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# PHENOL RED IN THE CULTURE MEDIUM STRONGLY AFFECTS THE SUSCEPTIBILITY OF HUMAN MCF-7 CELLS TO ROSCOVITINE

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**Abstract:** Estrogens play an important role in the growth and terminal differentiation of the mammary gland. Prolonged exposure to estrogens seems to predispose women to breast cancer. It recently became evident that not only the intrinsic hormonal status but also external factors such as the occurrence of pharmaceuticals and chemicals with hormone activity in the environment may put women at greater risk of developing breast cancer. We focused on the interference of endocrine disruptors in breast cancer therapy. We observed that phenol red added to the culture medium strongly promoted the cell proliferation and cell cycle progression of human cells expressing the estrogen receptor, and affected their susceptibility to chemotherapy.

**Key words:** Endocrine disrupters, Apoptosis, Cell cycle arrest, Cyclin-dependent inhibitors

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Abbreviations used: AIF – apoptosis inducing factor; CDK – cyclin-dependent kinase; EDC – endocrine disrupting compounds; ER – estrogen receptor; FCS – foetal calf serum; PBS – phosphate-buffered saline; ROSC – roscovitine; WCL – whole cell lysates; wt – wild-type

## INTRODUCTION

For years, the involvement of steroid hormones, especially estrogens, in the developmental growth and terminal differentiation of the mammary glands as well as in the development of breast cancer has been recognized. There is a body of evidence suggesting that the longer the exposure to ovarian estrogens, the greater the risk of breast cancer developing [1, 2].

The biological function of estrogens is primarily exerted via the stimulation of estrogen receptors (ERs). There are two main known ERs: the alpha [3, 4] and beta [5] estrogen receptors. They are hormone-stimulated transcription factors which belong to a large family of receptors, encompassing, among others, those for the thyroid and steroid hormones [6]. These proteins bind to specific hormone response elements in the promoter region of the regulated genes [7, 8]. However, the exact mechanism of action of the hormone receptors remains unclear. First, the hormone-receptor complexes are able not only to activate but also to suppress their responsive downstream genes [9]. Second, ER-mediated gene activation in the absence of an estrogen-responsive element has also been reported [10]. Third, the modulation of the functional status of ERs by other ligands such as anti-estrogens was observed. There is increasing evidence that conjugated fatty acids exerting anti-estrogenic effects may regulate the activity of ERs [11]. Considering the dominant role of ERs in the transduction of estrogen-signalling stimuli and in breast cancer etiology and progression, it is clear that ER status in breast cancer is both an important prognostic and therapeutic target.

Human MCF-7 cells expressing wild-type p53 protein are ER-positive breast cancer cells. They were reported to be resistant to the action of a variety of conventional cytostatic drugs [12, 13]. The decreased efficacy of some anti-cancer-drugs towards MCF-7 cells is attributable, at least partially, to the lack of caspase-3 expression due to a 47-base pair deletion within exon 3 of the *caspase-3* gene [14]. Therefore, treating MCF-7 cells with alternative drugs exhibiting low direct cytotoxicity on the one hand and concomitantly targeting the p53 response on the other hand could potentiate therapeutic efficacy by the induction of apoptotic steps independent of caspase-3 activity. The p53 tumor suppressor is known not only to initiate apoptosis [15, 16], but also to affect its execution at different stages [17]. The stimulated p53 protein enhances the activity of distinct pro-apoptotic genes such as *Bax*, *Apaf-1*, *Peg3*, *PUMA* and *caspase-9*, and is also able to repress some anti-apoptotic genes such as *Bcl-2*. A new p53-dependent pro-apoptotic gene, *p53-apoptosis inducing protein-1* (*p53AIP-1*) was recently identified [18]. p53AIP-1, a constituent of the mitochondrial membrane, was found to be regulated by p53 protein in a highly specific way [19]. Only wt p53 protein, phosphorylated at Ser46, was transcriptionally competent to induce p53AIP-1 protein synthesis [19]. It has been shown that upon severe DNA damage, Ser46 on p53 was phosphorylated, resulting in the induction of p53AIP-1 protein synthesis, followed by the

depolarization of the mitochondrial membrane and sequentially by the release of distinct mitochondrial proteins such as cytochrome C and apoptosis-inducing factor (AIF) [18, 19]. Ectopically expressed p53AIP-1 protein, which was localized in the mitochondria, led to apoptosis through the dissipation of the mitochondrial potential [18]. We observed that roscovitine (ROSC), a strong and specific inhibitor of cyclin-dependent kinases (CDKs), induced a cell cycle arrest of human MCF-7 breast cancer cells in a time- and dose-dependent manner [20]. The inhibition of DNA replication and the accumulation of $G_2$/M arrested cells coincided with a marked up-regulation of wt p53 protein and with the appearance of annexin-V positive cells. ROSC-induced cell cycle arrest preceded the onset of apoptosis. The main wave of apoptosis was observed after the exposure of MCF-7 cells to ROSC for 24 h [20].

In the course of our studies, we investigated by which pathway ROSC initiates apoptosis in human MCF-7 breast cancer cells [21]. We observed the decrease in the DeltaPsim of the mitochondrial membrane beginning 6 h after ROSC treatment, as detected by the stepwise loss of the formation of J-aggregates and by the release of distinct mitochondrial proteins such as cytochrome C and AIF. We also asked whether the ROSC-stimulated wt p53 is functionally linked to ongoing apoptosis. ROSC induced the phosphorylation of the p53 protein at Ser46 [21]. The strong site-specific phosphorylation of p53 occurred after just 4 h of ROSC treatment, and preceded the onset of mitochondrial depolarization by 2 h. The P-Ser46-activated tumor suppressor protein became transcriptionally competent and induced up-regulation of the p53AIP-1 protein, strongly implying that the activation of p53 is involved in the execution of the apoptotic program in MCF-7 cells exposed to ROSC [21]. More recently, we addressed the question whether the action of ROSC may depend on the presence of phenol red in the culture medium [22]. Phenol red mimics the action of estrogen, and, as a potential endocrine-disrupting compound (EDC), seems to be of particular concern, since endocrine disruptors not only have the capacity to interfere with the natural production and metabolism of hormones in the body, but also represent a serious carcinogenic risk. We cultivated cells in tissue medium with and without phenol red. Phenol red promoted the proliferation of MCF-7 cells [22]. However, within the first 24 h after cell plating, the difference in the growth kinetics was relatively low, implying that this time window would be suitable for experiments designed to evaluate the efficacy of CDK inhibitor therapy against breast cancer cells. Interestingly, ROSC action was negatively affected by the supplementation of the medium with phenol red. ROSC inhibited the proliferation of breast cancer cells more efficiently when cultivated in a phenol red-deprived medium [22].

In this study, we investigated whether supplementing the tissue culture medium with phenol red is able to restore the features of MCF-7 cells continuously cultivated in the tissue medium with phenol red. Moreover, we also tested the short-term effect of estrogen on cells cultivated in phenol red-deprived medium.

The supplementation of the tissue medium with phenol red slightly enhanced the proliferation rate of MCF-7 cells previously cultivated in a phenol red-deprived medium continuously for 6 months. Even the addition of phenol red at low concentrations increased the proliferation of cells cultivated in the presence of a low concentration of serum. The effect phenol red exerted on MCF-7 cells was comparable with that of estrogen. Our results show that short-term exposure to phenol red or to estrogen restores the features of MCF-7 cells continuously cultivated in a tissue medium with phenol red. These results are evidence that estrogen-like compounds may modulate the therapeutic effect of anti-cancer drugs, and that the presence of endocrine disruptors in the diet and drinking water could interfere with chemotherapy and reduce its efficacy.

## MATERIAL AND METHODS

### Cells

Human MCF-7 breast carcinoma cells were grown as a monolayer in Dulbecco's medium with and without phenol red supplemented with 10% FCS at 37ºC in an atmosphere of 8% $CO_2$ [20, 21]. In some experiments, phenol red or estrogen (E2) was added at the indicated concentration. Cells were grown up to 60% confluence and then treated with roscovitine (ROSC) at a final concentration ranging from 1-40 μM for the indicated periods of time. ROSC was dissolved as a 50 mM stock solution in DMSO and stored at -20ºC until used.

### Determination of the number of viable cells

The proliferation of human MCF-7 breast cancer cells and their sensitivity to increasing concentrations of ROSC and E2 was determined using the CellTiter-Glo™ Luminescent Cell Viability Assay (Promega Corporation, Madison, WI). As described recently in more detail [23, 24], the CellTiter-Glo™ Luminescent Cell Viability Assay generates luminescent signals and is based on the quantification of the cellular ATP levels. Tests were performed at least in quadruplicate. Luminescence was measured in a Wallac 1420 Victor, a multilabel, multitask plate counter. Each point represents the mean ± SD (bars) of replicates from one representative experiment.

### Measurement of the DNA of single cells by flow cytometry

The measurement of the DNA content was performed by flow cytometric analysis based on a slightly modified method [25] described previously by Vindelov *et al.* [26]. The cells were detached from the substratum by trypsinization, and then all the cells were harvested by centrifugation and washed in PBS. Aliquots of 1 x $10^6$ cells were used for further analysis. Cells were stained with propidium iodide as described previously, and then the fluorescence was measured using a Becton Dickinson FACScan after at least 2 h incubation at +4ºC in the dark.

**Statistical analysis**

Statistical analyses were performed using GraphPad Prism, and the significance levels were evaluated using Bonferroni's Multiple Comparison Test.

## RESULTS

### Human MCF-7 cells cultivated in the presence of phenol red have a higher proliferation rate

To examine the effect of phenol red on the proliferation of human ER-positive breast cancer cells, we compared the proliferation rate of MCF-7 cells maintained for a few months in phenol red-free tissue medium with that of cells cultivated in medium supplemented with phenol red. As shown in Fig. 1, MCF-7 cells grew faster in the medium containing phenol red. 53 hours after plating, the number of cells maintained in the medium containing phenol red increased approximately three-fold, whereas in the absence of phenol red, the number of cells increased only two-fold. The stronger increment in the cell number after cultivation for 53 h in the medium supplemented with phenol red was statistically significant.

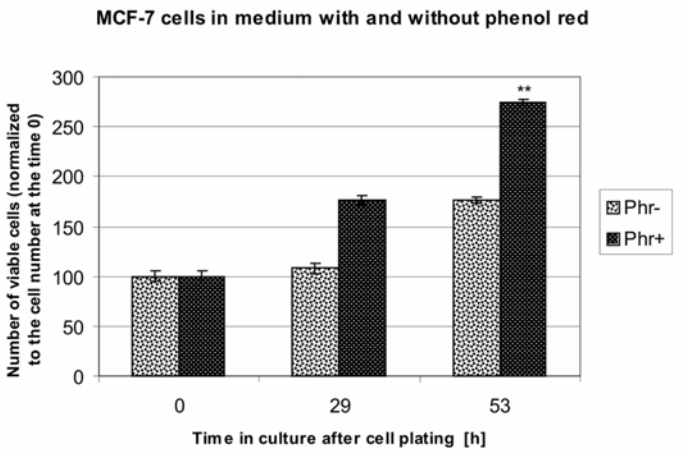


Fig. 1. Human MCF-7 cells cultivated in a medium supplemented with phenol red proliferate more rapidly. MCF-7 cells maintained in a tissue medium with or without phenol red were plated into microtiter plates ($5x10^3$ cells/well) and cultivated in the corresponding medium. At the indicated time points, the number of viable cells was determined using CellTiterGlo$^{TM}$ Luminescent Cell Viability Assay. Each column represents the mean of at least 4 replicates ± SD. The values were normalized against values at 0 h. The difference in cell number between cultures maintained for 53 h in a medium without or with phenol red was statistically significant ** ($p < 0.01$).

**Phenol red in the tissue medium affects the distribution of cells in distinct cell cycle phases**

The flow cytometric analysis of MCF-7 cells cultivated in medium with and without phenol red revealed the differences in the basal distribution of cells in the cell cycle phases. Generally, the frequency of the S-phase population was higher if MCF-7 cells were maintained in a medium containing phenol red (Fig. 2).

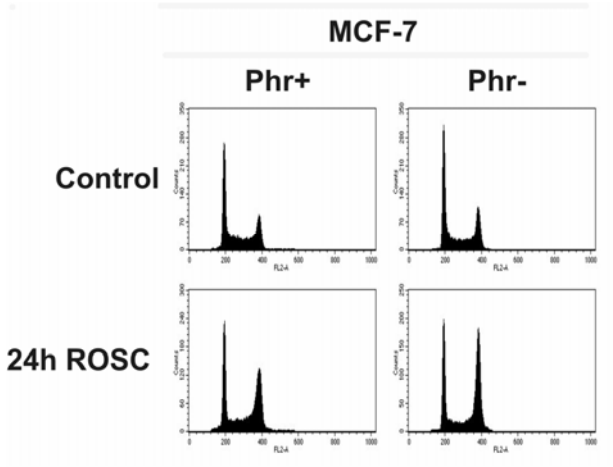


Fig. 2. The distribution of MCF-7 cells in distinct cell cycle phases in cells maintained in a medium with or without phenol red. MCF-7 cells cultivated in a medium with or without phenol red were treated with 20 μM ROSC for 24 h. Representative DNA profiles are shown. DNA histograms were generated using the ModFIT software.

**Supplementation of the tissue medium with phenol red positively affects the proliferation of MCF-7 cells**

Conventional Dulbecco's MEM contains 15 mg phenol red per 1 L medium (= 45 μM), whereas the phenol red concentration in the RPMI medium is 3-fold lower. To assess the effect of phenol red on the cell growth, we examined the effect of phenol red at a final concentration of 60 μM (20 mg/L) combined with different concentrations of foetal calf serum (FCS) (Fig. 3), or that of varying amounts of phenol red in combination with a constant concentration of FCS (Fig. 4). The addition of phenol red to a culture of MCF-7 cells previously continuously cultivated for 6 months in a phenol red-deprived medium increased the number of viable cells by approximately 50% after just 1 day. The observed increase was statistically significant. However, after 2 days, the dependence of the cells on FCS became obvious. Cells were able to divide rapidly if phenol red was combined with 15% FCS. On the other hand, in the presence of 20% FCS, the proliferation-supporting effect of phenol red became evident after 60 h. These results indicate that MCF-7 cells possess a limited capacity to divide, and this seems to depend on different intrinsic factors.

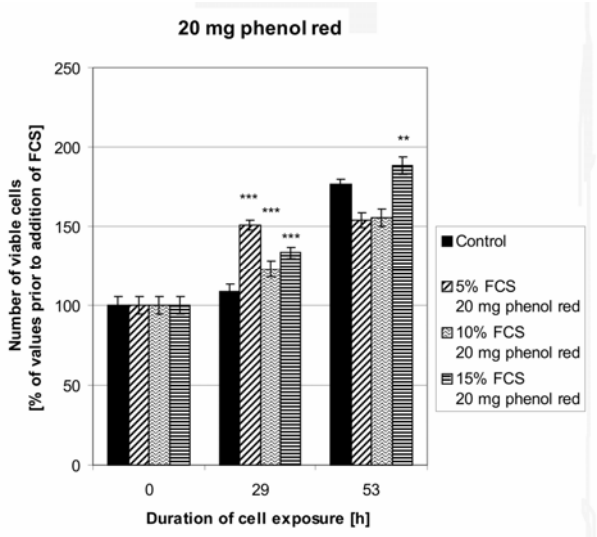


Fig. 3. Phenol red accelerates the growth of human MCF-7 cells in the presence of a low concentration of FCS. MCF-7 cells continuously cultivated in a tissue medium without phenol red for six months were plated into microtiter plates (5 x $10^3$ cells/well) and phenol red was added to the medium at a final concentration of 60 µM (20 mg/L). At the indicated time points, the number of viable cells was determined using the CellTiterGlo Assay. Each column represents the mean of at least 4 replicates ± SD. The values were normalized against the values at 0 h. After cell cultivation for 29 h, the difference in the cell numbers between the control culture and distinct supplementation conditions was statistically significant *** ($p < 0.001$).

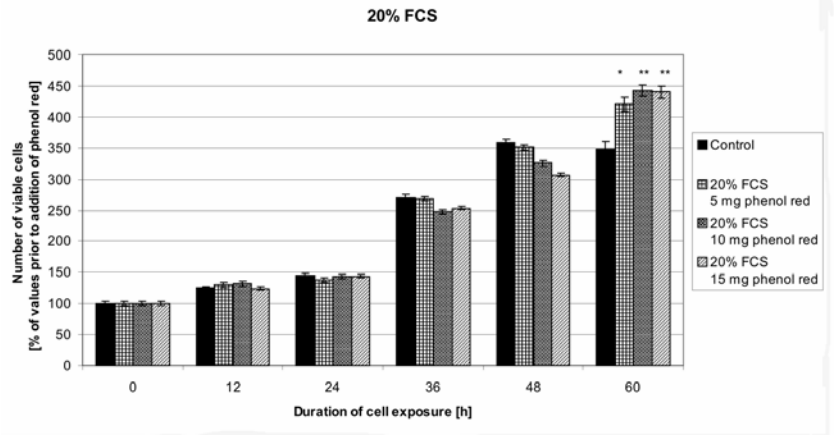


Fig. 4. Phenol red accelerates the growth of human MCF-7 cells in the presence of a low concentration of FCS. MCF-7 cells continuously cultivated in tissue medium without phenol red for six months were plated into microtiter plates (5 x $10^3$ cells/well) and phenol red was added to the medium at a final concentration ranging from 15 µM (5 mg/L) to 60 µM (20 mg/L). At the indicated time points, the number of viable cells was determined using the CellTiterGlo Assay. Each column represents the mean of at least 4 replicates ± SD. The values were normalized against the values at 0 h. At 60 h, the difference in the cell numbers between the control cultures and cells maintained in the medium with phenol red at higher concentrations was statistically significant ** ($p < 0.01$).

**Estrogen increases the proliferation of human MCF-7 cells**

In the next step, we addressed the question of how estrogen would affect the growth rate of MCF-7 cells previously continuously cultivated for 6 months in a phenol red-deprived medium. MCF-7 cells were plated into two microtiter plates. We added estrogen to the tissue medium to the final concentrations of

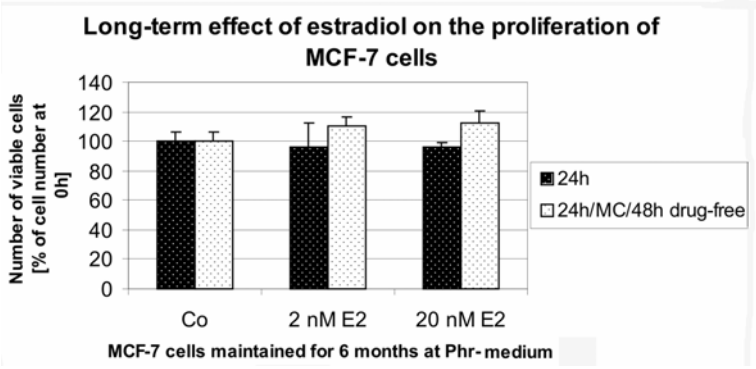


Fig. 5. Estrogen increases the proliferation of human MCF-7 cells. MCF-7 cells continuously cultivated in tissue medium without phenol red for 6 months were plated into microtiter plates (5 x $10^3$ cells/well) and estrogen (E2) was added to the tissue medium to a final concentration of 2 and 20 nM. At the indicated time points, the number of viable cells was determined using the CellTiterGlo Assay. Each column represents the mean of at least 4 replicates ± SD. The values were normalized against the values at 0 h.

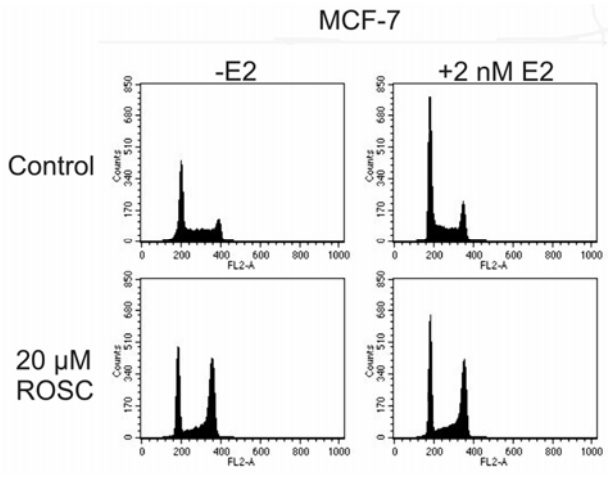


Fig. 6. The exposure of human MCF-7 cells to estrogen reduces the level of ROSC-mediated cell cycle arrest at $G_2$. MCF-7 cells cultivated in a medium without phenol red for 6 months were treated with 20 µM ROSC alone or in combination with 2 nM E2 for 24 h. The representative DNA profiles are shown. The DNA histograms were generated using the ModFIT software.

2 nM and 20 nM for 24 h. Then, the number of viable cells was determined directly after 24 h treatment, or the medium was changed (MC) and the cells were cultivated in fresh medium without estrogen for a further 48 h. Interestingly, the number of MCF-7 cells did not increase after 24 h estrogen treatment (Fig. 5). However, the estrogen effect became evident after the medium change and the cultivation of the MCF-7 cells in drug-free medium. Estrogen increased the number of cells by about 30%. This effect was even detected at low E2 concentrations. The exposure of MCF-7 cells to a ten-fold higher concentration of estrogen had no additional effect (Fig. 5). The analysis of the control and estrogen-treated cells by flow cytometry revealed that the 24 h treatment did not substantially affect the distribution of cells in the cell cycle (Fig. 6), and additionally confirmed the results of the cell proliferation assays.

**Phenol red reduces the cell cycle arrest induced by the pharmacological CDK inhibitor**

ROSC, a strong CDK inhibitor, induces a cell cycle arrest in human MCF-7 breast cancer cells in a concentration- and time-dependent manner. However, as reported recently, the exposure of MCF-7 cells cultivated in a tissue culture medium supplemented with phenol red to ROSC markedly reduced the frequency of the $G_2$ arrested cells (Figs 2 and 7).

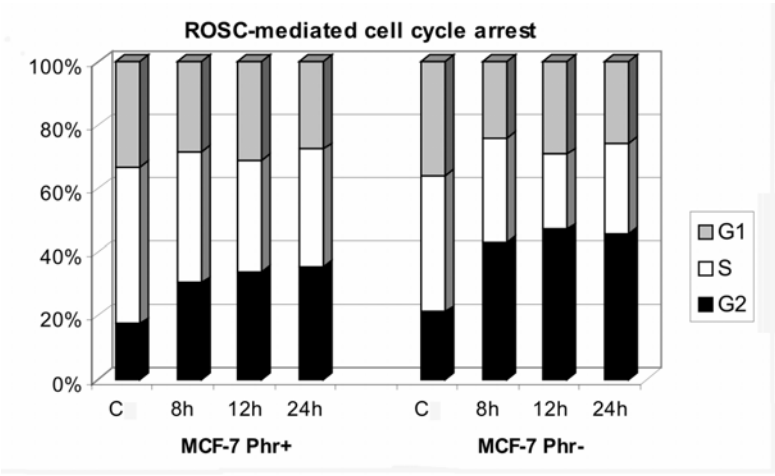


Fig. 7. Phenol red reduces the level of cell cycle arrest induced by the pharmacological CDK inhibitor. The distribution of distinct cell cycle phases in the control (C) and ROSC-treated for up to 24 hours cells.

**Estrogen partially abolishes the ROSC-mediated cell cycle arrest**

To prove whether the estrogen-like activity of phenol red may contribute to the observed diminution of the cell cycle block after the exposure to ROSC of MCF-7 cells maintained in phenol red-containing medium, we treated human MCF-7 breast cancer cells cultivated for a prolonged time in phenol red-deprived tissue

medium with ROSC alone or in combination with estrogen. As shown in Fig. 6, estrogen diminished the level of ROSC-mediated $G_2$ cell cycle arrest. These observations not only substantiate our recent finding that the presence of phenol red in the tissue medium negatively affected the therapy of MCF-7 cells by ROSC, but also explains how this observed effect is attributable to the estrogen-like activity of phenol red.

## DISCUSSION

Estrogens modulate gene expression via their specific receptors, ER-α and ER-β [3, 7]. Whereas the key role of ER-α in the development of breast cancer was clearly established and confirmed by considerable experimental and clinical data [27], the function of ER-β remains controversial [28]. ER-α is expressed in about 70% of all breast cancers [29], and therefore the determination of its expression status is important for therapeutic management. In the clinical routine, the presence of ER-α is an indication for selecting hormonal or aromatase inhibitor treatment. The mechanisms by which activated ERs mediate the transactivation of their responsive genes are complex and can be exerted by at least four distinct pathways [30].

Moreover, it has been proposed that estrogens might play a dual role in the development of breast cancer [31]. The promoting role of estrogens was frequently evidenced in multiple studies. However, it seems that in certain circumstances, estrogens might reduce the risk of cancer. This could be achieved via the estrogen-mediated induction of the tumor suppressor genes such as BRCA1, BRCA2 [32-34], p53, and retinoblastoma [35], and may be of importance during the early reproductive years.

Endocrine-disrupting compounds are of particular concern as they not only have the capacity to interfere with the natural production and metabolism of hormones in the body, but also represent a serious carcinogenic risk. The hormone-like action of drinking water contaminants could affect the proliferation and cell cycle of cells expressing hormone receptors. The action of this compound mimics that of hormones [36]. Moreover, the hormone-like agent modulated the susceptibility of cancer cells to chemotherapy. We observed that the efficacy of the chemotherapy of estrogen receptor positive cancer cells depends on the presence of phenol red. Phenol red reduced the therapeutic effect of the drug on MCF-7 cells. Its anti-apoptotic and proliferation-enhancing action reduced the levels of cell cycle arrest and apoptosis.

In this and in previous experiments [22], we observed that the proliferation of human MCF-7 breast cancer cells largely depends on the conditions of long-term cultivation. Cells divide more rapidly if maintained in the presence of phenol red, a hormone-like compound in the culture medium. Moreover, we found that the addition of phenol red to the culture medium strongly promoted cell proliferation and the cell cycle progression of human MCF-7 cells maintained for a long time in a phenol red-deprived medium. However, the promoting effect

of phenol red supplementation on cell proliferation became more pronounced after a short incubation, especially if it was combined with a low FCS concentration. One possible explanation for the observed effects is that during the maintenance of cells for a longer time in a medium containing a low FCS concentration, growth factors were exhausted. This could additionally positively affect the expression of distinct cell cycle regulators, e.g. p53 or $p21^{waf1}$ proteins. This assumption seems to be correct, because when cells were cultivated in a high FCS concentration, the cell growth-promoting effect of phenol red became evident after 60 h. Furthermore, the ability of ER to transactivate target genes is known to require the recruitment of additional co-activators such as cyclin D1 [37, 38]. One cannot exclude that the growth factors present in FCS at least partially require the same activators, and after the simultaneous administration of FCS at a high concentration and phenol red, the co-activators are limiting factors. This assumption is also supported by the fact that depriving MCF-7 cells of estradiol results in adaptative changes such as the up-regulation of ER-α or MAP kinase [39]. Moreover, the estradiol-deprived MCF-7 cells respond differently to the estradiol-mediated modulation of target genes as compared with their parental cells maintained under normal culture conditions [40].

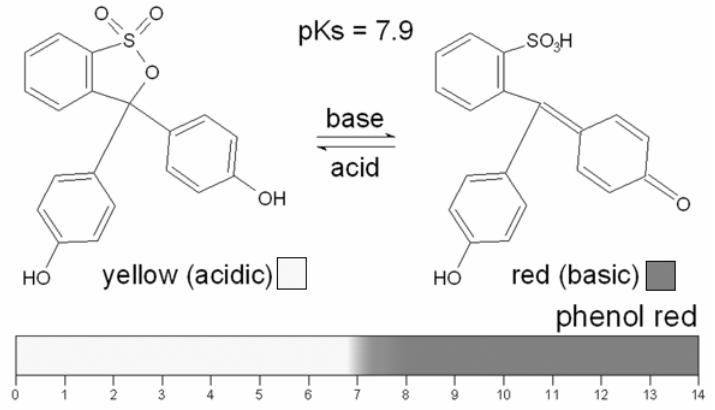


Fig. 8. The chemical structure of phenol red.

How can the effect of phenol red be explained? Phenol red is routinely used in tissue culture media as a pH indicator, turning red or yellow depending on the pH (Fig. 8). The concentration of phenol red in different tissue media differs strongly. Whereas in Dulbecco's MEM, the concentration of phenol red is relatively high (45 μM), its level is three-fold lower in RPMI medium. Since media differing in the concentration of phenol red are used for the cultivation of estrogen-responsive human breast cancer cell lines, the influence of the observed effects on cell proliferation cannot be ignored. Considering the key role of the activation of ER in the proliferation of breast cancer cells and their susceptibility to chemotherapy, the effects of xenoestrogens and their possible interference with the chemotherapy has to be investigated further.

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