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# Effect of Resveratrol and Mixtures of Resveratrol and Mitomycin C on Cancer Cells under Irradiation

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**Abstract.** *The radiation-biological effects of resveratrol (Res) alone or with mitomycin C (MMC) were investigated under various conditions in human breast cancer cells (MCF-7). The data of the survival curves obtained in aerated media (acting species: 42% OH, 54%* $O_2^{\bullet-}$*) showed that Res possesses antitumor activity and also acts as an efficient radical scavenger. This property was extremely enhanced in the presence of MMC. In media saturated with* $N_2O$ *(90% OH, 10% H) Res at low concentrations acted as a radiation-protecting agent, but at higher concentrations its cytostatic effect predominated. At the same time, the MMC-activity was reduced. In anaerobic media, Res demonstrated its radiation-protecting ability, but in mixtures the MMC-ability was reduced in comparison to that of pure MMC due to competition reactions between Res and MMC for the available free radicals. Finally, in a cell suspension containing formate as a specific scavenger for OH and H radicals (pH=7.4), Res successfully competed for these species and showed antitumor activity. Considering the reaction rate constants of the involved substrates and the implemented concentrations in each medium, the kinetic probability of each survival curve was calculated. Based on these data it was evident that the bifunctional property of Res (acting as radiation protector and also having antitumor activity) was based on an electron ejection process from its excited single state and on its reactivity with the primary radicals (OH, H,* $e_{aq}^-$*).*

Resveratrol (Res; trans-3,4',5-trihydroxystilbene) is found in a variety of plants, black and green tee, peanuts, berries, red wine, *etc.* (1). Results from a preview study have shown that the moderate consumption of red wine may offer some beneficial effects, which could be related to the anti-oxidant properties of the contained polyphenolic compounds, tannins, flavonoids, as well as phenolic acids (2). A large number of studies investigated the manifold properties of Res and the related substances. These investigations showed the anticancer action *in vitro* and *in vivo,* as well as the anti-inflammatory and antioxidant effects of Res (*e.g.,* 3-14). Murias *et al.* (15) synthesized five polyhydroxylated Res analogs and investigated their structure-activity relationships between pro- and anti-oxidant properties, as well as cytotoxity. Some of these compounds showed extremely high radical scavenging effect and very strong cytostatic activity. Based on the experimental data, the authors concluded, that the oxidation of ortho-hydroxystilbenes resulted in intermediates, which consume additional oxygen to form cytotoxic oxygen radicals. Wu *et al.* (16) investigated the interaction of six common polyphenols (quercetin, silymarin, resveratrol, naringenin, daidzein and hesperetin) with the multidrug-resistance-associated proteins. Their results suggested that these flavonoids enabled transport activities of MRP1, -4 and -5 proteins and influenced the bioavailability of anticancer and antiviral drugs *in vivo*.

As previously reported, anti-oxidant vitamins (C, E, β-carotene) act as electron donors and can contribute to the increase of the semiquinone formation of mitomycin C ($MMC^{\bullet-}$) and other cytostatics (17-20). Similar properties have also been established by the vitamins B1 (thiamine) (21), B2 (riboflavin) (22), B3 (nicotinamide) (23), B6 (pyridoxine) (24) and vitamin B11 (folic acid) (25). This capability is based on functional groups in the molecule, *e.g.* -OH, $-NH_2$, -SH, $-HPO_4$, *etc.*, which enable the excited states to eject or/and to transfer electrons to an electron acceptor (26). This process competes with the deactivation of the excited molecule by fluorescence (26). Since, the Res molecule contains three OH-groups (Figure 1), it is

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conceivable that it can affect the efficiency of cytostatics, *e.g.* MMC, under the influence of ionizing radiation, by ejecting one electron of its excited singlet state.

Taking into account the above mentioned properties of Res, its actions under irradiation with γ-rays, as well as its effect on MMC under various conditions, were also investigated. As a model for these studies, the human breast adenocarcinoma cell line MCF-7 was used.

## Materials and Methods

*General remarks.* A "Gammacell 220" (Nordion International Inc., Canada), providing a dose rate of 46 Gy/min was used as the irradiation source. For preparation of the aqueous media, triple distilled water and chemicals (Merck, Aldrich) with the highest purity available were used. Res (>99%, Sigma) and mitomycin C (MMC, Kyowa Hakko Kogyo Co. Ltd., Tokyo, Japan) were used as obtained. The media and supplements for the cells were obtained from Invitrogen GmbH (Lofer, Germany).

*Handling of the MCF-7 cells.* The human breast adenocarcinoma MCF-7 cells were maintained in a humidified atmosphere containing 5% $CO_2$ using an incubator (Cytoperm "Heraeus", Vienna, Austria). They were grown in DMEM High Glucose medium with 10% heat-inactivated fetal calf serum, 1% fugizone and 1% penicillin-streptomycin. The cells growing as monolayers were detached from the vessel surface using 2.5% trypsin-ethylenediamine tetraacetic acid (trypsin-EDTA) solution. The cells were then plated in sterilized plates (diameter 10 cm) and allowed to grow until they reached 60% confluence. The medium was then changed and the cells were incubated without additives (series A), in the presence of: 2 μmol Res (denoted as series B), 50 μmol Res (C), 1 μmol MMC (D), as well as with 2 μmol Res, + 1 μmol MMC (E) and 50 μmol Res, + 1 μmol MMC (F) at 37˚C.

After 48 h of treatment the medium was removed and the cells were washed with phosphate-buffered saline (PBS, pH=7.2).

In order to obtain a definite concentration of oxidizing (OH, $O_2^{\bullet-}$) or reducing species ($e_{aq}^-$, H), the aqueous media with cells were saturated either with argon, air or $N_2O$. In an additional series of experiments, 1 mmol formate was added (acting as scavenger for OH and H, as explained below) and the media were saturated with argon. Subsequently, the samples were irradiated with various γ-ray absorbed doses. The cells were then incubated with DMEM High Glucose medium containing 10% heat-inactivated fetal calf serum, 1% fugizone and 1% penicillin-streptomycin for 48 h. After staining with crystal violet, visibly colonies were counted using an inverted microscope (Olympus) at 40x-magnification. The results are calculated and presented as N/N0-ratio (N0 = number of cells before and N = number after treatment) as a function of the absorbed radiation dose. The experiments were performed at least in triplicate. The survival curves obtained under different experimental conditions were compared and discussed.

*Radiolysis of water.* The human organism is composed approximately by 70-75% of water. When water is subjected to ionizing radiation it is radiolyzed to reducing ($e_{aq}^-$, H) and oxidizing radicals (OH) in addition to molecular products ($H_2$, $H_2O_2$). The yields G-values: number of species formed or decomposed per 100 eV absorbed energy. 1 Gy (Gray)=100 rad=6.24x10$^{15}$ eV/g matter) of the primary products of water radiolysis at pH=6 to 8.5 are given in the general reaction, declaring the total summation of free radicals G($e_{aq}^-$+H+OH)=6.1=100%:

$$H_2O \rightarrow e_{aq}^-, H, OH, H_2, H_2O_2 \quad [1]$$
G-value: (2.7) (0.6) (2.8) (0.45) (0.72)

All radicals react in anaerobic aqueous solutions, but in the presence of oxygen, the $e_{aq}^-$ and H atoms are converted to peroxyl radicals:

$$H + O_2 \rightarrow HO_2^{\bullet} \; (k=2x10^{10}\ L.mol^{-1}.s^{-1}) \quad [2]$$
$$e_{aq}^- + O_2 \rightarrow O_2^{\bullet-} \; (k=1.9x10^{10}\ L.mol^{-1}.s^{-1}) \quad [3]$$
$$HO_2^{\bullet} \rightarrow H^+ + O_2^{\bullet-} \; (pK=4.8) \quad [4]$$

Hence, in such case 46% OH and 54% $O_2^{\bullet-}$ are involved in the radiation-induced process.

In aqueous media saturated with nitrous oxide ($N_2O$), all $e_{aq}^-$ are transformed into OH-radical, therefore, considering the G-values given in [1], 90% OH and 10% H will react:

$$e_{aq}^- + N_2O \rightarrow OH + OH^- + N_2 \; (k=0.91x10^{10}\ L.mol^{-1}.s^{-1}) \quad [5]$$

In solutions containing sufficient formate concentration, OH and H are scavenged and the resulting $^{\bullet}COO^-$ radical anion (27) can transfer one electron to an appropriate acceptor, *e.g.* MMC.

$$HCOO^- + OH \rightarrow {}^{\bullet}COO^- + H_2O \; (k=2.7x10^9\ L.mol^{-1}.s^{-1}) \quad [6]$$

$$HCOO^- + H \rightarrow {}^{\bullet}COO^- + H_2 \; (k=2.5x10^8\ L.mol^{-1}.s^{-1}) \quad [7]$$
$${}^{\bullet}COO^- + MMC \rightarrow CO_2 + MMC^{\bullet-} \text{ (biologically active species)} \quad [8]$$
and: $$e_{aq}^- + MMC \rightarrow MMC^{\bullet-} \; (k=2.2x10^{10}\ L.mol^{-1}.s^{-1}) \quad [9]$$

It should be mentioned that similar free radicals ($e_{aq}^-$, OH, $O_2^{\bullet-}$, *etc.*) are also produced in the human organism by enzymatic and various redox-processes. Therefore, using ionizing radiation, one can produce the desired kind and concentration of free radicals in order to study their specific biological effects.

## Results and Discussion

*Toxicity of resveratrol.* The toxicity of Res on the MCF-7 cells was investigated at two substrate concentrations (2 and 50 μmol Res) as a function of the incubation time (h). The results are presented in Figure 1. Obviously, in both cases Res acted as a promoter for cell growth. This effect proceeded linearly up to 50 h incubation time and thereupon is enhanced. It is interesting to note, that with an increase of substrate concentration, this ability decreased, suggesting a toxic effect. Consequently, low Res concentration promoted cell proliferation, but at higher concentrations (≥50 μmol Res) became toxic. In order to

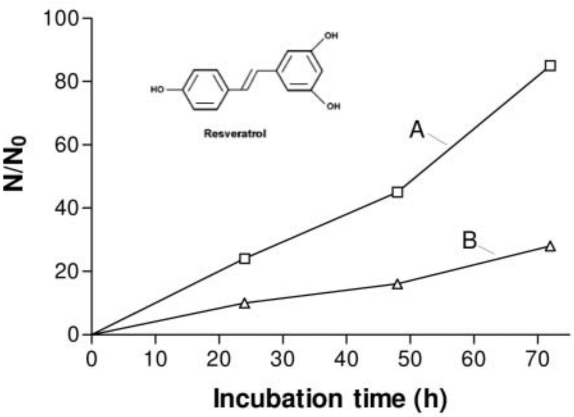


Figure 1. *$N/N_0$-ratio for the survival of human breast cancer cells (MCF-7) in media (pH= 7.4) containing 2 µmol (A) and 50 µmol (B) resveratrol in dependence of the incubation-time (h).*

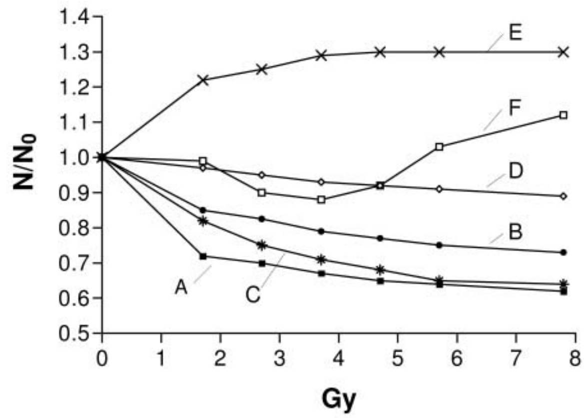


Figure 2. *Survival curves ($N/N_0$-ratio) of MCF-7 cells as a function of the absorbed irradiation-dose (Gy) in medium saturated with air (pH= 7.4, DL = 46 Gy/min) for: (A) buffer, (B) 2 µmol Res, (C) 50 µmol Res, (D) 1 µmol MMC, (E) 1 µmol MMC + 2 µmol Res and (F) 1 µmol MMC + 50 µmol Res.*

further investigate the role of Res concentration under irradiation, two concentrations, 2 and 50 µmol, were used in all further studies.

*Radiation effect on Res and Res/MMC in the presence of air.* As mentioned above, under these conditions, only oxidizing radicals (46% OH and 54% $O_2^{\bullet-}$) are reactive. The survival curves of the MCF-7 cells observed in buffer media at pH=7.4 (curve A), as well as in the presence of 2 and 50 µmol Res (curves B and C, respectively), for 1 µmol MMC (curve D) and the curves of the corresponding mixtures of Res and MMC curves (curves E and F, respectively), are shown in Figure 2.

As expected, the course of curve A ($N/N_0$-ratio) expresses the elimination of the cells as a function of absorbed radiation dose (Gy). The presence of 2 µmol Res acted as a radiation protector (curve B), but curve C for 50 µmol Res indicates a decrease in this protective effect. Here, the antitumor property of Res was evident. The course of survival curve D for 1 µmol MMC shows a very low antitumor effect. This appears as a consequence of a lack of $e_{aq}^-$, needed to produce $MMC^{\bullet-}$ (semiquinone, active form of the drug). Theoretically, no MMC effect should be expected under these conditions. The observed low antitumor efficiency (curve D) can be attributed to the formation of $MMC^{\bullet+}$ (radical cation) (28), which might act to some degree like the $MMC^{\bullet-}$ species. The course of survival curve E for 1 µmol MMC +2 µmol Res illustrated by an extraordinary radiation protection towards the reactive OH and $O_2^{\bullet-}$ radicals was unexpected. The mixture of 1 µmol MMC and 50 µmol Res under irradiation resulted in a rather unusual course (F). It is very likely that the produced radiolytic products of Res strongly influenced the co-operative action of both compounds. Moreover, in aerated media a relatively higher yield of $H_2O_2$ is formed by combination of $O_2^{\bullet-}$ species, which is certainly also involved in the process.

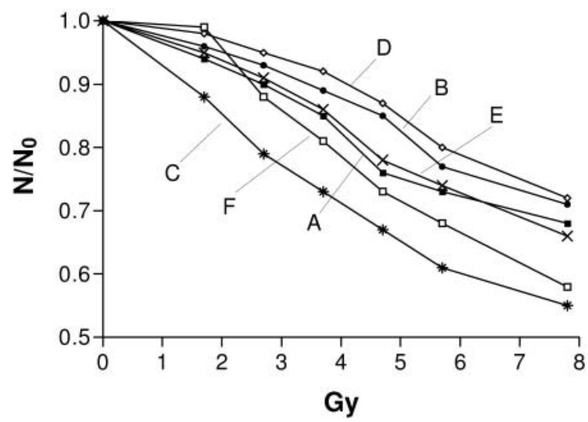


Figure 3. *Survival curves ($N/N_0$-ratio) of MCF-7 cancer cells as a function of the absorbed irradiation-dose (Gy) in medium saturated with $N_2O$ (pH= 7.4, $D_L$ = 46 Gy/min) for: (A) buffer, (B) 2 µmol Res, (C) 50 µmol Res, (D) 1 µmol MMC, (E) 1 µmol MMC + 2 µmol Res and (F) 1 µmol MMC + 50 µmol Res.*

*Radiation effect of Res and Res/MMC in the presence of $N_2O$.* In this case, the acting species were 90% OH radicals and 10% H atoms, leading to completely different survival curves, as presented in Figure 3. Whereas curve B corresponding to 2 µmol Res showed a slight radiation protection effect in comparison to the buffer-curve A, the survival curve C for 50 µmol Res demonstrated a very strong antitumor property. The 1 µmol MMC treatment (curve D) under the strong action of the OH radicals, resulted in a protective effect against the

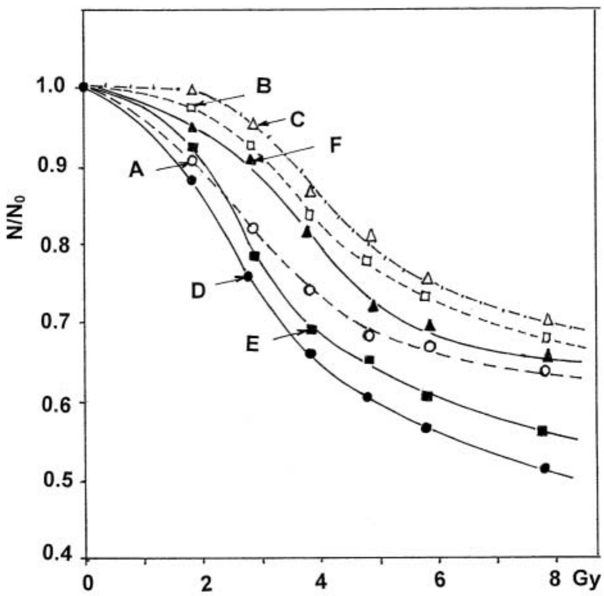


Figure 4. *Survival curves ($N/N_0$-ratio) of MCF-7 cancer cells as a function of the absorbed irradiation-dose (Gy) in medium saturated with Argon (pH= 7.4, $D_L$ = 46 Gy/min) for: (A) buffer, (B) 2 µmol Res, (C) 50 µmol Res, (D) 1 µmol MMC, (E) 1 µmol MMC + 2 µmol Res and (F) 1 µmol MMC + 50 µmol Res.*

radiation, as determined by the rather high reaction constant, k (OH + MMC) = $5.8 \times 10^9$ $L.mol^{-1}.s^{-1}$ (29). The mixture of 1 µmol MMC and 2 µmol Res (curve E) produced a slight radiation-protection effect, whereas the curve F for 1 µmol MMC and 50 µmol Res illustrated strong antitumor property, apparent when comparing curve D for 1 µmol MMC only with curve F. Res clearly exercised a synergistic effect towards MMC, and an intermolecular electron transfer process is likely involved in this case. Like other phenols, Res is able to eject electrons from the excited single state (26).

*Radiation effect of Res and Res/MMC in anaerobic media.* The implementation of 2 µmol Res (curve B, Figure 4) showed a pronounced radiation-protecting effect, compared to the buffer curve A. This radical scavenger ability of Res increased in the presence of 50 µmol Res (curve C). The survival curve D, corresponding to 1 mmol MMC demonstrated a very strong antitumor efficiency, as expected. However, the addition of 2 µmol Res to 1 µmol MMC (curve E) and particularly the addition of 50 µmol Res (curve F) resulted in a gradual decrease in the antitumor efficiency of MMC.

In order to interpret the individual survival curves, the interaction of the primary products of water radiolysis [1] with Res in competition with MMC should be considered. Under the present conditions, oxidizing radicals (46% OH) and reducing species (44% $e_{aq}^-$, 10% H) are involved in the radiation-induced processes. Based on the molecular structure

Table I. *Rate constants (k) for the reactions of MMC and Res with OH, H and $e_{aq}^-$ in neutral aqueous solution.*

| Reaction with | MMC k ($L.mol^{-1}.s^{-1}$) | Res k ($L.mol^{-1}.s^{-1}$) |
|---|---|---|
| OH | $2.7 \times 10^9$ (29) | $2.9 \times 10^{10}$ (31) |
| H | $3.1 \times 10^9$ (31) | $4.0 \times 10^9$ (31) |
| $e_{aq}^-$ | $2.2 \times 10^{10}$ (30) | $6.0 \times 10^7$ (31) |

of Res (composed of phenol-ethylene-resorcinol) its rate constants for the reactions with OH, H and $e_{aq}^-$ were calculated, and are presented in Table I together with those of MMC for comparison. Very recently the rate constant for the reaction of OH radicals with Res was determined by pulse radiolysis, k(OH+Res) = $6.9 \times 10^9$ $L.mol^{-1}.s^{-1}$. This value is lower compared to that given in Table I. Both values were considered in the calculations and the results were compared. The results of the second value (32) are given in brackets.

Based on the data of Table I, and the second value (ref. 32), the calculation of the reaction probability for curve E and F, Figure 4, of: (i) 2 µmol Res and 1 µmol MMC mixture and (ii) 50 µmol Res and 1 µmol MMC with OH, H and $e_{aq}^-$ led to the following conclusions:

- The OH in case (i) were 90% (70%) and in (ii) >99% (98%) consumed by Res, while the remainder reacted with MMC.
- The H atoms reacted 70% with Res and 30% with MMC in case (i), but 98% with Res in case (ii).
- The $e_{aq}^-$ were scavenged exclusively by MMC in case (i), but about 88% in case (ii), while the remainder reacted with Res.

These kinetic considerations show that the used substrate concentrations and the corresponding rate constants of the reactions with the primary products of water radiolysis were determining factors for the observed processes. The reaction probabilities also reflect the radiation-biological behavior of the *in vitro* experiments.

*Radiation effect of Res and Res/MMC in the presence of formate.* In the presence of sufficient formate concentration, the OH and H radicals are converted into $^{\bullet}COO^-$ species. The latter are capable of mediating one electron transfer to MMC to produce the active semiquinone form, $MMC^{\bullet-}$ [8]. Under these conditions the highest MMC efficiency was expected.

Taking into consideration the kinetic data presented in Table I and k(OH+Res)=$6.9 \times 10^9$ $L.mol^{-1}.s^{-1}$ (32), the probability of the competitive reactions between Res, MMC and formate with OH, H and $e_{aq}^-$ were calculated. It was established that in the survival curve B (Figure 5) corresponding to 2 µmol Res, about 98% OH (99.5% OH) and 97% H were scavenged by formate. However, in the presence of 50 µmol Res (curve C) 65% OH (89% OH) and 56% H were consumed by formate and remaining OH and H

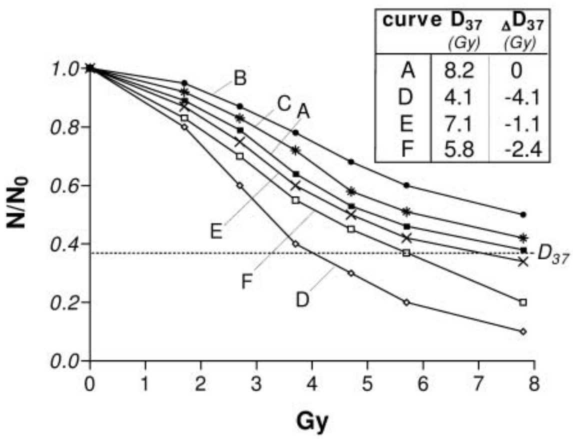


Figure 5. *Survival curves ($N/N_0$-ratio) of MCF-7 cancer cells in media containing 1000 μmol formate and saturated with Argon at pH=7.4 for: (A) buffer, (B) 2 μmol Res, (C) 50 μmol Res, (D) 1 μmol MMC, (E) 1 μmol MMC + 2 μmol Res and (F) 1 μmol MMC + 50 μmol Res.*

reacted with Res. In the survival curve D for 1 μmol MMC, practically all $e_{aq}^-$ were converted into $MMC^{\bullet -}$, as well as those originating from the electron transfer process from $^{\bullet}COO^-$ to MMC [8]. Hence, the highest yield of $MMC^{\bullet -}$ (G=6.1) was obtained. In the mixture of 2 μmol Res and 1 μmol MMC (curve E, Figure 5) approximately 98% OH (99.3%) and 96% H reacted with formate and were converted into $^{\bullet}COO^-$ species. In the case of 50 μmol Res and 1 μmol MMC (curve F, Figure 5) about 65% OH (88% OH) and 55% reacted with formate and resulted in $^{\bullet}COO^-$, approximately 85% $e_{aq}^-$ reacted directly with MMC, resulting in G ($MMC^{\bullet -}$) ≈ 2.3. In this case the expected total yield, G ($MMC^{\bullet -}$) ≈ 4.4, is much lower compared to that in case E. However, corresponding survival curves E and F in Figure 5 show an opposite character, since curve F was expected to be above curve E. This discrepancy can be explained by the fact that Res also acts as an electron donor, leading to additional formation of $MMC^{\bullet -}$ species. As mentioned before, phenol and related compounds, like Res, can eject electrons from their singlet excited states (26). Thus, upon increasing the concentration of Res, its antitumor ability also becomes more pronounced. This effect is illustrated by comparison of the $_{\Delta}D_{37}$-values given in Figure 5, indicating that survival curve D shows the greatest antitumor effect.

## Conclusion

Radiation-biological studies of Res, as well as of Res/MMC-mixtures, with breast cancer cells (MCF-7) as a model, were performed under various conditions in order to elucidate the interactions and the biological properties of the systems.

The $N/N_0$-ratio of 2 μmol Res registered as a function of the incubation-time (h) showed an increase on cell multiplication. But this effect was reduced by implementation of 50 μmol Res, indicating a bifunctional behavior of the substrate (Figure 1).

The survival curves (expressed also as $N/N_0$-ratio) for the MCF-7 cells in the presence of air (pH=7.4) as a function of absorbed radiation dose (Gy), where 46% OH and 54% $O2^{\bullet -}$ species were involved, indicated that both Res concentrations acted protective against radiation. This effect was greatly increased for 2 μmol Res + 1 μmol MMC (Figure 2).

In media saturated with $N_2O$ (90% OH, 10% H), th 2 μmol Res acted as an efficient radical scavenger, but 50 μmol Res showed an excellent antitumor efficiency. Similar behavior was observed with the corresponding Res/MMC- mixtures (Figure 3).

In media saturated with argon (acting species: 46% OH, 44% $e_{aq}^-$, 10% H, Figure 4), both Res concentrations showed a strong radiation-protection effect. The mixture of 2 μmol Res + 1 μmol MMC (curve E) illustrated an efficient cytostatic effect, nearly as good as that of MMC (curve D); but the curve F for 50 μmol Res + 1 μmol MMC predominantly showed a radiation-protective behavior.

Using formate as a specific scavenger for OH and H radicals in media saturated with argon (pH=7.4) under similar conditions with the above mentioned, both Res concentrations displayed a radiation protection effect (curves B and C, Figure 5). However, in the presence of MMC (curves E and F) the media showed a strong cytostatic efficiency. Naturally, curve (D) for MMC demonstrated a very strong antitumor effect. The course of the curves is discussed on the basis of the calculated kinetic probability for each survival curve. The radiation-induced antitumor ability of the corresponding systems is illustrated by the $_{\Delta}D_{37}$-values given as insert in Figure 5. (Positive $_{\Delta}D_{37}$-values express radiation protecting and the negative ones anticancer efficiency).

In conclusion, our results indicate that Res has bifunctional properties: it acts (i) as a radical scavenger (radiation protector) and (ii) as an antitumor agent, judging from its ability to eject electrons from the singlet excited state. Because of the radiation-induced competition process, the biological behavior of Res in neutral media depends on the concentration and on the reactivity of the present components with the primary species: $e_{aq}^-$, H, OH and $O_2^{\bullet -}$, produced either by radiation or enzymatically in human organism

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