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Figure 1

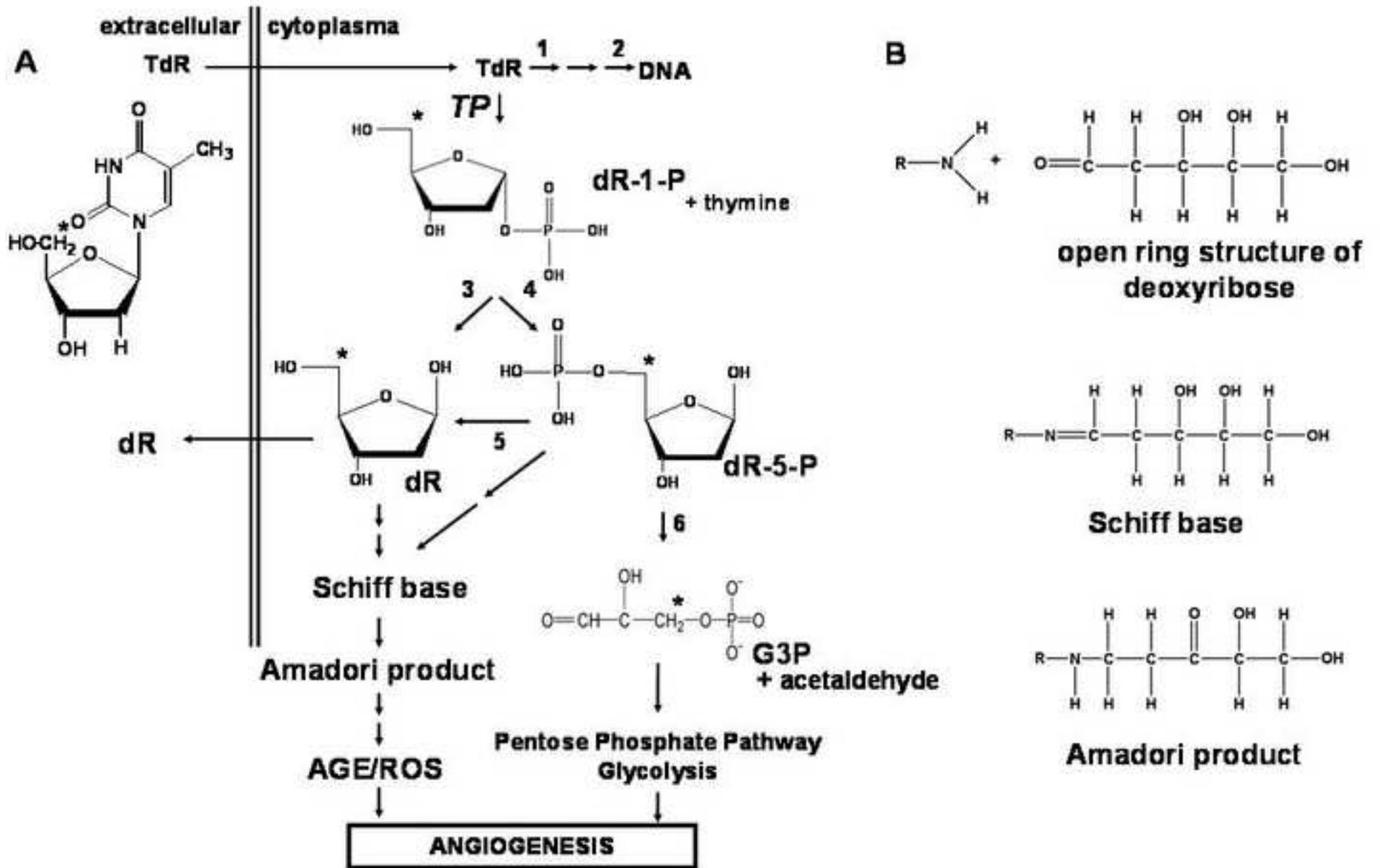
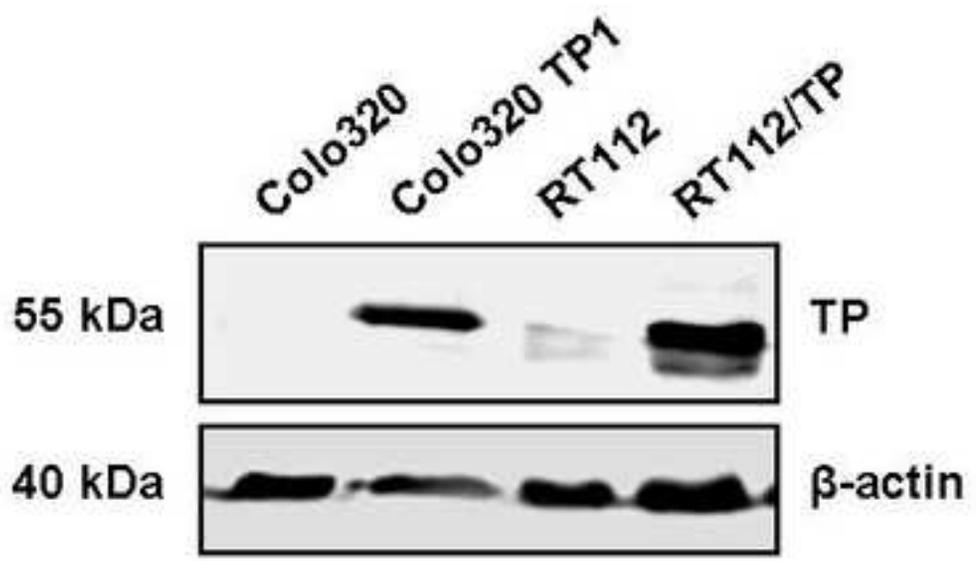
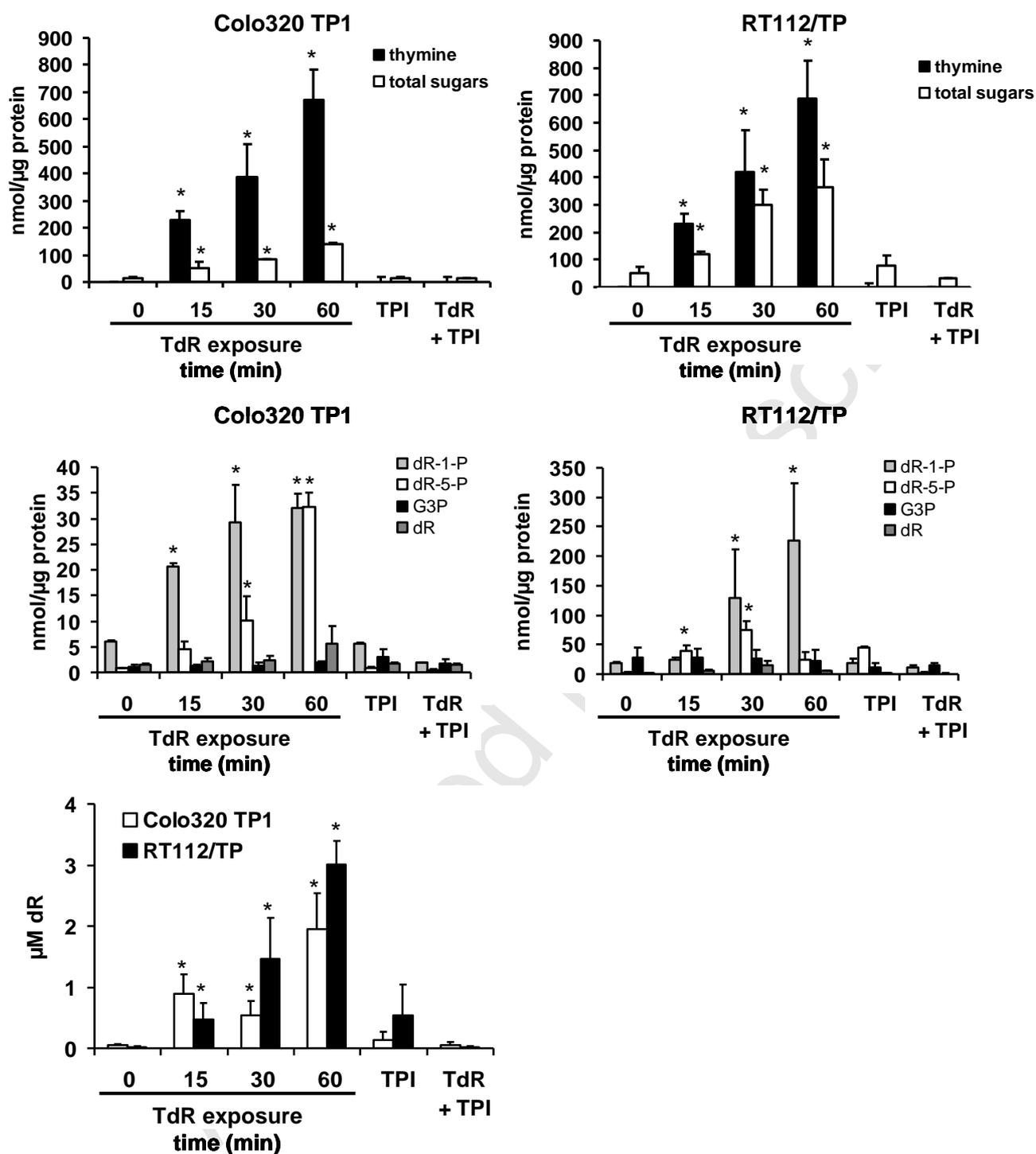
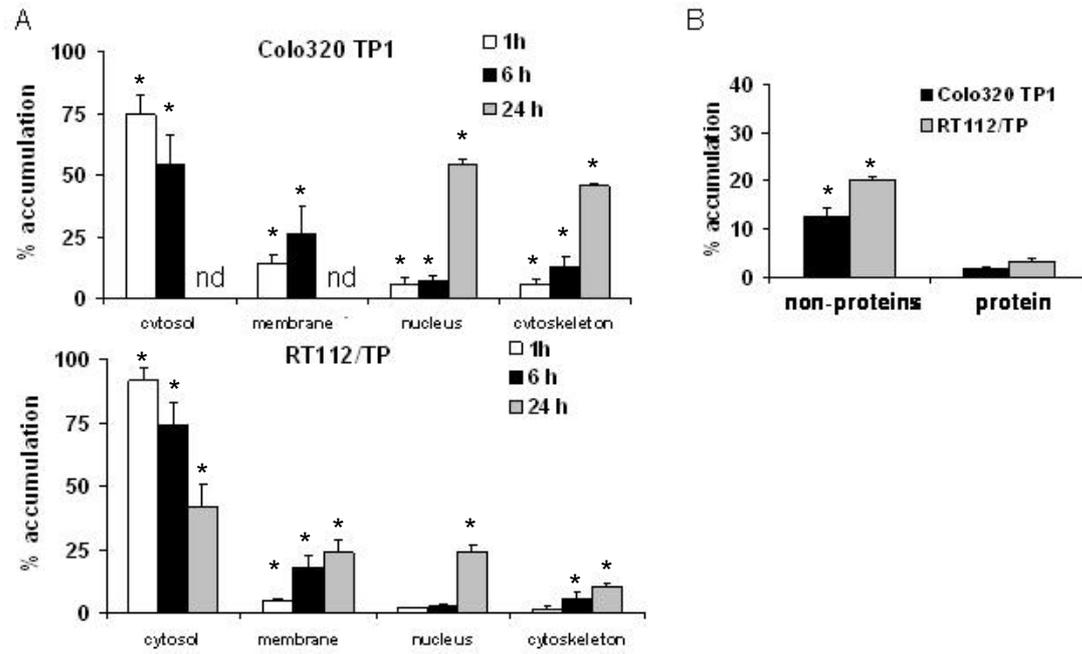
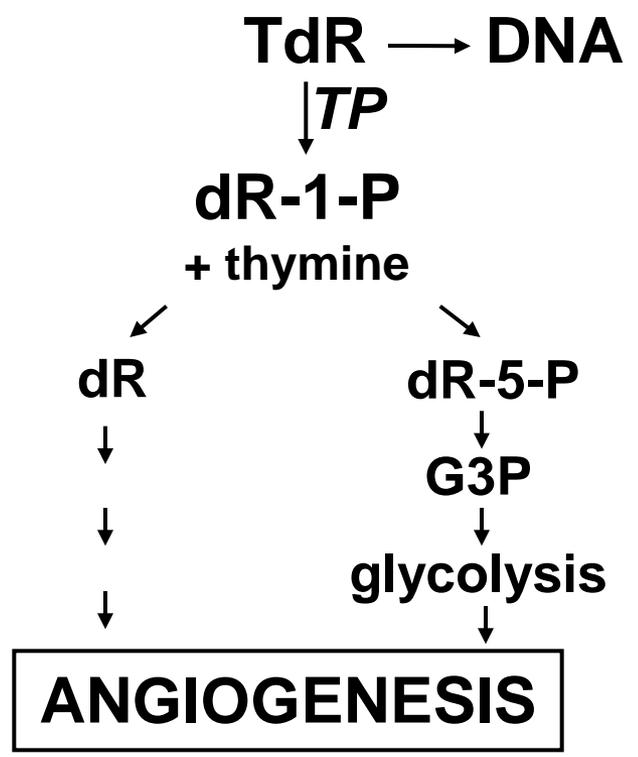


Figure 2 Bijnsdorp *et al.*









Thymidine (TdR) is converted by thymidine phosphorylase (TP) to deoxyribose-1-phosphate (dR-1-P), which can enter various metabolic pathways, which potentially contribute to TP mediated angiogenesis. dR=deoxyribose, dR-1-P=deoxyribose-1-phosphate, dR-5-P=deoxyribose-5-phosphate, G3P=glyceraldehyde-3-phosphate

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3 **Accumulation of thymidine-derived sugars in thymidine phosphorylase**  
4 **overexpressing cells**  
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46 **Category: Antibiotics and chemotherapeutics**  
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49 **Abbreviations:** TP: thymidine phosphorlylase; TdR: thymidine; G3P: glyceraldehyde-3-  
50 phosphate; dR: deoxyribose; dR-1-P: deoxyribose-1-phosphate; dR-5-P: deoxyribose-5-  
51 phosphate; TPI: thymidine phosphorylase inhibitor; AGE: advanced glycation endproducts;  
52 VEGF: vascular endothelial growth factor; IL-8: interleukin-8  
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57 <sup>i</sup> Employee of Taiho pharmaceuticals Co., ltd.

58 <sup>ii</sup> Received financial support from Taiho pharmaceuticals Co., ltd.  
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**ABSTRACT**

1  
2 Thymidine phosphorylase (TP) is often overexpressed in cancer and potentially plays a role in  
3  
4 the stimulation of angiogenesis. The exact mechanism of angiogenesis induction is unclear,  
5  
6 but is postulated to be related to thymidine-derived sugars. TP catalyzes the conversion of  
7  
8 thymidine (TdR) to thymine and deoxyribose-1-phosphate (dR-1-P), which can be converted  
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10 to dR-5-P, glyceraldehyde-3-phosphate (G3P) or deoxyribose (dR). However, it is unclear  
11  
12 which sugar accumulates in this reaction. Therefore, in the TP overexpressing Colo320 TP1  
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14 and RT112/TP cells we determined by LC-MS/MS which sugars accumulated, their  
15  
16 subcellular localization (using  $^3\text{H-TdR}$ ) and whether dR was secreted from the cells. In both  
17  
18 TP-overexpressing cell lines, dR-1-P and dR-5-P accumulated intracellularly at high levels  
19  
20 and dR was secreted extensively by the cells. A specific inhibitor of TP completely blocked  
21  
22 TdR conversion, and thus no sugars were formed. To examine whether these sugars may be  
23  
24 used for the production of angiogenic factors or other products, we determined with  $^3\text{H-TdR}$   
25  
26 in which subcellular location these sugars accumulated. TdR-derived sugars accumulated in  
27  
28 the cytoskeleton and to some extent in the cell membrane, while incorporation into the DNA  
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30 was responsible for trapping in the nucleus. In conclusion, various metabolic routes were  
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32 entered, of which the TdR-derived sugars accumulated in the cytoskeleton and membrane.  
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34 Future studies should focus on which exact metabolic pathway is involved in the induction of  
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36 angiogenesis.  
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## 1. Introduction

1  
2 The platelet derived endothelial cell growth factor (PD-ECGF) or thymidine phosphorylase  
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4 (TP) is often overexpressed in human cancers, which has been related to a higher microvessel  
5  
6 density, a higher tumor stage and the induction of metastasis [1,2,3,4]. In various *in vitro*  
7  
8 studies, TP had a chemotactic effect on endothelial cells [5,6,7,8]. *In vivo*, TP induced  
9  
10 angiogenesis using various cancer cell lines that were transfected with TP [9,10]. The role of  
11  
12 TP in angiogenesis seems evident, but its exact mechanism is still unknown and it is  
13  
14 postulated to be related to the sugars that are formed from thymidine (TdR) degradation by  
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16 TP.  
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21  
22 Deoxy- $\alpha$ -D-ribose-1-phosphate (dR-1-P) and thymine are the first products that are  
23  
24 formed by the phosphorylysis of TdR by TP (Fig. 1) [11]. After its formation, dR-1-P rapidly  
25  
26 disappears, possibly by conversion to 2-deoxy-D-ribose (dR) or 2-deoxyribose-5-phosphate  
27  
28 (dR-5-P) [12]. dR-5-P can be converted to glyceraldehyde-3-phosphate (G3P), which can  
29  
30 enter the glycolytic or pentose phosphate pathway. dR can form advanced glycation end  
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32 products (AGE) or reactive oxygen species (ROS) by Schiff base reactions [13]. The Schiff  
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34 base is formed from either dR or dR-5-P, which can be coupled to an intracellular protein by  
35  
36 nonenzymatic condensation between the sugar's aldehyde group and a lysine residue. This  
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38 Schiff base can subsequently rearrange to an Amadori product, such as an  $\alpha$ -hydroxyketone,  
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40 which may also form an enediol intermediate [14]. These unstable intermediates react via non-  
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42 enzymatic reactions to form AGEs. During these reactions, specifically in the transition  
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44 metal-catalyzed auto-oxidation, free radicals are produced [15,16,17]. The formation of AGE  
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46 from these reactions could be responsible for the angiogenic activity of TP [13].  
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54 The enzymatic activity of TP is indispensable for the angiogenic effect, since a  
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56 competitive inhibitor of TP could block the angiogenic effect [18]. Therefore, substrate and  
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58 metabolites from the reaction are often used for studying the angiogenic effect of TP [19].  
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1 The metabolite dR has previously shown angiogenic activity [6,10] and could elongate the  
2 sprouting in the aortic ring assay [19]. In addition, dR induced endothelial cell migration in  
3 various studies [5,8]. The association between TP activity and angiogenesis is based on the  
4 potential accumulation of TdR-derived sugars. However, little is known about the cellular  
5 metabolism of dR-1-P. Although dR is considered the main factor causing the angiogenic  
6 switch, the real identity and extent of formation of TdR-derived sugars remains unclear. dR-1-  
7 P rapidly disappears after its formation and its conversion could be reduced by addition of dR-  
8 5-P [11]. The aim of the present study was to characterize the specific sugars that can be  
9 formed by the TP reaction, their subcellular localization and whether they are secreted by the  
10 cancer cells, enabling endothelial cells to migrate and invade towards tumor sites.  
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## 26 **2. Materials and Methods**

### 27 *2.1 Cell culture and chemicals*

28 The human colon carcinoma cell line Colo320 TP1 was a variant of Colo320 (obtained from  
29 the ATCC), transfected with TP, as described previously [20]. RT112/TP was a kind gift of  
30 Dr. Bicknell (Oxford, UK). Cells were cultured as monolayers in DMEM, supplemented with  
31 10% heat inactivated FCS and 20 mM HEPES in 25 cm<sup>2</sup> culture flasks (Greiner Bio-One,  
32 Frickenhausen, Germany). Cells were maintained in a humidified 5% CO<sub>2</sub> atmosphere at  
33 37°C. TPI was provided by Taiho Pharmaceutical, Co., Ltd. (Tokushima, Japan). TdR,  
34 thymine, dR, dR-1-P, dR, dR-5-P and G3P were obtained from Sigma Aldrich Chemicals  
35 (Zwijndrecht, the Netherlands). These chemicals were dissolved in PBS in stock solutions of  
36 1-20 mM and stored at -20°C. <sup>13</sup>C<sub>6</sub>-glucose-6-phosphate (prepared as previously described  
37 [21]) and <sup>13</sup>C<sub>2</sub>-xylulose were obtained from Omicron biochemicals (South Bend, IN, USA).  
38 Ethoxyamine was obtained from Acros (Geel, Belgium) and pyridine from Merck (Darmstadt,  
39 Germany). 5'-<sup>3</sup>H-TdR (specific activity of 18.8 mCi/mmol, conc. 12.9 µg/ml) was purchased  
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1 from Moravek Biochemicals Inc. (Brea, CA, USA). The exact moiety where the sugar of the  
2 TdR is labelled is indicated in Fig. 1. The thymidine phosphorylase antibody was purchased  
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4 from R&D systems Inc. (Minneapolis, MN USA) and the thymidine kinase antibody was  
5  
6 purchased from QED Bioscience (San Diego, CA, USA). The secondary antibody, goat- $\alpha$ -  
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8 mouse-IRDye (800CW;#926-32210 and 680;#926-32220) was obtained from Westburg  
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10 (Leusden, the Netherlands).  
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### 17 *2.2 Treatment of the cells*

18  
19 For measurement of TdR and its sugars, Colo320TP1 and RT112/TP cells were seeded at  
20  
21  $2 \times 10^6$  cells/flask. After 48 h, cells were exposed to 100  $\mu$ M TdR for 0 min, 15 min, 30 min  
22  
23 and 60 min. TPI (10  $\mu$ M) was added 24 h prior to TdR exposure, after which samples were  
24  
25 incubated for 60 min. After treatment, cells were trypsinized, washed in PBS and centrifuged  
26  
27 for 5 min at 323 g at room temperature (RT). The medium and the cell pellets were stored  
28  
29 separately at  $-80^\circ\text{C}$  until sample preparation.  
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### 36 *2.3 Measurement of TP enzymatic activity and of TdR and thymine*

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38 HPLC measurement of TP enzymatic activity was performed as described previously [22] and  
39  
40 is based on the detection of both TdR and thymine [23]. After incubation with thymidine, 25  
41  
42  $\mu$ l 80% trichloroacetic acid (TCA) was added to the samples, which were left on ice for 20  
43  
44 min. Subsequently, samples were centrifuged at 14000 g at  $4^\circ\text{C}$  for 10 min. The supernatant  
45  
46 was transferred to a new vial, and the pH was neutralized. Samples were mixed and  
47  
48 centrifuged for 1 min at 14000 g. The upper aqueous layer was used for analysis of TdR and  
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50 thymine by HPLC analysis for nucleosides with UV detection as described previously [23].  
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#### 2.4 Measurement of sugar-phosphates

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2 For sample preparation, the cell pellet was dissolved in 500  $\mu\text{L}$  MQ and subsequently samples  
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4 were sonicated 3 x for 3 seconds on ice. 5  $\mu\text{M}$   $^{13}\text{C}_6$ -glucose-6-phosphate was used as an  
5  
6 internal standard. The samples were centrifuged through a filter with 10 kDa cut off (Ultracel  
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8 YM-10; #42407; Amicon Micron, Millipore, Billerica, MA, USA). Subsequently, the samples  
9  
10 were injected into the LC-MS/MS as described previously [24]. The amount of sugars was  
11  
12 expressed based on the total protein content of each sample that was measured before  
13  
14 centrifugation through the 10 kDa cut off filter. Protein concentration was determined using  
15  
16 the BioRad Protein assay (#500-0006; Bio-Rad Laboratories, Veenendaal, The Netherlands)  
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18 according to manufacture's instruction.  
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#### 2.5 Measurement of dR

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27 For preparation of the samples for the dR measurement, the cell pellet was dissolved in 150  
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29  $\mu\text{L}$  distilled water.  $^{13}\text{C}_2$ -xylulose was used as internal standard. 17  $\mu\text{L}$  of 20  $\mu\text{M}$   $^{13}\text{C}_2$ -xylulose  
30  
31 was added to 50  $\mu\text{L}$  of the sample. To deproteinize the sample, 200  $\mu\text{L}$  methanol was added.  
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33  
34 After 15 min, the sample was centrifuged for 10 min at 10000 g at 4°C. Following this, the  
35  
36 supernatant was transferred to a new vial and evaporated to dryness under a slight stream of  
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38 nitrogen. Ethoxime derivatives of dR were formed by treating the residue with 2 mg  
39  
40 ethoxyamine in 100  $\mu\text{L}$  pyridine at 60°C for 30 min. After cooling down to RT, the  
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42 hydroxygroups were acetylated by adding 100  $\mu\text{L}$  acetic anhydride at 80°C for 60 min. This  
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44 solution was evaporated to dryness and the residue was redissolved in ethylacetate. 1-2  $\mu\text{L}$  was  
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46 injected into the GC-MS operating under positive chemical ionization in the single ion  
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48 monitoring mode.  
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## 2.6 Separation of cell compartments

1  
2 Colo320 TP1 and RT112/TP cells ( $1.5 \times 10^6$  cells) were exposed to 200  $\mu\text{M}$  TdR (hot:cold  
3  
4 (1:21) of which the batch of  $[5' \text{-}^3\text{H}]$ -TdR was mixed with 1 mM unlabeled TdR). After  
5  
6 incubation for 1, 6 or 24 h or 1 h plus 24 h  $[5' \text{-}^3\text{H}]$ -TdR-free medium at  $37^\circ\text{C}$ , cell fractions  
7  
8 were separated using a ProteoExtract® Subcellular Proteome Extraction Kit according to  
9  
10 manufacture's instructions (Calbiochem, San Diego, CA). Of every cell fraction, including  
11  
12 from the medium above the cells after the designated incubation times, 5  $\mu\text{l}$  was counted. To  
13  
14 determine to which fraction (protein or non-protein fraction) secreted  $[5' \text{-}^3\text{H}]$ -TdR-derived  
15  
16 metabolites accumulated, 100  $\mu\text{l}$  of the medium above the cells after the retention was  
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18 precipitated with 60  $\mu\text{l}$  35% TCA for 20 min on ice. Subsequently, samples were centrifuged  
19  
20 for 5 min at 300 g at  $4^\circ\text{C}$  and 5  $\mu\text{l}$  of the supernatant (non-protein fraction) was counted. In  
21  
22 addition, the pellet (protein fraction) was recovered in 200  $\mu\text{l}$  MQ, of which 5  $\mu\text{l}$  was counted.  
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## 2.7 Western blotting

31  
32 Colo320, Colo320 TP1, RT112 and RT112/TP cells were washed twice with ice-cold PBS  
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34 and lysed in lysis buffer (Cell Signalling Technology Inc., Denver, USA). Cell lysates were  
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36 scraped, transferred into a vial and centrifuged at 11000 g at  $4^\circ\text{C}$  for 10 min. Supernatants  
37  
38 were transferred to a new vial and protein amounts were determined by the Bio-Rad assay,  
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40 according to the manufacturer's instruction (Bio-Rad Laboratories, Veenendaal, the  
41  
42 Netherlands). From each condition 30  $\mu\text{g}$  of protein was separated on a 10% SDS-PAGE and  
43  
44 electroblotted onto polyvinylidenedifluoride (PVDF) membranes (Millipore Immobilon™ –  
45  
46 FL PVDF, 0.45  $\mu\text{m}$ ). Subsequently, the membranes were blocked for 1 h at room temperature  
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48 (RT) in Odyssey blocking buffer (Odyssey blocking buffer #927-40003, Westburg, Leusden,  
49  
50 The Netherlands) and incubated overnight at  $4^\circ\text{C}$  with the primary antibodies (dilution  
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52 1:1000-10000 in Odyssey blocking buffer 1:1 diluted with PBS-T (PBS with 0.05% Tween-  
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20). The membrane was washed 5 times in PBS-T and incubated with the secondary antibodies (1:10000) for 1 h at RT in the dark. After incubation, the membrane was washed in PBS-T and followed by 5 min washing in PBS without Tween-20 to decrease the background signal. Subsequently, the bands were scanned using an Odyssey Infrared Imager (LI-COR Biosciences, Lincoln, Nebraska USA), 0 mm offset, 84  $\mu\text{m}$  resolution and with high quality [25].

### 2.8 Statistical analysis

Potential differences between controls and TdR exposed cells were evaluated using the two-tailed Student's *t*-test for paired data. Changes were considered significantly different when  $p < 0.05$ .

## 3 Results

### 3.1 TdR conversion to thymine

In order to determine the extent of TdR conversion, TdR and thymine levels were measured, after incubation with 100  $\mu\text{M}$  TdR for 15, 30 and 60 min. As expected, the parental Colo320 and RT112 cells did not or hardly convert TdR to thymine, since these cells do not or hardly express TP (Fig 2) [20] TP was highly expressed in both Colo320 TP1 and RT112/TP cells (Fig 2), while no band could be detected in Colo320 parental cells and a very faint band was found in RT112 cells. Colo320 and RT112 cells converted thymidine at a rate of  $0 \pm 0$  and  $3 \pm 0.04$  nmol/ million cells / h, respectively. Colo320 TP1 and RT112/TP converted thymidine at a rate of  $518 \pm 40$  nmol/million cells/h and  $418 \pm 34$  nmol/ million cells / h (Fig. 3A). After 1 h incubation of intact cells with TdR, the extracellular thymine concentrations were 24 and 30  $\mu\text{M}$ , respectively. The TK protein, which activates TdR for DNA synthesis, was similarly expressed in the TP-transfected cells (data not shown).

### 3.2 Intracellular accumulation of dR-5-P and dR-

TP degrades TdR into one molecule of thymine and one dR-1-P, which can be metabolized to other sugars (Fig. 1). We measured the intracellular levels of the potential metabolites dR-1-P, dR-5-P, dR and G3P in Colo320, RT112 and the transfected variants, Colo320 TP1 and RT112/TP. In the cells that did not express TP, no sugars accumulated at all (data not shown), which is in agreement with the absence of thymine formation. In general, the sugars accumulated to a much higher extent in RT112/TP cells, compared to Colo320 TP1 cells (Fig. 3A and 3B). dR-1-P increased rapidly, and was about 10 fold higher in RT112/TP cells compared to Colo320 TP1 cells. dR-1-P was rapidly metabolized to dR-5-P and dR. In both Colo320TP1 and RT112/TP cells, dR-1-P was the major intracellular metabolite (Fig. 3B). In RT112/TP cells, the accumulation of dR-5-P was lower than the level of dR-1-P. dR accumulated intracellularly to a low extent in Colo320TP1 cells and was only 4 fold increased compared to the control dR level. In RT112/TP cells, dR hardly accumulated. In Colo320 TP1 cells, G3P increased only at very low levels, with a 2 fold increase compared to the control. G3P did not accumulate in RT112/TP cells.

Addition of TPI alone to the cultures did not alter the production of the tested sugars. TPI completely blocked the conversion of TdR in Colo320 TP1 and RT112/TP cells (Fig. 3A), which is in agreement with the formation of the sugars, which were not formed at all (Fig. 3A and 3B).

### 3.3 dR is secreted from the cells

dR is an important angiogenic sugar that can be secreted by the cells [7,13]. Therefore, we determined the level of dR in the medium. dR-1-P and dR-5-P can not cross the membrane, because of the negative charge of the phosphate group on these sugars. In the medium above

1 Colo320 TP1 and RT112/TP cells, dR increased in time (Fig. 3C) and was found at higher  
2 levels than the other sugars intracellularly (Table 1). Of the total thymine detected, 10 and 13  
3  
4 % was measured as extracellular dR after 1 h by Colo320 TP1 and RT112/TP cells,  
5  
6 respectively (Table 1). TPI alone did not have any effect on the intracellular levels of dR,  
7  
8 while TPI prevented dR from being formed (Fig. 3C).  
9  
10

### 11 12 13 14 *3.4 Thymidine-derived sugars and accumulation levels*

15  
16 Since the thymine levels are representative for the total amount of sugars that are formed, we  
17 compared each value with the amount of thymine (in which thymine was set at 100 % for  
18  
19 each time point) (Table 1). In Colo320 TP1 cells, the total amount of measured sugars was  
20  
21 much lower than that of RT112/TP cells (Fig. 3A). In Colo320 TP1 cells, about 20% of the  
22  
23 total TdR-derived sugars presented intracellularly as dR-1-P, dR-5-P, dR and G3P and  
24  
25 extracellular dR. This did not increase after a longer exposure time to TdR, indicating that the  
26  
27 sugars are rapidly metabolized to other products. In RT112/TP cells, about 50 % of the TdR-  
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29 derived sugars were measured (Table 1), indicating a slower metabolism of these sugars into  
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31 other products compared to that in Colo320 TP1 cells.  
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### 41 *3.5 Accumulation in the cytoskeleton and nucleus*

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43 In order to determine in which cellular compartment the converted products of TdR  
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45 accumulated, Colo320 TP1 and RT112/TP cells were exposed to [5'-<sup>3</sup>H]-TdR for different  
46  
47 time periods. Subsequently, subcellular cell fractions were separated and the amount of  
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49 radioactivity was determined in each compartment (Fig. 4).  
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53 In Colo320 TP1 cells, the level of [5'-<sup>3</sup>H]-products in the cytosolic compartment  
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55 decreased in time, until it was hardly detectable after 24 h. Products in the membrane fraction  
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57 increased to some extent after 6 h, while after 24 h hardly any radioactivity was detectable in  
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1 this fraction. In the nuclear fraction, the radioactivity was increased after 24 h, which was  
2 about 55% of the total intracellular radioactivity. This is possibly in part due to metabolic  
3 activation of TdR by thymidine kinase, resulting in incorporations into the DNA and nuclear  
4 trapping. Interestingly, TdR-derived radioactivity was present in the cytoskeletal protein  
5 fraction, which increased in time up to about 45% after 24 h.  
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11 In RT112/TP cells, the radioactivity in the cytosolic fraction decreased in time, with  
12 about 45% of [5'-<sup>3</sup>H]-products still detectable after 24 h (Fig. 4). This is in agreement with  
13 the lower metabolism of the sugars in these cells, as measured by LC-MS/MS (Table 1).  
14 Comparable to Colo320 TP1 cells, the radioactive products in the nuclear fraction increased  
15 in RT112/TP cells, although at lower levels; up to 25 % after 24 h. Both the membrane and  
16 cytoskeleton fraction increased in time, with a maximum of about 25 % and 10 % after 24 h  
17 incubation, respectively.  
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28 When the [5'-<sup>3</sup>H]-TdR was washed away following 1 h incubation, the radioactivity  
29 was found to a high extent in the membrane (9.6 and 13.6 %) and cytoskeletal (73.5 and 39.4  
30 %) compartments of both Colo320 TP1 and RT112/TP cells, respectively (data not shown).  
31 Hardly any of the radioactive label retained in the nuclear fraction, which may be related to a  
32 lack of activation of TdR by TK within 1 h exposure. In summary, the TdR-sugars are used in  
33 the cytoskeletal and membrane, which may possibly be related to protein-glycation.  
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### 46 *3.6 Secreted products are in the non-protein fraction*

47 To determine whether products were secreted after conversion of TdR, Colo320 TP1 and  
48 RT112/TP cells were exposed to [5'-<sup>3</sup>H]-TdR for 1 h, after which the medium was refreshed  
49 and cells were washed to remove the excess of [5'-<sup>3</sup>H]-TdR. The amounts of secreted  
50 products were determined by analyzing the amount of radioactivity in the medium fraction.  
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Both Colo320 TP1 and RT112/TP cells secreted radioactive products, with higher levels for RT112/TP (24%) than for Colo320 TP1(15%) cells.

To analyze whether the secreted products were in the protein or in the non-protein fraction, proteins were precipitated by adding TCA (Fig. 4B). In both Colo320 TP1 and RT112/TP cells, the largest part of the secreted radioactive products was found in the non-protein fraction. This indicates that the sugars may not be used to a high extent for protein-glycation of secreted proteins, but may be more important for processes intracellularly.

#### 4. Discussion

In this study we describe TdR phosphorylysis to dR-1-P, which is rapidly followed by its isomerization to dR-5-P or degradation to dR of which the latter was extensively secreted. To our knowledge, the formation of these sugars using human (eukaryotic) cells has not been described earlier. We observed different levels of accumulation of TdR-derived sugars in the two cell types. In addition, the converted TdR was found in the cytoskeleton and to some extent in the cell membrane.

Previously we demonstrated that dR-1-P formed from TdR rapidly disappeared from Colo320 TP1 cells [11]. dR-1-P can be isomerized to dR-5-P mediated by phosphopentomutase (E.C. 5.4.2.7) and is degraded to dR by a phosphatase [10]. dR-5-P can be split into G3P plus acetaldehyde by deoxyriboaldolase (E.C. 4.1.2.4) [12,27]. The formed acetaldehyde may be converted into acetyl-CoA by aldehyde oxidase and acetyl-CoA synthetase [27]. G3P can enter several metabolic pathways, including the glycolytic and the pentose phosphate pathway, and possibly therefore did not accumulate in the cells in our study. Initially, these steps were only described in *e.g. Bacillus cereus* [28]. Enzymatic activity needed for this pathway was also identified in epithelial amoebic WISH cells [12]. The sugars may also be substrate in the formation of AGE (unpublished data), which may be

1 used for protein glycation, e.g. adding sugar moieties to proteins by non-enzymatic reactions  
2 [29,30]. However, whether protein glycation of AGEs are formed in the cells used in our  
3 study, remains to be identified. Brown et al. previously reported a possible Schiff base  
4 reaction in RT112 cells, and demonstrated the presence of reactive oxygen species by  
5 expression levels of the oxidative stress marker heme oxygenase-1 (HO-1) [31].  
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11 dR-1-P can also be formed by conversion of purine deoxynucleosides by purine  
12 nucleoside phosphorylase (PNP) [32]. Therefore theoretically, PNP may also be an  
13 angiogenic enzyme. However, to the best of our knowledge, a role for PNP in angiogenesis  
14 has never been described before. Important differences between TP and PNP involve their  
15 function: TP overexpression in tumor sites has clearly been related with angiogenesis [2],  
16 while PNP is involved in immune function [33,34]. In addition, PNP is hardly overexpressed  
17 in cancer [33,34,35], making it less likely to be an angiogenic enzyme. It might be possible  
18 that other products than the sugars are involved in angiogenesis as well, such as  $\beta$ -Amino-  
19 iso-butyric acid, which is another downstream metabolite of thymine [19]. However, several  
20 enzymes involved in thymine catabolism (e.g. DPD and  $\beta$ -ureidopropionase) are usually low  
21 or not detectable in many tumors [36,37,38]. In our and other panels of colon cancer cell  
22 lines, DPD activity was low to not detectable [39, 40], in contrast to pancreatic and breast  
23 cancer [40,41,42]. Therefore, it is unlikely that  $\beta$ -Amino-iso-butyric acid is involved in the  
24 angiogenic activity of TP.  
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45 In the present study, we used cancer cells to determine the sugar metabolism in order  
46 to stimulate angiogenesis. TP expression in endothelial cells may also be important for their  
47 angiogenic properties. However, TP expression is very low in endothelial cells [43,44]. In  
48 addition, TP expression is often reported to be in either cancer cells or other tumor stromal  
49 cells such as fibroblasts. The angiogenic role of TP is possibly more related to the secretion of  
50 angiogenic factors (i.e. dR or chemoattractants such as IL-8) by cancer cells, rather than an  
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1 autocrine stimulation by endothelial cells themselves. Therefore, the model that we used in  
2 the present study was intended to reflect the stimulation of angiogenesis by cancer cells.  
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4 Although TP is clearly associated with angiogenesis, the exact link between TdR-  
5 related sugars and angiogenesis is still missing. Cells with a high TP can secrete angiogenic  
6 factors at high levels, including the vascular endothelial growth factor (VEGF) and  
7 interleukin-8 (IL-8), but the role of sugars in the activation of these factors remains unclear  
8 [6,13]. TdR-derived sugars accumulated in the cell fractions of the membrane and  
9 cytoskeleton, which may be related to glycation of proteins by the formation of AGE. This  
10 may lead to the hypothesis that protein glycation plays a role in the activation of the  
11 transcription of angiogenic factors, such as IL-8 and VEGF [13] which can subsequently be  
12 secreted. The secreted fraction consisted of non-proteins. This indicates that dR may be the  
13 main TdR-sugar-product that is secreted by TP-expressing cells. Possibly, protein-glycation  
14 of secreted proteins is not the main regulator of angiogenesis. Thus, glycation of proteins that  
15 remain intracellularly are possibly important for angiogenesis, by activating transcription of  
16 angiogenic enzymes. Since dR can be secreted by the cancer cells, it can be taken up by the  
17 endothelial cells, in which it can also undergo Schiff base reactions, forming AGE inside the  
18 endothelial cells, stimulating their angiogenic properties (Fig. 1).  
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41 A possible role of sugars in the angiogenic potential of TP was studied through its  
42 metabolite dR, which can stimulate endothelial cell migration and angiogenesis. dR is a  
43 strongly reducing sugar that can generate oxygen radical species during the early stages of  
44 protein glycation [13]. dR can bind to an amino group of proteins by a non-enzymatic  
45 reaction. This leads to the formation of a Schiff base, which can subsequently rearrange to an  
46  $\alpha$ -hydroxyketone. During this reaction, free oxygen radicals are produced. Thus, through the  
47 formation of dR, TP induces oxidative stress in TP-overexpressing tumor cells. This may  
48 result in the secretion of angiogenic factors, such as VEGF or IL-8 [13]. dR secreted by the  
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1 cancer cells may in this way directly or indirectly (by its conversion to other sugars) stimulate  
2 angiogenesis. dR was secreted by the cancer cells in our study, indicating that dR can directly  
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4 play a role in the angiogenic potential of TP.  
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7 In conclusion, dR-1-P and subsequently dR-5-P and dR are formed from TdR  
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9 conversion by TP. Both metabolites may stimulate angiogenesis, e.g. by the stimulation of  
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11 angiogenic factors, while secreted dR may attract endothelial cells to form new blood vessels  
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13 in the tumor tissue. The sugars formed from TdR will accumulate in the membrane and  
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15 cytoskeleton of the cells, indicating that the sugars are used for further cellular metabolism,  
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17 possibly mediating angiogenesis in a more indirect manner.  
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