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Clavilactones, a Novel Class of Tyrosine Kinase Inhibitors of Fungal Origin

Giuliana Cassinelli, Cinzia Lanzi,*† Tiziana Pensa,* Romolo A. Gambetta,* Gianluca Nasini,‡ Giuditta Cuccuru,* Marco Cassinis,* Graziella Pratesi,* Donatella Polizzi,* Monica Tortoreto* and Franco Zunino**

*Oncologia Sperimentale B, Istituto Nazionale per lo Studio e la Cura dei Tumori, 20133 Milan; and ‡Dipartimento di Chimica del Politecnico, Centro del CNR per le Sostanze Organiche Naturali, 20131 Milan, Italy

ABSTRACT. Targeting of deregulated protein tyrosine kinases has been proposed as a new approach in the therapeutic intervention against pathological processes including proliferative disorders and cancer. Using a screening approach based on a comparative evaluation of antiproliferative effects in a panel of tumor cells with differential expression of protein tyrosine kinases, three benzoquinoid macrolidic fungal metabolites produced by *Clitocybe clavipes*, clavilactones A, B, and D (CA, CB, and CD) and two semisynthetic derivatives of these products, diacetyl-CA and dimethyl-CA, were identified as inhibitors of protein tyrosine kinases. Naturally occurring CA, CB, and CD showed inhibitory activity in kinase assays against the Ret/ptc1 and epidermal growth factor receptor (EGF-R) tyrosine kinases, while being less effective against the v-Abl tyrosine kinase and $p34^{cdc2}$ serine/threonine kinase (IC_{50} 2.8, 5.5, 81.3, and 128 μM respectively, for the most potent compound CD). CB was shown to be a non-competitive inhibitor of EGF-R with respect to ATP or poly(Glu_6Ala_3Tyr). CD also preferentially inhibited the growth of A431 cells, which overexpress a constitutively active EGF-R, as opposed to IGROV-1 and SKOV-3 cells, which express low levels of the receptor. Further, EGF-R was shown to be a target for clavilactones in A431 cells, since EGF-induced receptor autophosphorylation was inhibited in the presence of CB, CD, and diacetyl-CA. Both CD and diacetyl-CA displayed weak activity when administered daily (i.p.) to mice bearing ascitic A431 tumor. These findings indicate that clavilactones represent the prototypes of a new structural class of tyrosine kinase inhibitors deserving further investigation. BIOCHEM PHARMACOL **59**;12:1539–1547, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. protein tyrosine kinase; phosphotyrosine; EGF receptor kinase; oncogene proteins; enzyme inhibitors; fungi

Protein tyrosine kinases play a key role in signal transduction pathways regulating essential cellular processes such as growth, differentiation, and death [1, 2]. A role in the etiology of different human diseases including proliferative disorders, inflammatory responses, and tumors has been ascribed to proteins of this family pathologically endowed with a deregulated constitutive activity [3, 4]. Such aberrant function can be the result of various alterations, namely gene rearrangements [5–7], point mutations [8], amplification/overexpression [8, 9], or inappropriate expression triggering an autocrine loop [10]. On the basis of this background, several protein tyrosine kinases have been intensively investigated as possible therapeutic molecular targets [4, 11, 12]. The EGF-R§ is one of the most widely studied targets of this type. Its overexpression, frequently observed in epithelial tumors [13], has been correlated to poor clinical outcome in breast and squamous cell carcinomas of various tissue origin [14–16], and different approaches have been shown to effectively block a deregulated EGF-R-mediated signal [12]. Ret-derived oncoproteins, specifically involved in the development of thyroid cancers, represent further attractive targets. In such malignancies, the Ret receptor tyrosine kinase is frequently found in a constitutively active form following somatic rearrangements or germline point mutations of the RET gene [17].

The tyrosine kinase reaction, consisting in the transfer of a phosphoryl group from ATP to a specific tyrosine residue in a protein substrate, can be targeted by chemical agents. In recent years, several products displaying inhibitory activity against specific tyrosine kinases have been designed and for some, antitumor activity on experimental tumors has been described and clinical trials are in progress [4, 11, 18]. Many of the earliest identified tyrosine kinase inhibitors were natural products. In particular, compounds such as

† Corresponding author: Dr. Cinzia Lanzi, Oncologia Sperimentale B, Istituto Nazionale Tumori, via Venezian 1, 20133 Milan, Italy. Tel. +39-2-2390627; FAX +39-2-2390692; E-mail: Lanzi@istitutotumori.mi.it

§ *Abbreviations:* EGF, epidermal growth factor; EGF-R, epidermal growth factor receptor; CA, clavilactone A; CB clavilactone B; CD, clavilactone D; polyGAT, poly(Glu_6Ala_3Tyr); TCA, trichloroacetic acid; SRB, sulforhodamine B; and ptc, papillary thyroid carcinoma.

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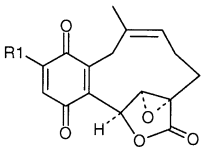
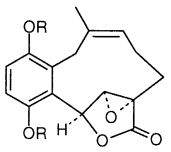
TABLE 1. Structure of clavilactones and summary of IC_{50} (μM) values in antiproliferative tests

Compound		R	R_1	A431*	IGROV-1*	SKOV-3*	NIH^{PTC1}†	$NIH^{H\text{-}RAS}$†	NIH^{HER1}†
	CD	—	OH	0.1 ± 0.05	0.8 ± 0.22	1 ± 0.44	0.1	0.2	0.02
	CB	—	H	3.6 ± 1.31	6.1 ± 1.66	21.9 ± 10	2	1.3	ND
	CA	H	—	ND‡	ND	ND	2	2.8	ND
	Dimethyl-CA	CH_3	—	inactive§	inactive	inactive	inactive	ND	ND
	Diacetyl-CA	$COCH_3$	—	15.8 ± 0.79	22.4 ± 11.8	41.2 ± 13	inactive	ND	8.3

* SRB assay. Cells were incubated in the presence of clavilactones for 96 hrs. IC_{50} is defined as the concentration of compound producing 50% inhibition of cell growth. Values are the means ± S.D. of two to seven independent experiments performed in eight replicates.

† Soft agar assay using NIH3T3 fibroblasts expressing the oncoproteins: Ret/ptc1 tyrosine kinase (NIH^{PTC1}), $p21^{H\text{-}RAS}$ small G protein ($NIH^{H\text{-}RAS}$), or EGF-R tyrosine kinase (NIH^{HER1}). Cells were incubated in semisolid medium in the presence of clavilactones. NIH^{HER1} cell medium contained 20 ng/mL of EGF. IC_{50} is the concentration of compound producing 50% inhibition of colony growth. Values are the means of two independent experiments performed in duplicate.

‡ ND, not determined.

§ Compounds were defined as inactive when the IC_{50} was not reached at the maximum concentration tested, i.e. 100 μM in the SRB assay and 20 μM in the soft agar assay.

genistein, herbimycin A, K-252a, and radicicol were isolated from fungal extracts [19–22]. In some cases, lead compounds of a structural class of inhibitors were synthetic derivatives of naturally occurring protein tyrosine kinase inhibitors. For example, tyrphostins of the first generation, characterized as inhibitors of EGF-R kinase, were designed on the styrene-based structure of the actinomycete metabolite erbstatin [23]; the pharmacore of the broad spectrum natural kinase inhibitors quercetin, lavendustin A, and staurosporin served as a template for the synthesis of more potent and selective tyrosine kinase inhibitors [24–26].

Clavilactones are fungal metabolites endowed with an unusual structure based on a 10-membered macrolide fused to a benzoquinoid ring and a 2,3-epoxy-γ-lactone (Table 1). They were isolated from the culture medium of the non-toxigenic basidiomycetae *Clitocybe clavipes* and were originally characterized as antifungal and antibacterial agents [27, 28]. In the present study, i.e. in the context of a mechanism-based drug discovery strategy, EGF-R and the Ret/ptc1 oncoprotein were investigated as possible targets of these compounds. The data presented demonstrate that clavilactones have inhibitory properties and represent a novel class of tyrosine kinase inhibitors.

MATERIALS AND METHODS

Clavilactones

The benzoquinoid macrolide structure of clavilactones under study is reported in Table 1. On the basis of the oxidation status of the aromatic ring, these molecules can be assigned to one of two groups, the first including the quinone derivatives CB and CD and the second the hydroquinones CA, dimethyl-CA, and diacetyl-CA. CA, CB, and CD were extracted from the *C. clavipes* culture medium as previously described [27, 28]. Dimethyl-CA and diacetyl-CA are semisynthetic derivatives [28]. CA and CB were found by HPLC analysis to be in equilibrium in serum-containing medium. Clavilactones were extracted with EtOAc and analyzed on a LiChrograph L-600A (Merck-Hitachi) equipped with an L-4000 Detector (λ = 225 nm), using Si60 silica gel column 0.45 × 25 cm with exane-EtOAc (3:1) as eluent at a nominal flow rate of 1.6 mL/min. The retention times were 10.80 and 4.66 min for CA and CB, respectively. The analysis of CB extracts following 18 hr in serum-containing medium showed the presence of the hydroquinone CA as the prevalent form. TLC analysis confirmed these results. A similar redox balance is to be expected for the quinone CD. For experiments, compounds were dissolved in DMSO and then diluted in cell medium or buffer. The maximum concentration used in each test was dictated by the solubility of the compound.

Antibodies

The rabbit anti-ret antibody recognizing a COOH-terminal sequence of the Ret/ptc1 protein was described previously [29]. The preparation of the rabbit anti-phosphotyrosine antibody (anti-Ptyr) was also previously reported [5].

Cell Lines and Culture Conditions

The human epidermoid carcinoma cell line A431 overexpresses EGF-R, which is also involved in a transforming growth factor α - mediated autocrine loop [30]. In this cell line, EGF-R is endowed with constitutive enzyme activity and has been found to be a good target for antiproliferative tyrosine kinase inhibitors in both *in vitro* and *in vivo* systems [31, 32]. EGF-R is also expressed at low levels in the two human ovarian cell lines IGROV-1 and SKOV-3, while the cognate erbB-2 receptor, present at a low level in IGROV-1

cells, is overexpressed in SKOV-3 cells as determined by Western blotting (not shown). All three carcinoma cell lines were grown in RPMI medium containing 10% fetal bovine serum at 37° in 5% CO_2.

Transformed NIH3T3 mouse fibroblasts expressing the RET/PTC 1 ($NIH3T3^{PTC1}$) or H-RAS ($NIH3T3^{H\text{-}RAS}$) oncogenes were obtained by transfection assay with human DNA from a papillary thyroid carcinoma and a bladder carcinoma, respectively. RET/PTC1 is a rearranged oncogenic version of the RET gene expressed in papillary thyroid carcinomas [33]. Unlike the Ret receptor tyrosine kinase, the Ret/ptc1 chimeric oncoprotein is endowed with constitutive tyrosine kinase activity [5]. The unrelated H-RAS oncogene encodes for a small G protein [34]. Both fibroblast cell lines form colonies in soft agar and were routinely grown as adherent cultures in Dulbecco's modified Eagle medium (DMEM) supplemented with 5% ($NIH3T3^{PTC1}$) or 10% ($NIH3T3^{H\text{-}RAS}$) bovine serum (Colorado Serum Company) at 37° in a 10% CO_2 atmosphere.

$NIH3T3^{HER1}$ cells, which overexpress EGF-R following transfection with EGF-R cDNA [35], were maintained in RPMI medium containing 10% fetal bovine serum at 37° in 5% CO_2. The transformed phenotype of these cells was EGF-dependent.

Antiproliferative Assays

The SRB colorimetric assay [36] was used to test the antiproliferative effects on A431, IGROV-1, and SKOV-3 cells. Cellular monolayers were treated with dilutions of clavilactones in solvent (final DMSO 0.1–0.2%). Cell growth was evaluated after 96 hr or throughout 7 days of incubation in the presence of test compounds. Each drug concentration was tested in eight replicates.

A soft agar assay was used to evaluate the effect of clavilactones on the growth of $NIH3T3^{PTC1}$, $NIH3T3^{H\text{-}RAS}$, and $NIH3T3^{HER1}$ cells. In this assay, only viable transformed cells, maintaining the property of growing independently from anchorage, can form colonies [37]. Assays with NIH^{HER1} cells were performed in the presence of 20 ng/mL of EGF. Each drug concentration was assayed in duplicate. After 8–10 days, colonies were stained with *p*-iodonitrotetrazolium violet (Sigma) for 24 hr and then counted by the aid of a magnifying projector.

Kinase Assays

The EGF-R autophosphorylation assay was performed using cell membranes from the EGF-R-overexpressing A431 cell line as a source of enzyme, essentially as described by Levitzki *et al.* [38]. After 30 sec at 4° in the presence of [γ-^{32}P]ATP, the reaction was stopped by concentrated Laemmli solution. Samples were then subjected to 7% SDS–PAGE. EGF-R phosphorylation was evaluated by PhosphorImager (Molecular Dynamics).

Autophosphorylation of the Ret/ptc1 oncoprotein was evaluated by immunokinase assay. $NIH3T3^{PTC1}$ cell monolayers were rinsed twice with PBS containing 0.1 mM sodium orthovanadate and then lysed with cold RIPA buffer (20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, 1 mM sodium orthovanadate, 10 μg/mL of aprotinin, 10 μg/mL of pepstatin, 10 μg/mL of leupeptin, 2 mM phenylmethylsulfonyl fluoride). After 20 min on ice under agitation, clarified cell lysates were incubated with anti-ret antibody for 2 hr at 4° and then dispensed equally in Eppendorf tubes containing 30 μL of protein A–agarose (Sigma). After 30 min of incubation at 4°, immunocomplexes were washed in 50 mM HEPES, pH 7.2, 20 mM $MnCl_2$, 5 mM $MgCl_2$ and left in the same buffer plus 0.5 mM 1,4 dithiothreitol (incubation buffer) for 10 min at 30°. The kinase assay was performed in 20 μL of incubation buffer containing solvent (1% DMSO, 4.5% ethanol final concentrations) or clavilactone diluted in solvent, and 1.3 μM [γ-^{32}P]ATP (>5000 Ci/mmol, Amersham) at 140 nCi/pmol. Reactions were carried out at 30° for 10 min and stopped by adding 10 mM phosphate buffer, pH 7, 1% Triton X-100, 0.1% sodium deoxycholate, 1 mM sodium orthovanadate, 1 mM ATP, 5 mM EDTA, and 5 μg/mL of aprotinin. After two washings with the same buffer, proteins were eluted by boiling in 50 μL of Laemmli buffer for 5 min and subjected to 10% SDS–PAGE. Ret/ptc1 phosphorylation was analyzed by PhosphorImager densitometry.

$p34^{cdc2}$ enzyme activity was measured in cellular extracts as the ability to phosphorylate a peptide based on simian virus 40 large T antigen, specific to the cell division cycle (cdc)-2 kinase, according to Amersham's Biotrak $p34^{cdc2}$ kinase enzyme assay system.

v-Abl kinase activity was evaluated by a modified exogenous substrate kinase assay (ESKA) as described by Buzzetti *et al.* [39]. Recombinant catalytically active v-Abl-derived protein (provided by Dr. Paolo Caccia, Pharmacia and Upjohn) was assayed in the presence of clavilactones and angiotensin II as an exogenous substrate. The reaction mixture contained 40 ng of purified v-Abl kinase, 5 μL of 1 mM [Val^5]-angiotensin II (Sigma), 5 μL of solvent (1% DMSO, 4.5% ethanol final concentrations) or clavilactone in solvent, and buffer (25 mM Tris–HCl, pH 8, 10 mM $MgCl_2$, 0.1 mM 1,4 dithiothreitol) to 45 μL. The reaction, initiated by the addition of 5 μL of [γ-^{32}P]ATP (5 μM, 0.5 μCi/sample), was driven for 20 min at 30° and terminated by adding 50 μL of cold 5% TCA. After incubation on ice for 10 min, 90 μL of each sample was spotted on phosphocellulose paper squares (Whatman P-81) and extensively washed in acetic acid. Filters were dried and bound radioactivity measured by liquid scintillation counting. Each drug concentration was tested in triplicate in the EGF-R assay and in duplicate in the other kinase assays.

Kinetic Analysis

Enzyme kinetics in the presence of CB and [γ-^{32}P]ATP were carried out using lectin-purified EGF-R [23] and

polyGAT (Sigma) as an exogenous substrate, based on the method described by Levitzki *et al.* [38]. For the competition experiments in which ATP concentrations varied from 1.3 to 9.6 μM, a fixed concentration of polyGAT (0.5 mM) was used corresponding to ten times the K_m value. When polyGAT was the variable component in the reaction mixture, ranging from 25 to 180 μM, ATP was set at 21.4 μM (= 8 K_m). ^{32}P radioactivity incorporated into the polymer substrate, precipitated on Whatman 3MM paper, was finally determined by liquid scintillation β-counting.

Western Blot Analysis

A431 cell monolayers were incubated with the indicated concentrations of clavilactones or solvent (0.3% DMSO) for 18 or 24 hr at 37° and then stimulated with 0.1 μg/mL of EGF (Sigma) for 2 min at 4°. After two washings with ice-cold PBS containing 0.1 mM sodium orthovanadate, whole-cell extracts were prepared as previously described [5], and equal amounts of proteins were analyzed by 7% SDS–PAGE followed by transfer to nitrocellulose paper and immunoblotting with anti-Ptyr antibody. The phosphorylation of EGF-R bands was estimated by ^{125}I-Protein A (Amersham) labeling and PhosphorImager densitometry.

In Vivo *Studies*

Experiments were carried out using athymic Swiss nude mice, 10–12 weeks old (Charles River). Mice were maintained in laminar flow rooms at constant temperature and humidity. Experimental protocols were approved by the Ethics Committee for Animal Experimentation of the National Cancer Institute (Milan) according to the U.K. Co-ordinating Committee on Cancer Research Guidelines [40]. For chemotherapy experiments, CD and diacetyl-CA were dissolved in DMSO/Tween 80/0.9% NaCl solution (3/5/92%). Mice received a volume of 10 mL/kg of body weight. Control groups were treated with the solvent solution. Each experimental group included 7/8 mice inoculated i.p. with A431 cells (10^7 cells/mouse). Drugs were delivered i.p. and treatments started on the day of tumor inoculum, with a daily schedule for 12 (diacetyl-CA) or 18 (CD) days. For CD, an intermittent schedule every 3rd–4th day was also tested (q3/4d × 6). Mice survival time was observed. Mice which died before the first control mouse were ascribed to drug toxicity.

RESULTS

Antiproliferative Activity of Clavilactones towards Tumor and Oncogene-Transformed Cell Lines

We focused on EGF-R and the Ret/ptc1 oncoprotein as possible target tyrosine kinases. To select inhibitory compounds, we adopted a cell and mechanism-based approach previously described [41, 42]. As first step of the screening, we compared the antiproliferative activity profile of test compounds against a variety of cell lines characterized by differential expression of these two enzymes. The antiproliferative effects of clavilactones after 96-hr incubation of the three human cell lines A431, IGROV-1, and SKOV-3 are reported in Table 1. CD was the most potent inhibitor, displaying submicromolar IC_{50} values and a preferential effect against A431 cells which, differently from the others, overexpress a constitutively active EGF-R [30]. Compounds CB and diacetyl-CA had a somewhat similar profile of activity with lower potency and selectivity, while dimethyl-CA was totally inactive.

Clavilactones were further evaluated for their ability to inhibit the anchorage-independent growth of NIH3T3 fibroblasts transformed by oncogenes (Table 1). The drugs did not show any preferential effect against cells expressing the Ret/ptc1 target tyrosine kinase (NIH3T3^{PTC1}) with respect to cells expressing the small G protein p21$^{H\text{-}RAS}$ (NIH3T3$^{H\text{-}RAS}$). CD was again the most potent antiproliferative agent. The derivatized dimethyl- and diacetyl-CA did not inhibit NIH3T3^{PTC1} cell colony formation. EGF-dependent colony formation of NIH3T3^{HER1} cells, which overexpress EGF-R, was strongly inhibited by both CD and diacetyl-CA, with IC_{50} of 0.02 and 8.3 μM, respectively.

Effects on Protein Kinase Activities

Clavilactones were evaluated for inhibitory activity against EGF-R and Ret/ptc1 kinases *in vitro*. An additional protein tyrosine kinase, the v-Abl oncoprotein, and the serine/threonine kinase p34^{cdc2} were also assayed in the presence of these compounds. Results of the kinase assays are reported in Table 2. The natural clavilactones CA, CB, and CD were active and displayed a preferential effect on tyrosine kinases, particularly Ret/ptc1 and EGF-R. The activity of the hydroquinone CA was, as expected, similar to that of its redox partner CB. Compound CD was the most potent inhibitor. It is noteworthy that the additional hydroxyl group on the aromatic ring of CD (R_1 in Table 1) conferred higher potency with respect to compounds CB and CA both in the antiproliferative (Table 1) and kinase assays performed in the presence of Ret/ptc1 and EGF-R target kinases (Table 2). Dimethyl-CA, evaluated in the EGF-R kinase assay, was inactive; diacetyl-CA was shown to be inactive towards all protein kinases. These data indicate that tyrosine kinase inhibitory activity is retained, *in vitro*, only by clavilactones carrying redox-cycling groups on the aromatic ring.

Effects on EGF-R Autophosphorylation in Living Cells

Since the antiproliferative activity of clavilactones CB and CD suggested a preferential target in A431 cells (Table 1) and because both compounds inhibited purified EGF-R kinase (Table 2), we examined their effects on the receptor tyrosine kinase in living A431 cells. Diacetyl-CA, which also showed an antiproliferative effect (Table 1), was included in these experiments. Following cell exposure to the drugs for 18 and 24 hr and to EGF for 2 min,

TABLE 2. Inhibition of protein kinases by clavilactones*

Compound	Enzyme			
	EGF-R†	Ret/ptc1‡	v-Abl§	$p34^{cdc2}$‖
CD	5.5	2.8	81.3	128‡‡
CB	27	7.7	67.5	120‡‡
CA	26	10.2¶	ND**	ND
Dimethyl-CA	Inactive¶††	ND	ND	ND
Diacetyl-CA	Inactive¶	Inactive‡‡	Inactive‡‡	Inactive‡‡

* Data are presented as means of two or three independent kinase assays performed in duplicate or in triplicate (EGF-R) except where indicated. Values are expressed as concentrations of compound that inhibited enzymatic activity by 50% (IC_{50}).

† Autophosphorylation of EGF-R tyrosine kinase performed with lectin-purified receptor from A431 cells.

‡ Autophosphorylation of the Ret/ptc1 tyrosine kinase performed by immunokinase assay.

§ Exogenous substrate kinase assay (ESKA) performed with the catalytic domain of v-Abl tyrosine kinase and angiotensin II as an exogenous substrate.

‖ Phosphorylation of a peptide based on simian virus 40 large T antigen as an exogenous substrate and A431 cell extract as a source of enzyme, according to the Amersham kit for $p34^{cdc2}$ Ser/Thr kinase.

¶ Single observation performed in triplicate.

**ND, not determined.

†† Compounds were defined as inactive when IC_{50} was not reached at the maximum concentration tested, which varied from 60 to 150 μM.

‡‡ Single observation performed in duplicate.

EGF-induced receptor autophosphorylation was evaluated in cell extracts by Western blotting with anti-Ptyr antibody. The results, illustrated in Fig. 1, showed a dose-dependent inhibitory activity for the two quinone derivatives CB and CD as well as for hydroquinone diacetyl-CA, in spite of the latter's inactivity in the cell-free system (Table 2). This finding suggested that, under the cell culture conditions, diacetyl-CA might be transformed into active metabolite/s. CD was the most potent inhibitor of EGF-R autophosphorylation, exhibiting a submicromolar IC_{50} (0.17 μM) following 18-hr exposure (Fig. 1). Since this compound was also the most potent and selective inhibitor of A431 cell proliferation (Table 1), EGF-R is likely to be a major cellular target for this clavilactone. For all three compounds tested, inhibition of receptor tyrosine phosphorylation at 18 hr (IC_{50}: 0.17, 22, and 100 μM for CD, diacetyl-CA, and CB, respectively) (Fig. 1B) was higher than inhibition observed at 24 hr (IC_{50}: 20, 100, and >100 μM for CD, diacetyl-CA, and CB, respectively) (Fig. 1C), indicating a reversible effect. Overall EGF-R levels were not affected in treated cells, as evaluated in parallel immunoblots using anti-EGF-R antibody (not shown).

Effect of Clavilactones on A431 Cell Growth

A431 cells were exposed to CB, CD, and diacetyl-CA at the respective IC_{50} calculated after 96 hr, and cell growth inhibition was followed throughout. While the antiproliferative activity of CB and CD was found to be reversible after 96 hr of treatment, the inhibitory effect of diacetyl-CA was observed to be the most persistent, being present up to 168 hr (Fig. 2). These results support the hypothesis that clavilactone A diacetate could function as a prodrug.

Mechanism of Inhibition of the EGF-R Tyrosine Kinase

Competition experiments were carried out to evaluate the effect of clavilactone CB on the utilization of the phosphate donor ATP or the tyrosine-containing polymer substrate polyGAT in the EGF-R kinase reaction. As shown by kinetic patterns in Fig. 3, the analysis of enzyme inhibition by the drug revealed a non-competitive mechanism of action with respect to both ATP and the peptide substrate (Fig. 3).

Evaluation of In Vivo *Activity*

Clavilactone CD was selected for *in vivo* studies because of its high inhibitory potency in cell and cell-free systems (Tables 1 and 2, Fig. 1). Diacetyl-CA was selected for its long-lasting antiproliferative activity (Fig 2). The two compounds were evaluated for their effect on the survival of nude mice bearing ascitic A431 tumors. Because of the reversible character of EGF-R kinase inhibition (Fig. 1), a daily treatment was preferred, with the exception of the highest CD dose. Drugs were delivered i.p. Table 3 summarizes the results of these experiments. Regarding CD effects, in spite of a marginal increase in the median survival time (MST) of treated mice, 37% survived longer than control mice. In fact, 4/8 mice died after the last control mouse in the group receiving either the total dose of 18 mg/kg by daily injections or 30 mg/kg by intermittent treatments, and 1/8 in the group receiving the total dose of 45 mg/kg. In regard to diacetyl-CA, long-term surviving mice were observed only in the group receiving the higher dose tested, i.e. 600 mg/kg, which, however, proved to be toxic. One mouse surviving over 52 days was killed and showed no tumor at the necropsy.

DISCUSSION

In an attempt to identify novel natural inhibitors of protein tyrosine kinases, we evaluated the three fungal metabolites, clavilactones CA, CB, and CD, and the two semisynthetic derivatives, diacetyl- and dimethyl-CA (structures in Table 1) [27, 28], by a cell and mechanism-based approach which previously allowed the identification of a new EGF-R

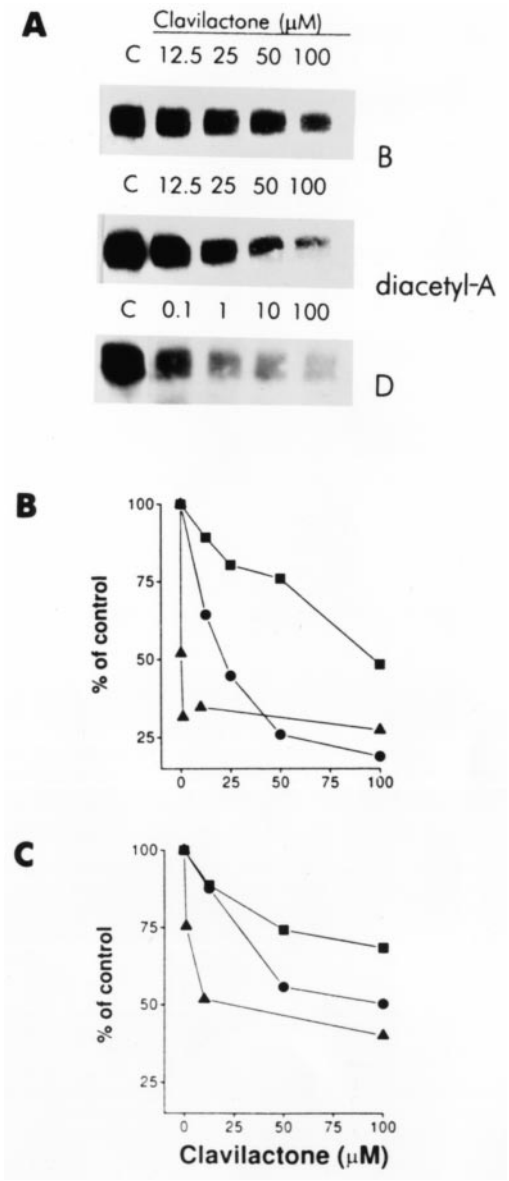


FIG. 1. Inhibition of EGF-R autophosphorylation by clavilactones in A431 cells. Cells were incubated in the absence (C) or presence of the indicated concentrations of compounds CB (B, ■), diacetyl-CA (diacetyl-A, ●), or CD (D, ▲) at 37° for 18 (A and B) or 24 hr (C). After stimulation with EGF (0.1 μg/mL) for 2 min at 4°, cells were lysed in boiling Laemmli sample buffer, and equal amounts of cell lysate proteins were subjected to SDS–PAGE and immunoblotting with anti-Ptyr antibody. Tyrosine phosphorylation of the 170 kDa EGF-R bands was evidenced by ^{125}I-Protein-A labeling followed by autoradiography (A) and then estimated by PhosphorImager densitometry referring to the band of untreated cells as 100% (B and C).

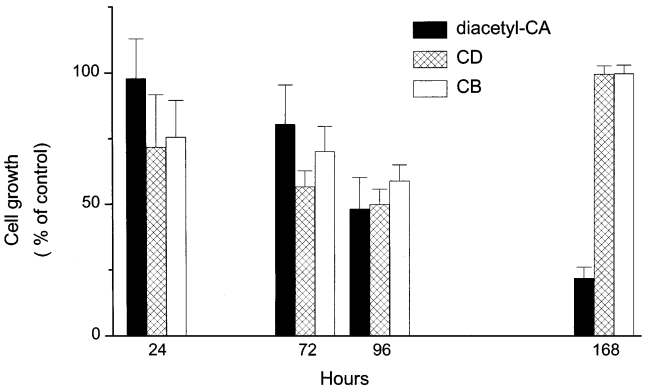


FIG. 2. Time–course of clavilactone antiproliferative effect on A431 cells. Cells were incubated in the presence of compounds CB (open columns), CD (crossed columns), or diacetyl-CA (closed columns) at the concentrations of 3, 0.125, and 12.5 μM, approximately corresponding to the respective IC_{50} after 96 hr. At the indicated times, cell monolayers were processed for the SRB assay to evaluate the cell growth relative to untreated cells (100%). A representative experiment is shown. Data are means ± SD (N = 8).

inhibitor [41] and inhibitors of the Ret/ptc1 oncoprotein [42]. The comparison of the antiproliferative activity of these compounds against cell lines differentially expressing the target tyrosine kinase indicated a preferential effect of the natural clavilactone CD against A431 cells overexpressing a constitutively active EGF-R. None of the natural compounds discriminated between NIH3T3^{PTC1} cells, transformed by the Ret/ptc1 tyrosine kinase, and NIH3T3$^{H\text{-}RAS}$ transfected cells in the soft agar assay, while CD strongly inhibited EGF-dependent colony formation of NIH3T3^{HER1} cells (Table 1). In the kinase assays, CA, CB, and CD displayed inhibitory activity towards both the EGF-R and Ret/ptc1 tyrosine kinases while being less effective against another tyrosine kinase, v-Abl, and the Ser/Thr kinase p34^{cdc2} (Table 2). This series of experiments demonstrated that naturally occurring clavilactones have tyrosine kinase inhibitory properties associated with preferential antiproliferative activity on cell lines depending on EGF-R for growth. Inhibition of other tyrosine kinases and/or further unknown cellular effects cannot be excluded and might also account for the similar antiproliferative effect of these compounds on NIH3T3^{PTC1} and NIH3T3$^{H\text{-}RAS}$ cells.

Results obtained with the two semisynthetic clavilactones evidenced the essential role of hydroxyl substituents in the aromatic ring for inhibitory activity. Indeed, a highly stable derivatization of hydroquinone, such as methylation in dimethyl-CA, inactivated the molecule either in the antiproliferative tests and in the kinase assays. Otherwise, diacetyl-CA, characterized by the easily cleavable acetyl group, was inactive in kinase assays but maintained antiproliferative activity (Tables 1 and 2). In A431 cells, EGF-R was shown to be a target for diacetyl-CA as well as for CD and CB, since EGF-induced receptor phosphorylation was inhibited by these compounds (Fig. 1). These findings confirmed that natural clavilactones can interfere with EGF-R-mediated signaling and suggested that the activity of diacetyl-CA requires the release of an active compound, probably involving deacetylation favored under the cell culture conditions. Indeed, TLC analysis of solvent extracts from culture medium and treated cell lysates revealed the presence of metabolites, including CA (not shown). Since CB was found to be mostly converted to its hydroquinone counterpart CA in serum-containing me-

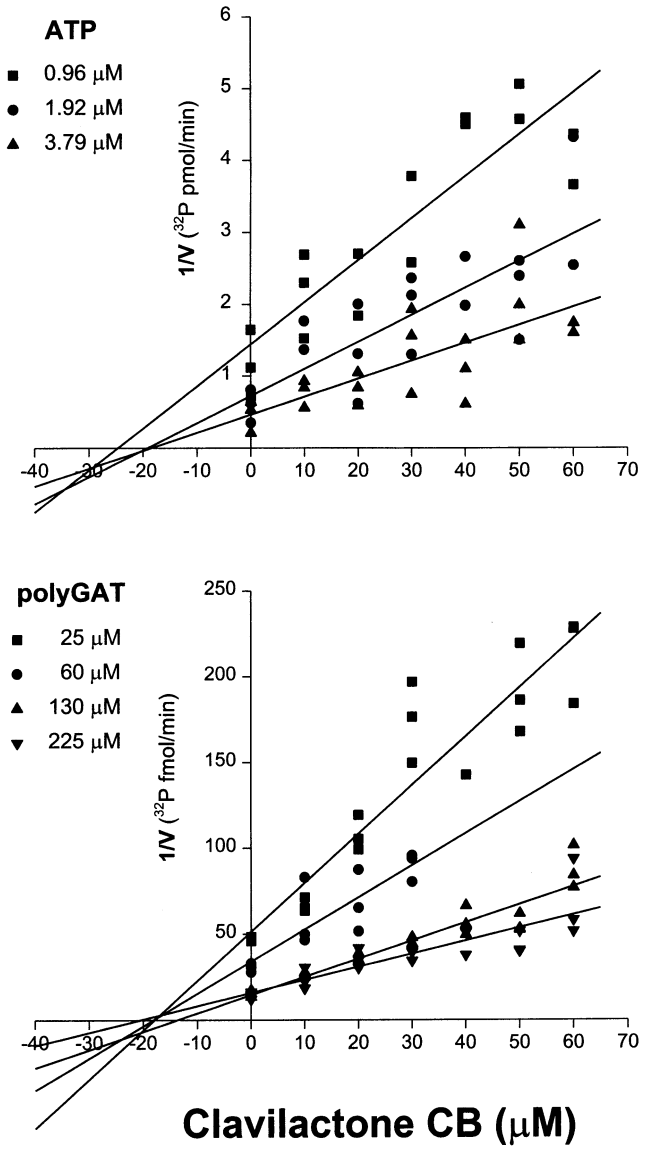


FIG. 3. Kinetics of inhibition of EGF-R by clavilactone B showing non-competition with respect to ATP or polyGAT. Kinase assays were carried out, in triplicate for each experimental point, with purified EGF-R in the presence of 0–60 μM compound CB, [γ-^{32}P]ATP, and polyGAT as an exogenous substrate. In ATP competition experiments (upper panel), the polyGAT concentration corresponded to 10 K_m and ATP concentrations varied as indicated. In polyGAT competition experiments (lower panel), the ATP concentration was 8 K_m and polyGAT concentrations were those indicated. Incorporation of ^{32}P into the polymer substrate was measured by liquid scintillation β-counting.

dium, the lower potency of CB with respect to diacetyl-CA as inhibitor of EGF-R autophosphorylation in living cells (Fig. 1) might be ascribed to different kinetics of drug metabolism and uptake. For diacetyl-CA and CD, the correspondence of concentrations reducing A431 cell growth (Table 1) and EGF-R autophosphorylation to 50% in the same cells (Fig. 1B) implies that EGF-R could be a major target for these compounds, while inhibition of EGF-R by CB might contribute only marginally, or may not be relevant, to A431 cell growth inhibition. As further suggested by reversible inhibition of A431 cell growth (Fig. 2) and EGF-R autophosphorylation in the same cells (Fig. 1), chemical instability affects the biological activity of clavilactones. It is also conceivable that redox transformation and/or metabolism might confer some specificity of effects to each molecule. For example, cellular effects contributing to the persistent antiproliferative activity of diacetyl-CA are not superimposable to those of CB and CD at equitoxic concentrations (IC_{50} at 96 hr) (Fig. 2) and do not correlate with reversibility of the inhibition of EGF-R function (Fig. 1), thus suggesting additional targets for the diacetyl compound. Redox transformations and metabolism of the drugs, as well as pharmacokinetics, might also explain the delayed inhibition of EGF-R autophosphorylation after treatment of the cells with clavilactones. Further elucidation of clavilactone cellular effects will need an extensive analysis of redox and metabolism products. Nonetheless, the possibility of modulating clavilactone biological activity through chemical modification and the potentiality of these molecules to function as prodrugs are interesting characteristics which deserve attention for further development.

Tyrosine kinase inhibitors are generally classified as competitors with respect to one or both substrates of the kinase reaction, ATP and the phosphorylable protein. The analysis of EGF-R enzyme kinetics in the presence of clavilactone CB revealed that this agent does not compete with ATP or with the polyGAT substrate (Fig. 3). Such a mechanism, although not unprecedented [43], is uncommon for a tyrosine kinase inhibitor and indicates the presence on the EGF-R molecule of a clavilactone binding site distinct from those of ATP and of the protein substrate recognizing polyGAT. Although it has been demonstrated only for compound CB at present, this feature could represent a further argument of interest for inhibitors of this novel class.

In vivo experiments were performed with clavilactone CD, the most potent compound in the series (Table 1, Fig. 2), and diacetyl-CA, which displayed the longest-lasting antiproliferative effect against A431 cells (Fig. 2). A431 tumor cells were grown in the peritoneal cavity of the mouse and drug treatments were delivered i.p. to allow the best conditions for drug–cell interactions. A modest activity of the two clavilactones could be detected, as indicated by a marginal increase in survival in treated animals (Table 3). Further studies will need to determine the correlation between these effects and inhibition of EGF-R kinase although, given the observed reversibility of biological effects (Figs. 1 and 2), a different treatment modality might be worthwhile to evaluate *in vivo* activity of clavilactones. For example, regimen treatments (every 8 hrs) assuring the continuous blocking of the target phosphorylating activity have been demonstrated to be essential for the antitumor activity of other tyrosine kinase inhibitors [18, 32]. Preliminary attempts to allow a continuous drug release by Alzet

TABLE 3. Effect of clavilactones CD and diacetyl-CA on survival time of nude mice bearing ascitic A431 human tumor (10^7 cells/mouse)

Compound	Schedule	Dose (mg/kg)*		MST†	Days of death‡
		single	total		
Solvent	qd§ × 18			25	18,20,21,25,25,25,28,29
CD	qd × 18	1	18	28.5	22,23,25,27,30,33,33,49
CD	qd × 18	2.5	45	23.5	17,19,22,23,24,25,28,43
CD	q3/4d × 6	5	30	29	18,23,27,28,30,30,30,33
Solvent	qd × 12			22	16,17,19,22,22,22,23
Diacetyl-CA	qd × 12	10	120	18.5	15,15,16,18,19,22,22,22
Diacetyl-CA	qd × 12	50	600	16	2, 4,15,15,17,18,31,>52‖

* Intraperitoneal treatments started the same day (day 1) of cell inoculum. Control mice received solvent solution.

† Median survival time.

‡ Days of death of each single mouse calculated from the day of cell inoculum.

§ qd, treatment every day; q3/4d, treatment every 3/4 days.

‖ This mouse was still alive at day 52. Necroscopic examination showed no presence of tumor.

pumps were hampered by the low solubility of clavilactones in pump-compatible solvents.

Even though obtained in a small panel of Tyr- and Ser/Thr kinases, our data support a non-selective tyrosine kinase inhibitory activity for the class of clavilactones (Table 2). A potential therapeutic utility for compounds with such characteristics has been suggested for proliferative disorders involving multiple aberrantly functioning tyrosine kinases [4]. For example, tumors involving multiple tyrosine kinase-driven autocrine loops or blood vessel diseases such as atherosclerosis and restenosis might benefit from treatments with broadly acting inhibitors.

In conclusion, we have presented evidence that clavilactones, naturally occurring fungal metabolites, represent a new class of tyrosine kinase inhibitors characterized by a peculiar structure based on a benzoquinoid 10-membered macrolide incorporating a 2,3-epoxy-γ-lactone. The present data indicate that their biological activity is susceptible of modulation by chemical modifications of the basic structure, while more extended pharmacological investigations will help to assess their potential interest as therapeutic agents.

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