STUDIES IN PLANT TISSUE CULTURE. SYNTHESIS AND BIOSYNTHESIS OF CLINICALLY IMPORTANT ANTI-TUMOUR AGENTS

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It is well established that higher plants provide a fertile source of important medicinal agents. In many instances, however, these plants are not readily accessible due to geographical location or alternatively the yield of the desired natural product is extremely low, subject to seasonal variation, etc. Attempts to alleviate these situations have led numerous laboratories to consider studies with tissue cultures derived from such plants. Successful research in these directions would clearly provide methodology for a controlled and hopefully reproducible laboratory source for such compounds.

Plant tissue cultures have also been employed in biosynthetic investigations where incorporation levels of proposed precursors are generally higher than in the living plant and they also provide potentially important media for biotransformation studies. This lecture will summarize the most recent experiments which have been completed in our program on tissue cultures generated from a variety of plant species which have produced interesting compounds of importance in the cancer area.

The specific experiments which we have conducted concern the following plant species: 1) Catharanthus roseus L. G. Don; 2) Tripterygium wilfordii; 3) Cephalotaxus harringtonia.

Medicinal agents from higher plants continue to provide important avenues of research directed toward the utilization and developments of effective drugs for clinical treatment in man. Unfortunately, in selected instances, there are problems associated with the use of living plants as sources of such drugs. These are: 1) the desired agent is often present in minute amounts in the plant; 2) separation from other co-occurring natural products may be difficult and costly; 3) the concentration of the target compound varies in the plant and often depends upon what season the plant is harvested, and 4) the required plant species may grow in inaccessible regions of the world. Attempts to alleviate such situations have led our and other laboratories to initiate studies with plant tissue cultures in the hope that better overall sources of such medicinal drugs can be provided. The obvious advantages of tissue culture techniques when compared to living plants are: 1) growth conditions of the cultures are laboratory controlled and therefore reproducible.

Fig. 1. Plant species under study and the target compounds involved.
2) culture growth parameters could be properly evaluated so as to provide optimal production conditions and, in turn, higher yields of the target compound. 3) cloning could provide selected cell lines for reliable production of the desired agent; 4) tissue cultures provide excellent media for biosynthetic studies in order to understand the production of the secondary metabolite, and 5) isolation and studies of enzymes should be more easily achieved for a more rigorous understanding of the biosynthetic process.

A number of excellent books1-4 and recent reviews5,6 summarize the methods employed for the propagation of plant tissue cultures and also provide an overall summary of previous studies relating to the above areas. As a result this lecture will focus entirely on recent studies in our laboratory and concerned with tissue cultures propagated from plant species known to produce active anti-cancer agents either in clinical use or in pre-clinical development.

The specific experiments under study concern the following plant species: 1) Catharanthus roseus L. G. Don; 2) Tripterygium wilfordii; 3) Cephalotaxus harringtonia and Fig. 1 summarizes the desired anti-cancer agents which are involved. Results within the Catharanthus and Tripterygium areas are now presented.

1) Studies in Catharanthus roseus

The direction of our program was influenced considerably by our earlier studies on the synthesis and biosynthesis of bisindole alkaloids within the vinblastine family. Vinblastine (3) one of the clinically important anti-tumour agents isolated from C. roseus, represents an important member of these complex natural products and synthetic routes to (3) from more readily available starting materials have been under study for some years. The development of the "biogenetic" approach in our7,9 and other8,12 laboratories and involving the coupling of catharanthine N-oxide (6) with vindoline afforded an important route to the bisindole system. Fig. 2 summarizes our initial studies in which the three bisindole products (7) - (9) were isolated. Under optimum conditions, 3',4'-anhydrovinblastine (7) was obtained in respectable yield (>60%) and its role as an intermediate toward a variety of bisindole alkaloids and derivatives is established9,12. In a parallel study in our laboratory13,15, and utilizing cell free extracts from C. roseus, we were able to demonstrate that 3',4'-anhydrovinblastine (7) is also formed enzymatically from catharanthine (1) and vindoline (2) (Fig. 3) and that (7) is subsequently transformed to the alkaloids vinblastine (12, R = CH3), leurosine (10) and catharine (11) (Fig. 4). An independent and simultaneous study by Scott16 provided results analogous to those outlined in Fig. 3.
Studies in plant tissue culture

The above studies clearly demonstrated the importance of the two monomeric alkaloids, vindoline and catharanthine, and these compounds became prime targets in our tissue culture studies. The following discussion summarizes our experiments in this area. Details of these studies are available in various recent publications.\textsuperscript{17-26}

Our initial study\textsuperscript{17,18} was undertaken to delineate the variability of serially cultured callus and cell suspension cultures derived from highly uniform explants, i.e. anthers of buds identical in developmental stage. The only variables introduced were the use of 3 periwinkle cultivars and treatment of buds with a mutagen. In a supplementary study the synthesis and accumulation of alkaloids was related to the growth of those periwinkle cultures which were selected for particular alkaloid content.

1) General growth conditions\textsuperscript{17,18}

Callus grown from anthers generally originated at the cut of the filament and in the anther walls, i.e. diploid tissue. When grown to a size of 1 - 2 g freshweight, about 2 cm in diameter, the callus was cut into small pieces and serially subcultured on fresh agar medium or transferred to liquid medium (Gamborg's B5 medium) giving rise to a cell suspension. For large scale production Zenk's alkaloid production medium was employed.

The alkaloid production varied with the cell line and age of the subculture and ranged from 0.1 - 1.5% of cell dryweight. The relative amounts of alkaloids produced was fairly constant under conditions given and appeared cell line specific.

All subcultures of cell lines grown in 7.5 liter Microferm bioreactors followed essentially the pattern shown in Fig. 5. After incubation with actively growing cell suspension the mitotic index (MI) dropped to zero within 24 hours and remained there for 2 to 3 days. Thereafter the index rose sharply and reached its maximum (MI 1.8 - 3.0) within 2 days and declined again gradually over the following 10 - 15 days to zero. The cell dryweight over the culture period increased by a factor of 8 to 10 while the variation in pH stayed within half a unit.
During an 8 week culture period alkaloids have been found as soon as 2 weeks after inoculation. Most cell lines showed a maximum accumulation of alkaloids in the third to fifth week of culture.

ii) Alkaloid production

A large number of experiments have been conducted in order to delineate the various parameters essential for optimum alkaloid production and to relate the spectrum of alkaloids produced in the culture versus the conditions of culture growth, etc. A brief summary of the most salient features of such studies is provided here as general background information but detailed accounts of our results with two C. roseus culture lines coded as 953 and 200GW are published elsewhere.

The 953 line

Studies were performed in shake flasks and bioreactors utilizing first the 1B5 medium for inoculum growth and then Zenk's alkaloid production medium. The alkaloid yields versus culture method (shake flask or bioreactor) are summarized in Table 1 while yields versus

TABLE 1. Alkaloid yields from batches of 953 line C. roseus cell cultures.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Culture method</th>
<th>Weight of freeze dried cells (g)</th>
<th>Weight of basic fraction (g)</th>
<th>% Alkaloid</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Bioreactor (10 days)</td>
<td>90.5</td>
<td>0.168</td>
<td>0.185</td>
</tr>
<tr>
<td>2</td>
<td>Bioreactor (11 days)</td>
<td>110.0</td>
<td>0.178</td>
<td>0.16</td>
</tr>
<tr>
<td>3</td>
<td>Bioreactor (22 days)</td>
<td>26.9</td>
<td>0.058</td>
<td>0.21</td>
</tr>
<tr>
<td>4</td>
<td>Shake flask (14 days)</td>
<td>40.6</td>
<td>0.065</td>
<td>0.16</td>
</tr>
<tr>
<td>5</td>
<td>Shake flask (21 days)</td>
<td>49.66</td>
<td>0.182</td>
<td>0.37</td>
</tr>
</tbody>
</table>

TABLE 2. Alkaloid yields from 953 line C. roseus shake flask cultures.

<table>
<thead>
<tr>
<th>Cultivation time</th>
<th>Weight of freeze dried cells (g)</th>
<th>Weight of basic fraction (g)</th>
<th>% Alkaloid</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 Weeks</td>
<td>65.9</td>
<td>0.15</td>
<td>0.23</td>
</tr>
<tr>
<td>4 Weeks</td>
<td>51</td>
<td>0.15</td>
<td>0.29</td>
</tr>
<tr>
<td>5 Weeks</td>
<td>87.6</td>
<td>0.24</td>
<td>0.28</td>
</tr>
<tr>
<td>6 Weeks</td>
<td>19.8</td>
<td>0.125</td>
<td>0.63</td>
</tr>
<tr>
<td>7 Weeks</td>
<td>19.7</td>
<td>0.1</td>
<td>0.51</td>
</tr>
</tbody>
</table>
cultivation time are presented in Table 2. High performance liquid chromatography (HPLC) analysis of the resultant alkaloid mixtures obtained from different growth periods of the 953 line, when grown in a bioreactor, is shown in Fig. 6. The latter illustrates that, in general, the same spectrum of alkaloids is obtained but their relative concentrations alters with age of culture.

![Fig. 6. Reverse phase HPLC of alkaloid mixtures obtained after different growth periods.](image)

In summary, these studies show that percentage of alkaloid per gram of cell weight increases with time, with optimum production at 3 - 4 weeks and coinciding with a zero value of the mitotic index.

Harvesting of the culture from a larger scale bioreactor (15—30 liters) allows the isolation and characterization of the following alkaloids: ajmalicine (13), yohimbine (14), iso-sitsirikine (15), vallesiachotamine (16), strictosidine lactam (17), lochnericine (18), hbrhammericineline (19), hbrhammerinine (20), vindolinine (21), 19-epivindolinine (22), 19-acetoxy-11-methoxytabersonine (23), 19-hydroxy-11-methoxytabersonine (24) and dimethyltryptamine (25).
The 200GW line

This cell line is uniquely different from the 953 line and produces a somewhat different spectrum of alkaloids (Table 3). Of particular interest is the alkaloid catharanthine (1, 0.005% dry cell wt) isolated for the first time in our studies. This line provides this alkaloid in amounts ca. 3-5 times that normally obtainable from C. roseus plant material.

### Table 3. Alkaloids isolated from the 200GW cell line.

<table>
<thead>
<tr>
<th>Alkaloid</th>
<th>% Yield from dry cell wt.</th>
<th>% of crude alkaloid mixture</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.005</td>
<td>1.35</td>
</tr>
<tr>
<td>16</td>
<td>0.015</td>
<td>4.05</td>
</tr>
<tr>
<td>epimer of 16</td>
<td>0.026</td>
<td>7.02</td>
</tr>
<tr>
<td>13</td>
<td>0.006</td>
<td>1.62</td>
</tr>
<tr>
<td>19</td>
<td>0.002</td>
<td>0.54</td>
</tr>
<tr>
<td>20</td>
<td>0.005</td>
<td>1.44</td>
</tr>
<tr>
<td>21</td>
<td>0.002</td>
<td>0.54</td>
</tr>
<tr>
<td>22</td>
<td>0.002</td>
<td>0.54</td>
</tr>
<tr>
<td>17</td>
<td>0.224</td>
<td>60.48</td>
</tr>
</tbody>
</table>

% figures refer to isolated yields

Very recent studies with the 200GW line have involved the use of bioregulators, as general inducers of isoprenoids, for the purpose of increasing the yield of the target compound catharanthine. The structure of the five organic amines (26 - 30) under investigation are presented in Fig. 7. Results of these experiments will be presented in the lecture.

![Fig. 7. Bioregulators for study in catharanthine production by 200GW cell line.](image)

### iii) Biotransformation studies

As noted above, alkaloid production from nutrients in the growth medium generally takes 2 - 3 weeks and unless this time period can be reduced, the commercial viability of such a process remains questionable. An alternative approach which we have undertaken in order to possibly increase yields of the target compounds and, in particular, reduce the production time period concerns biotransformation of synthetic precursors which are believed to be on the biosynthetic pathway to the desired compounds. For this purpose we have initiated studies with appropriate C. roseus cell lines and selected synthetic bisindole alkaloid analogues.

The substrate 3',4'-anhydrovinblastine (7), synthesized by the method outlined in Fig. 1, was evaluated employing a cell line coded as 916. This unique line did not produce any alkaloids and thus allowed for a less complicated system in terms of end product isolation.
The results of this experiment are summarized in Tables 4 and 5. Based on the amount of recovered substrate, the transformation of 3',4'-anhydrovinblastine (7) to leurosine (10) and catharine (11) was 25.5 and 16.3% respectively, or, approximately 42% of 7 had been utilized by the cells. It should be noted, however, that no attempts have yet been made to optimize the yields of specific products.

**TABLE 4. Biotransformation of 3',4'-anhydrovinblastine (7, 300 mg\(^a\)) in C. roseus suspension cell culture in Microferm bioreactor, cell line 916, 48 h**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Weight of sample (g)</th>
<th>Weight of methanol extract (g)</th>
<th>Weight of neutral ethyl acetate extract (g)</th>
<th>Weight of basic ethyl acetate extract (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell material</td>
<td>10.25</td>
<td>3.5</td>
<td>0.469</td>
<td>0.065</td>
</tr>
<tr>
<td>Supernatant</td>
<td>ca 140</td>
<td>-</td>
<td>0.143</td>
<td>0.187</td>
</tr>
</tbody>
</table>

\(^a\) added as the hydrogensulfate salt

**TABLE 5. Alkaloids isolated from biotransformation of 3',4'-anhydrovinblastine (7, 300 mg\(^a\)) in C. roseus suspension cell culture in Microferm bioreactor, cell line 916, 48 h**

<table>
<thead>
<tr>
<th>Alkaloid isolated</th>
<th>Weight of alkaloid isolated (mg)</th>
<th>% of the substrate added</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Basic supernatant extract</td>
<td>Basic cell material extract</td>
</tr>
<tr>
<td>3',4'-anhydrovinblastine (7)</td>
<td>28.8</td>
<td>30.0</td>
</tr>
<tr>
<td>catharine (11)</td>
<td>28.1</td>
<td>3.1</td>
</tr>
<tr>
<td>leurosine (10)</td>
<td>17.0</td>
<td>8.2</td>
</tr>
</tbody>
</table>

\(^a\) added as the hydrogensulfate salt

Thus, it has been established that short time periods (24 - 48 h) are required for such biotransformations - clearly an advantage when compared to 2 - 3 weeks in experiments where metabolites are being produced from media nutrients. Further results will be presented in the lecture.

iv) Studies with cell free systems

As noted earlier, plant tissue cultures should provide excellent media for biosynthetic investigations since incubation and growth periods, precursor administration, etc. can be carefully controlled. Such experiments could involve whole cells or enzyme mixtures available from cell free extracts. It was mentioned earlier (Fig. 3 and 4) that we have performed a series of experiments with cell free systems and obtained important information concerning the biosynthesis of the bisindole alkaloids of the vinblastine family. Details of these studies are published\(^{13-15,30}\) so only a brief summary is provided here.

The purification procedure employed in our studies with leaves and/or tissue cultures is summarized in Fig. 8. The crude enzyme thus obtained was used to obtain the results summarized in Fig. 3 and 4. The HPLC profile, employing the developed HPLC procedure\(^{30}\), of
C. roseus leaves (or tissue cultures)

↓ homogenized in 0.1 M potassium phosphate buffer, pH 6.3

↓ centrifuged at 30,000 x g for 20 min

↓ crude enzyme

↓ ammonium sulfate precipitation (70% saturation) dialysis

↓ DEAE-cellulose chromatography

↓ Sephadex G-200 chromatography

↓ partially purified enzyme

Fig. 8. General procedure for preparation of cell free systems from C. roseus leaves and/or tissue cultures.

this enzyme mixture is shown in Figure 9. Subsequent purification by DEAE cellulose and Sephadex G-200 chromatography afforded two fractions (II-1 and II-2) with HPLC profiles shown in Fig. 10 and 11. It was then shown by incorporation of radiolabelled alkaloids that the enzyme(s) involved in the coupling of catharanthine (1) and vindoline (2) (Fig. 3) are in fraction II-2 (A in Fig. 10) with HPLC retention times of 11 - 20 minutes and suggesting proteins of molecular weight greater than 25,000.

Fig. 9. HPLC analysis of cell-free extract prepared from C. roseus leaves

Employing the methodology described above, crude enzyme preparations were obtained from various C. roseus tissue culture lines and HPLC analyses were performed. Figure 12 summarizes the results of four well-developed C. roseus lines from which various alkaloids have been isolated and characterized. The spectrum of alkaloids from the lines coded as 953 and 200GW was discussed earlier. The 916 line is somewhat unique in that it exhibits normal growth characteristics but does not produce any of the alkaloids normally found in the other lines. A subline coded as 91601 and developed from 916 does produce alkaloids and the HPLC enzyme profiles, particularly in the region of 7-15 minutes retention time, are strikingly different. Such data are of considerable value in developing tissue culture lines with optimum production of target compounds and are also useful in biosynthetic investigations.
Fig. 10. HPLC analysis of fraction II-1.

Fig. 11. HPLC analysis of fraction II-2.

Fig. 12. HPLC profiles of crude enzyme extracts from several C. roseus tissue culture cell lines.
Studies in Tripterygium wilfordii

Triptolide (Td, 4, R = OH) and triptolide (Tl, 4, R = H), isolated from the African plant, Tripterygium wilfordii, in low concentrations (0.001%) are novel diterpene triepoxides with significant in vivo activity against L-1210 and P-388 leukemias in the mouse and also exhibit high KB activity. Their low availability provided some stimulation toward the development of plant tissue culture cell lines which are capable of producing these compounds. Earlier studies have been published so only a brief summary is provided here in order to serve as a background for the most recent studies.

1) General growth conditions

Stem and leaf explants obtained from T. wilfordii plants were placed on B5 and PRL-4 media and supplemented with numerous combinations of the following compounds: 2,4-dichlorophenoxyacetic acid (D) (2 mg/l), kinetin (K) (0.1 mg/l), 1-naphthaleneacetic acid (NA) (0.15 mg/l), indole-3-acetic acid (I) (2 mg/l), 6-benzylamino purine (B) (2 mg/l), 4-amino benzoic acid (P) (2 mg/l), and coconut milk (Co) (100 ml/l). The explants and resulting calli were incubated at room temperature in darkness. Subculture occurred about every four weeks. Preliminary selection of promising cell lines was based on growth vigor as well as qualitative TLC and KB cytotoxic activity analyses. The cell line, designated TRP 4a, selected for further investigations was initiated as a leaf explant on PRI2 Co100 agar (i.e. PRL-4 medium supplemented with indole-3-acetic acid (2 mg/l) and coconut milk (100 ml/l)), transferred to PRD2 Co100 agar, and maintained on the latter medium. Suspension cultures of TRP 4a were generated in PRD2 Co100 broth and were maintained as stock cultures by regular subculture using 10% inoculum at three week intervals.

2) Metabolite production

Studies were conducted in shake flasks and bioreactors and the presence of the target compounds (Td and Tl) was assayed by fluorimetric detection. In order to ascertain the spectrum of metabolites produced, large scale experiments in shake flasks and bioreactor (28-50 liters) were analyzed by the usual chemical and/or spectroscopic methods to afford the compounds shown in Fig. 13. Tripdiolide (Td, 4, R = OH) is obtained in levels 20 times higher than in the plant. The isolation of dehydroabiatic acid (31) and the unsaturated acid 39 may be biosynthetic precursors of the cytotoxic agent.

Fig. 13. Natural products isolated from T. wilfordii tissue cultures.
Although we had established that formation of Td by TRP 4a cells in PRNA0.5K0.5 medium can be achieved with a peak level of over 2 mg/l, it was of interest to examine different production media in the continuing effort to improve the yield of the desired compounds. The effects of two other basal media (those of Murashige and Skoog (MS) and Schenk and Hildebrandt (SH)) on growth and Td production of TRP 4a cells were compared with PRNA0.5K0.5 medium in a series of parallel experiments. Results are shown in Fig. 15. Good cell growth was observed in all three media, but a superior level of Td (4.0 mg/l) was produced in MSNA0.5K0.5 (2% sucrose) after 35 days of incubation. Furthermore, production of Td in the modified MS medium reached the maximum in a shorter time than it did in cultures in the other two media (see Fig. 15), thereby suggesting that this is a more effective Td production medium than similarly supplemented PRL-4 or SH medium for cultivation of TRP 4a suspension cultures.

Comparison of the three basal media (PRL-4, MS, SH) reveal several major differences in their compositions. Some possible key components are: (a) Concentration of available nitrogen in the forms of NH₄⁺ ion or NO₃⁻ ion. Both are present in much higher concentrations in the MS medium. (b) Concentration of calcium chloride (CaCl₂·2H₂O) is also higher in MS (440 mg/l). (c) Concentration of thiamine is much lower in MS (0.1 mg/l) than in the other two media (10 mg/l and 5 mg/l). (d) Glycine (2 mg/l) is only present in MS. In addition there are other differences in the micronutrients (e.g. Mn²⁺, Zn²⁺, Cu²⁺ and Co²⁺).

The effect of different levels of some of these components (NH₄NO₃, sucrose, CaCl₂) was studied using MSNA0.5K0.5 as the basal medium. Results of these studies are summarized in Fig. 16.

Fig. 16A indicates Td production is highest in medium with 1650 mg/l of NH₄NO₃ while biomass yield is greatest at a lower NH₄NO₃ concentration (850 mg/l). Fig. 16B illustrates the effect of sucrose concentration, that is, cultures grown in 4% sucrose showed the best biomass and Td yields, while Fig. 16C shows that biomass yield was very similar for all three CaCl₂ concentrations with maximum Td production at 880 mg/l CaCl₂.
**Fig. 16.** The effects on Td and dry biomass production of different medium concentrations of NH₄NO₃ (Panel A), sucrose (Panel B) and CaCl₂ (Panel C). MSNA₀₅K₀₅ was the basic medium and inocula were grown for 20 days in PRD₂CO₁₀₀.

### iii) Biotransformation studies

As in the C. roseus area, it was considered essential to evaluate appropriate synthetic precursors for the purpose of biotransformation to tripdiolide and/or triptolide. It was hoped that such experiments would afford higher yields and reduce the time period for metabolite production. For this purpose, chemical syntheses of appropriate precursors were developed from the readily available dehydroabietic acid (31) and a summary of the completed chemical routes is provided in Fig. 17 and 18. The utilization of compounds 44, 45, 48 and 49 in biotransformation studies with resting cell suspensions of T. wilfordii are presently underway.

In summary, this lecture illustrates some of the interesting avenues of research in which tissue culture techniques can be employed to advantage over studies with living plant systems. It is clear that in addition to studies within academic laboratories, the use of tissue cultures for commercial production of important and costly pharmaceutical drugs represents an area of considerable potential.

### Acknowledgements:

The author serves merely as a spokesman for the enthusiastic and hard working group of researchers who have made possible the above presentation. The studies with C. roseus represent a collaborative program between the author's laboratory and the National Research Council of Canada, Plant Biotechnology Institute, in Saskatoon. The senior collaborators in Saskatoon are Drs. W. G. W. Kurz and F. Constabel, and they along with their associates, K. B. Chatson, K. A. Busch, H. Evans, P. Gaudet-La Prairie, L. Leland, G. Mickelson, J. Parkes, S. Rambold and B. P. Vierheilig are responsible for all of the development of the cell culture methods and large scale production of the natural products. Development of all the analytical methodology (HPLC, etc.), the subsequent isolation and characterization of the various alkaloids discussed, and the isolation of cell free systems was performed in the author's laboratory by B. Aweryn, L. S. L. Choi, T. Honda, P. Kolodziejczyk, N. G. Lewis, T. Sato, S. K. Sleigh, K. L. Stuart and B. R. Worth.

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Fig. 17. Synthetic diterpene analogues as precursors for biotransformation studies in *T. wilfordii* cell cultures.

It is a particular pleasure to acknowledge the dedicated efforts of these colleagues.

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