The anti-malarial artesunate is also active against cancer

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Abstract. Artesunate (ART) is a semi-synthetic derivative of artemisinin, the active principle of the Chinese herb *Artemisia annua*. ART reveals remarkable activity against otherwise multidrug-resistant *Plasmodium falciparum* and *P. vivax* malaria. ART has now been analyzed for its anti-cancer activity against 55 cell lines of the Developmental Therapeutics Program of the National Cancer Institute, USA. ART was most active against leukemia and colon cancer cell lines (mean GI_{50} values: 1.11 ± 0.56 μM and 2.13 ± 0.74 μM, respectively). Non-small cell lung cancer cell lines showed the highest mean GI_{50} value (25.62 ± 14.95 μM) indicating the lowest sensitivity towards ART in this test panel. Intermediate GI_{50} values were obtained for melanomas, breast, ovarian, prostate, CNS, and renal cancer cell lines. Importantly, a comparison of ART's cytotoxicity with those of other standard cytostatic drugs showed that ART was active in molar ranges comparable to those of established anti-tumor drugs. Furthermore, we tested CEM leukemia sub-lines resistant to either doxorubicin, vincristine, methotrexate, or hydroxyurea which do not belong to the N.C.I. screening panel. None of these drug-resistant cell lines showed cross resistance to ART. To gain insight into the molecular mechanisms of ART's cytotoxicity, we used a panel of isogenic *Saccaromyces cerevisiae* strains with defined genetic mutations in DNA repair, DNA checkpoint and cell proliferation genes. A yeast strain with a defective mitosis regulating BUB3 gene showed increased ART sensitivity and another strain with a defective proliferation-regulating CLN2 gene showed increased ART resistance over the wild-type strain, wt644. None of the other DNA repair or DNA check-point deficient isogenic strains were different from the wild-type. These results and the known low toxicity of ART are clues that ART may be a promising novel candidate for cancer chemotherapy.

Introduction

Chemical structures with biological activity in micro-organisms, plants, and animals have been developed during evolution of life and may be exquisite targets for drug research. Biogenic drugs are indispensable parts of the pharmacological repertoire to combat human diseases. Natural compounds can serve as lead structures and derivative compounds can be inferred from large screenings with chemical drug libraries. *Vinca* alkaloids (vincristine, vinblastine), epipodophyllotoxins (etoposide, teniposide), and taxanes (paclitaxel, taxotere) are important plant-derived drugs in cancer chemotherapy. Unfortunately, chemotherapy of tumors is frequently hampered by the emergence of drug resistance and the occurrence of severe adverse side effects. Hence, the development of less toxic drugs which retain activity against otherwise drug-resistant tumor cells is urgently warranted.

The Chinese herb *Artemisia annua* has been used in traditional medicine for more than 2,000 years to treat febrile symptoms associated with malaria. Since the isolation of artemisinin as the active principle of *Artemisia annua* in the early 1970s by Chinese scientists (1), the drug has attracted attention in the Western world. The inhibitory activity of artemisinin towards *Plasmodium falciparum* and *P. vivax* malaria has been corroborated in a number of investigations (2,3). Semi-synthetic artemisinin derivatives with improved pharmacological features have been generated. Out of the different artemisinin derivatives, arteether, artemether, and artesunate, the latter one is the most potent one *in vitro* (4). The World Health Organization recommends the use of these compounds in geographical areas with multidrug resistant malaria. Propitious features of artesunate (ART) are the activity against otherwise multidrug-resistant *Plasmodium strains* (5), its good tolerability and the lack of significant adverse side effects (6).

Previously, we demonstrated that ART, apart from its anti-malarial activity, inhibits the growth of leukemic cells

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and induces apoptosis (7). In the present investigation, ART has been analyzed for its activity against 55 cell lines of different tumor types in collaboration with the Developmental Therapeutics Program of the National Cancer Institute of the USA. As drug resistance is a severe limitation of many established cytostatic agents, we then analyzed whether ART reveals cross-resistance in cell lines resistant to either doxorubicin, vincristine, methotrexate, or hydroxyurea. In an effort to gain insight into the molecular mechanisms of ART's cytotoxicity, we examined a recently described panel of isogenic *Saccharomyces cerevisiae* strains with defined genetic defects (8) and identified two putative target genes, BUB3 and CLN2 (cyclin G1).

Materials and methods

Chemicals. Artesunate (ART) was obtained from Saokim Co. Ltd. (Hanoi, Vietnam). The chemical structure of this sesquiterpene is depicted in Fig. 1.

Cell lines of the anti-cancer drug screen. The origins and processing of the cell lines of the Developmental Therapeutics Program of the National Cancer Institute of the USA were as previously described (9). The panel for the present investigations consisted of 55 human tumor cell lines representing leukemia (CCRF-CEM, HL-60, K-562, MOLT-4, RPMI-8226, SR), melanoma (LOX IMVI, M14, SL-MEL-2, SK-MEL-28, SK-MEL-5, UACC-257, UACC-62), non-small cell lung cancer (A549, EKVX, HOP-62, HOP-92, NCI-H322M, NCI-460), colon cancer (COLO 205, HCC-2998, HCT-116, HCT-15, HT29, KM12), renal cancer (786-0, A498, ACHN, CAKI-1, SN12C, TK-10, UO-31), ovarian carcinoma (IGROV1, OVCAR-3, OVCAR-4, OVCAR5, OVCAR-8, SK-OV-3), and tumors of the central nervous system (SF-268, SF-295, SF-539, SNB-19, SNB-75, U251).

Cell lines selected for drug resistance. Human CEM-CCRF leukemia cells were maintained in RPMI medium (Gibco, Eggenstein, Germany) supplemented with 10% fetal calf serum in a 5% CO_2 atmosphere at 37°C. Cells were passaged twice weekly. All experiments were performed with cells in the logarithmic growth phase. The development of drug-resistant sublines has been described (10-12). Drug-resistant cell lines were maintained in 5000 ng/ml doxorubicin (CEM-ADR5000), 100 ng/ml vincristine (CEM-VCR100), 2.0 μM MTX plus 10 nM leucovorin (CEM-MTX1500LV), or 90 μM hydroxyurea (CEM-HUR90), respectively.

Sulforhodamin B testing procedure. Cell lines of the Developmental Therapeutics Program of the National Cancer Institute of the USA were added in aliquots of 100 μl per well into 96-well microtiter plates in a density of 5,000 to 40,000 cells according to the growth characteristics of the particular cell type. Inoculates were allowed to incubate for 24 h at 37°C prior to the addition of ART (10^{-8} to 10^{-4} M). After a further 48-h incubation period, cells were assayed by means of a sulforhodamine B assay (13). The optical densities read by a plate reader were processed by a micro-computer. The drug concentration required to inhibit cell growth by 50% (GI_{50} value) was calculated according to the
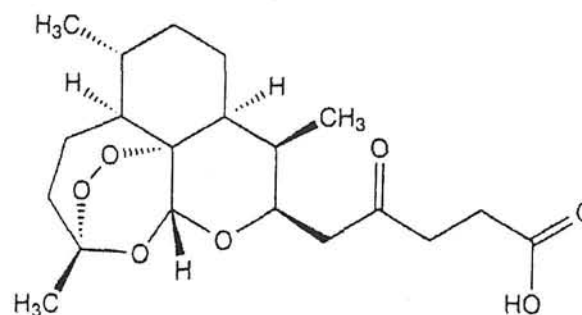

Figure 1. Structural formula of artesunate.

formula 100 x $(T - T_0)/(C - T_0) = 50$, where T is the optical density of the test well after a 48 h period of exposure to test drug, T_0 the optical density at time zero, and C the control optical density (14).

Growth inhibition assay. The *in vitro* response to cytostatic drugs was evaluated by means of a growth inhibition assay (15). Aliquots of $5x10^5$ cells ml^{-1} were seeded in culture medium and drugs were added at different concentrations. Cells were counted 10 days after application. Cell numbers were determined each in triplicate independent determinations.

Saccharomyces cerevisiae mutant strains. A panel of isogenic strains of *Saccharomyces cerevisiae* defective in certain genes has been described in detail (8). Yeast strains without (wt644, wt780) or with genetic defects in cell proliferation, growth arrest, or damage repair genes (bub, CLN, mec, mgt, mlh, r14, r18, r50, r50+, r52, sgs) were used for the present investigation. The toxicity of ART towards these strains was determined according to a test assay described previously (8). Two independent experiments were performed. First, we tested 3-fold dilutions with a high concentration of 100 μM ART. This is the standard range for yeast toxicity assays. ART was toxic to yeast in the 10 to 100 μM range. In the second experiment, we tried to resolve minor differences using 2-fold dilutions. Aliquots of 135 μl yeast cultures ($7.4x10^4$ cells/ml) were placed in 96-well microtiter plates. Fifteen μl of each ART concentration was added in triplicate to the yeast-containing wells. Plates were incubated for 18 h at 30°C and the absorbance (A660) was read in a microplate reader. The percentage of surviving cells was calculated relative to unirradiated control plates.

Results

Dose response curves of artesunate (ART) have been determined over a dose range from 10^{-8} to 10^{-4} M in 55 cell lines of different cancer types and GI_{50} values have been calculated thereof. Among the tumor cell types tested, leukemia and colon cancer cell lines revealed the lowest mean GI_{50} values (1.11±0.56 μM and 2.13±0.74 μM, respectively; Fig. 2), while non-small cell lung cancer cell lines showed the highest mean GI_{50} value (25.62±14.95 μM). Thus, ART was most active against leukemia and colon
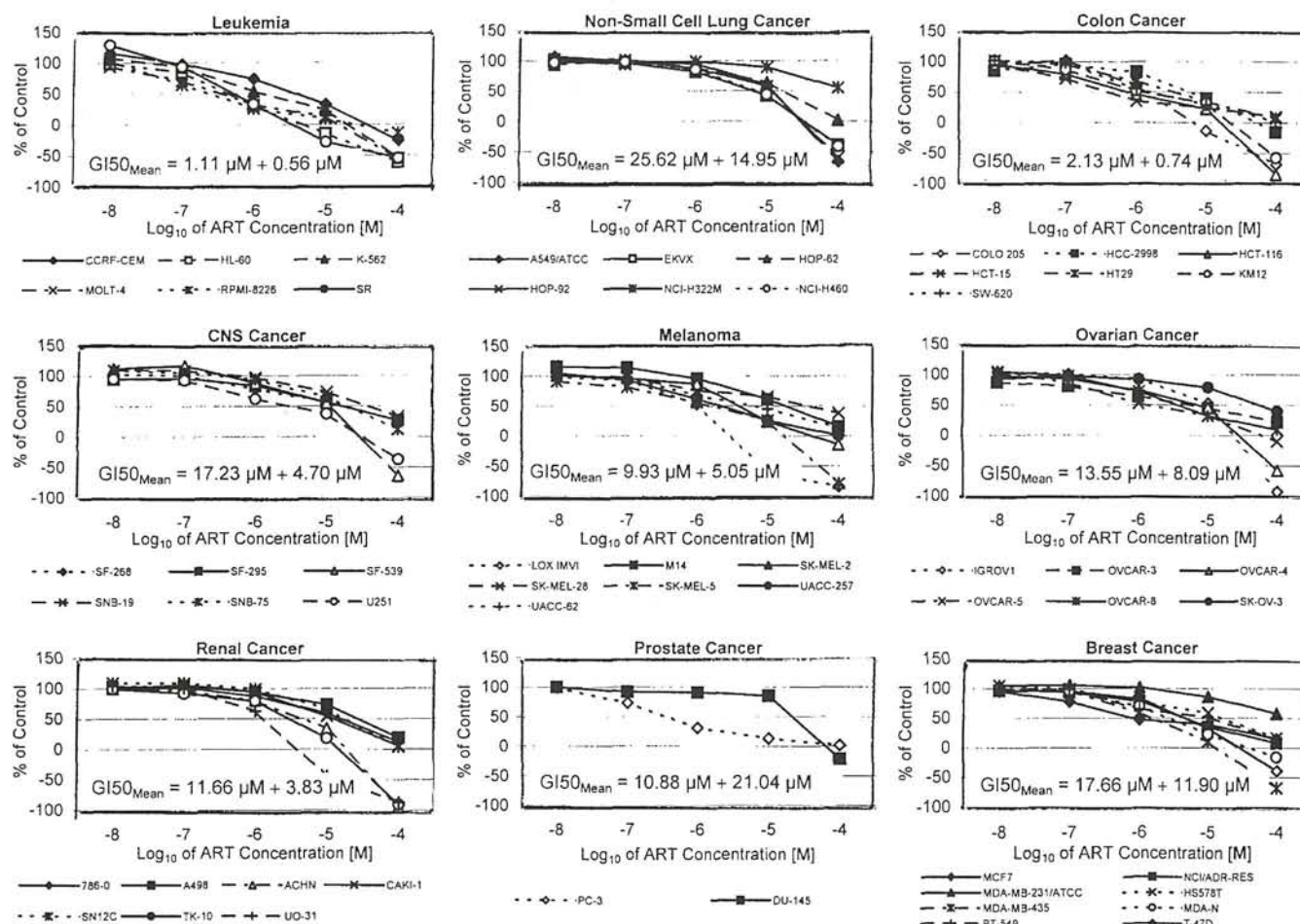
Figure 2. Anti-cancer activity of artesunate. Dose response curves of 55 cell lines of the Developmental Therapeutics Program of the National Cancer Institute, USA, are shown. Artesunate was tested over a dose range from 10^{-8} to 10^{-4} M using a sulforhodamine B assay (9). GI_{50} values were calculated from dose response curves derived thereof and are depicted as mean values for each tumor type.

cancer cells *in vitro*. Interestingly, the drug was also active against otherwise chemoresistant tumors like renal cancers, melanomas, or CNS tumors.

Next, we compared ART's anti-cancer activity with those of other established anti-neoplastic agents in the cell line panel. Fig. 3 shows the GI_{50} values of ART and of drugs included in the N.C.I.'s Standard Agents Database of the Developmental Therapeutics Program for the 55 tumor cell lines. Drugs were selected, if they were either part of standard treatment protocols or if they are under investigation in recent clinical studies (16-18). In all tumor types investigated, ART was as active, in comparable molar ranges, as established cytostatic drugs. Interestingly, ART was the most active compound in colon cancer and was more active than drugs of the approved cyclophosphamide-methotrexate-vincristine regimen in breast cancer treatment.

The effect of ART on drug-resistant tumor cells was studied in CCRF-CEM leukemia cell sublines. We used four different sublines which revealed resistance to either doxorubicin (Fig. 4a), vincristine (Fig. 4b), methotrexate (Fig. 4c), or hydroxyurea (Fig. 4d). Pleasingly, none of the drug-resistant sublines exerted cross-resistance to ART (Fig. 4e-h).

To gain insight into the molecular basis of ART's cytotoxicity, we used a panel of isogenic *Saccaromyces cerevisiae* mutant strains. While most of the strains have sensitizing mutations erg6, pdr1 pdr3, we included an ERG6 PDR1 PDR3 rad 50 strain (r50+) in every experiment and compared this strain to the rad 50 erg6 pdr1 pdr3 (r50) to gauge the importance of these sensitizing mutations. ART was about equally sensitive in both backgrounds indicating that the analyses were not dependent on using the sensitized background. Among the nine defects represented in our panel, only the BUB3 strain (bub), lacking the mitotic background, showed a slight but reproducible increased sensitivity over the wild-type strain wt644 (Fig. 5). The strain over-expressing the G1 cyclin CLN2 (CLN) reproducibly appeared to be slightly resistant to ART. None of the DNA repair or DNA checkpoint defective strains are reproducibly more sensitive than wild-type.

Discussion

Though artesunate (ART) and other artemisinin derivatives have initially been described as anti-malaria drugs (1), their activity is not restricted to protozoans. The present investigation
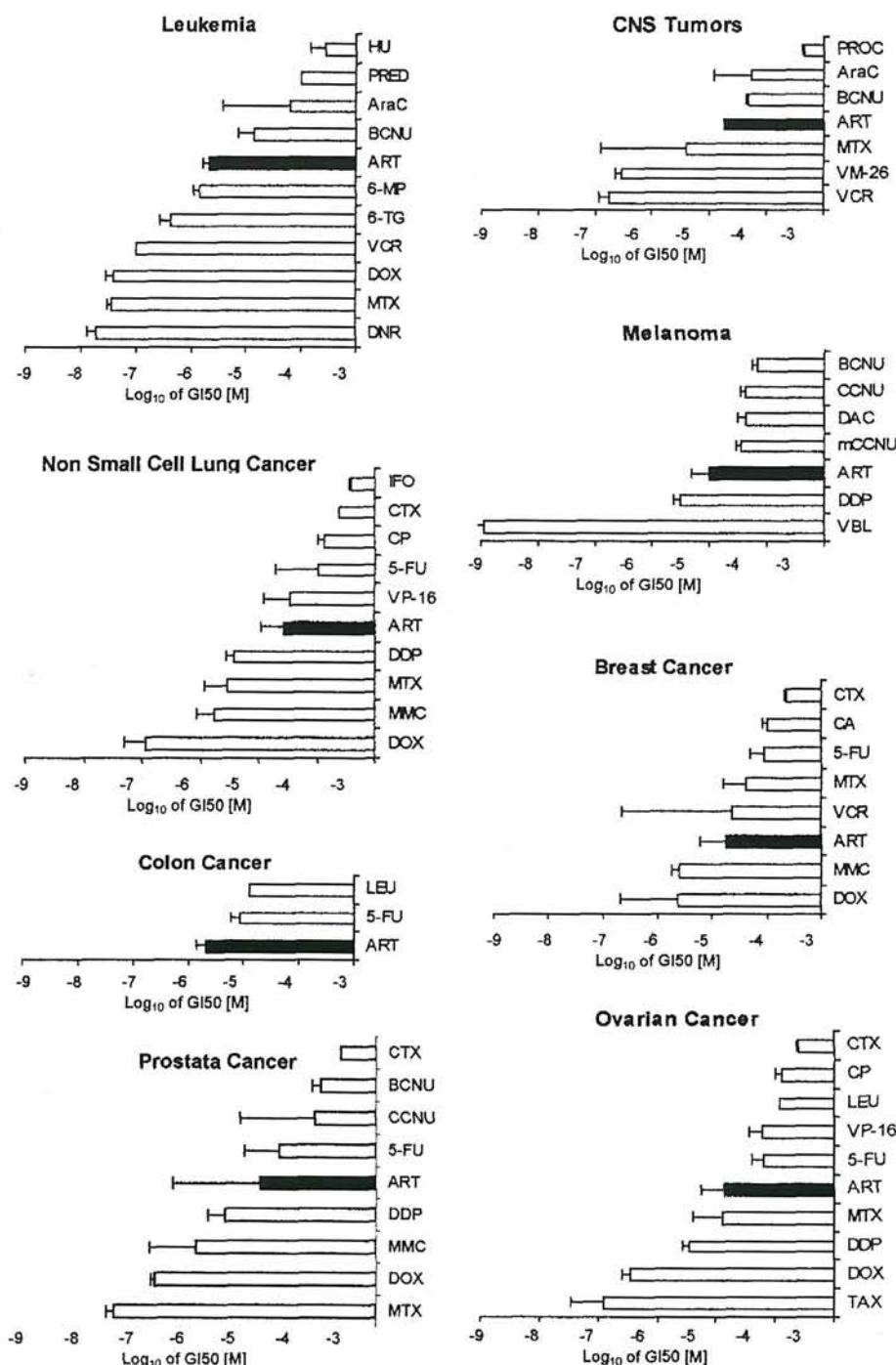
Figure 3. GI$_{50}$ values of artesunate in comparison to GI$_{50}$ values of common cytostatic drugs determined in 55 cell lines of the N.C.I. tumor panel. AraC, cytosine-arabinoside; ART, artesunate; BCNU, carmustine; CA, chlorambucil; CCNU, lomustine; CP, carboplatin; CTX, cyclophosphamide; DAC, dacarbazine; DDP, cisplatin; DNR, daunorubicin; DOX, doxorubicin; 5-FU, 5-fluorouracil; HU, hydroxyurea; IFO, ifosfamide; LEU, leucovorin; mCCNU, semimustine; MMC, mitomycin C; Mitox, mitoxantrone; 6-MP, 6-mercaptopurine; MTX, methotrexate; PROC, procarbazine; PRED, prednisone; TAX, paclitaxel; 6-TG, 6-thioguanine; VBL, vinblastine; VCR, vincristine; VP-16, etoposide; VM-26, teniposide.

demonstrates the anti-cancer activity of ART on cell lines of different tumor types, e.g. leukemia, melanoma and cancers of the colon, lung, prostate, breast, ovarian, and CNS. The anti-leukemic activity has previously been reported by us for ART (7) and by Woerdenbag *et al* for artemisinin (19).

ART affects multidrug-resistant *Plasmodium* strains as shown by Looareesuwan *et al* (6) as well as multidrug-resistant cancer cells as shown in the present investigation.

Our results indicate that ART may not be a substrate of the multidrug resistance gene product P-glycoprotein. CEM-ADR5000 and CEM-VCR100 cells express P-glycoprotein (20) and reveal the classical multidrug resistance phenotype with cross-resistance between *Vinca* alkaloids, taxanes, epipodophyllotoxins, and anthracyclines. The chemical structure of ART differs from other cytotoxic compounds involved in multidrug resistance. For example, essential
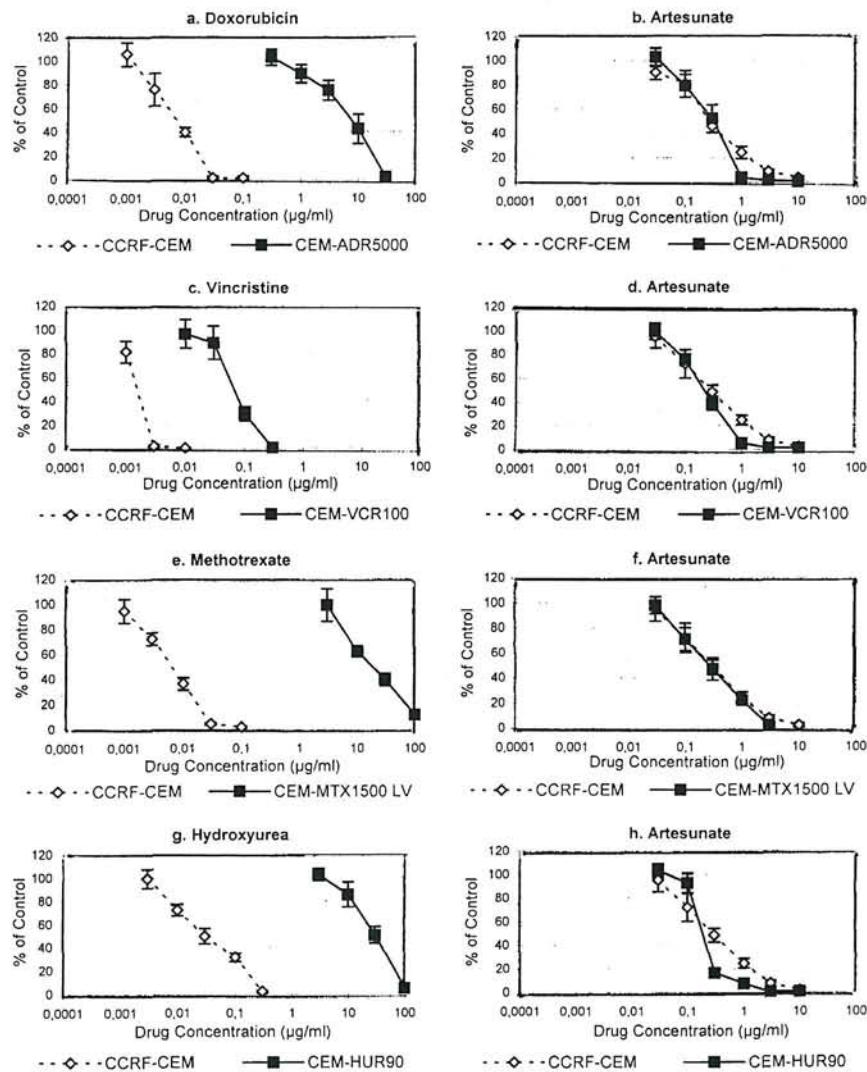
Figure 4. Growth inhibition assays of drug-sensitive and drug-resistant CEM leukemia cell lines. (a), sensitive and doxorubicin-resistant cells tested with doxorubicin; (b), sensitive and vincristine-resistant cells tested with vincristine; (c), sensitive and methotrexate-resistant cells tested with methotrexate; (d), sensitive and hydroxyurea-resistant cells tested with hydroxyurea. ART was tested in sensitive and (e), doxorubicin-resistant; (f), vincristine-resistant; (g), methotrexate-resistant; and (h), hydroxyurea-resistant leukemia cells. Control (100%) for each cell type represents cell growth without drug addition (mean ± SEM of each three independent determinations).
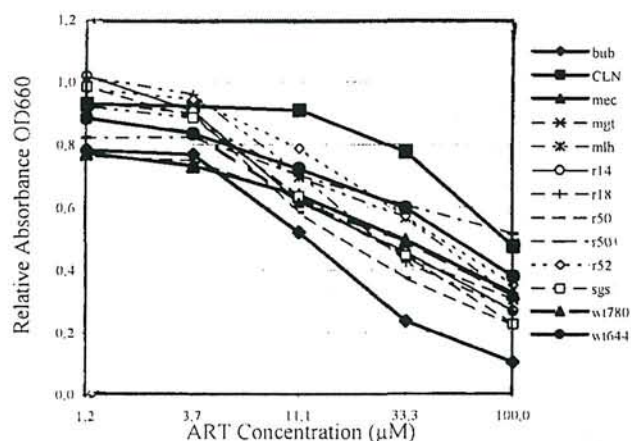

Figure 5. Toxicity of ART towards isogenic *Saccharomyces cerevisiae* mutant strains. Dose response curves of two wild-type (wt 644, wt780) and 11 different strains with defined defects (see insert in the figure) are shown. Control (100%) for each strain represents growth without drug addition. Values shown represent means of triplicate determination of one experiment. A repeated experiment revealed similar results.

structural features required for a drug to bind to P-glyco-protein are the presence of a tertiary amine (21,22). ART lacks this feature. Likewise, dihydrofolate reductase which is responsible for resistance to methotrexate was not able to protect from the detrimental effects of ART. CEM-MTX1500LV cells which amplify the DHFR gene (11) had similar responsiveness to ART as the sensitive parental CEM cells. As hydroxyurea-resistant cells were not cross-resistant to ART in our experiments, ribonucleotide reductase, which is over-expressed in these cells (12), must also be excluded as target molecule for ART. Since cancer chemotherapy is frequently hampered by the emergence of drug resistance, the treatment of refractory and otherwise drug-resistant tumors with ART represents an attractive prospect. As P-glycoprotein is part of the blood-brain barrier (23), ART may be valuable for the treatment of CNS tumors.

As these mechanisms of drug resistance seem to be irrelevant for ART's cytotoxic action, the question for the molecular targets of ART arises. The mode of action of ART

may be of a general anti-proliferative type. The iron-catalyzed generation of a free oxygen radical from the bridged endoperoxide group (Fig. 1) appears to be crucial for the antimalarial activity of artemisinin derivatives (24). Although unproven as of yet, it may be assumed that ART's anti-cancer activity is caused by a similar mechanism. ART's anti-malarial action is linked to protein damage (25). This raises the question as to whether ART may target specific proteins in cancer cells. To prove this possibility, we tested ART in a panel of isogenic *Saccharomyces cerevisiae* mutant strains. The use of yeast strains with genetic defects in cell proliferation, growth arrest, and damage repair genes is suitable to investigate the molecular target genes of anti-tumor drugs (8). We found that a BUB3 defective *S. cerevisiae* strain was more sensitive and a CLN2 defective strain was more resistant than the wild-type wt644 strain. The human homologue of yeast BUB3, hBUB3, is a mitotic spindle assembly checkpoint gene. It interacts with hBUB1 and hBUBR1. These three genes may be part of a protein complex which localizes to kinetochores before chromosome alignment. As hBUB3, hBUB1, and hBUBR1 dissociate from kinetochore in metaphase, they seem to modulate timing of anaphase initiation (26). The human homologue of yeast CLN2 is cyclin G1. Its upregulation in G1 phase and constitutive expression throughout the cell cycle implicates a role in growth regulation (27). Since cyclin G1 is a transcriptional target of the tumor suppressor p53, it may be part of a pathway leading to growth arrest and/or apoptosis (28). Although a possible role of BUB3 and CLN2 genes for the action of ART needs to be corroborated in further experiments with human tumor cells, the identification of two growth-related genes by this means substantiates earlier observations of the anti-proliferative action of ART in pro- and eukaryotes (2,7).

As shown in the present investigation, the molar ranges of GI_{50} values of ART and other approved cytostatic agents are comparable in the cell lines of the N.C.I. screening panel. It remains to be proven, whether dose ranges of ART necessary to affect cancer cells can be reached *in vivo*. A comparison of pharmacokinetic data with the *in vitro* data of the present investigation do indeed speak for this possibility. Concentrations of ART applied for the treatment of malaria (e.g. 2 mg/kg intravenously) reach peak plasma drug concentrations of 2640 ± 1800 μg/l (= 6.88 ± 4.69 mM) (29). The overall mean GI_{50} value of all 55 cell lines tested in the present study is three orders of magnitude lower (4.68 μM). As shown in clinical studies with more than 1,000 malaria patients, ART and other artemisinin derivatives are well tolerated with few and insignificant adverse side effects (30-32). Effects which may be disease- rather than treatment-related are transient fewer, dizziness, itching, and vomiting (33-35). Neurotoxicity has been found in animal studies using supra-therapeutic concentrations (36). It can be speculated that ART may also be well tolerated in cancer treatment.

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