A Novel Class of Antitumor Antibiotics Containing a Cyclodiynene Skeleton : Activity and Mechanism of Action

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Search for the cytotoxic agents in the microbial metabolites began some 30 years ago largely by Japanese researchers and yielded several clinically useful anticancer drugs including actinomycin D, mitomycin C, neocarzinostatin and bleomycin. Although these agents are being widely used for the cancer chemotherapy, a large majority of solid tumors such as the lung, intestinal, and liver cancers remain unresponsive, and even at first responsive cancers later develop resistance to the anticancer drugs primarily treated. There is, consequently, an even enhanced need to develop novel types of anticancer drugs for future chemotherapy. The fermentation-derived compounds, antitumor anti-

otics, are considered to be unique resources of the novel chemotypes of antitumor agents.

Recently, a new class of antitumor antibiotics possessing the unprecedented bicyclodiynene system has been discovered. The first examples of this class of antibiotics were esperamicins^{1,2)} and calicheamicins³⁾ which were published at the same time in 1987. Subsequently, dynemicins^{4,5)}, a hybrid of an anthraquinone and a 1,5-diyn-3-ene, was isolated from *Micromonospora chersina* as a violet colored metabolites. Neocarzinostatin chromophore⁶⁾, previously characterized as the active principle of anticancer peptide antibiotic neocarzinostatin, was also shown to be a member of this group

Fig. 1. Chemical structures of the cyclodiynene group of antibiotics

Table 1. Producing strains of cyclodiynene antibiotics

Antibiotic	Producing organism
Esperamicins	<i>Actinomadura verrucosospora</i> H964-92 (ATCC 39332)
Veractamycins (PD-114759 and -115028)	<i>Actinomadura verrucosospora</i> subsp. <i>veractimycis</i> (ATCC 39364)
FR-900405 and -900406 (WS-6049)	<i>Actinomadura pulvercea</i> sp. nov. No. 6049
Calicheamicins	<i>Micromonospora echinospora</i> sp. <i>calichensis</i> (NRRL 15839)
Dynemicins	<i>Micromonospora chersina</i> M956-1 (ATCC 53710)
Deoxydynemicin	<i>Micromonospora globosa</i> MG331-HF6
Neocarzinostatin chromophore (Neocarzinostatin)	<i>Streptomyces carzinostaticus</i> var. F-41

(Fig. 1). The intriguing chemical structures coupled with their extraordinary potency against a variety of bacteria and tumor cells and their unusual mechanism of DNA-cutting action have generated globally the greatest interest of a number of chemists, biochemists and oncologists. This review is summarized the progress of studies on the cyclodiynene antibiotics with emphasis on the activity and mechanism of action.

1. Discovery, production and chemical structure of the cyclodiynene antibiotics.

Esperamicins, calicheamicins and dynemicins were discovered in the fermentation broths by using the detection systems highly sensitive to the DNA-interacting compounds. Recombination deficient mutant strains of *Bacillus subtilis* and *Escherichia coli* (Rec⁻) are known to be more sensitive to the DNA-damaging agents than the wild strains (Rec⁺). The assay system has been used for detection of the chemical mutagens and is called Rec-assay⁷⁾. Esperamicins and dynemicin A were found to exhibit much stronger growth inhibition to the Rec⁻ mutant of *B. subtilis* than those to the parent strain. The primary *in vitro* detection system used for calicheamicins was the prophage induction assay (BIA)⁸⁾. The assay uses a strain of *Escherichia coli* lysogenic for a γ -*lacZ* fusion phage and the induction of β -galactosidase is detected by colorimetry. Practically, all the cyclodiynene class of antibiotics show extremely potent growth inhibition against bacteria and fungi, particularly against Gram-positive bacteria, and the antimicrobial assay was most

conveniently used for detection, isolation and fractionation of the activities. Antitumor potential of these antibiotics were readily detected with their crude extracts as they demonstrated extremely potent *in vivo* activity against experimental murine tumors such as P388 leukemia and B16 melanoma.

Interestingly, all the three new cyclodiynene antibiotics were isolated from so-called rare actinomycetes, either *Actinomadura* or *Micromonospora* strains (Table 1). FR900405 and FR900406⁹⁾ and veractamycins¹⁰⁾ were found as the products of *Actinomadura* species and later identified to be one of the esperamicin components. Recently deoxydynemicin A was also reported to be produced by *Micromonospora* species¹¹⁾. Neocarzinostatin was, however, discovered in 1965 as an antitumor antibiotic produced by a *Streptomyces* strain, the most popular soil actinomycetes. As often observed in the case of potent antibiotics, productivity of the cyclodiynene antibiotics is extremely low. Together with the mutation studies, media improvement and refinement of fermentation conditions were performed for increasing the productivity. A dramatic increase of antibiotic titer was observed when sodium or potassium iodide was supplemented to the fermentation media of esperamicins, calicheamicins and dynemicins. When sodium bromide was added to the media, production of calicheamicins was stimulated. The addition of potassium iodide remarkably promoted a yield of the activities and changed the products from the natural bromine-containing components to new iodine-containing components (Table 2)¹²⁾.

The cyclodiynene antibiotics are neutral to

Table 2. Effects of halide addition on calicheamicin production

Addition	Calicheamicin content ($\mu\text{g/ml}$)			
	γ_1^{Br}	β_1^{Br}	γ_1^{I}	β_1^{I}
none	<0.05	0.1	<0.05	<0.05
NaBr (0.05%)	2.3	0.3	<0.05	<0.05
KI (0.01%)	<0.05	<0.05	9.8	2.6

weakly basic, lipophilic compounds and are unstable to acid, heat and light. Neocarzinostatin chromophore was separated from neocarzinostatin apo-protein fraction by extraction with acidic aqueous methanol solution in a dark room.

The unique structures of the cyclodiynene antibiotics were determined by a combination of careful stepwise degradations and spectroscopic analyses (Fig. 1). The heart of the molecules is a bicyclo [7.3.1 or 7.3.0] diynene system which is substituted with a DNA-interacting group (trisaccharide or aromatic nucleus) and a trigger group for activation of the enediyne (allylthiolsulfide, electrophilic double bond or anthraquinone). In the structural studies of esperamicins, an inactive co-product named esperamicin X played a vital role. The X-ray analysis demonstrated that esperamicin X possess a 1,2-disubstituted benzene in place of the 1,5-diyne-3-ene of the active esperamicin²). A similar aromatized compound, dynemicin M was also discovered in the fermentation broth of dynemicin⁴) (Fig. 2). These aromatized analogues also served to elucidate the activation mechanism of cyclodiynene antibiotics as discussed in the later section.

2. Biological activity of the cyclodiynene antibiotics

2.1 Antimicrobial activity

The cyclodiynene group of antibiotics endows a broad spectrum of antimicrobial activity with extraordinary potency. They are extremely active, pg/ml order, against Gram-positive bacteria and highly active, $\mu\text{g/ml}$ order, against Gram-negative bacteria and fungi. The antimicrobial activity of esperamicins is shown in Table 3-1, and that of four calicheamicin components in Table 3-2¹³). Esperamicin A2, the positional isomer of the anthranilate of esperamicin A1, is ca. 1/4 - 1/8 as active as esperamicin A1. The antimicrobial activity of calicheamicin β and γ components are similar to those of esperamicins A1 and A2. The iodine-containing components are slightly more active than the bromine-containing components. The neocarzinostatin chromophore showed only weak antimicrobial activity (MIC: 12.5 - 50 $\mu\text{g/ml}$) probably due to the instability. When the chromophore was re-united with the apo-protein, the antimicrobial activity jumped up ca. 100 fold to the MIC values of 0.1 - 3.1 $\mu\text{g/ml}$. The MIC values of dynemicin A and its triacetate are lower (MIC: 0.000006 - 0.1 $\mu\text{g/ml}$) than those of esperamicins and calicheamicins. Interestingly, dynemicin A cured the mice infected with lethal inoculum of *Staphylococcus aureus* Smith by intramuscular injection (PD_{50} : 0.13 mg/kg). The aromatized dynemicin M exhibited significant *in vitro* (MIC: 0.2 - 0.4 $\mu\text{g/ml}$) and *in vivo* activity (PD_{50} vs *S. aureus* Smith, 1.8 mg/kg, im) against Gram-positive bacteria, which is quite

Fig. 2. Naturally-occurring aromatized compounds

Table 3-1. Antimicrobial activity of esperamicins

Organism	MIC ($\mu\text{g/ml}$)	
	A ₁	A ₂
<i>Staphylococcus aureus</i> 209P	<0.0008	0.0063
<i>S. aureus</i> Smith	<0.0008	0.0031
<i>Bacillus subtilis</i> PCI219	<0.0008	0.005
<i>Micrococcus luteus</i> 1001	0.0016	0.0063
<i>M. flavus</i>	<0.0008	0.0016
<i>Mycobacterium</i> 607	0.05	0.1
<i>Escherichia coli</i> NIHJ	0.1	0.8
<i>Klebsiella pneumoniae</i> D11	0.4	0.8
<i>Pseudomonas aeruginosa</i> D15	0.8	1.6
<i>Bacteriodes fragilis</i> A20928	0.2	1.6
<i>Clostridium difficile</i> A21675	0.4	0.8
<i>C. perfringens</i> A9635	0.05	0.8
<i>Candida albicans</i> IAM 4888	0.4	0.4
<i>Cryptococcus neoformans</i>	1.6	3.1

MIC values were determined by the standard agar dilution method in nutrient agar medium (Gram-positive and Gram-negative bacteria) and Sabouraud dextrose medium (fungi).

contrast to the bio-inactive nature of esperamicin X. This observation implies that the anthraquinone moiety of dynemicin plays an important role in expressing the activity.

2.2 *In vitro* cytotoxic activity

The cyclodiynene antibiotics show exceptional

potency, and broad spectrum *in vitro* and *in vivo* inhibitory activities against various tumors. On a molar basis, these antibiotics are approximately 1000 - 4000 times more potent than adriamycin and 3 - 10 times more potent than CC-1065 which had been considered as the most potent antitumor antibiotic¹⁴⁾.

When tested by a dye binding assay, esperamicin A1 exerted extremely powerful cytotoxicity to various murine and human cell lines with IC₅₀ values in the pg - ng/ml range (Table 4)¹⁵⁾. No cross resistance was seen against the vincristine resistant P388 leukemia cells. The same level of unusually strong *in vitro* cytotoxicity was also reported for veractamycins, FR-900405 and calicheamicins. Esperamicin C, a partial hydrolysis product of esperamicin A1 lacking the anthranyl-2-deoxyfucose moiety, was 1/200th as potent as esperamicin A1 against HCT-116, Moser and B16 cells in culture. When the methylthiohexose moiety was removed from esperamicin C (esperamicin D), the cytotoxicity dropped to ca. 1/1000th of esperamicin A1. Further removal of the alkylaminosugar from esperamicin D resulted in nearly complete loss of cytotoxicity and the aromatized esperamicin X became inactive.

Dynemicin A and its tri-*O*-acetate derivative exhibited marked cytotoxic activity comparable to esperamicin A1. Dynemicin M retained significant cytotoxicity against B16 melanoma and Moser

Table 3-2. Antimicrobial activity of calicheamicins

Organism (strains tested)	MIC ($\mu\text{g/ml}$)			
	β_1^{Br}	β_1^{I}	γ_1^{Br}	γ_1^{I}
<i>Staphylococcus aureus</i> (7)	≤ 0.00025	≤ 0.000031	≤ 0.000031	≤ 0.000031
<i>S. epidermidis</i> (2)	≤ 0.00025	≤ 0.000031	≤ 0.000031	≤ 0.000031
<i>Enterococcus</i> sp. (1)	0.0038	0.031	0.062	0.0078
<i>Bacillus subtilis</i> (1)	≤ 0.00025	≤ 0.000031	≤ 0.000031	≤ 0.000031
<i>Escherichia coli</i> (3)	0.12 - 0.5	0.25 - 0.5	0.25 - 0.5	0.25
<i>Klebsiella pneumoniae</i> (2)	0.12 - 0.25	0.5	0.5	0.25
<i>Enterobacter</i> sp. (2)	0.25 - 0.5	0.25 - 0.5	0.5	0.5
<i>Serratia</i> sp. (2)	0.12	0.25 - 0.5	0.25 - 0.5	0.12 - 0.25
<i>Citrobacter</i> sp. (2)	0.12	0.25 - 0.5	0.25 - 0.5	0.12 - 0.25
<i>Acinetobacter</i> sp. (2)	0.06 - 0.12	0.25	0.25	0.06 - 0.12
<i>Pseudomonas aeruginosa</i> (2)	0.25 - 0.5	0.25 - 0.5	0.5 - 1	0.12 - 0.25

MIC value were determined by the standard agar dilution method in Mueller-Hinton medium.

Table 4. Cytotoxicity of esperamicins and dynemicins

Antibiotic	IC ₅₀ (μg/ml)					
	B16-F10	Moser	HCT-116	P388	P338/VCR	K562
Esperamicin A ₁	0.0018	0.000016	0.00030	0.00005	0.00008	0.00024
Esperamicin C ₁	0.34	0.003	0.067			
Esperamicin D	1.84	> 0.02	0.87			
Dynemicin A	0.0041	0.000003	0.00028	0.00002	0.00002	0.00008
Triacetyldynemicin A	0.0027	0.000004	0.00018	0.00002	0.00002	0.00002
Dynemicin M	0.12	0.40				

B16-F10 (murine melanoma), Moser (human colon carcinoma), HCT-116 (human colon carcinoma), P388 (murine leukemia), P338/VCR (vincristine-resistant P388), K562 (human myelogenous leukemia).

colon carcinoma cells, although the activity was much weaker than dynemicin A.

2.3 Antitumor activity

In *in vivo* antitumor tests, esperamicin A₁ administered intraperitoneally (ip) produced significant prolongation of life span of mice inoculated ip with P388 leukemia, L-1210 leukemia, B16 melanoma, M109 lung carcinoma, C26 colon carcinoma, M5076 sarcoma or Lewis lung carcinoma at extremely low doses¹⁶⁾. In addition, by intravenous (iv) administration it was effective against iv implanted P388 (T/C : 125 - 294 %) and L1210 (T/C : 175%) leukemia, as shown in Table 5. Esperamicin A₁ was also active against the MX-1 human mammary tumor xenograft implanted in the subrenal capsule of nude mice, achieving over 85% inhibition of the tumor growth. Calicheamicin γ₁^I was described to show strong antitumor activity against

experimental murine tumors such as P388 leukemia and B16 melanoma at dose of 0.15-10.0 μg/kg with some cured mice at the optimal doses.

Dynemicin A showed *in vivo* antitumor activity against various experimental tumors at a fairly wide dose range (4-250 μg/kg). The T/C values were, however, relatively low, not exceeding 150 %. The weaker antitumor activity and lower T/C values of dynemicin A compared to other cyclodiynene antibiotics are presumably due to its pharmaceutical properties such as low water-solubility.

As discussed on the antimicrobial activity, neocarzinostatin chromophore, the active principle of neocarzinostatin is greatly stabilized by non-covalent binding to the apo-protein. Thus, *in vivo* antitumor activity of the chromophore was much increased in the presence of apo-protein (Minimum effective dose : 5 μg/kg, ip).

Table 5. Antitumor activity of esperamicin A₁

Tumor (site)	Schedule,	Route	Effective dose (μg/kg/inj)	Maximum T/C% (cure/treated)
P388 (ip)	day 1,	ip	0.1 - 30.0	152 - > 300 (4/6)
" (ip)	day 1→9,	ip	0.02 - 3.0	206
" (iv)	day 1,	iv	0.3 - 10	294 (3/6)
L1210 (ip)	day 1	ip	1.0 - 4.0	157
B16 (ip)	day 1,5,9,	ip	0.03 - 10	146 - 210 (3/10)
LL (ip)	day 1→11,	iv	0.1 - 1.0	286
M109 (ip)	day 1→4,	ip	0.1 - 1.0	200
C26 (ip)	day 1→4,	ip	0.1 - 1.0	176
M5076 (ip)	day 1,5,9,13	ip	- 1.28	156

L1210 (murine leukemia), LL (Lewis lung carcinoma), M109 (Madison 109 lung carcinoma), C26 (colon 26 carcinoma), M5076 (reticulum cell sarcoma).

2.4 DNA breakage activity

The antineoplastic action of the cyclodiynene antibiotics has been shown to be associated with the interaction to and cleavage of DNA by the antibiotics. In the macromolecular synthesis inhibition with esperamicin A₁, specific and strong inhibition of DNA synthesis was observed (Table 6). Dynemicin A also inhibits DNA synthesis specifically, with the potency being about 1/1000th that of esperamicin A₁. No specific inhibition of DNA, RNA or protein syntheses was seen for dynemicin M, indicating that the cytotoxic mechanism of dynemicin M would be different from those of the cyclodiynene antibiotics.

3. Mechanism of activation of the cyclodiynene antibiotics

Together with their outstanding biological activity and unprecedented chemical structures, the cyclodiynene antibiotics have elicited the highest attention with their attractive activation mechanism.

The mechanism of action studies to date on these class of agents have identified the following common features for their mode of activation and interaction with DNA. First, they intercalate base-specifically between DNA base pairs using a part of their molecules. They also possess a chemical triggering mechanism which transforms the enediyne core to a high energy intermediate capable of forming biradicals under physiological conditions. The biradical induces either single or double strand DNA scission depending upon the orientation of the core moiety in the minor groove of DNA.

The action mechanism proposed for esperamicins and calicheamicins involves four steps: (1)

thiol compounds such as dithiothreitol and glutathione cleave the allylic methyl trisulfide of the antibiotics, (2) the resulting thiol induces intramolecular Michael type addition to the bridgehead double bond forming a dihydrothiophene, (3) the saturation of the double bond eases the ring strain and decreases the distance between the transannular triple bond terminals, which induces Masamune-Bergman type cyclization of the 1,5-diyne-3-ene moiety to form 1,4-dehydrobenzene diradical species, and (4) the diradical abstracts hydrogens from the deoxyribose backbone of nearby DNA (Fig. 3-1)¹².

In the neocarzinostatin chromophore, thiol compounds are believed to attach to the C12 position by nucleophilic addition. This generates the double bond shift and epoxide opening producing an ene-yne-cumulene intermediate which in turn cyclizes to a highly reactive indacene biradical¹⁷. As in the case of esperamicins and calicheamicins, the biradical abstracts hydrogen from the DNA backbone to produce DNA strand breaks (Fig. 3-2).

With combining characteristics of both the enediyne and anthracycline class of antibiotics, dynemicin A is considered to be activated by a different mechanism. It was evidenced to be greatly activated by NADPH and other reducing agents. These agents appear to reduce the anthraquinone of dynemicin A to the hydroquinone form, which rearranges to the quinonemethide with concomitant opening of the epoxide. Further rearrangement to the quinone form allows approach of the triple bonds and induces cycloaromatization to a dehydrobenzene radical. This causes oxidative DNA strand scission resulting in DNA damage and cell death (Fig. 3-3)¹⁸.

Another interesting mechanistic feature is the base-specific DNA single and double strand cleavages observed in the cyclodiynene antibiotics. This is supposed due to a result of a unique binding of the compounds to DNA base pairs. The extensive DNA cleavage studies using a variety of oligonucleotides indicated that the preferred cutting sites are T>C>>A>G for esperamicins, C>>T>A=G for calicheamicins and T>A>C>G for neocarzinostatin chromophore. The 3'-sides of purine bases are specifically attacked by dynemicin A. Among them, calicheamicins show the greatest sequence selectivity.

Table 6. Inhibition of macromolecular biosynthesis (L1210)

Antibiotic	IC ₅₀ (μg/ml)		
	DNA	RNA	Protein
Esperamicin A ₁	0.0000017	0.013	0.17
Dynemicin A	0.0022	8.5	14
Dynemicin M	>10	8.2	>10

L1210 cells were incubated with ³H-thymidine, ¹⁴C-uridine or ³H-leucine, and the incorporated radioactivities into acid-insoluble fraction were measured by a scintillation counter.

1 - Esperamicin -

2 - Neocarzinostatin Chromophore -

3 - Dynemicin -

Fig. 3. Proposed mechanism of activation

ty in its cleavages of double stranded DNA. Recent studies demonstrated that the antibiotics recognize 5'-AGGA/3'-TCCT pair exclusively and cause strong strand breaks at the underlined cytidine and the 3 base-pair apart base of the opposite strand¹⁹⁾. This specificity is considered to be due to the close association of the thiobenzoate-carbohydrate of calicheamicins with the minor groove of DNA. Significant alteration of the cleavage site by pretreatment with a minor groove binder such as netropsin and distamycin substantiated the assumption. Lacking the thiobenzoate-rhamnoside moiety at the trisaccharide terminal, esperamicins are less base-specific than calicheamicins and break both single and double strands. The trisaccharide moiety, however, has been proved to play a key role in the binding of esperamicins to DNA. Neocarzinostatin chromophore breaks single and double strands through binding to double stranded DNA via intercalation of its naphthoate moiety into the minor groove.

The hydroxyanthraquinone of dynemicin A is believed to serve as a DNA recognition site in addition to as a bio-activation trigger. Dynemicin A causes typical double-strand cuts, and the preferential cutting sites of the antibiotic (3'-side of purine bases such as 5'-AT and 5'-GT) are clearly different from those of other cyclodiynene antibiotics. It also breaks the 3 base-pair apart bases on the 3'-side of complementary strand, which are characteristic of double strand breakage by the minor groove interacting agents. The strong inhibition of the dynemicin-induced DNA cleavage was seen by the pretreatment with distamycin and anthramycin, supporting the minor groove intercalation of dynemicin.

SUMMARY

The cyclodiynene class of antibiotics esperamicins, calicheamicins and dynemicins represent a new type of antitumor agents possessing highly unusual chemical, biological and mechanistic properties. The extensive studies done so far on these antibiotics gave us a new understanding of how the novel molecules work to kill tumor cells. Neocarzinostatin, without the knowledge of its chemical structure and action mechanism, has been

marketed as an anticancer drug since 1977 in Japan and esperamicin A1 is now under clinical trial in Europe and the United States. With their extreme antitumor potency, esperamicins and calicheamicins are considered to become an ideal targeting drug in combination with a monoclonal antibody, and the works toward such directions are in progress.

Inspired by their structural novelty and fascinating mechanism of action, a number of research groups have challenged the total synthetic studies of neocarzinostatin chromophore, esperamicins, calicheamicins and dynemicins. Although considerable progress has already been made, none of these interesting molecules has been totally synthesized so far. However, a great deal of the model compounds having a cyclo-diyne nucleus have been synthesized, and some of them were proved to have DNA-cleaving activity and even cytotoxicity. More interestingly, several appropriately substituted acyclic diyne models are also effective in cleaving DNA. These synthetic works and further mechanistic studies on the cyclodiynene antibiotics are expected to provide us novel antitumor drugs for the future cancer chemotherapy.

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