SELECTIVE KILLING OF HUMAN T CELL LYMPHOTROPIC VIRUS TYPE I-TRANSFORMED CELL LINES BY A DAMAVARICIN Fc DERIVATIVE

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7-Pentyl ether of damavaricin Fc (n-pentyl DvFc) preferentially killed human T-cell lymphotropic virus type I (HTLV-I)-transformed cell lines. The mechanism of action of the drug was investigated using MT-4 cells. Cytotoxic action was diminished by the removal of n-pentyl DvFc from the culture or by the addition of sulfhydryl compounds such as 2-mercaptoethanol and dithiothreitol. The killing activity of n-pentyl DvFc was also diminished by membrane-acting agents including quinidine and diphenylhydantoin. Influx and subsequent efflux of Ca²⁺ were observed when either HTLV-I infected (MT-4 cells) or uninfected cells were treated with n-pentyl DvFc. An efflux of K⁺ was observed in HTLV-I infected MT-4 cells immediately after the exposure of the cells to n-pentyl DvFc. The K⁺ efflux, however, was not observed in the uninfected T cells. n-Pentyl DvFc seems to act primarily on the cell surface of MT-4 cells, leading to the perturbation of membrane function. The restoration of cell growth, however, is critically dependent on the presence of dithiothreitol and 2-mercaptoethanol, implying a role for a free sulfhydryl group in the killing activity.

Damavaricin Fc is an atropisomeric mixture of two compounds produced by treatment of streptovaricin C with oxygenated concentrated ammonia - methanol. Damavaricin Fc, in which the ansa bridge lies above the aromatic nucleus, has the P helicity as well as streptovaricin C, whereas atropisodamavaricin Fc, in which the ansa bridge lies below the aromatic nucleus, has the M helicity (Fig. 1)°. To avoid complicating nomenclature, hereafter we will use damavaricin Fc to refer to this atropisomeric mixture. Streptovaricins belong to the ansa-ring class of antibiotics and in addition to antibiotic activity, they also inhibit retrovirus reverse transcriptase activity in vitro. We have previously reported the biological activities of several derivatives of damavaricin Fc including those which have different alkyl ether linkage at the C-19 position of the naphthoquinone ring of the molecule. Some of these derivatives inhibited proliferation of a mouse retrovirus°.

Recently, it has been discovered that several retroviruses can cause severe human diseases°-°. Human T-cell lymphotropic virus type I (HTLV-I) is the causative agent of adult T-cell leukemia°, one of the most aggressive human leukemias, as well as HTLV-I associated myelopathy (HAM)°,
Fig. 1. Structure of damavaricin Fc.

[Diagram showing the structure of damavaricin Fc and its relationship to Streptovaricin C.

Streptovaricin C

\[ \quad \]

Damavaricin C

\[ \quad \]

Damavaricin Fc

\[ \quad \]
and tropical spastic paraparesis\(^7\)). However, no effective therapy for HTLV-I-related disease is currently known.

We wished to determine whether damavaricin Fc derivatives would inhibit the growth of HTLV-I-transformed cells. In the present study, we describe the selective killing of HTLV-I-transformed cells by the \(n\)-pentyl ether of damavaricin Fc (\(n\)-pentyl DvFc), and its mode of action.

### Materials and Methods

#### Cells

Peripheral blood lymphocytes (PBL) were obtained after Ficoll-Hypaque centrifugation of peripheral blood from normal healthy donors. The established cell lines used in this study were the HTLV-I-negative MOLT-4\(^8\), SKW, and NALM16 cells\(^9\); and the HTLV-I-positive T-cell lines MT-1\(^10\), MT-2, MT-4\(^11\), 467\(^12\), KAN\(^13\) and TLOml cells\(^14\). FUK cells were established from PBL of an adult T-cell leukemia (ATL) patient by culturing with interleukin-2 (IL-2) (unpublished). They were cultured in RPMI 1640 medium (Gibco Co.) supplemented with 10% or 20% fetal calf serum (FCS) (M.A. Bioproduct, Walkersville, MD), benzylpenicillin (100 U/ml) and streptomycin (100 \(\mu\)g/ml).

#### Chemicals

\(n\)-Pentyl DvFc was prepared as previously described\(^15\). 5,5-Diphenylhydantoin (5,5-DPH) and quinidine hydrochloride were purchased from Sigma Chemical Company. The radioactive compounds \([\text{methyl}^3\text{H}]\text{thymidine} (20 \text{Ci/mm})\), \([5\text{-}[\text{H}]\text{uridine} (30 \text{Ci/mm})\), \([1,4,5,\text{H}]\text{leucine} (63 \text{Ci/mm})\), \(\text{Ca}^{4+} (40 \text{mCi/mg calcium})\) and \(\text{Rb}^{86+} (8 \text{mCi/mg rubidium})\) were obtained from Amersham Co., Ltd. Dithiothreitol and 2-mercaptoethanol were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

#### Sensitivity of the Cells to \(n\)-Pentyl DvFc

1 ml of cell suspension (6 x 10\(^5\) cells/ml) was added to 1 ml of medium containing various concentration of \(n\)-pentyl DvFc, and the mixtures were incubated at 37°C in a CO\(_2\) incubator. Growth and viability of the cells were monitored by cell counting and by Trypan blue dye exclusion.

#### Assay for Alkaline Phosphodiesterase

MT-4 cells were ruptured in ice-cold phosphate-buffered saline (PBS, 10 mM potassium phosphate, pH 7.4, 135 mM NaCl) with a Dounce homogenizer and the extract was used as a source of a typical sulfhydryl (SH) enzyme. The reaction mixture (1 ml) contained: Tris-HCl (pH 9.0) 50 mM, MgCl\(_2\) 5 mM, thymidine-5-monophosphate-\(p\)-nitrophenyl ester 1 mM, extract from 5 x 10\(^6\) MT-4 cells, and various concentrations of \(n\)-pentyl DvFc. After incubation at 37°C for 45 minutes, the enzyme reaction was terminated by the addition of 300 \(\mu\)l of TCA. The deproteinized supernatant was added to 400 \(\mu\)l of 2.5 \(\text{N}\) NaOH, and the optical density at 400 nm was compared with standard \(p\)-nitrophenol to determine the amount of substrate hydrolyzed.

#### Biosynthesis of DNA, RNA, and Proteins

MT-4 cells (3 x 10\(^6\) cells/ml) were incubated with various concentration of \(n\)-pentyl DvFc in culture medium (total volume; 400 \(\mu\)l) containing labeled precursors \([\text{H}]\text{thymidine, [H}]\text{uridine, or [H}]\text{leucine, all at 4 \(\mu\text{Ci/ml})}\) for 40 minutes. Cells were washed with PBS, and treated with 400 \(\mu\)l of 10% cold TCA. Acid-insoluble fractions were collected on glass fiber filters (Advantec GC50) and washed with 5% cold TCA, and the radioactivity was counted.

#### Rubidium Efflux Measurements

HTLV-I infected (MT-4) or uninfected (MOLT-4) cells (2.4 x 10\(^6\) cells/ml) were loaded overnight with 10 \(\mu\text{Ci/ml of }\text{Rb}^{86+}\) in RPMI 1640 containing 10% FCS. Cells were diluted 10-fold into non-radioactive medium, spun down (400 \(\times g\), 5 minutes) and resuspended in the same medium at 6 x 10\(^6\) cells/ml. \(n\)-Pentyl DvFc (5 \(\mu\)g to 3 x 10\(^6\) cells) was added to the culture and incubated at 37°C in a CO\(_2\) incubator. At the indicated times, 100 \(\mu\)l of cell suspension was removed and layered over 500 \(\mu\)l
of a corn oil and dibutylphthalate (3:10) mixture\textsuperscript{15}, and centrifuged in an Eppendorf microfuge (15,000 rpm, 40 seconds). The tip of tube containing the cell pellet was cut off and the radioactivity was determined.

**Calculated Flux Measurement**

MT-4 or MOLT-4 cells (6 × 10\textsuperscript{6} cells/ml) were incubated in the presence of \textsuperscript{45}CaCl (50 μCi/ml). After 10 minutes, \textit{n}-pentyl DvFc (5 μg to 3 × 10\textsuperscript{5} cells) was added to the culture. At the indicated times, 120 μl of cell suspension was transferred into 1 ml of simplified medium (NaCl 145 mm, KCl 5 mm, Na\textsubscript{2}HPO\textsubscript{4} 1 mm, MgSO\textsubscript{4} 0.5 mm, glucose 5 mm, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) 10 mm, ethylene glycol bis(2-aminoethylether)tetracetic acid (EGTA) 1 mm, pH 7.4). Cells were spun down (10,000 × g, 30 seconds) and resuspended in simplified medium, transferred to a membrane filter (pore size, 0.45 μm), washed, and the radioactivity was determined.

**Results**

**Sensitivity of HTLV-I-transformed Cells to \textit{n}-Pentyl DvFc**

All HTLV-I-positive cell lines tested were highly sensitive to \textit{n}-pentyl DvFc as compared

![Fig. 2. Effect of \textit{n}-pentyl DvFc on the growth of MT-4 cell.](image-url)

The concentrations of DvFc were 0 (●), 1 (○), 5 (△), 10 (◆), and 25 (□) μg/ml.

![Table 1. Effect of \textit{n}-pentyl DvFc on cell growth.](image-url)

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Minimum concentration of \textit{n}-pentyl DvFc inhibiting cell growth by 50% (μg/ml)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBL (resting)</td>
<td>44</td>
</tr>
<tr>
<td>PBL (PHA-activated)</td>
<td>32</td>
</tr>
<tr>
<td>HTLV-I-negative cell lines</td>
<td></td>
</tr>
<tr>
<td>MOLT-4</td>
<td>16</td>
</tr>
<tr>
<td>SKW</td>
<td>25</td>
</tr>
<tr>
<td>NALM16</td>
<td>27</td>
</tr>
<tr>
<td>HTLV-I-positive T-cell lines</td>
<td></td>
</tr>
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<td>FUK</td>
<td>1</td>
</tr>
<tr>
<td>467</td>
<td>3</td>
</tr>
<tr>
<td>KAN</td>
<td>6</td>
</tr>
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</tr>
<tr>
<td>MT-2</td>
<td>7</td>
</tr>
<tr>
<td>MT-4</td>
<td>2</td>
</tr>
</tbody>
</table>

* The values present the concentration of \textit{n}-pentyl DvFc showing 50% cell growth compared with DvFc-free control after incubation for 72 hours.

![Fig. 3. Effect of the removal of \textit{n}-pentyl DvFc on the growth of \textit{n}-pentyl DvFc-treated MT-4 cells.](image-url)

MT-4 cells (3 × 10\textsuperscript{6} cells/ml) were incubated in the presence of \textit{n}-pentyl DvFc (5 μg/ml). At indicated times (presented by arrow heads), viable cells were determined by Trypan blue dye exclusion (●), 1 ml of culture was taken and cells were washed twice with 1 ml of drug-free medium by centrifugation (400 × g, 3 minutes). Cells were resuspended in medium, incubated for 46 hours, and viable cells were determined (○).
Fig. 4. Recovery of cell killing by \( n \)-pentyl DvFc with SH-compound.

MT-4 cells (3 x 10^5 cells/ml) were incubated in the presence of \( n \)-pentyl DvFc (5 \( \mu \)g/ml) and various concentrations of dithiothreitol (A) or 2-mercaptoethanol (B).

![Graph A](image1)

(A)

![Graph B](image2)

(B)

Incubation time (days)

Cell growth and viability were monitored with the Trypan blue dye exclusion test. The values in the figure are the concentration (\( \mu \)M) of SH-compound.

with HTLV-I-negative cell lines (Table 1). Among the sensitive cell lines, MT-4 was chosen to study the mode of action of \( n \)-pentyl DvFc. To determine the time course of cytotoxic action of \( n \)-pentyl DvFc, logarithmically growing MT-4 cells were exposed to various concentrations of \( n \)-pentyl DvFc and the cell number was measured at various times. MT-4 cells showed no growth in the presence of \( n \)-pentyl DvFc at concentrations of more than 5 \( \mu \)g/ml under these conditions (Fig. 2). However, these MT-4 cells were viable for at least 12 hours after exposure to \( n \)-pentyl DvFc (5 \( \mu \)g/ml) as indicated by the following experiments. MT-4 cells were incubated in the presence of \( n \)-pentyl DvFc (5 \( \mu \)g/ml), and at various times, were washed with \( n \)-pentyl DvFc-free medium, and were incubated in the absence of the drug. The washed cells were able to resume growth in drug-free medium even after 42 hours (Fig. 3). Similar results were obtained in experiments using 10 \( \mu \)g/ml of \( n \)-pentyl DvFc. These results imply that \( n \)-pentyl DvFc binds to MT-4 cells reversibly.

Effect of Sulfhydryl Compounds

It was reported that the activity of naphthomycin, an antibiotic of the ansamycin group, was prevented by SH compounds\(^{16} \). Therefore we examined whether the cytotoxic action of \( n \)-pentyl

Fig. 5. Inhibitory effect of \( n \)-pentyl DvFc on the membrane bound enzyme, alkaline phosphodiesterase of MT-4 cells.

![Graph](image3)

MT-4 cells were ruptured and the extract was used as a source of SH enzyme.
DvFc might also be prevented by SH compounds. Both dithiothreitol and 2-mercaptoethanol diminished the action of n-pentyl DvFc on MT-4 in a dose-dependent manner (Fig. 4). These results indicate that n-pentyl DvFc may act on the SH groups of proteins. To confirm the action of n-pentyl DvFc on the SH groups of proteins, we measured the inhibitory effect of n-pentyl DvFc on alkaline phosphodiesterase, a known SH enzyme located in the cell membrane of lymphocytes\(^{17}\). n-Pentyl DvFc inhibited this enzymic activity in vitro (Fig. 5). 50% inhibition was seen at a n-pentyl DvFc

Fig. 6. Effect of membrane-acting agents on the cytotoxic activity of n-pentyl DvFc in MT-4 cells.

Logarithmically growing MT-4 cells were incubated for 24 hours in the presence of both n-pentyl DvFc (5 μg/ml) and membrane-acting agents (○), or of the later only (●).

(A) Effect of DPH. 100% corresponds to 7.2 × 10⁵ cells/ml. (B) Effect of quinidine. 100% corresponds to 8.8 × 10⁵ cells/ml.

Fig. 7. Effect of n-pentyl DvFc on the \(^{86}\text{Rb}^+\) efflux from MT-4 cells.

● n-Pentyl DvFc-free control, ○ n-pentyl DvFc-treated.

Logarithmically growing MT-4 (HTLV-I infected) (A) and MOLT-4 (uninfected) (B) cells were loaded with \(^{86}\text{Rb}^+\), and cellular \(^{86}\text{Rb}^+\) was measured as described in the Materials and Methods.
concentration of 10 μg/ml. These results suggest that n-pentyl DvFc may react reversibly with the SH groups of the membrane proteins of MT-4 cells.

Effect of n-Pentyl DvFc on Macromolecular Synthesis

To determine the effect of n-pentyl DvFc on macromolecular synthesis, we measured the incorporation of radiolabeled precursors by MT-4 cells into DNA ([3H]thymidine), RNA ([3H]uridine), and protein ([3H]leucine). DNA synthesis was inhibited by n-pentyl DvFc in a dose dependent manner (data not shown). Neither RNA nor protein synthesis was affected significantly.

Reversal of the Action of n-Pentyl DvFc by Membrane-acting Agents

The results described above indicate that n-pentyl DvFc might act directly on the cell membrane of MT-4 cells. We investigated the effect of membrane-active agents such as quinidine and DPH. These drugs are a K+ channel blocker and an anticonvulsant, respectively. Both types of drugs overcame the inhibitory effects of n-pentyl DvFc (Fig. 6). However, another K+ channel blocker, 4-aminopyridine (4-AP) did not show this effect. Verapamil, a Ca2+ channel blocker, was also effective at concentration of 10^{-7} to 10^{-4} M (data not shown).

Effect of n-Pentyl DvFc on Ion Permeability

To confirm whether n-pentyl DvFc actually caused K+ efflux in MT-4 cells, 86Rb\(^+\)-loaded MT-4 cells were exposed to n-pentyl DvFc and the cellular 86Rb level was examined. Efflux of 40% of cellular 86Rb was observed within 6 minutes after the exposure of cells to n-pentyl DvFc. Efflux of 86Rb was not observed in the HTLV-I negative MOLT-4 cells under the same condition (Fig. 7). The two drugs which reversed the killing of HTLV-I infected cells by n-pentyl DvFc, DPH\(^{18}\) and verapamil\(^{19}\), are known to inhibit Ca\(^{2+}\) influx into cells. Therefore Ca\(^{2+}\) influx into MT-4 cells might be changed by the action of n-pentyl DvFc. MT-4 cells were loaded with \(^{41}\)Ca and then exposed to n-pentyl DvFc in the presence of \(^{42}\)Ca\(^{2+}\). There was a transient increase in intracellular \(^{45}\)Ca ion followed by a decrease within 10 minutes (Fig. 8). Since this phenomenon was also observed in MOLT-4 cells, it cannot be responsible for the selective killing of MT-4 cells.

Discussion

We have been engaged in a series of studies to elucidate the relationship between the structure and the biological activity of streptovaricin C derivatives (damavaricin Fc and damavaricin C)\(^{1,20-22}\).
The damavaricin Fc as well as damavaricin C were produced from streptovaricin C\textsuperscript{23}. Whereas damavaricin C retained antibacterial activity, damavaricin Fc lost it\textsuperscript{17}. It is suggested that the lactone-ring formed between C-7 and C-10 is closely related to the loss of antibacterial activity. The low toxicity of damavaricin Fc has been demonstrated on mammalian cells in vitro\textsuperscript{12} and also in animals when orally administered (unpublished data). Therefore, damavaricin Fc may have a potential for the therapy of adult T cell leukemia or HTLV-I-related diseases. This drug may be applicable to the treatment of ATL by adoptive immunotherapy such as lymphokine-activated killer cells\textsuperscript{24}.

Since \(n\)-pentyl DvFc has shown the most effective inhibitory activity against focus formation by mouse sarcoma virus/mouse leukemia virus complex\textsuperscript{15}, we selected this derivative for study. We have shown the selective killing of the HTLV-I-transformed cells by \(n\)-pentyl DvFc, and have given circumstantial evidence for the mechanism of cell-killing. The results suggest that the drug acts on the cell membrane. It has been reported that HTLV-I-transformed cells have an altered cell surface: They express the IL-2 receptor\textsuperscript{25} and La antigen\textsuperscript{26}. In the present study, we demonstrated that K\textsuperscript{+} efflux occurred in HTLV-I-transformed cells immediately after exposure to \(n\)-pentyl DvFc. Since K\textsuperscript{+} efflux was specific for HTLV-I infected cells, K\textsuperscript{+} efflux seemed to be an essential event at an early phase in the course of the killing action. The killing action by \(n\)-pentyl DvFc, however, was not reversed by 4AP, which is a blocking agent of voltage-sensitive K\textsuperscript{+} channel\textsuperscript{26}. This suggests that the K\textsuperscript{+} efflux caused by \(n\)-pentyl DvFc was not through the voltage-sensitive channel but through the Ca\textsuperscript{2+}-dependent one. In fact, Ca\textsuperscript{2+} influx seems to occur in MT-4 cells immediately after the addition of DvFc, but MOLT-4 cells also showed a similar pattern of Ca\textsuperscript{2+} influx, suggesting that drug-induced Ca\textsuperscript{2+} influx was not specific to infected cells. We speculate that if the level of intracellular Ca\textsuperscript{2+} was similar between these two cell lines (MT-4 and MOLT-4) after exposure to \(n\)-pentyl DvFc, the Ca\textsuperscript{2+}-dependent K\textsuperscript{+} channel of MT-4 cell is more sensitive to the level of Ca\textsuperscript{2+} than that of MOLT-4 cell.

What is the target site for the action of \(n\)-pentyl DvFc? From the results in which the killing is prevented by the removal of \(n\)-pentyl DvFc or by the addition of SH-compounds, we consider that \(n\)-pentyl DvFc interacts with membrane proteins containing SH groups in MT-4 cells. HTLV-I antigens expressed on the cell membrane are unlikely to be the target molecules, because TLOml cells which are also HTLV-I-infected but do not express HTLV-I antigen in vitro\textsuperscript{15} were also killed by \(n\)-pentyl DvFc. DNA synthesis was inhibited after addition of DvFc to HTLV-I infected cells. The inhibitory activity of \(n\)-pentyl DvFc on SH enzymes may effect DNA synthesis, but this is probably a secondary effect.

From the results described above, we conclude that \(n\)-pentyl DvFc acts directly on the membrane and that the membranes of HTLV-I-transformed cells respond differently from the membranes of uninfected cell lines. The precise mechanism is not clear at this moment. The relationship between the Ca\textsuperscript{2+} channel and the K\textsuperscript{+} channel in HTLV-I-transformed T cells as compared to untransformed cells appears to be different.

References