INHIBITION OF COLLAGEN PEPTIDASE IN HELA CELLS AND
HUMAN TUMOURS BY COMPOUNDS INCLUDING DRUGS
USED IN CANCER THERAPY

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Summary.—Collagen-peptidase activity in extracts of HeLa cells and human tumours
is inactivated by Razoxane (ICRF-159), cyclophosphamide, 5-fluourouracil, thiotepa,
aprotinin, EDTA and phenanthroline. As this activity, in association with other
enzymes, may contribute to tissue lysis in cancers, chemical intervention may
reduce invasiveness and modify the processes of infiltration and metastasis. Accord-
ingly, some drugs used in therapy or for the prevention of metastasis may produce
their observed effects by a combination of factors including enzyme inhibition.

Following study of collagenase en-
zymes with synthetic peptides by Nagai
et al. (1960), introduction of the chromo-
phore substrate p-phenyl-azo-benzoxycarbonyl-pro-leu-gly-pro-D-arginine by
Wunsch and Heidrich (1963) enabled
collagen-peptidase activity, specific for
apolar peptide sequences analogous to
those of the collagen molecule, to be
identified and assayed in mouse and chick
embryo and in human HeLa cells by
Strauch (1967), Strauch and Vencelj (1967)
and Strauch et al. (1968). Although
assays of tumour peptidase with Pz-sub-
strate by Keiditsch and Strauch (1970)
corresponded closely with results obtained
by the method of Nagai et al. (1966) using
14C-labelled collagen as substrate, collagen
peptidase and collagenase when isolated
and purified are not identical. A similar
distribution of the enzymes suggesting a
close functional relationship was also
recorded by Robertson and Williams
(1969) in an invasive rat epithelioma where
collagen-peptidase and true collagenolytic
activity were found in corresponding
regions of the tumour periphery.

Langer et al. (1968) and Keiditsch and
Strauch (1968, 1970) found high levels of
collagen peptidase in human tumours, and
in 1972 Strauch showed benign and non-
invasive tumours to be moderately in-
creased, and malignant tumours of both
epithelial and mesenchymal origin to be
much increased compared with tumour-
free tissue. Invading margins of malignant
tumours had high activity, with lower
values in the centre, still higher than sur-
rounding normal tissues. Other workers
have identified neutral collagenase activity
in tumours, corresponding to that des-
cribed by McCroskery et al. (1975).

Although collagen- and reticulin-conta-
ining structures are among the most
persistent of mammalian tissues, growth
of cancers frequently accompanies exten-
sive breakdown of adjacent connective
tissue, which takes the form of swelling
followed by disorganization and dissolu-
tion (Birbeck and Wheatley, 1965;
Hamperl, 1967; Frithiof, 1972). Such
tissue destruction, associated with eleva-
ted levels of hydrolytic enzymes from
neoplastic and host cells of the cancer and
its stroma, is believed to contribute to
infiltration, invasiveness and possibly met-
astasization (Pearse et al., 1961; Sylven,
1961; Cameron, 1966; Latner et al., 1974;
Carter, 1976; Easty and Easty, 1976).

In 1969, Creighton et al. showed ICRF-
154 and ICRF-159 (Razoxyane) to have anti-tumour activity apparently related to their potential chelating properties. The correlation between collagen-peptidase activity and malignancy expressed by the histological classification of the tumour (Strauch, 1972) suggested that inhibition of the responsible enzymes might control and possibly reduce neoplastic infiltration (Ras-segna, 1971; Strauch, 1972). Protease inhibitors restore contact inhibition (Goetz et al., 1972) and reduce malignant invasion. Latner et al. (1974) using the protease inhibitor Trasylol (aprotinin) observed reduced invasiveness and tumour size. Boggust (1976) found that some drugs used in cancer therapy (including ICRF-159) were inhibitory in human tumour extracts capable of hydrolysing collagen and reticulin as well as Pz-substrate. EDTA and phenantheroline are also inhibitory of proteolytic or peptidase activity in tumours (Seifter and Gallop, 1962; Werb et al., 1974; Boggust, 1976).

This paper shows that several drugs used clinically as cytostatic or cytotoxic agents inhibit the collagen peptidase of HeLa cells and human tumours.

MATERIALS AND METHODS

HeLa cells, cultured 4 days at 37°C in Eagle's minimum medium with 10% calf serum, 1% glutamine and antibiotics, were harvested during log phase, washed in Ringer's solution and counted.

For enzyme activity, cells were extracted 30 min at 37°C with veronal-acetate buffer pH 7.2 (Documenta Geigy, 1962) diluted to 1-6 vols with water (1 ml to 10⁵ cells) and centrifuged 10 min at 35,000 g. The pellet was twice dispersed and re-extracted at 37°C, the combined supernatant being stored at −25°C before standardization with p-phenyl-azo-benzyl - oxy carbonyl - pro - leu - gly - pro - D- arginine.

Tumours from surgery, examined histologically, included adenocarcinomas of colon, rectum and kidney, squamous carcinomas of vulva, seirrhous carcinomas of breast, invasive squamous carcinoma of ear and differentiated squamous carcinoma of finger. Tumour (0.5 g), free from normal tissue, fat, necrotic or infected material, was disintegrated by 4 passes at −25°C in the Biotec X-press, dispersed in diluted veronal-acetate buffer, pH 7.2 (1 ml) and centrifuged for 10 min at 5°C and 30,000 g. The residue was suspended ×4 and re-extracted in 0.5 ml portions of cold buffer, the combined supernatants being stored at −25°C. Only a limited number of assays could be performed with the extract from each tumour.

For assay, mixtures containing serial dilutions of cell or tissue extract, or buffer control (0-25 ml) and buffer (0-5 ml) were pre-incubated at 37°C for 1 h before adding substrate solution (0.4 mg/ml in buffer) or buffer (0-5 ml) with incubation for 15 min. After adding 0.5% citric acid (2-5 ml) extracts in ethyl acetate (2×2-5 ml) combined and dried over anhydrous Na₂SO₄ gave optical densities at λ=320 nm.

For assay of inhibition, mixtures containing cell or tissue extract (0-25 ml) serial dilutions of test substance in buffer prepared with minimal warming or control buffer (0-25 ml) and buffer (0-25 ml) were set up in duplicate and preincubated at 37°C for 1 h; substrate solution in buffer (0-5 ml) was added to one series and buffer (0-5 ml) to the other as controls: all were incubated for 15 min at 37°C and treated as described above. Inhibition of collagen-peptidase was calculated from the dilution/optical-density curve obtained by assay of serially diluted untreated extracts.

RESULTS

Representative findings are presented as % inhibition of collagen-peptidase activity in HeLa extracts (Table I) and tumour extracts (Table II) by drugs at stated concentrations in each complete incubation mixture. Peptidase activities in OD 320 units are given for comparison of the extracts and molar concentrations of drugs producing 50% inactivation are computed from the data.

HeLa extracts were generally reproducible in character and activity, but tumour extracts showed considerable variations in assayable activity, and especially in their total solutes, which possibly modified the action and effective concentration of test drugs.
### Table I.—Inhibition of HeLa collagen peptidase by drugs at stated concentrations in each incubation mixture. Peptidase activities for each extract are given in OD units for comparison together with computed concentrations of drugs producing 50% inhibition.

| Drug            | Extract, mg/ml | OD/320 | μ M for 0·6 0·2 0·06 0 50% inhib. | % inhibition |
|-----------------|----------------|--------|---------------------------------|--------------|
| Razoxane        | 0·031          | 97·0   | 75·0 21·0 0·0 0·43              |              |
|                 | 0·030          | 91·0   | 65·0 22·0 0·0 0·43              |              |
|                 | 0·030          | 87·0   | 70·0 22·0 0·0 0·48              |              |
| Cyclophosphamide| 0·054          | 64·0   | 46·0 34·0 3·0 0                |              |
|                 | 0·050          | 61·0   | 71·0 40·0 1·15                 |              |
|                 | 0·038          | 69·0   | 42·0 1·15                      |              |
| 5-Fluorouracil  | 0·032          | 70·0   | 50·0 29·0 7·7 0                |              |
|                 | 0·032          | 93·0   | 79·0 39·0 2·7                 |              |
|                 | 0·031          | 79·0   | 57·0 21·5 5·8                 |              |
| Thiotepa        | 0·032          | 70·0   | 22·0 0·8 0·7                  |              |
|                 | 0·030          | 68·0   | 39·0 22·0 0·8 0·7              |              |
| Aprotinin       | 0·033          | 99·0   | 57·0 0 0                     |              |
|                 | 0·063          | 74·0   | 57·0 13·0 0                 |              |
|                 | 0·065          | 80·0   | 70·0 17·0 0                  |              |
| EDTA            | 0·033          | 79·0   | 69·0 55·0 10·0 0·06           |              |
|                 | 0·035          | 100·0  | 100·0 91·0 80·0 0·02          |              |
| Phenanthroline  | 0·035          | 100·0  | 95·0 70·0 0·22               |              |
|                 | 0·035          | 100·0  | 58·0 80·0 0·22               |              |
|                 | 0·031          | 71·0   | 45·0 0 1·33                 |              |

### Table II.—Inhibition of tumour collagen peptidase by drugs at stated concentrations in each incubation mixture. Peptidase activities for each extract are given in OD units for comparison together with computed concentrations of drugs producing 50% inhibition.

| Drug            | Extract, mg/ml | OD/320 | μ M for 0·6 0·2 0·06 0 50% inhib. | % inhibition |
|-----------------|----------------|--------|---------------------------------|--------------|
| Razoxane        | 0·070          | 75·0   | 59·0 48·0 0·28                 |              |
|                 | 0·137          | —      | 49·0 40·0 0·78                 |              |
|                 | 0·053          | 46·0   | 36·0 0·12                      |              |
|                 | 0·158          | 26·0   | 19·5 6·5 2·24                 |              |
| Cyclophosphamide| 0·045          | 91·0   | 71·0 47·0 15·0 0·88           |              |
|                 | 0·040          | 87·5   | 13·0 — 0·10 — 0·06 0·02       |              |
| 5-Fluorouracil  | 0·023          | 87·5   | 75·0 9·0 6·15                 |              |
|                 | 0·029          | 74·0   | 56·0 — 0·9 0·22               |              |
|                 | 0·026          | 37·0   | 24·0 — 0·22                    |              |
| Thiotepa        | 0·037          | 96·0   | — — 0·95                      |              |
|                 | 0·021          | 84·0   | 73·5 12·0 1·27                 |              |
| Aprotinin       | 0·045          | 63     | 33 0 — 0·22                    |              |
| EDTA            | 0·041          | —      | 39·0 15·0 0·12                 |              |
| Phenanthroline  | 0·167          | 50·0   | 21·0 10·0 — 0·68               |              |
Excepting aprotinin, for which no comparison can be expressed in molar units, decreasing inhibition of tumour peptidase was observed in the order phenanthroline, EDTA, Razoxane, cyclophosphamide, thiotepa, 5-FU. With HeLa extracts, EDTA and 5-FU were rather more effective than phenanthroline and thiotepa respectively.

The results show better than 50% inactivation of tumour collagen peptidase at concentrations of inhibitor substantially less than upper levels for therapy cited for Razoxane (ICRF-159) (Wasserman et al. 1973) and for cyclophosphamide, aprotinin and EDTA (Martindale, 1972). 5-FU produced variable levels of inhibition and not all tumour extracts were inhibited. Phenanthroline, a potent inhibitor of collagen peptidase, produces a neuromuscular reaction in mice at levels above 5 μg/g (Peters, 1975).

No inhibition was seen with N-acetylglucosamine, bleomycin, colchicine, cytarabine, daunomycin, daunorubicin, iodoacetamide, levamisole, methotrexate, phosphocreatine, vinblastine or vincristine.

No overall biochemically acceptable reaction mechanism can be proposed, as the lack of specificity in the inhibitory compounds indicates that the biological situation is complex and may involve more than the blockade of a single enzyme-substrate reaction. As incubation of test substances in buffer showed no significant change of pH, reduced hydrolysis of substrate is not due to enzyme denaturation or to the effect of a less favourable pH upon activity.

DISCUSSION

Certain cytotoxic drugs have been shown to inactivate the collagen peptidase from HeLa cells in culture as well as in extracts of spontaneous human tumours. In addition to malignant cells, tumours may contain non-neoplastic host cells infiltrated into the stroma, including leucocytes, macrophages and fibroblasts. Although such cells have been reported, by various authors, to contain collagenolytic enzymes, no information is available specifically about their collagen-peptidase activity or its inhibition. Accordingly the overall contribution of such drugs to the control of tissue lysis around tumours has yet to be assessed in full.

The role of collagen peptidase in relation to intact macromolecules of tissue structures in the environment of tumours is considered to involve the continuation and extension of lysis begun by the action of true proteases with which they are closely associated.

The relevance of collagen-protease and collagen-peptidase activity to invasiveness and metastasization is related to the weakening of, or creation of defects in, physical barriers to tumour growth. Natural substrates include components and degraded forms of glycoprotein or mucopolysaccharide such as are found in collagen, reticulin, fibrin and the ground substance in matrix of basement membrane, connective tissue, intercellular cement, or in the perithelial structure of the micro-vascular system round the tumour. Lysis of these materials may release cells from the tumour mass, open up pathways for invasion into surrounding areas, or allow passage of cancer cells into the lumen of adjacent lymphatic or blood capillaries for transport to metastatic sites.

Inhibitors of collagenase and collagen-peptidase activity modify the environment of cancers by reducing the hydrolysis of substances which prevent tumour-cell release and movement. By constraining the tumour within the surrounding stroma, blockade of expansive and invasive growth may produce a state like basal-cell carcinomas, which form an enveloping capsule usually staining for reticulin. Such tumours are potentially invasive but, if unable to lyse their capsule, remain benign and undergo maturation and keratinization. By reducing lysis of reticulin-like components in capillary perithelium, enzyme inhibition may con-
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CONTRIBUTIONS TO THE BIOLOGY OF TUMOURS

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