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Differential *In Vitro* Inhibitory Effects of Anticancer Drugs on Tumor-Associated Carbonic Anhydrase Isozymes CA IX and CA XII

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SUMMARY

Carbonic anhydrase IX (CA IX) and, to a lesser extent, carbonic anhydrase XII (CA XII) are highly overexpressed in hypoxic tumors. In this study, the inhibitory effects of 11 different anticancer drugs including paclitaxel, amethopterin, etoposide, irinotecan, gemcitabine, 5-fluorouracil, oxaliplatin, epirubicin, cisplatin and carboplatin on the tumor-associated carbonic anhydrase isozymes CA IX and CA XII and cytosolic carbonic anhydrases I and II have been investigated. SX.18MV-R Applied Photophysics stopped-flow instrument was used for measuring the initial velocities for the CO_2 hydration reaction catalyzed by different CA isozymes, by following the change in the absorbance of a pH indicator. CA IX and CA XII were the most affected by carboplatin and cisplatin amongst the panel of anticancer drugs. Moreover, the cytosolic carbonic anhydrases I and II can also be affected. Consequently, CA IX and CA XII are interesting targets for anticancer drug development, although more selective and powerful CA inhibitors could prove useful for elucidating the role of the protein in hypoxic cancers, for controlling the pH imbalance in tumor cells and for developing diagnostic or therapeutic applications for the management of hypoxic tumors, generally unresponsive to classical chemo- and radiotherapy. Copyright 2008 Prous Science, S.A.U. or its licensors. All rights reserved.

Key words: Hypoxia - Carbonic anhydrase - hCA I - hCA II - hCA-IX - hCA-XII - Hypoxia - Inhibition

INTRODUCTION

Hypoxia is a pathological condition in which the body as a whole (generalized hypoxia) or a region of the body (tissue hypoxia) is deprived of adequate oxygen supply. Low oxygen content in the blood is referred to as hypoxemia. Hypoxia in which there is complete deprivation of the oxygen supply is referred to as anoxia (1).

Tumor hypoxia is the situation in which tumor cells have been deprived of oxygen. This is relevant in the study of radiation therapy as such cells can be made more susceptible to treatment by increasing the amount of oxygen (2).

Tumor growth involves complex interactions between cells and their unique microenvironment, wich is characterized by low extracellular acidification (pHe) and altered hydrostatic and oxygen pressures. Tight control of pH homeostasis in tumors is achieved by using proton extrusion mechanisms that include plasma membrane proton pumps, proton channels/proton wires, sodium/proton exchangers and monocarboxylic acid transporters. Regarding the implications for tumor growth and spread, it would seem that tumor microenvironmental acidity could play a predominant promoting role in tumor growth and metastasis and could also underlie resistance to radiotherapy, chemotherapy and other nonsurgical treatments (3, 4).

Catalysis of a reversible conversion of carbon dioxide to bicarbonate and proton, participation in gas exchange, ion transport and acid–base balance across the cell membrane and in different intracellular compartments are mediated by the carbonic anhydrase family (5). The expression levels of human tumor-associated carbonic anhydrase isozymes IX and XII (hCA IX and XII) are elevated in response to hypoxia, and research on the involvement of these isozymes in cancer has progressed considerably in recent years (6-9). It has been reported that these enzymes are responsible for the low pHe of the tumor microenvironment. Multiple downstream effects of this reduced pHe are associated with tumor progression and poor prognosis (10-12).

In this study, our goal is to investigate the inhibitory effects of some anticancer drugs on cytosolic human carbonic anhydrases I and II (hCA I and hCA II) and tumor-associated human carbonic anhydrases IX and XII (hCA IX, hCA XII).

The presence of an H^+ gradient across the membrane of tumor cells also has interesting implications for chemotherapy (13). Acidification of the solid tumor milieu might decrease the uptake of weak basic anticancer drugs, leading to chemoresistance (14). Most anticancer drugs are transported by either active transport or passive diffusion into cells, where they frequently undergo further metabolism (15). Because all of these processes are pH-sensitive, the cytotoxic activity of anticancer drugs could depend on both intracellular pH (pHi) and pHe.

There are several studies in which the hCA IX and hCA XII isozymes were found to be prominently associated with and overexpressed in many tumors in crucial processes associated with cancer progression and response to therapy (16-20).

Anticancer clinical trials test many types of treatments such as new drugs, new approaches to surgery or radiation therapy, new combinations of treatments or new methods such as gene therapy. The goal of this research is to find better ways to treat cancer and help cancer patients by investigating the anticancer drug effects on hCA I, hCA II, hCA IX and hCA XII.

MATERIALS AND METHODS

Materials

Sepharose 4B-L-tyrosine sulfonamide, protein assay reagents and other chemicals were obtained from Sigma Chemicals (Milan, Italy). Medical drugs were provided by a local pharmacy in Balikesir, Turkey.

CA IX and CA XII genes were a gift from Dr. Claudiu T. Supuran, Florence University, Italy.

Methods

CA catalytic domain

The final CA domains of CA IX and CA XII were further purified by Sepharose 4B–L-tyrosine–sulfonamide affinity gel (21) with 129.15- and 92.29-fold purifications for CA IX and CA XII, respectively. The amount of enzyme was determined by spectrophotometric measurements and its activity by stopped-flow experiments, with CO_2 as substrate (19).

Preparations of enzyme solutions

Cytosolic human carbonic anhydrases I and II were purchased from Sigma Chemicals (Milan, Italy), and had concentrations of 1×10^{-6} M and 1×10^{-7} M for hCA I and hCA II, respectively (19).

Tumor-associated carbonic anhydrase isozymes were further purified by Sepharose 4B–L-tyrosine–sulfonamide affinity gel (21), and had a concentration of 4×10^{-6} M for both hCA IX and hCA XII (19).

CA enzyme assay

SX.18MV-R Applied Photophysics stopped-flow instrument was used for measuring the initial velocities for the CO_2 hydration reaction catalyzed by different CA isozymes, by following the change in the absorbance of a pH indicator. Phenol red (at a concentration of 0.2 mM) was used as an indicator, working at the absorbance maximum of 557 nm, with 10 mM Hepes (pH 7.5) as buffer, 0.1 M Na_2SO_4 (for maintaining constant the ionic strength), and following the CA-catalyzed CO_2 hydration reaction for a period of 10–100 s. Saturated CO_2 solutions in water at 20 ºC were used as substrate. The CO_2 concentrations ranged from 1.7 to 17 mM for the determination of the inhibition constants. For each inhibitor, at least six traces of the initial 5–10% of the reaction were used for determining the initial velocity. The uncatalyzed rates were determined in the same manner and subtracted from the total observed rates.

Stock solutions of anticancer drugs were prepared at a concentration of 1–3 mM (in DMSO/water 1:1, v/v), and dilutions up to 0.01 nM were done with the assay buffer mentioned earlier. The inhibition constants were obtained by nonlinear least-squares methods using PRISM 3, from Lineweaver–Burk plots, as reported earlier (19). The mean was represented from at least three different determinations.

RESULTS AND DISCUSSION

Anticancer drugs are directed toward specific signaling pathways responsible for tumor growth and metastasis and target specific antigens, growth factors, receptors or other molecules in the signaling pathway of interest (22). These drugs, such as bifunctional alkylating agents, inhibitors of DNA synthesis and inhibitors of topoisomerases, induce homologous recombination in mammalian cells, which suggests the possibility of developing secondary tumors, and thus poses the question of whether cytostatic drugs should be additionally tested for adverse effects in cancer chemotherapy. Figure 1 shows the structures of the anticancer drugs (23) discussed in this study.

In spite of the tremendous advances in technology, imaging and genomic information, cancer is unfortunately still one of the leading causes of death in developed countries, as it is often diagnosed and treated too late, mainly because it is very difficult to monitor and predict the progress of metastasis. The most commonly used anticancer cytostatic drugs were designed to target DNA by affecting its replication and causing cell death.

The first comprehensive study on the correlation between gene expression and drug activity was reported by Scherf *et al*. (24). They used microarrays to assess gene expression profiles in the NCI-60 used at the National Cancer Institute to screen compounds for anti-

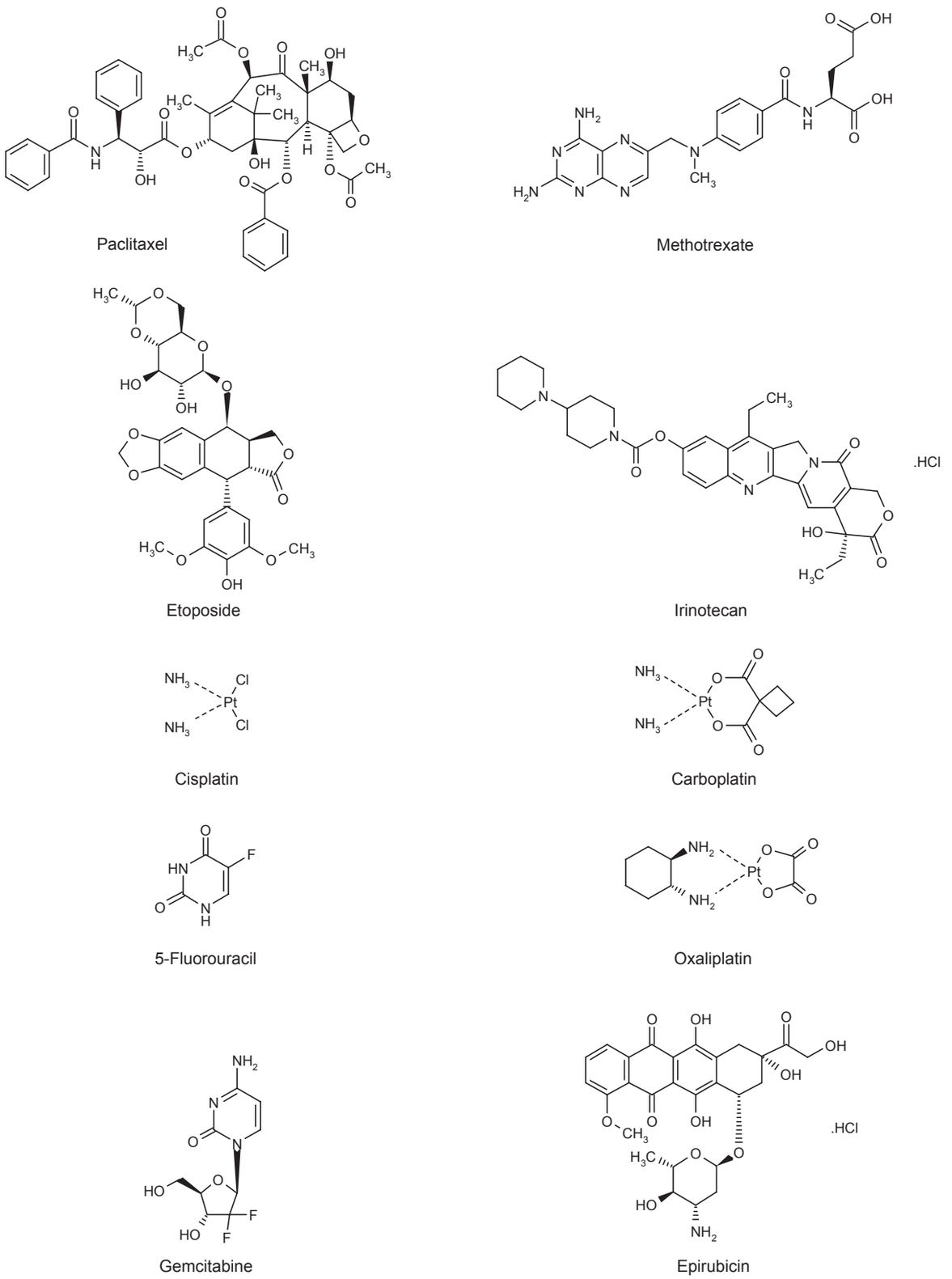

FIG. 1. The chemical structures of paclitaxel, etoposide, cisplatin, 5-fluorouracil, gemcitabine, methotrexate, irinotecan, carboplatin, oxaliplatin and epirubicin (23).

TABLE 1. IC_{50} values of CA isozymes.

Anti-cancer drugs	IC_{50} M			
	hCA I x 10^{-5}	hCA II x 10^{-5}	hCA IX x 10^{-5}	hCA XII x 10^{-5}
Paclitaxel 30 mg/5 mL	3.91	1.16	2.84	4.34
Methotrexate 50 mg/2 mL	6.15	2.72	1.26	5.36
Etoposide 100 mg/5 mL	14.01	5.27	2.50	1.64
Irinotecan 100 mg/5 mL	2.70	1.80	2.75	2.28
Carboplatin 50 mg/5 mL	3.35	8.98	0.58	0.68
Cisplatin 10 mg /10 mL	3.36	2.15	0.67	0.93
Gemcitabine 1 g	7.24	3.25	3.09	5.10
5-Fluorouracil (Ebewe) 1000 mg/20 mL	14.47	2.40	0.83	1.60
5-Fluorouracil (Biocyn) 1000 mg/20 mL	4.56	4.15	1.19	0.85
Oxaliplatin 100 mg/1 mL	2.92	2.65	2.35	3.04
Epirubicin 50 mg/5 mL	10.74	2.87	1.56	3.62

cancer activity. Clustering cell lines based on gene expression yielded relationships very different from those obtained by clustering the cell lines on the basis of their response to a total of 118 anticancer drugs.

Carboplatin, like cisplatin, produces predominantly interstrand DNA crosslinks rather than DNA–protein crosslinks (25). This effect is apparently cell cycle nonspecific. The aquation of carboplatin, which is thought to produce the active species, occurs at a slower rate than in the case of cisplatin. Despite this difference, it appears that both carboplatin and cisplatin induce equal numbers of drug–DNA crosslinks, causing equivalent lesions and biological effects. The differences in potencies for carboplatin and cisplatin appear to be directly related to the difference in aquation rates (26). In this study, both cytosolic and tumor-associated carbonic anhydrases were affected by carboplatin and cisplatin. Table 1 shows the IC_{50} values for both CA isozymes, and the greatest inhibition was seen for carboplatin against the tumor-associated isozymes hCA IX and hCA XII (IC_{50} = 0.58 x 10^{-5} and 0.68 x 10^{-5} M, respectively). Following carboplatin, cisplatin also showed the greatest inhibition for hCA IX and hCA XII, with IC_{50} values of 0.67 x 10^{-5} and 0.93 x 10^{-5} M, respectively. Also with this drug, hCA II was affected more than with carboplatin. Another anticancer drug used in this study is 5-fluorouracil (Ebewe and Biocyn), a well-known anticarcinogenic drug, which is a fluorinated pyrimidine antimetabolite and is mostly employed in the palliation of inoperable malignant neoplasms (27). The IC_{50} values are shown in Table 1. The drug content is the same, but the manufacturers are different, as indicated (Ebewe and Biocyn), and they had different inhibitory profiles on hCA isozymes. Interestingly, hCA IX was the most affected by 5-fluorouracil (Ebewe), with an IC_{50} value of 0.83 x 10^{-5} M. On the other hand, hCA XII showed the greatest inhibition with 5-fluorouracil (Biocyn), with an IC_{50} value of 1.19 x 10^{-5} M.

Oxaliplatin is an organoplatinum complex (23, 28). *In vivo* studies have shown antitumor activity of oxaliplatin against colon carcinoma (29). In combination with 5-fluorouracil, oxaliplatin exhibits *in vitro* and *in vivo* antiproliferative activity greater than either compound alone in several tumor models: HT29 (colon), GR (mammary) and L1210 (leukemia) (30). Platinum also binds irreversibly and accumulates (approximately twofold) in erythrocytes, where it appears to have no relevant activity. Oxaliplatin undergoes rapid and extensive nonenzymatic biotransformation. There is no evidence of cytochrome P450-mediated metabolism *in vitro*. Up to 17 platinum-containing derivatives have been observed in plasma ultrafiltrate samples from patients, including several cytotoxic species (monochloro DACH platinum, dichloro DACH platinum, and monoaquo and diaquo DACH platinum) and a number of noncytotoxic conjugated species (31). Here, like cisplatin and carboplatin, oxaliplatin also showed inhibitory effects on hCA IX and hCA XII, as seen in Table 1. Cytosolic isozymes hCA I and hCA II were affected by all platinum-containing anticancer drugs in this work.

Irinotecan is a derivative of camptothecin (23). Camptothecins interact specifically with the enzyme topoisomerase I, which relieves torsional strain in DNA by inducing reversible single-strand breaks (32). Possible pharmacokinetic interactions of irinotecan with other concomitantly administered medications have not been formally investigated (33). In this study, with this drug the inhibitory effects on hCA isozymes were similar, with hCA II inhibited the must, with an IC_{50} value of 1.80 x 10^{-5} M.

Gemcitabine HCl is a nucleoside analogue that exhibits antitumor activity (23). Gemcitabine exhibits cell phase specificity, primarily killing cells undergoing DNA synthesis (S phase) and also blocking the progression of cells through the G1/S phase boundary. Gemcitabine is metabolized intracellularly by nucleoside kinases to the active diphosphate (dFdCDP) and triphosphate (dFdCTP) nucleosides (34). hCA IX showed less inhibition with this drug compared to the other anticancer drugs (IC_{50} = 3.09 x 10^{-5} M), but hCA II was inhibited to a similar extent as hCA IX (IC_{50} = 3.25 x 10^{-5} M).

Amethopterin (methotrexate) is an antimetabolite used in the treatment of certain neoplastic diseases, severe psoriasis and adult rheumatoid arthritis (35). This drug interferes with DNA synthesis, repair and cellular replication (23). Actively proliferating tissues such as malignant cells, bone marrow, fetal cells, buccal and intestinal mucosa, and cells of the urinary bladder are in general more sensitive to this effect of methotrexate (36). When cellular proliferation in malignant tissues is greater than in most normal tissues, methotrexate may impair malignant growth without irreversible damage to normal tissues. In this study, hCA IX showed the greatest inhibition by this anticancer drug, with an IC_{50} of 1.26×10^{-5} M. *CA9* is one of the genes highly upregulated by hypoxia that encodes isozyme IX of carbonic anhydrase. The levels of this enzyme, which catalyzes CO_2 hydration to bicarbonate and H^+ ions, increase in response to hypoxia via direct transcriptional activation of the *CA9* gene by the hypoxia-inducible factor HIF-1 (37).

Paclitaxel is a novel antimicrotubule agent that promotes the assembly of microtubules from tubulin dimers and stabilizes microtubules by preventing depolymerization (23, 38). hCA II and hCA IX are most affected by this drug among the isozymes ($IC_{50} = 1.16 \times 10^{-5}$ M and 2.84×10^{-5} M, respectively).

Among the other anticancer drugs, epirubicin (23), an anthracycline drug used for chemotherapy, also showed the greatest inhibition of hCA IX and hCA XII, as seen in Table 1, but hCA I was not affected by this drug at all. Another antineoplastic agent, etoposide (23), did not have a significant inhibitory efect on hCA I, like epirubicin, but it showed good inhibition of hCA IX and hCA XII. Similar to other anthracyclines, it acts by intercalating DNA strands and results in complex formation, which inhibits DNA and RNA synthesis (39).

Inhibition of the CA enzymatic activity by specific inhibitors, such as the sulfonamide compounds, explains the tumorigenesis related with hCA IX and hCA XII. Thus, it is obvious by the mechanisms by which these drugs may interact with hCA IX, hCA XII, hCA I and hCA II that they do not bind to metal ions as do classical CA inhibitors of the sulfonamide or sulfamate/sulfamide type. Hydrophobic interactions probably play a major role in the observed inhibition.

CONCLUSIONS

Selective hCA IX and hCA XII inhibitors could prove useful for elucidating the role of tumor-associated CA isozymes in hypoxic cancers, for controlling the pH imbalance in tumor cells and for developing diagnostic or therapeutic applications for tumor management. Indeed, fluorescent inhibitors and membrane-impermeant sulfonamides have recently been used as proof-of-concept tools, demonstrating that CA IX is an interesting target for anticancer drug development (16, 40, 41).

The results of the present study show that anticancer drugs also have an important role in inhibiting CA isozymes such as hCA I, hCA II, hCA IX and hCA XII. It thus appeared of interest to further explore the connections between CAs and tumors, and the development of specific inhibitors for some of the isozymes presumably involved in such processes would be highly beneficial for both obtaining novel types of drugs and for a better understanding of the physiology of the CAs. To clarify the role of tumorigenesis in cancer treatment, further studies on chemotherapy or other treatment modalities in patients are needed, and investigations along these lines may yield an important new approach to cancer therapy.

Consequently, this manuscript describes the interaction of various types of antitumor drugs with CA isoforms I, II (cytosolic) and IX and XII (transmembrane, tumor-associated). The levels of inhibitory activity are rather low, but may be significant *in vivo* when high doses of such compounds are used in chemotherapy.

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