat later than VM, again based on results in the L1210 model and on the fact that the ethylidene derivative is, in contrast to VM, effective also when given p.o.

VM and VP have been tested by ourselves and others in a number of preclinical systems (6, 37, 38, 41). Besides inhibition of cell proliferation *in vitro*, where VM is about 10 times more potent than VP, the fate of cells treated for restricted periods has been investigated; it was found that, depending on drug concentration and exposure time, a large proportion of P-815 mastocytoma and L1210 leukemia cells is permanently prevented from further multiplication without being immediately damaged as assayed by the dye exclusion test (42, 43). Efficacy in mouse leukemia L1210 was high and among the best obtained with an anticancer drug; a considerable schedule dependency was found and many mice could be cured, particularly

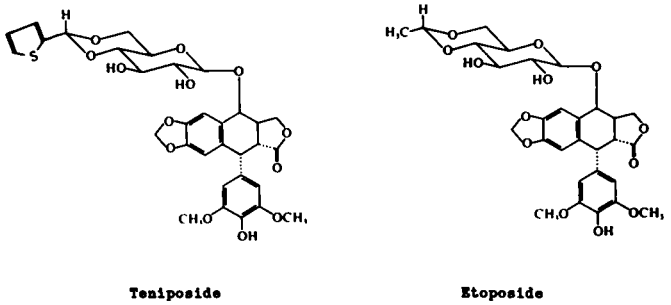

Fig. 10. The two aldehyde condensation products of 4′-demethylepipodophyllotoxin glucoside selected for clinical testing, VM 26 and VP 16.

when the tumor was inoculated i.p. and the drug was administered by the same route. In most systems, VP was somewhat superior to VM, except in Lewis lung carcinoma and in L1210 inoculated i.c. VP exhibits a considerable immunosuppressive activity[6] and almost completely prevents the secondary joint swellings in Freund's adjuvant arthritis,[5] a rat model for rheumatoid arthritis. Apparently of little or no clinical consequence is the observation that VM and VP, upon repeated i.p. administration in mice and rats, cause a chronic chemical peritonitis which leads to liver damage and death (44); no comparable effects have been reported in humans. Other toxicological findings with the epipodophyllotoxins were rather unremarkable, the significant pathological changes being those to be expected from a cytostatic compound.

The low water solubility of the epipodophyllotoxins posed some problems. For i.v. administration, it was necessary to find a formulation which would allow dilution of the ampuls with an aqueous medium without precipitation occurring. Solvents had to be used with a high capacity to prevent precipitation of hydrophobic compounds when water is added, namely polysorbate 80 and polyethoxylated castor oil. It was also found that solvents which do not prevent this precipitation are not optimal for the preparation of galenical forms for p.o. administration of podophyllum compounds, presumably because precipitation in the gastrointestinal tract leads to poor enteral absorption. This had already been observed with SP-G (14) and was again crucial in overcoming absorption difficulties with cyclosporin A (45).

Results with the epipodophyllotoxins in the treatment of human malignancies have been related in numerous reports since 1970 and will not be discussed here in any detail. Reviews (*e.g.*, 46–53) have summarized them. Activities in different types of cancer have been found, among the most important being small cell lung cancer (54), testicular cancer, lymphomas, leukemias and, with VM, brain tumors. While VP has thus far been investigated much more extensively, newer results with VM seem to suggest that this compound may have similar merits (see, *e.g.*, Ref. 55). One of the positive aspects of the epipodophyllotoxins is that severe toxicity is largely restricted to the bone marrow; for this reason, VP is being used in the treatment of leukemias, lymphomas, or disseminated cancers with very high doses which is then followed by bone marrow transplantation (see *e.g.*, Refs. 56 and 57). Of course, both drugs are used today, mostly in combination with other anticancer compounds.

In the late 1970s, when VM had already been commercialized in some countries and preparations for the registration of VP were at an advanced stage, our company, Sandoz, handed over further development of these drugs to the United States company Bristol-Myers, since they had more know-how and infrastructure than Sandoz for such an undertaking. Indeed, this company, with its extensive involvement in the cancer chemotherapy area, has pursued the clinical and commercial development of both epipodophyllotoxins in a professional and successful manner.

Mechanism of Action

Since 1946 it has been known that podophyllin depresses cell proliferation by inhibiting the formation of the mitotic spindle (18). Cells can still enter mitosis and perform a normal prophase, but separation of the chromosomes, which is dependent on formation of the spindle fibers consisting of microtubules, cannot take place and the cells entering mitosis accumulate in metaphase with clumped chromosomes until they die and disintegrate after several hours. In stained cultures of multiplying fibroblasts this is very easy to recognize (see Fig. 2 in Ref. 58). All *Podophyllum* compounds known up to 1965 exhibited this mechanism. Therefore, the finding of a derivative (DEPBG) which prevented the entry of cells into mitosis and reduced the mitotic index was a great surprise. Still, most of the cells looked normal (see Fig. 3 in Ref. 58), namely those which had not gone into mitosis during the 6 h of treatment. Time course analysis in tissue culture then showed that the disappearance of mitoses begins less than 1 h after drug addition, which means that the compound acts in late S or G_2 phase of the cell cycle, and that, when using high concentrations, a few c-mitoses arrested in metaphase appear at the beginning, but later disappear. This sequence of events has been studied mainly by using one of the first analogues of DEPBG synthesized and tested, namely VM 26 (38). Thus, by the simple method of using different incubation times and different drug concentrations in fibroblast cultures it was possible to conclude that this new type of *Podophyllum* compounds has acquired a new mechanism, namely arrest of the cells in late S or in G_2 phase of the cell cycle, but has still retained the old mechanism, the spindle poison activity; the latter, however, has become irrelevant for practical purposes because it is effective only at much higher drug concentrations than the new mechanisms.

However, these studies provided evidence for the new mechanism at the cellular level only. Therefore, in the late 1960s and early 1970s we made some attempts to elucidate the basis of the arrest in G_2 phase at the biochemical level. An inhibition of the incorporation of labeled thymidine (but barely of that of [^{3}H]uridine and [^{3}H]leucine) by cells treated with VM was found (59), in contrast to spindle poisons (60). More detailed analysis later revealed that, despite reduced uptake of thymidine into DNA of cells treated with VM or VP, DNA synthesis continues and the DNA content per cell increases (61, 62). Inhibition of incorporation (transport?) of externally supplied nucleosides was apparently not correlated with the inhibition of cell proliferation. These investigations also revealed that the early biochemical effects of the two epipodophyllotoxins on proliferating cells *in vitro* differ from those of other cytostatic agents (spindle poisons, alkylating agents, antimetabolites and others), but that there is an astonishing similarity to the effects of X-rays (62). Our investigations thus did not provide definite clues to a biochemical basis for the cellular effects of the epipodophyllotoxins.

A breakthrough came in 1974, when Loike *et al.* (63) reported fragmentation of DNA in HeLa cells (but not of purified DNA) by VP and VM. The lowest VP concentration which produced a detectable fragmentation in HeLa cells (1 μM) (64) was not much higher than the ID_{50} for inhibition of the multiplication of these cells (0.26 μM) (41); this made a causal relationship between the two effects more likely than, *e.g.*, between inhibition of nucleoside transport (65) and of cell proliferation.

Several years later, this fragmentation could be correlated, first by Long and Minocha (66), with the inhibition of topoisomerase type II activity and it was proposed that VP produces enzyme-DNA cross-links. These findings were corroborated by many other investigators and it appeared that the epipodophyllotoxins share this mechanisms with other anticancer drugs, *e.g.*, the anthracyclines, for which a topoisomerase inhibition

[6] S. Lazary, unpublished results.

had been shown previously. However, the latter are intercalating agents while VM and VP do not interact with purified DNA[7] at all or only at a low level (67, 68). The interference of VM and VP with topoisomerase II and its consequences for cell proliferation and cell death have not yet been elucidated completely; it does not seem to be a simple, straightforward process. Intensive research efforts are ongoing in many laboratories regarding the role of topoisomerases in cellular functions and in mediating cytostatic and cytotoxic effects, but it is not clear whether interference of VM and VP with topoisomerase activity is able to explain all relevant pharmacological effects of these drugs (for review see Ref. 69). Recently, it has been suggested that VM 26, due to its effect on topoisomerase II, may influence gene expression indirectly by blocking the periodic spacing of nucleosomes (70).

Another aspect of the mechanism of action of the epipodophyllotoxins is the question whether they act as such or whether metabolites, generated in the treated organisms, are responsible for or at least contribute to the cytostatic effect. The fact that the parent molecules, VM and VP, have a quick onset of action at low concentrations in cell cultures speaks against a major contribution of metabolites, such as those generated by oxidative transformations in the dimethoxyphenol ring (ring C in the nomenclature used by us); they have not yet been shown to be as potent as or more potent than the parent molecules and/or to be formed in the organism in large enough amounts. Some of these aspects have been discussed by van Maanen *et al.* (67) and Saulnier *et al.* (68); the former authors also point out the similarities between the effects of epipodophyllotoxins and ionizing radiation (see also Refs. 71 and 72).

Connected to the mechanisms of action is the problem of resistance of the tumors to the effects of drugs. VM and VP seem to be subject to the development of two main types of decreased sensitivity of tumor cells. One of them is related to topoisomerase. Cell lines have been developed which exhibit altered catalytic activity of topoisomerase and are resistant to VM (73, 74); such cell strains usually also exhibit reduced susceptibility to other cytostatic agents which act by interfering with topoisomerase activity. Another type of resistance involves a group of different chemotherapeutic agents and is called multidrug resistance; the reduced sensitivity is brought about by an increased production of a glycoprotein (gp170) which transports these drugs (epipodophyllotoxins, colchicine, *Vinca* alkaloids, anthracyclines, and others) out of the cell. A certain reversal of this type of multidrug resistance can be brought about by several compounds, making the tumors again more susceptible to chemotherapy; to these compounds belong cyclosporin A (75) and some of its derivatives (76).

Structure-Activity Relationships

As a consequence of the extensive derivatization program which was carried out in our company with *Podophyllum* compounds, a large number of questions regarding structure-activity relationships arose. Some of them have been mentioned in or can be deduced from an earlier review (6). Here, only very few shall be discussed, namely those relating to the mechanism of action, in particular the structural features which determine whether the molecule inhibits cell proliferation mainly by preventing formation of microtubules (spindle poison) or by inhibiting the entry into mitosis (presumably, at least partially, by interfering with topoisomerase II activity). The latter mechanisms, which may be called G_2 activity, is apparently the more interesting one from the point of view of clinical (and preclinical) tumor chemotherapy. Conflicting results have been reported regarding the structural requirements for these effects of *Podophyllum* compounds.

In 1972, we listed the four chemical alterations which, starting from podophyllotoxin, bring about the change from a "pure" spindle poison to an (almost) pure "G_2 poison" (58): demethylation in position 4'; epimerization in position 1 (sometimes designated position 4); presence of glucose in position 1; and aldehyde condensation to the glucose. Glucose with an aldehyde condensed to it may be replaced by some nitrogen containing residues (see above), an area which has not yet been explored sufficiently. Our decision whether a compound is a spindle poison or a "G_2 poison" was based on the number and appearance of mitotic figures in fibroblast cultures treated with the minimal concentration of the compound which prevents completion of mitosis in all cells. After 6 h of incubation with a pure spindle poison, the mitotic index in such cultures increases at least 6-fold over controls. If the increase is less than that, the compound must be assumed to also affect entry into mitosis, and if the mitotic index is lower than in controls, a predominant inhibition in interphase (*e.g.*, in G_2) can be implied. The fibroblast test is very sensitive particularly for spindle poisons; podophyllotoxin, *e.g.*, produces accumulation of c-mitoses down to a concentration of about 10 nM, while in a tubulin binding assay (77) the minimal effective concentration is about 50 times higher and that required for inhibition of microtubule assembly is roughly 500 times greater (65).

In Table 5, compounds with all possible combinations of the four alterations mentioned above are listed, together with some relevant experimental data. From Table 5, it is possible to deduce the effect on cellular and antitumor activity of the different substitutions on the podophyllotoxin molecule. All derivatives with one or two alterations are predominantly or exclusively spindle poisons, albeit with large differences in potency; some of them, at higher concentrations, also produce DNA breaks and must therefore be assumed to interact with topoisomerase. Of the derivatives with three alterations, two

Table 5 *Structure-activity relationships regarding 4'-demethylation (D), 1-epimerization (E), glucosidation (G) and benzaldehyde condensation (B) of podophyllotoxin (P)*

	IC_{50} P-815 (μm)[a]	RMI[b]	Tubulin assembly IC_{50} (μm)[c]	Colchicine binding K_i (μm)[d]	DNA breaks (μm)[e]	L1210 ILS (%)[a]
P	0.012	12	0.6	0.51	>100	35
DP	0.018	14	0.5	0.65	10	10
EP	0.082	9.0	5	12	>100	11
DEP	0.14	9.5	2		1	7
PG	10	10		180		0
DPG	3.6	7.9			100	0
EPG	>35					7
DEPG	7.8	26			100	34
PBG	5.7	14		39		5
DPBG	1.2	3.7			1	29
EBPG	0.50	10				60
DEPBG	0.010	0.10			0.1	97
VM 26	0.0076	0.07	>100		0.1	121
VP 16	0.078	0	>100		1	167

[a] Values taken from Stähelin (38, 41, 58); for explanation, see also Table 2. IC_{50}, 50% inhibitory concentration.

[b] For explanation of RMI see Table 3; values taken from Stähelin (58) and unpublished results.

[c] Values [taken from Loike *et al.* (78)] represent IC_{50}s for microtubule assembly.

[d] Values [taken from Kelleher (77)] are K_i values for inhibition of colchicine binding to mouse brain tubulin.

[e] Values [adapted from data of Long *et al.* (79)] represent the lowest concentrations increasing single-strand break frequency to an evaluable level.

[7] J. Ostrowski and H. Stähelin, unpublished results, 1970.

Table 6 *Coincidences between etoposide and cyclosporin*

	Etoposide	Cyclosporin
Isolation and structure, group of	von Wartburg	von Wartburg
Biological work, group of	Stähelin	Stähelin
Approved by Food and Drug Administration	Nov. 10, 1983	Nov. 10, 1983
Mechanism of action involves	(Topo) isomerase	(*cis-trans*)-Isomerase
Effect on immune system	Suppression	Suppression
Special problem during development	Galenics	Galenics
Clinically used in, *e.g.*,	Leukemia	Leukemia
	Bone marrow transplantation	Bone marrow transplantation

are predominantly spindle poisons, while DPBG only moderately elevates the mitotic index in fibroblast cultures and also induces DNA breaks at low concentrations; DPBG is thus a typical example of a compound with a mixed mode of action. In accordance with this is the result in leukemia L1210 where the drug produces a moderate but significant increase in life span.

The most dramatic effect, however, is brought about by one modification, the attachment of an aldehyde to the glucose moiety of DEPG. This latter compound has a cytostatic ID_{50} in mastocytoma cultures of 7.8 μM and increases the mitotic index more than 20-fold. When thiophene aldehyde is condensed to the glucose (resulting in VM 26), cytostatic potency is 1000 times higher and the mitotic index almost zero; at the same time, the concentration required for DNA breaks is 1000 times lower than without the aldehyde. The decisive role of the aldehydes has been somewhat neglected in many investigations dealing with the mechanism of action and structure-activity relationships in the *Podophyllum* area.

The balance between spindle poison activity and "G_2 activity" seems to be very important for efficacy in leukemia L1210, since the former apparently contributes only little to this antitumor effect but adds to the toxicity (probably due, at least partially, to an effect on the nervous system with its abundant tubulin). The side effects of compounds acting predominantly by interference with topoisomerase, as, *e.g.*, VM and VP, are largely restricted to proliferating tissues like bone marrow and intestinal epithelium. Therefore, of two derivatives with about equal DNA scission potency, the one with less spindle poison activity is more valuable in treating L1210 (see Table 5) and presumably also for human malignancies.

Serendipity, Coincidences with Cyclosporin

Among the large number of *Podophyllum* compounds studied in our laboratories, the most successful, etoposide, was about the 500th to be tested. On the other hand, the immunosuppressant cyclosporin A (Sandimmun), found originally in a microbiological, then in a pharmacological screening, was the first of this class of chemical structures encountered by us and has thus far not been surpassed by more than 500 tested derivatives. The epipodophyllotoxins were found due to systematic investigations of many (semi-) synthetic *Podophyllum* compounds and plant extracts, but an element of serendipity was involved: condensation of aldehydes to the glucoside of podophyllotoxin was performed in order to stabilize the PG molecule and to improve its pharmacokinetic behavior, which it did; application, for reasons of economy, of this procedure to a crude plant extract (resulting in SP-G, see above) produced a completely unexpected effect, namely that a then unknown substance in the mixture was converted into a compound (DEPBG) of much higher therapeutic value. This is a case of that special type of chance which is called serendipity, namely arriving at something interesting when not in search of it. The immunosuppressive activity of cyclosporin, on the other hand, was found in a screening in which we were specifically looking for, among many other effects, immunosuppression; this discovery is therefore not serendipity [although the course of events preceding, and leading to, it involved some serendipity (45)].

On the other hand, there are an astonishing number of coincidences between etoposide and cyclosporin (Table 6). The first coincidence was that etoposide and cyclosporin were both found and developed on the chemical and biological side by the same groups, those of the present authors (45, 80–83) and their specific biological effects were discovered by one of us (84, 85); second, both compounds were approved by the United States Food and Drug Administration on the same day in November 1983, although they had been submitted by different companies; furthermore, both drugs act via an effect on an intranuclear isomerase, topoisomerase, and peptide *cis-trans*-isomerase, respectively; both compounds are potent immunosuppressants; etoposide as well as cyclosoporin are used in the treatment of leukemias or other malignancies, the latter after bone marrow transplantation to prevent graft-*versus*-host disease, the former also being used in conjunction with bone marrow transplantation; sometimes, the two compounds are used concomitantly, exploiting the capacity of cyclosporin to reduce certain types of multidrug resistance (75) or to modify immunity against tumors cured by VP (86); in the development of both drugs, galenical problems arose, related to poor water solubility and absorption from the intestinal tract, and experience gained with etoposide in this area was crucial for overcoming, several years later, difficulties of a similar type with cyclosporin. It is left to the reader to make assumptions about the heuristic aspects of these surprising coincidences.

Conclusions

The introduction of etoposide and teniposide into cancer chemotherapy is one example of the way by which, starting from old folk remedies, new single chemical entities of therapeutic value are developed. The path leading to these drugs was long and involved many windings and loops; some of this was mentioned here, a more extensive report has been published previously (6).

What can we expect from the future? Clinical evaluation and application of etopside and teniposide will still make further progress. Investigations are ongoing in several institutions exploring the possibility of finding *Podophyllum* compounds of higher clinical utility. There is certainly room for improvement by enhancing the therapuetic index, and one of the aims of this report and the previous review (6) is to put the reader in a better position to decide where to look and where not for such improvement.

Acknowledgments

This paper is not intended to be a complete review on *Podophyllum* compounds but is offered as a personal account of research conducted in our laboratories, including references to work of many other investigators. A more detailed, but still not complete, report has been presented recently (6). It is a pleasure to acknowledge the help of our collaborators, too numerous to be named all individually, over the more than 20 years of endeavors, who contributed with their careful work to the final outcome. We also would like to pay tribute to the research directors of our company who provided encouragement and technical facilities, J. Renz, J. Rutschmann, M. Taeschler, and the late A. Cerletti.

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